Investigating the effects of bredemolic acid on selected markers of some prediabetes-associated dysfunctions in diet-induced prediabetic rats

By

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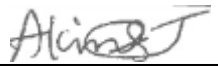
Signature 

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I, Akinjide Moses Akinnuga (Student no: 217081429), hereby declared that the thesis entitled **"Investigating the effects of bredemolic acid on selected markers of some prediabetes-associated dysfunctions in diet-induced prediabetic rats"**, is the original result of my own investigation and research, and that this thesis has not been submitted for any degree or examination at any other university. Where use was made of the work of others, they were duly acknowledged in the text.

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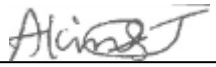
A.M. Akinnuga  **3/5/2020**

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DEDICATION

This thesis is dedicated to God Almighty, my lovely wife, my beautiful daughters, my dad and my late mum.

ACKNOWLEDGEMENTS

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α	Alpha
ADP	Adenosine diphosphate
AGE	Advanced glycated end product
AgRP	Agouti related protein
AKT	Protein kinase B
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
Ang	Angiotensin
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AT_1R	Angiotensin 1 receptor
B	Beta
BA	Bredemolic acid
BMI	Body mass index
C	Celsius
CD36	Fatty acid translocase
cDNA	Complementary Deoxyribonucleic acid
ChREBP	Carbohydrate responsive element binding protein
CKD	Chronic kidney disease
CPT	Carnitine palmitoyl transferase
CVD	Cardiovascular disease
DAG	Diacylglycerol
dl	Decilitre
DKD	Diabetic kidney disease
DMSO	Dimethyl sulphoxide
DN	Diabetic nephropathy
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
eNOS	Endothelial nitric oxide synthase
FATP	Fatty acid transport protein
FBG	Fasting blood glucose
FFA	Free fatty acid
FOXO1	Forkhead Box O1
GFR	Glomerular filtration rate

GK	Goto-Kakizaki
GLUT	Glucose transporter
GPx	Glutathione peroxidase
g	gram
h	hour
hrs	hours
HBA1c	Glycated haemoglobin
HDL	High density lipoprotein
HFHC	High fat high carbohydrate
HOMA2-IR	Homeostasis model assessment
hs-CRP	High sensitive C-reactive protein
HRP	Horseradish peroxidise
IFG	Impaired fasting glucose
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IL	Interleukin
IRS	Insulin receptor substrate
Kcal	Kilocalorie
kg	Kilogram
KIM	Kidney injury molecule
K^+	Potassium ion
LDL	Low density lipoprotein
L	Litre
MDA	Malondialdehyde
MET	Metformin
µ	Micro
µL	Microlitre
mg	Milligram
min	Minutes
mL	Millilitre
mm	Millimetre
mmol	Millimole
mol	Mole
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate

NAFLD	Non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
ND	Normal diet
ng	Nanogram
nm	Nanometre
NO	Nitric oxide
NPD	Non-prediabetic
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
PAI	Plasminogen activator inhibition
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD	Prediabetic
PI3K	Phosphoinositol-3-kinase
PKC	Protein kinase C
PTP	Protein tyrosine phosphate
PVDF	Polyvinylidene difluoride
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SGK	Serum/glucocorticoid-regulated kinase
SGLT	Sodium/glucose cotransporter
SOD	Superoxide dismutase
SREBP	Sterol regulatory element binding protein
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglyceride
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrotic factor
TOAC	Total antioxidant capacity
tPA	tissue type plasminogen activator
TTBS	Tris-buffered saline with 0.1% Tween 20
V	Volt
VLDL	Very low-density lipoprotein
WC	Waist circumference
ZDF	Zucker diabetic fatty

PUBLICATIONS AND PRESENTATIONS

Published Manuscripts

Akinnuga, A.M., Siboto, A., Khumalo, B., Sibiya, N.H., Ngubane, P., & Khathi, A. (2019). Evaluation of the effects of bredemolic acid on selected markers of glucose homeostasis in diet-induced prediabetic rats. Archives of Physiology and Biochemistry. DOI:10.1080/13813455.2019.1680697. https://doi.org/10.1080/13813455.2019.1680697. See chapter 2 and appendix III

Akinnuga, A.M., Siboto, A., Khumalo, B., Sibiya, N.H., Ngubane, P., & Khathi, A. (2020). Bredemolic acid ameliorates selected liver function biomarkers in a diet-induced prediabetic rat model. Canadian Journal of Gastroenterology and Hepatology. Volume 2020: Article ID: 2475301, page 1 - 9. doi:10.1155/2020/2475301. See chapter 3 and appendix IV.

Akinnuga, A.M., Siboto, A., Khumalo, B., Sibiya, N.H., Ngubane, P., & Khathi, A. (2020). Bredemolic acid improves cardiovascular function and attenuates endothelial dysfunction in diet-induced prediabetes: Effects on selected markers. Cardiovascular Therapeutics. Volume 2020: Article ID:1936406, Page 1 - 9. doi:10.1155/2020/1936406. See chapter 4 and appendix V.

Akinnuga, A.M., Siboto, A., Khumalo, B., Sibiya, N.H., Ngubane, P., & Khathi, A. (2020). Ameliorative effects of bredemolic acid on markers associated with renal dysfunction in a diet-induced prediabetic rat model. Oxidative Medicine and Cellular Longevity. Volume 2020: Article ID:2978340, Page 1 - 12. See chapter 5 and appendix VI.

Conference presentations

Akinnuga, A.M., Siboto, A., Khumalo, B., Sibiya, N.H., Ngubane, P., & Khathi, A. Evaluation of the effects of bredemolic acid on selected markers of glucose homeostasis in diet-induced prediabetic rats. 47th Conference of the Physiological Society of Southern Africa (PSSA), 18th– 21st August 2019, East London, South Africa. See Appendix VII.

Akinnuga, A.M., Siboto, A., Khumalo, B., Sibiya, N.H., Ngubane, P., & Khathi, A. Bredemolic acid, a pentacyclic triterpene, ameliorates selected liver function biomarkers in high fat high carbohydrate diet-induced pre-diabetic rat model. Annual Laboratory Medicine and Medical Sciences (LMMS) Research Symposium 2019, 6thof September 2019, Westville, Durban, South Africa. See Appendix VIII.

THESIS LAYOUT

The current thesis is presented and submitted in a manuscript format. The data and findings in this research study are prepared and presented in seven chapters in which four are presented as manuscripts in the following manner:

Chapter 1: This chapter provides an introduction to the study, literature review, justification, aim and objectives of the study

Chapter 2: This chapter is the first manuscript which provides information on the effects of bredemolic acid on selected markers of glucose homeostasis in diet-induced prediabetic rat. This manuscript has been published in the Archives of Physiology and Biochemistry. https://doi.org/10.1080/13813455.2019.1680697. See Appendix III

Chapter 3: This chapter is the second manuscript that presents reports on the effects of bredemolic acid on liver function biomarkers in a diet-induced prediabetic rat model. This manuscript has been published in the Canadian Journal of Gastroenterology and Hepatology, Volume 2020: Article ID: 2475301, Page 1-9. See Appendix IV

Chapter 4: This is the third manuscript which provides information on the effects of bredemolic acid on cardiovascular and endothelial functions in diet-induced prediabetic rats. This manuscript is currently published in Cardiovascular Therapeutics, Volume 2020: Article ID:1936406, Page 1-9. See Appendix V

Chapter 5: This chapter presents the fourth manuscript and provides a report on the ameliorative effect of bredemolic acid on renal dysfunction in diet-induced prediabetic rats. This manuscript is in press in the journal: Oxidative Medicine and Cellular Longevity, Volume 2020: Article ID:2978340, Page 1-12. See Appendix VI

Chapter 6: This chapter described the synthesis, conclusions, limitations and future studies.

Chapter 7: This chapter presents the appendices.

ABSTRACT

Introduction

Prediabetes is an abnormal glycaemic state between normoglycaemia and chronic hyperglycaemia which is currently prevalent in developing and developed countries due to increased consumption of high caloric diet coupled with sedentary lifestyle. Prediabetes is associated with abnormal glucose metabolism. Additionally, the risk of developing prediabetes-associated complications such as non-alcoholic fatty liver disease (NAFLD), cardiovascular and renal diseases is not only present in overt diabetes mellitus but also in prediabetes. Management of prediabetes involves the combination of dietary and pharmacological interventions, however there is reported low compliance among patients as they tend to become overly dependent on the pharmacological interventions. Consequently, the pharmacological intervention efficacy is reduced as patients still progress to having overt diabetes. Therefore, managing prediabetes with anti-diabetic agents that will remain effective even in the absence of dietary intervention is considered necessary. Triterpenes have been found to have potential as anti-diabetic agents. Bredemolic acid (BA), a pentacyclic triterpene, has been reported to have increased biological activity relative to some other triterpenes. In this study, we sought to investigate the effects of BA on selected markers of some prediabetes-associated dysfunctions such as abnormal glucose homeostasis, hepatic, cardiovascular and renal dysfunctions in a prediabetic rat model in both the presence and absence of dietary intervention.

Materials and Methods

Thirty six (36) Sprague Dawley male rats that weighed 150 – 180g were divided into two groups: the non-prediabetic (n=6) and the prediabetic groups (n=30) which were fed a normal diet (ND) and high fat high carbohydrate (HFHC) diet respectively for 20 weeks to induce prediabetes. At 20^{th} week, prediabetes was confirmed by assessment of fasting blood glucose (FBG) and oral glucose tolerance test (OGTT). The prediabetic rats were further sub-divided into five groups (n=6) and treated with either BA (80 mg/kg) or metformin (MET, 500 mg/kg) in the presence and absence of diet intervention for 12 weeks. Every 4 weeks of treatment, all the animals were placed in metabolic cages to determine caloric and fluid intake as well as urine output. Also, the body mass index (BMI), waist circumference (WC), blood pressure and heart rate were measured at every 4 weeks of treatment. After the 12 weeks of treatment, the animals were sacrificed, blood samples were collected into EDTA sample bottles and centrifuged to obtain plasma. Also, the skeletal muscle, liver, heart and kidney were collected, weighed, snapped frozen with liquid nitrogen and stored at -80°C before the biochemical analysis of selected markers of glucose homeostasis, hepatic, cardiovascular and renal functions.

Results

In the first study, the untreated diet-induced prediabetic rats had a significantly increased body weight, increased caloric intake, elevated glycated haemoglobin, increased ghrelin plasma concentration, decreased muscle glycogen concentration, insulin resistance and hyperinsulinaemia compared to the non-prediabetic rats. However, BA treatment with or without diet intervention ameliorated the body weight, caloric intake, glycated haemoglobin, muscle glycogen, glucose tolerance, plasma insulin and increased the expression of glucose transporter 4 (GLUT 4) in the skeletal muscle by comparison to the untreated prediabetic rats.

Prediabetic induction in the second study resulted into elevated plasma concentration of liver enzymes, increased liver glycogen and triglyceride concentrations, increased oxidative stress in the liver and decreased sterol regulatory element binding protein (SREBP1c) by comparison to the non-prediabetic animals. Conversely, administration of BA with or without dietary intervention ameliorated liver functions by decreased oxidative stress, decreased liver enzymes, decreased liver glycogen and triglyceride as well as increased hepatic SREBP1c concentration in comparison to the untreated prediabetic animals.

The results in the third study showed that the untreated prediabetic rats had a significantly increased body mass index (BMI), waist circumference (WC), blood pressure, heart rate, lipid profile, oxidative stress and inflammatory markers with significantly decreased endothelial nitric oxide synthase (eNOS) by comparison to the non-prediabetic control rats. On the other hand, the administration of BA with or without diet intervention improved cardiovascular functions by a decrease in BMI, WC, total cholesterol concentration, triglyceride concentration, blood pressure, heart rate, oxidative stress and inflammation with significant increase in eNOS plasma concentration in comparison to the untreated prediabetic rats.

In the fourth study, the untreated prediabetic rats had a significantly increased fluid intake, urine output, sodium retention, potassium loss, aldosterone concentration, albuminuria, proteinuria, kidney injury molecule (KIM-1) and urinary podocin mRNA expression in comparison to non-prediabetic control and BA treated rats with or without diet intervention. Also, the untreated prediabetic rats presented increased albumin, total protein, urea, uric acid, creatinine and oxidative stress markers concentrations with a significant decrease in glomerular filtration rate (GFR). However, administration of BA with or without diet intervention attenuated oxidative stress, decreased urinary podocin mRNA expression and the aforementioned renal dysfunctions parameters.

Conclusion

This study showed that long term consumption of high caloric diet-induced prediabetes and resulted in abnormal glucose homeostasis, hepatic, cardiovascular and renal dysfunctions. Also, the results of this study showed that these dysfunctions are not only present during overt type 2 diabetes mellitus but

already present at the prediabetic stage due to insulin resistance or hyperinsulinaemia that triggered oxidative stress in the physiological systems that we examined in this study. However, due to amelioration of insulin resistance via improved insulin sensitivity and earlier reported antioxidant activities that are common to all pentacyclic triterpenes, administration of BA significantly ameliorated the prediabetes-associated dysfunctions (abnormal glucose homeostasis, hepatic, cardiovascular and renal dysfunctions) with or without diet intervention in the prediabetic stage.

CHAPTER 1

Introduction and Literature Review

1.0 Introduction

Type 2 diabetes (T2DM) is a prevalent metabolic disorder in Africa and the death rate due to this disease is expected to double between 2016 and 2030 (Bos & Agyemang, 2013, WHO, 2016). Before the onset of type 2 diabetes mellitus, an individual may be in a prediabetic state. Prediabetes is a condition where fasting blood glucose concentrations are above the homeostatic range but below the threshold for diagnosis of T2DM (Tabak *et al.,* 2012, Bansal, 2015). This stage is also characterized by impaired glucose tolerance and elevated glycated haemoglobin (Tabak *et al.,* 2012, ADA, 2017, Brannick and Dagogo-Jack, 2018). As prediabetes progresses, prediabetic individuals are at risk of developing T2DM and predisposed to complications such as renal dysfunction, non-alcoholic fatty liver disease and cardiovascular diseases due to increased insulin resistance (Tabak *et al.,* 2012, Edwards and Cusi, 2016, Melsom *et al.,* 2016, Brannick *et al.,* 2016). Presently, the combination of dietary and pharmacological intervention has been the main approach to prevent the progression of prediabetes to T2DM and the associated complications (Edward and Cusi, 2016, Brannick and Dagogo-Jack, 2018). Despite this approach, many prediabetic individuals continue to develop T2DM. Consequently, low patient compliance in using both dietary and pharmacological interventions as most patients only adhere to the pharmacological interventions has been reported (Ramachandran *et al.,* 2006, Gamede *et al.,* 2018, Glechner *et al.,* 2018). This in turn leads to the efficacy of pharmacological intervention being reduced (Gamede *et al.,* 2018). Therefore, there is a need for anti-diabetic agents with the ability to manage prediabetes even in the absence of dietary intervention.

Currently, research on natural products such as triterpenes has gained recognition due to their anti-diabetic properties and ameliorative potentials in diabetes associated complications (Nazaruk and Borzym-Kluczyk, 2015, Gamede *et al.,* 2018). Pentacyclic triterpenes specifically belong to a vital class of natural products that have been reported as anti-diabetic compounds without any side effects (Sanchez-Gonzalez *et al.,* 2013, Putta *et al.,* 2016). Maslinic acid is an example of pentacyclic triterpenes that has been found to have anti-inflammatory, antioxidant and anti-diabetic properties (Mkhwanazi *et al.,* 2014, Putta *et al.,* 2016). A structural isomer of maslinic acid, known as bredemolic acid, was reported to have increased biological activity relative to maslinic acid (Wen *et al.,* 2006, Cheng *et al.,* 2008). The effects of bredemolic acid on the progression of prediabetes, however, remain unknown.

In our laboratory, we have developed a prediabetic rat model that accurately mimics the prediabetic condition in humans (Luvuno *et al.,* 2017, Gamede *et al.,* 2018, Mabuza *et al.,* 2019). We have further demonstrated that this prediabetic rat model develops diabetes-associated complications such as hepatic, cardiovascular and renal dysfunctions (Mkhwanazi *et al.,* 2014, Gamede*et al.,* 2018, Mabuza *et al.,* 2019, Gamede *et al.,* 2019). In this study, we sought to investigate the effects of bredemolic acid

on glucose homeostasis in a diet-induced prediabetic rat model. We further investigated the effects of this triterpene on selected markers associated with some diabetic complications, namely, hepatic, cardiovascular and renal dysfunctions.

1.1 Literature review

1.1.1 Prediabetes

Prediabetes is an intermediary state of glucose metabolism between normoglycaemic and diabetic states which has been characterized by impaired glucose tolerance (IGT), impaired fasting blood glucose (IFG) and elevated glycated haemoglobin (Edwards and Cusi, 2016, Huang *et al.*, 2016). Prediabetes can also be described as a stage that often precedes the onset of overt T2DM (Watson, 2017). Therefore, prediabetes is not a disease state but a risk state of developing T2DM and its complications if left untreated (Tabák *et al.*, 2012, Brannick *et al.*, 2016). The increased prevalence of this condition is directly correlated to the increased consumption of high caloric diets as well as sedentary lifestyles in modernized and urbanized cities (Lam and Leroith, 2012, Edward and Cusi, 2016). Apart from chronic consumption of high caloric diets and sedentary lifestyles, other risk factors associated with prediabetes include age, obesity and other pathological conditions that can result into insulin resistance (Lam and LeRoith, 2012, Reinehr, 2013, Watson, 2017).

Prediabetes is generally observed in developed countries; however, the prevalence of this condition is increasing in developing countries as well (Edward and Cusi, 2016). The prevalence of prediabetes is expected to increase from 280 million to 398 million in the year 2030 (Aguiree *et al.*, 2013, Edward and Cusi, 2016). The greatest increases are expected in Africa, South-East Asia and Western Pacific region of the world (IDF, 2011, Tabak *et al.*, 2012). Prediabetes has been identified as a therapeutic target to prevent the onset of T2DM as this could potentially reduce the prevalence of T2DM and its associated complications (Tabak *et al.*, 2012). However, prediabetes is an asymptomatic condition hence a lot of cases go undiagnosed.

1.1.2 Diagnosis of prediabetes

Early diagnosis of prediabetes is important to prevent the progression of prediabetes to T2DM (Watson, 2017). According to the American Diabetes Association (ADA), impaired glucose tolerance (IGT) remains the most accurate method for diagnosing prediabetes (ADA, 2016, Watson, 2017). A plasma glucose concentration of 7.8 – 11.0 mmol/L after a 2h oral glucose tolerance test is diagnosed as being prediabetic. However, the cut-off points for impaired fasting blood glucose (IFG) as recommended by ADA and WHO seems controversial (Huang *et al.*, 2016). The ADA recommended IFG cut-off point of 5.6 – 6.9 mmol/L while WHO defines the cut-off point for prediabetes as fasting blood glucose concentration of 6.1– 6.9 mmol/L (WHO, 2006, ADA, 2011, Weiss *et al.*, 2017). The ADA cut-off point for IFG has been used in animal and human research and has been shown to predispose individuals to increased risk of developing metabolic complications (Huang *et al.*, 2016, Sörensen *et al.*, 2016).

Similarly, the ADA recommended a glycated haemoglobin concentration (HbA1c) of 39 – 47 mmol/mol (5.7–6.4%) as another potential biomarker for diagnosis of prediabetes while the National Institute for Health and Care Excellence (NICE) as well as the International Expert Committee (IEC) recommended a higher cut-point of 42 – 47 mmol/mol (6.0 – 6.4%) for prediabetes (IEC, 2009, Chatterton *et al.*, 2012, Huang *et al.*, 2016, Weiss *et al.*, 2017). The IFG is more sensitive than the HbA1c but less sensitive than the IGT for diagnosis of prediabetes (Watson, 2017). The HbA1c assay revealed the glycaemic loads of over two or three months, however, there may be poor agreement among the three biomarkers in diagnosing prediabetes (Incani *et al.*, 2015). Consequently, levels of the aforementioned markers associated with diagnosis may not all be present during the prediabetic stage, but all the three markers are fully present in the diabetic stage (ADA, 2016, Watson, 2017). In this study, the three recommended markers by ADA and WHO were used to confirm prediabetes in the diet-induced prediabetic rats. In addition, in this study, we also assessed impaired glucose tolerance by oral glucose tolerance test (OGTT) and homeostasis model assessment index (HOMA2-IR) as additional parameters associated with prediabetes.

1.1.3 Pathophysiology of prediabetes

Under homeostatic conditions, blood glucose concentrations are strictly regulated within a narrow range in fasting and postprandial states (Abdul-Ghani *et al.*, 2006, Tabak *et al.*, 2012). This regulation of blood glucose concentration is determined by a variety of factors within the narrow range of hormonal, neural, and metabolic activities (Weiss *et al.*, 2017). The pathogenesis of prediabetes is associated with insulin resistance in the skeletal muscle and the liver (Kahn, 2003, Tabak *et al.*, 2009, Brannick *et al.*, 2016).

Insulin resistance appears to be the first abnormality and is evident in IFG or IGT individuals (Kanat *et al.*, 2015). In the British Whitehall study, it was shown that insulin resistance had already occurred and increased for 13 years before the diagnosis of overt diabetes (Tabak *et al.*, 2009). In the study, blood glucose remained normal due to the compensatory mechanism of increased insulin production by β-cell until 2 – 6 years before the time of diagnosis when sustained hyperglycaemia was found (Tabak *et al.*, 2009, Tabak *et al.*, 2012). Furthermore, the study revealed that insulin resistance begins before the development of T2DM, while β-cell dysfunction is already present in the prediabetic stage (Tabak *et al.*, 2009). The individuals with isolated IFG or IGT have different fasting blood glucose and 2h post-load glucose concentrations with different curve patterns of glucose concentration in oral glucose tolerance test (Tabak *et al.*, 2012). However, both IFG and IGT individuals have insulin resistance but the site of insulin resistance is different (Ferrannini *et al.*, 2011). An individual with IFG has normal skeletal muscle insulin sensitivity but the hepatic insulin sensitivity is impaired (Ferrannini *et al.*, 2011, Basu *et al.*, 2013, Edward and Cusi, 2016). Conversely, in IGT individuals, the skeletal muscle insulin sensitivity is impaired with a modest change in hepatic insulin sensitivity (Ferrannini *et al.*, 2011). In addition, in the IFG individuals, early insulin response is impaired during glucose tolerance test, but

their second phase of insulin secretion improves while in IGT individuals the early and late phase of insulin secretion is impaired (Ferrannini *et al.,* 2011).

1.2 Changes in glucose homeostasis during prediabetes

Glucose, being the primary substrate for metabolism, energy production and stimuli for insulin release, needs to be regulated (Bogan, 2012, Fu *et al.,* 2012). In prediabetes, there is moderate hyperglycaemia and impaired glucose tolerance in fasting and postprandial states (Rizza, 2010, Titchenell *et al.,* 2017). The hyperglycaemia has been shown to be caused by a variety of factors such as increased hepatic and renal glucose release as well as decreased glucose uptake in the skeletal muscle (Wilding, 2014, Samuel and Shulman, 2016). On the other hand, the impaired glucose tolerance is said to be due to decreased suppression of the hepatic and renal glucose release (Agius, 2010, Wilding, 2014). However, insulin resistance is the major cause of the decreased suppression of hepatic and renal glucose release (Wilding, 2014, Petersen, 2017). Consequently, the insulin resistance leads to increase in blood glucose concentration above homeostatic range but lower than the threshold for diagnosis of diabetes (Ciccone *et al.,* 2014).

Chronic consumption of high caloric diets as well as sedentary lifestyles has been shown to induce a 2.5-fold increase in plasma insulin concentrations (DeFronzo, 2004, Sharabi *et al.,* 2015). At fasting blood glucose concentrations of about 7.8 mmol/L, the pancreatic β-cell can no longer sustain the elevated insulin release, hence, the plasma concentration of insulin drops slightly and hepatic glucose output increases (Sharabi *et al.,* 2015). In this condition, the glucose homeostasis is impaired with subsequent increases in fat deposition in insulin-dependent peripheral tissues followed by peripheral insulin resistance, decreased glucose uptake, hyperinsulinaemia, visceral adiposity and increased body weight (Samuel and Shulman, 2012, Kowalski and Bruce, 2014, Sharabi *et al.,* 2015).

Furthermore, the increased fat deposition in the skeletal muscle leads to increased intramyocellular lipid deposition followed by intramuscular diacylglycerol accumulation and skeletal muscle insulin resistance (Szendroedi *et al.,* 2014). Due to skeletal muscle insulin resistance, the insulin signalling pathway is impaired leading to activation of protein kinase C (PKCθ) (Samuel and Shulman, 2016). Consequently, the activation of PKCθ leads to decreased phosphorylation of insulin receptor (IRS1/2) which in turn leads to decreased translocation of GLUT 4 to the plasma membrane and subsequently, decreased glucose uptake (Bogan, 2012, Samuel and Shulman, 2016). The decreased glucose uptake is associated with decreased glycogenesis in the skeletal muscle (Bogan, 2012). However, the decrease in glucose uptake potentiates decreased availability of glucose in the peripheral cells (Hardie *et al.,* 2012, Chabot *et al.,* 2014). Therefore, ghrelin hormone is secreted by the oxyntic gland of the stomach as a compensatory mechanism to activate the hypothalamic orexigenic signalling pathway of neuropeptide Y (NPY) and agouti related protein (AgRP) neurons in the arcuate nucleus (Briggs & Andrews 2011, Chabot *et al.,* 2014). Hence, the activation of arcuate nucleus of the hypothalamus stimulates increased caloric intake and body weight (Castañeda *et al.,* 2010, Chabot *et al.,* 2014, Luvuno *et al.,* 2016). An

inverse relationship exists between ghrelin and insulin secretion under homeostatic conditions but in diabetic conditions, ghrelin and insulin plasma concentrations are constantly high (Barazzoni, 2014, Alamri *et al.*, 2016, Luvuno *et al.*, 2016). The increased plasma concentration of ghrelin and insulin leads to increased hyperphagia and hyperinsulinaemia associated with the diabetic condition (Chabot *et al.*, 2014, Samuel and Shulman, 2016). Furthermore, due to hyperinsulinaemia, glucose is diverted to the liver with a subsequent increase in *de novo* lipogenesis and gluconeogenesis (Flannery *et al.*, 2012, Sharabi *et al.*, 2015). In addition, the increased *de novo* lipogenesis promotes hepatic lipid accumulation which is activated by sterol regulatory element binding protein (SREBP1c) and carbohydrate regulatory element binding protein (ChREBP) (Herman and Samuel, 2016, Reccia *et al.*, 2017).

Furthermore, bioactive compounds such as maslinic acid and oleanolic acid have been reported to ameliorate glucose homeostasis and the associated complications of glucose metabolism in the skeletal muscle and the liver in both prediabetic and diabetic states (Mkhwanazi *et al.*, 2014, Gamede *et al.*, 2018). However, the effects of bredemolic acid on glucose homeostasis in prediabetic state have not been shown. Hence, in Chapter 2 of this study, the effects of bredemolic acid are investigated on the aforementioned glucose homeostasis biomarkers, as well as expression of GLUT 4 in the skeletal muscle in a diet-induced prediabetic rat model.

1.3 Complications of prediabetes

Although, prediabetes is not a disease, it is associated with some complications due to abnormal glycaemia which affects the cells and tissues of various organs in the physiological system (Brannick *et al.*, 2016). Prior to its progression to T2DM, prediabetes is associated with increased risk of developing cardiovascular diseases, renal diseases, hepatic dysfunction and other complications (Brannick *et al.*, 2016, Melsom *et al.*, 2016, Wasserman *et al.*, 2018).

Apart from abnormal glycaemia and insulin resistance associated with prediabetes, subsequent metabolic complications such as increased oxidative stress, inflammation, dyslipidaemia and endothelial dysfunction contribute to the onset of the diseases associated with prediabetes (Wasserman, 2018). Literature has shown that bioactive compounds such as maslinic acid, oleanolic acid ameliorated renal, cardiovascular and hepatic dysfunctions in diabetic animal model (Mkhwanazi *et al.*, 2014, Gamede *et al.*, 2019). The effects of bredemolic acid, however, have not been established. Hence, in Chapter 2 to Chapter 5 of this study, the effects of bredemolic acid were examined on selected markers of glucose homeostasis as well as cardiovascular, hepatic and renal functions. The following section describes the effects of prediabetes on the functioning of the aforementioned organ systems.

1.3.1 Effects of prediabetes on hepatic function

High carbohydrate and high fat diets coupled with sedentary lifestyles have been reported to be associated with prediabetes and hepatic dysfunction (Lozano *et al.*, 2016, Reccia *et al.*, 2017). However, the link between prediabetes and hepatic dysfunction is mainly via hepatic insulin resistance which is associated with high caloric diets and has been described in various ways (Sung and Kim, 2011, Lozano *et al.*, 2016, Hazlehurst *et al.*, 2016). Firstly, high caloric diets cause hepatic insulin resistance through the activation of toll-like receptor 4 (TLR4) pathway (Galbo *et al.*, 2013, Reccia *et al.*, 2017). Activation of TLR4 induces *de novo* synthesis of ceramide, ceramide accumulation as well as ceramide-mediated activation of protein phosphatase 2A (Galbo *et al.*, 2013). This in turn directly inhibits insulin signalling at AKT phosphorylation level, leading to hepatic insulin resistance (Holland *et al.*, 2011, Galbo *et al.*, 2013). Secondly, high caloric diets induce mitochondrial dysfunction which leads to overproduction of reactive oxygen species (ROS) followed by oxidative stress in hepatocytes (Nassir and Ibdah, 2014). The mitochondrial dysfunction or oxidative stress causes insulin resistance due to overproduction of toxic lipid metabolites (such as ceramides, diacylglycerol) which further impair insulin action in hepatocytes resulting in hepatic insulin resistance (Nassir and Ibdah, 2014, Reccia *et al.*, 2017). Thirdly, hepatic insulin resistance can also be caused by increased lipolysis in the white adipose tissue due to impaired insulin lipogenic action in the adipose tissue (Perry *et al.*, 2015, Samuel and Shulman, 2016). The increased lipolysis leads to increased flux of fatty acids to the liver which results in increased hepatic acetyl CoA (Perry *et al.*, 2015). Subsequently, the increased hepatic acetyl CoA leads to glycogenesis, accumulation of triglyceride and fatty acid metabolites (such as fatty acyl CoA, diacylglycerol (DAG), ceramides, glycosphingolipids) which further activates protein kinase Cε (PKCε) in the liver (Nagle *et al.*, 2009, Perry *et al.*, 2015, Reccia *et al.*, 2017). The activation of PKCε inhibits phosphorylation of hepatic insulin receptors (IRS1/IRS2) which in turn impairs insulin signalling cascades of reactions, thereby leading to hepatic insulin resistance (Samuel *et al.*, 2004, Reccia *et al.*, 2017).

Perhaps more significantly, hepatic insulin resistance alters hepatic glucose metabolism due to reduced sensitivity of hepatocytes to insulin action (Wiernsperger *et al.*, 2013, Petersen *et al.*, 2017). Insulin is a crucial regulator of hepatic glucose metabolism from glucose production to glucose storage (Samuel and Shulman, 2016). Insulin controls hepatic glucose storage (glycogenesis) through AKT by activation of glycogen synthase to enhance hepatic glycogenesis (Wan *et al.*, 2013). On the other hand, insulin suppresses hepatic glucose production (gluconeogenesis) via inactivation of FOXO1 (Forkhead Box O1) to decrease transcription of the gluconeogenic enzyme as well as via inhibition of glycogen phosphorylase enzyme to inhibit gluconeogenesis and glycogenolysis respectively (Titchenell *et al.*, 2017, Petersen *et al.*, 2017).

Moreover, during insulin-resistant conditions such as T2DM, insulin fails to adequately suppress hepatic glucose production at pre-prandial and postprandial states (Rizza, 2010, Titchenell *et al.*, 2017). Therefore, the resultant excessive hepatic glucose production due to the insulin failure coupled with

impaired hepatic glucose uptake leads to hyperglycaemia in diabetic individuals even during the pre-prandial state (Rizza *et al.*, 2010, Titchenell *et al.*, 2017). Also, due to impaired suppressive action of insulin on hepatic glucose production, the influx of gluconeogenic substrates into the liver is increased to promote more gluconeogenesis (Sharabi *et al.*, 2015). The increased hepatic glucose production activates increased expression of SREBP1c which caused hepatic *de novo* lipogenesis that promotes increased re-esterification of fat resulting in excessive hepatic fat accumulation known as non-alcoholic fatty liver disease (NAFLD) (Lambert *et al.*, 2014).

Several pieces of literature have established that NAFLD is associated with prediabetes or T2DM (Sung and Kim, 2011, Birkenfeld and Shulman, 2014, Hazlehurst *et al.*, 2016). Studies have shown that 70% of newly diagnosed diabetic patients have NAFLD while the risk of developing diabetes in NAFLD patients is approximately 5-fold (Williamson *et al.*, 2011, Choi *et al.*, 2013). In NAFLD, liver enzymes, hepatic lipid and lipoprotein metabolism are severely impaired as a result of insulin resistance (Fon Tacer and Rozman, 2011). Therefore, due to insulin resistance and hyperinsulinaemia, hepatic fat uptake is increased via increased expression of fatty acid transport proteins (FATP) and fatty acid translocase (CD36) as well as increased lipolysis in white adipose tissue (He *et al.*, 2011, Kawano and Cohen, 2013). As a result of the increased hepatic fat uptake, the beta-oxidation of fatty acid is impaired via increased activity of acetyl-CoA carboxylase 2 which enhances the production of malonyl-CoA which in turn inhibits mitochondria beta-oxidation of fatty acid via negative inhibition of carnitine palmitoyl transferase 1 (CPT1) enzyme (FonTracer *et al.*, 2011). Impaired beta-oxidation of fatty acid in the mitochondria leads to mitochondrial dysfunction which further leads to overproduction of ROS that culminates into oxidative stress (Nassir and Ibdah, 2014). However, it has been reported that oxidative stress activates the Jun N-terminal kinase (JNK) which subsequently stimulates inflammatory reactions that leads to fibrotic hepatic damage known as non-alcoholic steatohepatitis (NASH) (Kodama and Brenner, 2009, Gautheron *et al.*, 2014, Reccia *et al.*, 2017). Recently, bioactive compound such as maslinic acid has been shown to ameliorate hepatic dysfunction in obesity-induced non-alcoholic fatty liver disease via the regulation of Sirt1/AMPK pathway (Liou *et al.*, 2019). The effects of bredemolic acid on hepatic function in prediabetic stage have not been shown. Therefore, in Chapter 3 of this study, the effects of bredemolic acid, in both the presence and absence of dietary intervention, were investigated on liver function in diet-induced prediabetic rat model. However, increased hepatic lipogenesis is one of the features of prediabetes which contributes to dyslipidaemia and subsequently leads to cardiovascular dysfunction. The cardiovascular dysfunctions that are associated with prediabetes are described in the following section.

1.3.2 Effects of prediabetes on cardiovascular function

Prediabetes is associated with an increased risk for developing arteriosclerosis and other cardiovascular diseases (Ford *et al.*, 2010, Huang *et al.*, 2017). Prediabetes is also a risk factor for cerebrovascular diseases such as transient ischaemic attack, stroke and recurrent stroke (Roquer *et al.*, 2014). However,

a study by Qiao and colleagues demonstrated that a strong predictor of stroke and future cardiovascular disease is hyperglycaemia post-load OGTT level (Qiao *et al.,* 2002, Brannick and Dagogo-Jack, 2018). Other studies further indicate that individuals with fasting blood glucose above 5.6 mmol/L have a high risk of developing coronary heart disease (Sarwar *et al.,* 2010, Huang *et al.,* 2017). According to the Heart Outcome Prevention Evaluation (HOPE) study, every 1 mmol/L increase in fasting blood glucose concentration increased the risk of cardiovascular diseases by almost 9% in the following 4.5 years (Gerstein *et al.,* 2005, Huang *et al.,* 2017). Furthermore, the study showed that with a relative risk of 1.07, the tendency of developing cardiovascular diseases increased with every 1% increase in HbA1c. In addition, the Diabetes Epidemiology Collaborative analysis of Diagnostic criteria in Europe (DECODE) revealed that the correlation between fasting blood glucose and cardiovascular disease associated mortality is "J-shaped" curve without any threshold effect at elevated glucose concentration (DECODE, 2003, Ford *et al.,* 2010). The weight gain and abdominal adiposity have been associated with increased body mass index (BMI) and waist circumference (WC) which are additional risk factors that promote cardiovascular diseases in prediabetic condition (Abraham *et al.,* 2015).

However, the relationship between increased fasting blood glucose and the development of cardiovascular disease involves some molecular mechanisms and pathways. It has been reported that increased plasma glucose concentration activates reactive oxygen species (ROS) which inactivate nitric oxide (NO) and subsequently lead to endothelial dysfunction (Paneni *et al.,* 2013). Also, an increase in ROS production has been shown to contribute to cardiovascular diseases by stimulating the activation of PKC (Huang *et al.,* 2017). The activation of PKC alters vascular homeostasis and causes a predisposition to cardiovascular diseases (Huang *et al.,* 2017). The activation of PKC also leads to inactivation of NO and induction of vasoconstrictor (endothelin-1) synthesis (Geraldes and King, 2010, Huang *et al.,* 2017). Consequently, the combination of decreased NO production and increased vasoconstrictor production culminates into vascular changes that result in increased blood pressure, heart rate and arteriosclerosis (Huang *et al.,* 2017). Besides the activation of PKC, hyperglycaemia also activates the polyol and hexosamine pathways which contribute to cardiovascular system damage (Graves and Kayal, 2011, Wasserman *et al.,* 2018). Moreover, insulin resistance has also been reported to be one of the major factors associated with endothelial dysfunction in prediabetes (Wasserman *et al.,* 2018). Insulin is a vasodilator for skeletal muscle and coronary vessels (Laakso *et al.,* 1990, Lautamäki *et al.,* 2006, Wasserman *et al.,* 2018). In an insulin-resistant state, the insulin-induced NO-dependent vasodilatation in skeletal muscle is impaired, therefore, the cascade of phosphorylation from insulin receptors to AKT (protein kinase B) is altered (Montagnani *et al.,* 2002, Artunc *et al.,* 2016). Subsequently, the phosphorylation of endothelial nitric oxide synthase (eNOS) is also impaired, and this results in vasoconstriction which further leads to increased blood pressure as well as heart rate (Artunc *et al.,* 2016, Wasserman *et al.,* 2018). In contrast, insulin stimulates endothelin-1 in an insulin resistance state, thereby causing vasoconstriction (Cardillo *et al.,* 1999, Artunc *et al.,* 2016). However,

vascular insulin resistance causes down-regulation of insulin receptors, AKT and eNOS but the endothelin pathway remains intact (Potenza *et al.,* 2005, Symons *et al.,* 2009, Artunc *et al.,* 2016).

Glycation of haemoglobin leads to advanced glycation product (AGE) formation and is a contributor to the development of cardiovascular disease (Graves and Kayal, 2011, Chilelli *et al.,* 2013, Sörensen *et al.,* 2016, Huang *et al.,* 2017). AGEs increase the expression of adhesion molecules on vascular endothelial cells to promote migration of monocytes which subsequently form macrophages (Schmidt *et al.,* 1995, Huang *et al.,* 2017). AGEs also stimulate the monocytes to release cytokines such as interleukin 6 and tumour necrosis factor (TNFα) which are mediators of inflammatory reactions associated with cardiovascular diseases (Schmidt *et al.,* 1995, Keane *et al.,* 2015). Furthermore, in obese patients with prediabetes, increased plasma concentration of free fatty acid contributes to insulin resistance and endothelial dysfunction (Wasserman *et al.,* 2018). High fat diets have been reported to induce endothelial dysfunction in mice and decrease brachial artery reactivity in humans (Wasserman *et al.,* 2018). Additionally, high fat diets decrease tyrosine phosphorylation of insulin receptor (IRS-1/2) which leads to inhibition of PI3K-AKT pathway and subsequently decreases the phosphorylation of eNOS (Wang *et al.,* 2006, Wasserman *et al.,* 2018). However, apart from vascular endothelial dysfunction, the endothelial fibrinolytic function is also impaired in prediabetes (Wasserman *et al.,* 2018). Vascular fibrinolytic dysfunction contributes to the risk of developing cardiovascular diseases in prediabetes (Wasserman *et al.,* 2018). In non-diabetic conditions, the endothelium stores tissue-type plasminogen activator (tPA) which protects against vascular thrombosis (Emeis *et al.,* 1997, Fattah *et al.,* 2013). The primary inhibitor of tPA *in vivo* is plasminogen activator inhibitor-1 (PAI-1) (Alessi *et al.,* 2007). The increased production or circulation of PAI-1 has been reported to be associated with thrombosis, myocardial infarction as well as stroke (Thögersen *et al.,* 1998, Wasserman *et al.,* 2018). Studies further indicate that, hyperglycaemia, very low-density lipoprotein (VLDL), insulin, angiotensin II, aldosterone and inflammatory cytokines (interleukin 6 and TNFα) stimulate the expression of PAI-1 (Wasserman *et al.,* 2018). In the prediabetic state however, the endothelial tPA release is decreased thereby leading to thrombotic events that promote endothelial fibrinolytic dysfunction (Van Guilder *et al.,* 2008, Wasserman *et al.,* 2018). In addition, one of the functions of insulin is to increase fibrinolysis and inhibit thrombosis (Chaudhuri *et al.,* 2004, Huang *et al.,* 2017). However, under insulin-resistant conditions, calcium accumulates in platelets and platelet aggregation is formed, and this subsequently leads to cardiovascular disease development (Vinik *et al.,* 2001, Huang *et al.,* 2017). Pentacyclic triterpenes such as maslic acid and oleanolic acid has been reported to ameliorate markers of cardiovascular function in diet-induced prediabetic rats (Mkhwanazi *et al.,* 2014, Gamede *et al.,* 2019). The effects of bredemolic acid on cardiovascular function in prediabetic condition have not been reported.

Therefore, in Chapter 4 of this study, the effects of bredemolic acid, in both the presence and absence of dietary intervention, were investigated on selected markers of cardiovascular function in a diet-induced prediabetic rat model. Moreover, literature has established that high caloric diet triggers

oxidative stress and results in renal dysfunction in an insulin-resistant condition such as prediabetes (Chou & Fang 2010, Odermatt 2011). Hence, the following section described the renal dysfunctions that are associated with prediabetic condition.

1.3.3 Effects of prediabetes on renal function

Intermediate hyperglycaemia and insulin resistance, as common features of prediabetes, are precursors for developing renal dysfunction or diabetic kidney disease (DKD) (Tabak *et al.,* 2012, Melsom *et al.,* 2016). However, decreased insulin sensitivity and β cell dysfunction are associated with glucotoxicity which has been reported to be a risk factor for cell and tissue damage in organs such as the kidney (Ritz *et al.,* 2011, Echouffo-Tcheugui *et al.,* 2016, De Nicola *et al.,* 2016). The peripheral cells such as skeletal muscle cells are not susceptible to damage by glucose toxicity in hyperglycaemic or insulin resistance state due to their expression of insulin-dependent glucose transporters (Powell *et al.,* 2013). More significantly, glomerular endothelial cells which express insulin-independent glucose transporters are not affected by impaired insulin signalling in prediabetic or diabetic conditions (Artunc *et al.,* 2016). Therefore, down-regulation of glucose transport in the presence of insulin resistance does not occur in the endothelial cells (Powell *et al.,* 2013). Insulin signalling does not stimulate glucose uptake in the glomerular endothelial cells or remodel its actin cytoskeletons (Artunc *et al.,* 2016). However, several studies have shown that renal cells respond to insulin signalling differently because they express different members of the glucose transporter family (Heilig *et al.,* 1995, Powell *et al.,* 2013). In addition, the glomerular endothelial and mesangial cells express insulin-independent glucose transporters while the podocytes express insulin-dependent glucose transporters (Powell *et al.,* 2013, Artunc *et al.,* 2016). Unlike the glomerular endothelial cells, the mesangial cells and podocytes respond to changes in insulin plasma concentration (Artunc *et al.,* 2016).

In the prediabetic state, since mesangial cells are insulin independent, glucose utilization in mesangial cells increased with subsequent mesangial matrix production (Powell *et al.,* 2013). Also, due to hyperinsulinaemia, formation of homodimeric insulin-like growth factor receptor (IGF-1R) and increased signalling of insulin-like growth factor (IGF-1) are enhanced in mesangial cells (Kong *et al.,* 2016, Artunc *et al.,* 2016). The IGF-1 signalling enhances the synthesis of fibronectin and collagen IV which promotes mesangial cell growth, proliferation, hypertrophy, as well as matrix deposition (Yano *et al.,* 2012). Podocytes respond to insulin stimulation in a manner similar to that of skeletal muscle since it is insulin-dependent (Coward *et al.,* 2005). However, it has been established that insulin stimulates expression of nephrin, a transmembrane protein, on the podocyte (Artunc *et al.,* 2016). Nephrin constitutes part of the podocyte slit diaphragm and is associated with the podocyte actin cytoskeleton (Coward *et al.,* 2007, Chou and Fang, 2010). When insulin signalling is impaired, nephrin expression on the podocyte is decreased with subsequent loss of podocyte foot processes and actin cytoskeleton (Welsh *et al.,* 2010). The loss of podocyte integrity leads to loss of other transmembrane proteins such as neph 1 and podocin in the urine (Nakamura *et al.,* 2000, Camici, 2007, Lioudaki *et al.,*

2015). As a result of the loss of podocyte foot processes, the structural arrangement of the glomerular filtration barriers is altered, the negatively charged glycosaminoglycans are lost, and hence, albuminuria occurs (Nakamura *et al.*, 2000, Powell *et al.*, 2013, Mora-Fernandez *et al.*, 2014).

In Chapter 5 of this study, we investigated changes in the filtration barrier by determination of albumin and gene expression of podocin in the urine of bredemolic acid-treated prediabetic rats.

Furthermore, another deleterious effect of hyperglycaemia and insulin resistance on the renal system is the activation of local renal renin-angiotensin-aldosterone system (RAAS) in the mesangial, podocyte and tubular cells (Luther and Brown, 2011, Mora-Fernandez *et al.*, 2014). Hyperglycaemia activates RAAS and induces production of angiotensin II (Ang II) as well as angiotensin II type 1 receptors (AT_1R) expression in podocyte and mesangial cells (Giunti *et al.*, 2006, Jaikumkao *et al.*, 2017). The activated Ang II stimulates mesangial cell proliferation and the expression of growth factors (such as transforming growth factor β, TGFβ) as well as cytokines (such as TNFα) which contribute to inflammation, fibrosis and apoptosis (Chawla *et al.*, 2010, Jaikumkao *et al.*, 2017). However, increased Ang II concentration does not only cause increased cell growth or hypertrophy but also a direct stimulation of vasoconstriction of the intraglomerular capillaries followed by increased intraglomerular capillary pressure and glomerular hyperfiltration (Chawla *et al.*, 2010, Artunc *et al.*, 2016). Moreover, glomerular hyperfiltration leads to increased glomerular filtration rate (GFR) (Ruggenenti *et al.*, 2012, De Nicola *et al.*, 2016). It is one of the features of the early stages of diabetes mellitus that has been associated with impaired fasting blood glucose and insulin resistance (Melsom *et al.*, 2011, Okada *et al.*, 2012, Echouffo-Tcheugui *et al.*, 2016).

The glomerular filtration rate is an important marker to assess renal function, therefore in Chapter 5 of this study, we examined the effects of bredemolic acid on selected renal function markers such as GFR, creatinine, urea, electrolytes, albumin, total protein and uric acid in a diet-induced prediabetic rat model. Moreover, hyperglycaemia-induced activation of RAAS stimulates secretion of aldosterone from glomerulosa cells in the adrenal gland through AT_1R (Luther and Brown, 2011). In turn, aldosterone stimulates up-regulation of serum/glucocorticoid-regulated kinase 1 (SGK1) which increases the expression of epithelial sodium channel (ENaC) in the distal tubule cell to increase sodium reabsorption and potentiate potassium loss (Artunc *et al.*, 2016). It has been reported that even without the activation of RAAS, hyperglycaemia and hyperinsulinaemia also increase SGK1 and ENaC gene expression to stimulate sodium reabsorption in the distal tubule (Artunc *et al.*, 2016).

In the proximal tubule, hyperglycaemia and hyperinsulinaemia upregulate sodium/glucose co-transporter 2 (SGLT 2) expression which also increases the reabsorption of sodium, and glucose (Novikov and Vallon, 2016). A study has shown, however, that the up-regulation of SGLT is not affected by insulin resistance in prediabetic or diabetic conditions (Wilding 2014). Therefore, due to the increased sodium reabsorption through the ENaC expression and SGLT 2 up-regulation in distal and proximal tubule respectively, prediabetic or diabetic individuals are prone to sodium retention as well as hypertension (Bakris *et al.*, 2009, Alsahli and Gerich, 2017). In addition, studies have shown

that increased proximal tubule reabsorption of sodium leads to a decrease in sodium delivery to the macula densa with subsequent deactivation of tubuloglomerular feedback followed by an increase in GFR (Cherney *et al.,* 2014, De Nicola *et al.,* 2016). Hence, tubuloglomerular feedback and glomerulotubular balance are altered as a result of increased proximal tubule reabsorption of sodium in diabetic conditions (De Nicola *et al.,* 2016).

In Chapter 5 of this study, the effects of bredemolic acid, in both the presence and absence of dietary intervention, were investigated on selected markers of renal function in a diet-induced prediabetic rat model

1.4 Management of prediabetes

Upon diagnosis, the combination of lifestyle and pharmacological intervention has been the main therapeutic approach recommended in the management of prediabetes (Ramachandran *et al.,* 2009, Brannick and Dogogo-Jack, 2018). Therefore, in the subsequent paragraphs, the contributions of each intervention on management of prediabetes are elucidated.

1.4.1 Lifestyle intervention

Lifestyle interventions are modifications that involve recommended guidelines on diet regimen and physical activities such as exercise in order to manage prediabetes or prevent its progression to T2DM (Gæde *et al.,* 2016). According to ADA, lifestyle modification is the first approach to prevent prediabetes or T2DM (ADA, 2017). In Diabetes Prevention Program (DPP) and Finnish Diabetes Prevention Study (FDPS) studies, it was demonstrated that the development of T2DM from prediabetes decreased by 60% through lifestyle changes and decreased by 31% with the use of metformin (Tuomilehto *et al.,* 2001, DPP, 2002, Knowler *et al.,* 2002, Stefan *et al.,* 2015, Hostalek *et al.,* 2015). The studies also showed that every 1kg loss in weight resulted in 16% reduction in the risk of developing diabetes (Tuomilehto *et al.,* 2001, DPP, 2002). In addition, the Da Qing IGT and diabetes study have also revealed that lifestyle changes decreased cardiovascular complications and other causes of mortality after 23years follow-up (Li *et al.,* 2008, Li *et al.,* 2014).

Furthermore, the management of prediabetes through lifestyle intervention involves dietary changes and physical activities (Schwarz *et al.,* 2012, Dunkley *et al.,* 2014). A diet rich in vegetables, whole grains, fruits, low animal fats or trans fats as well as simple sugar along with maintenance of body weight, BMI and active lifestyle has been recommended for prediabetes (Dunkley *et al.,* 2014, Ley *et al.,* 2014). However, exercise and physical activity form a significant part of a lifestyle intervention in the management of prediabetes (Schwarz *et al.,* 2012). Studies show that moderate exercise for at least 150 minutes per week has been recommended for obese children and prediabetes susceptible adults to decrease glycaemia and improve insulin sensitivity (Roglic, 2014, ADA, 2016, Watson, 2017). Additionally, a combination of diet modification and increased physical activity has been reported to cause weight loss, improve insulin sensitivity and glycaemic control (Watson, 2017). It has also been

shown to improve lipid profile and decrease mean arterial blood pressure (Hansen *et al.*, 2010, Abraham *et al.*, 2015).

However, lifestyle modification has been associated with some shortfalls in the management of prediabetes (Li *et al.*, 2009, Li *et al.*, 2014). Firstly, maintenance of diet modification is expensive, and due to variation in socio-economy status, the compliance of patients to following recommended diet by healthcare providers is low (Govil *et al.*, 2009, DPP 2012, Glechner *et al.*, 2018). Secondly, there is low patient compliance in terms of changing to a more active lifestyle (Li *et al.*, 2014, Glechner *et al.*, 2018).

1.4.2 Pharmacological intervention

Pharmacological intervention is another adjunct treatment for prediabetic patients who are unable to lose weight through lifestyle intervention (Watson, 2017). However, the pharmacological approach can be an indication in the case of women with a history of gestational diabetes and high-risk individuals that are unresponsive to lifestyle modification (ADA, 2017). Evidence has shown that pharmacological intervention is efficient in patients who are susceptible to prediabetes, under the age of 60 and have a BMI score above 35 (ADA, 2016, Watson, 2017). The pharmacological approach is less effective in preventing prediabetes progression to T2DM but may achieve a greater risk reduction when used alongside with lifestyle modification (ADA, 2016).

Metformin, as a recommended anti-diabetic drug for T2DM, has been reported to lower BMI and lipid levels (Salpeter *et al.*, 2008, Tabak *et al.*, 2012). Also, it decreases fasting blood glucose by inhibition of hepatic glucose output and improvement of insulin sensitivity in peripheral muscle tissue (DeFronzo *et al.*, 2014, Abraham *et al.*, 2015). However, several anti-diabetic drugs (metformin, thiazolidinedione, alpha-glucosidase etc.) with different mechanisms of action have been examined in different studies and reported to have variable efficacy in preventing prediabetes or T2DM (approximately 25% reduction vs placebo) but most of them are presented with side effects (Daniele *et al.*, 2014). According to trial evidence in prediabetes people, metformin was reported to lower the risk of developing T2DM by 45% but gastrointestinal tract disruption was observed as a side effect (Lilly and Godwin, 2009, Tabak *et al.*, 2012). Additionally, two thiazolidinedione drugs, troglitazone and rosiglitazone, were withdrawn from the European market for hepatotoxicity and increased risk of heart failure as serious side effects (Nathan *et al.*, 2009, Tabak *et al.*, 2012). Apart from the adverse side effects, the pharmacological intervention as a therapeutic approach to prediabetes is expensive. Consequently, most patients fail to comply with their medications, and the progression of prediabetes to T2DM remains prevalent.

According to DPP-2 study in prediabetic people, it was observed that no difference existed in the rate of development of T2DM between lifestyle intervention alone and lifestyle supplemented with pioglitazone in a 3-year trial (Ramachandran *et al.*, 2009). Currently, the combination of diet and pharmacological intervention is the preferred approach to managing prediabetes, however, the compliance of patients to the combination of diet and pharmacological intervention is low as most

patients merely use pharmacological intervention without any change of lifestyle. Hence, the efficacy of the pharmacological intervention is reduced. Also, despite the combination of lifestyle and pharmacological intervention as a treatment for prediabetes, some prediabetes patients still progress to overt diabetic stage or develop liver, cardiovascular and renal complications. Therefore, since the combined therapy has failed in the management of prediabetes with all the shortfalls discussed above, the need for alternative medicine without diet modification is necessary. In this study, the effects of bredemolic acid with and without diet intervention were examined in diet-induced prediabetic rats.

1.4.3 Alternative therapeutic approach

Several studies propose that alternative compounds from plant products have therapeutic potentials to mitigate diabetes and its complications (Ngubane *et al.,* 2011, Khathi *et al.,* 2013, Mkhwanazi *et al.,* 2014, Nazaruk and Borzym-Kluczyk, 2015, Putta *et al.,* 2016). However, among the alternative compounds from natural origin, the anti-diabetic compounds called triterpenes are of interest in this study. Triterpenes are anti-diabetic compounds with high therapeutic potential in terms of amelioration of complications associated with diabetes in streptozotocin-induced diabetic rats (Khathi *et al.,* 2013, Mkhwanazi *et al.,* 2014, Nazaruk and Borzym-Kluczyk, 2015).

Triterpenes are secondary metabolites found in leaves, stems, fruits, roots and are widely distributed within the plant kingdom (Jager *et al.,* 2009). Pentacyclic triterpenes such as oleanolic acid and maslinic acid have been shown in many studies to demonstrate anti-diabetic, antioxidant, anti-inflammatory, anti-obesity, antiviral as well as anti-cancerous properties (Baltina *et al.,* 2003, Ramachandran and Prasad, 2008, Laszczyk, 2009, Khathi *et al.,* 2013, Mkhwanazi *et al.,* 2014, Gamede *et al.,* 2018). However, in contrast to synthetic drugs, pentacyclic triterpenes have been found to exhibit low pharmacokinetic activity of three days without any side effects (Sanchez-Gonzalez *et al.,* 2013, Nazaruk and Borzym-Kluczyk, 2015). Studies indicate that pentacyclic triterpenes inhibit enzymes associated with abnormal glucose metabolism and ameliorate insulin resistance (Nazaruk and Borzym-Kluczyk, 2015, Putta *et al.,* 2016). Maslinic acid is a pentacyclic triterpene that consists of 30 carbon atoms grouped into five cycles which have several substitutes and has two hydroxyl groups bound to C2 and C3 atoms as well as one carboxyl group bound to C17 atom (Putta *et al.,* 2016). In addition, maslinic acid has been extensively reported to exhibit anti-diabetic property by reducing blood glucose via inhibition of glycogen phosphorylase enzyme in the liver and skeletal muscle (Nazaruk and Borzym-Kluczyk, 2015). It has also been shown that maslinic acid with IC_{50} (99 µM) is a more potent hepatic glycogen phosphorylase inhibitor than caffeine, a positive control with IC_{50} (648 µM) (Wen *et al.,* 2008). However, studies have shown that the carboxylic group in maslinic acid structure is the first active site which is responsible for the inhibition of glycogen phosphorylase enzyme (Wen *et al.,* 2006, Mkhwanazi *et al.,*2014). In addition, the hydroxyl active site at the C2 and C3 has also been shown to cause inhibitory expression of protein tyrosine phosphatase 1 (PTP1) and to exhibit antioxidant properties (Li *et al.,* 2004, Mkhwanazi *et al.,* 2014).

Bredemolic acid is a 2β, 3α structural isomer of maslinic acid. This compound was first described as early as 1960 by Rudolf Tschesche (Tschesche and Sen Gupta, 1960). Bredemolic acid was isolated from *Bredemeyera floribunda* in small amounts, although a lengthy partial synthesis was described by the same group in 1963 (Tschesch *et al.*, 1963) and more recently by Cheng Keguang in 2008 (Cheng *et al.*, 2008). Few years ago, bredemolic acid was shown to be synthesized from oleanolic acid (Sommerwerk *et al.*, 2015). Another study indicated that bredemolic acid is a more potent anti-diabetic compound than maslinic acid due to different structural arrangement of its hydroxyl group and its inhibitory effect on glycogen phosphorylase enzyme in skeletal muscle (Cheng *et al.*, 2008).

Based on these observations, it is possible that bredemolic acid is a more potent glycogen phosphorylase inhibitor and has more glucoregulatory effects in the skeletal muscle and liver compared to maslinic acid.

In this study, based on the aforementioned anti-diabetic properties of pentacyclic triterpenes, bredemolic acid was investigated as our alternative anti-diabetic compound in diet-induced prediabetic rats, particularly directing much effort on glucose metabolism, hepatic cardiovascular and renal functions. We had envisaged that the outcomes of this study may shed some light on the therapeutic value of this triterpene in prediabetes.

Molecular structure of bredemolic acid and maslinic acid

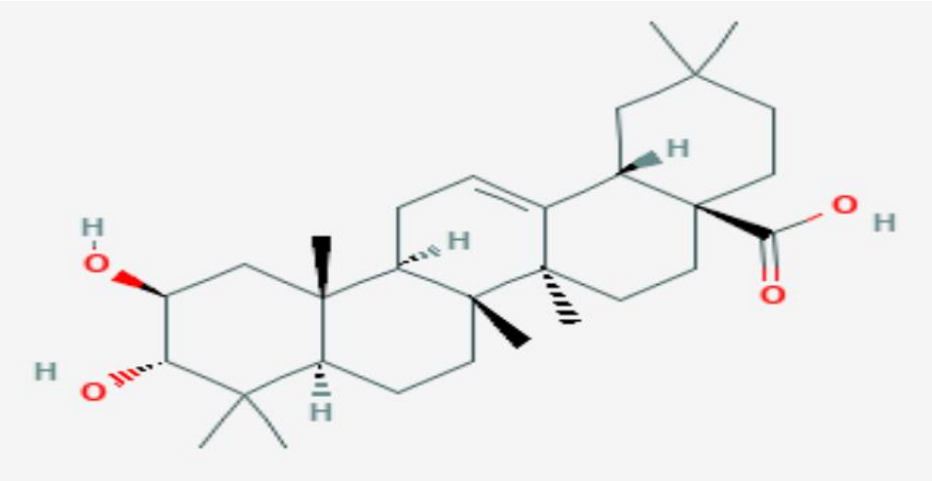

Figure 1: Chemical structure of bredemolic acid. Adopted from Cheng *et al.*, 2008

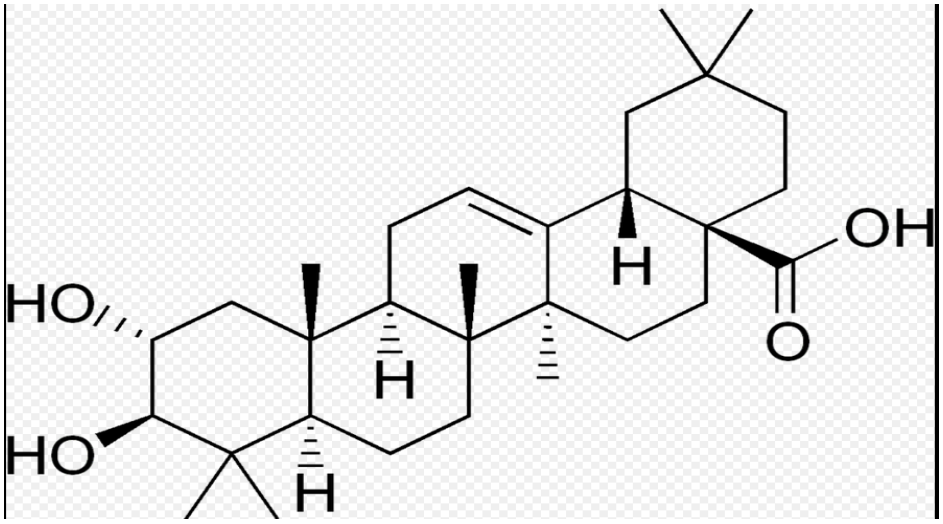

Figure 2: Chemical structure of maslinic acid. Adopted from Mokhtari *et al.,* 2015

1.5. Animal models associated with diabetes

Diabetes mellitus is a complex metabolic disorder that may not be fully understood in humans except through translational research from animals to human. This translational research involves the use of animal models that mimic the two main types of diabetes mellitus, type 1 and type 2 diabetes mellitus. The type 1 diabetes mellitus (T1DM) is an autoimmune disease that is most common in children and young adults (Hyttinen *et al.,* 2003, Al-awar *et al.,* 2016). However, to fully elucidate the pathogenesis of T1DM which is characterized by deficient insulin production, chemically induced and genetically induced animal models that mimic human T1DM have been established (Al-awar *et al.,* 2016).

The chemically induced animal model of diabetes involves the administration of streptozotocin (STZ) and alloxan which are diabetogenic chemicals that damage the pancreatic β-cells (Lenzen, 2008). Both chemicals are cytotoxic and accumulate in the pancreatic β-cells via GLUT 2 (Lenzen, 2008). STZ mode of action is due to the transfer of its methyl-nitrosourea moiety to the β-cell DNA molecule resulting in DNA damage and fragmentation (Lenzen, 2008). Intraperitoneal administration of 60 mg/kg of STZ induced T1DM in fasted animals after 72 hours. The modes of action of alloxan are in two forms, formation of ROS leading to selective necrosis of β-cell, and selective inhibition of β-cell glucokinase which in turn leads to inhibition of glucose-induced insulin secretion that mimic the pathogenesis of the human T1DM (Lenzen, 2008). Chemically induced T1DM has been shown to occur in fasted rats by subcutaneous administration of 125 mg/kg of alloxan (Lenzen, 2008, Al-awar *et al.,* 2016). Consequently, the chemically induced animal model of T1DM has been associated with increased mortality rate in animals and it is a short-term experimental model with limited availability of investigating T1DM complications (Al-awar *et al.,* 2016). Therefore, based on this shortfalls, the genetically induced animal model of T1DM (such as Biobreeding rats, Lewis-insulin dependent diabetes mellitus rats, Akita mice and Nonobese diabetic rats or mice) were developed.

T2DM is associated with insulin resistance and relative insulin deficiency. It is the common type of diabetes mellitus in the adult (DeFronzo, 2004). Model of T2DM has been developed by a combination of a high caloric diet with a chemically induced model. The combination of high caloric diet with chemically induced model involves feeding the animal a high fat or high fructose diet for 10 weeks followed by injection of various low dose of STZ (15-30 mg/kg) and the same diet continued for 22 weeks (Ionut *et al.,* 2009, Islam and Venkatesan, 2016). The high caloric diet/STZ-injected model develops mild T2DM coupled with increased body weight, visceral and subcutaneous fat as well as reduced insulin sensitivity (Islam and Venkatesan, 2016). Consequently, this model has not been evaluated by pharmacological screening via the use of anti-diabetic drugs. Apart from this animal model of T2DM, genetically induced animal models of T2DM such as the Zucker Diabetic Fatty (ZDF) rats, Goto-Kakizaki (GK) rats have also been established. However, these animal models are expensive and not widely available for diabetes research (Islam and Venkatesan, 2016).

However, with the shortfalls of animal models of T2DM, and to understand the pathogenesis of T2DM and its complications, animal models of prediabetes can be the better models compared to T2DM models. In addition, since the prediabetic stage precedes overt T2DM, developing animal models of prediabetes may prevent the progression of prediabetes to T2DM.

1.6. Diet-induced animal models of prediabetes

Genetically induced animal models of prediabetes have been developed in various laboratories around the world. However, due to non-availability and unevenly distribution of these animal models worldwide, an experimentally induced animal model of prediabetes through high caloric diet has been developed. Diet-induced animal models of prediabetes have been reported to be developed by feeding animals high fat diet, high fructose or sucrose diet, high fat high fructose/sucrose diet (Islam and Venkatesan, 2016, Gamede *et al.*,2018).

High fat diet-induced animal model of prediabetes was developed in C57BL/6J mice by feeding the mice high fat diet for a duration of 16 weeks (Obrosova *et al.,* 2007). This model of prediabetes was characterized by obesity, increased plasma concentration of free fatty acids (FFA), hyperinsulinaemia and impaired glucose tolerance (Obrosova *et al.*, 2007). Also, high fat diet that contains 24.5% lard and 2.5% soybean oil was used to induce prediabetes in 4 weeks old C57BL/6J mice (Jin *et al.,* 2013). After 12 weeks of feeding, non-significantly higher blood glucose, glucose intolerance, high serum concentration of triglyceride, total cholesterol and low HDL serum concentration were observed in the mice (Jin *et al.,* 2013). However, this model was not evaluated by anti-diabetic drugs except by anti-diabetic plant extract (Islam and Venkatesan, 2016).

High fructose diet-induced animal model of prediabetes is another model of prediabetes. However, several literatures have shown that a high fructose diet contributes to insulin resistance, obesity, prediabetes and T2DM (Basciano *et al.*, 2005, Miller and Adeli, 2008). High fructose diet-induced animal model of prediabetes was developed to mimic the occurrence of prediabetes or its complications

due to chronic consumption of beverages in developing and developed countries (Lozano *et al.,* 2016). The model was developed by feeding rhesus monkeys diet that contained 30% protein, 11% fat and 59% carbohydrate coupled with 15% fructose drinking water for 12 months (Bremer *et al.,* 2011). The prediabetic model was characterized by insulin resistance, central obesity, dyslipidaemia and inflammation. However, this model was not evaluated by anti-diabetic drugs and the period of induction was long. High fructose diet-induced animal model was also developed by feeding Sprague Dawley rats 60% fructose-containing diet along with fibre-free refined wheat flower for a period of 8 weeks (Amin and Gilani, 2013). This model was characterized by hyperglycaemia, hyperinsulinaemia and reduced HDL concentration at 4th week period (Amin and Gilani, 2013). This animal model of prediabetes has a shorter duration period however, prediabetes may be reversed to normal (Amin and Gilani, 2013).

In addition, sucrose-fed animal model of prediabetes has been developed in rodents by feeding Wistar rats 35% sucrose *ad libitum* for a period of 9 weeks (Soares *et al.,* 2013). This model was characterized by normoglycaemia, hyperinsulinaemia and hypertriglyceridaemia (Soares *et al.,* 2013). Similarly, in another study, the period of induction was extended to 16 weeks, hyperinsulinaemia, hypertriglyceridaemia, insulin resistance, glucose intolerance with the absence of hyperglycaemia and obesity was observed (Nunes *et al.,* 2013). However, this model has not been evaluated by anti-diabetic drugs.

Furthermore, both high fat and high fructose diets are used for induction of prediabetes model in animals but literature has shown that high fat diet can develop a better model of prediabetes (Zaman *et al.,* 2011). However, it has been reported that the combination of high fat high fructose diet is more suitable to induce prediabetes model in rodents than high fat or high fructose diet alone (Charlton *et al.,* 2011). High fat high fructose diet-induced prediabetes model was developed by feeding mice high fat diet (60% calorie from fat) along with 23.1 g/L of high fructose-containing water for 24 weeks (6 months) (Charlton *et al.,* 2011). The prediabetes model was characterized by obesity, insulin resistance, liver fibrosis, inflammation and endoplasmic reticulum stress. In this prediabetes model, the percentages of diet combination did not actually mimic high caloric diet that predisposes humans to prediabetes in developed countries.

In our laboratory, an animal model of prediabetes was induced by feeding rats high fat high carbohydrate diet (55% carbohydrate, 35% fats and 15% protein) that was supplemented with 5% milk and 15% fructose drinking water for 20 weeks (Luvuno *et al.,* 2017, Gamede *et al.,* 2018). In addition, we have established through research and publications from our laboratory that this prediabetes model presented hyperglycaemia, insulin resistance, glucose intolerance, increased glycated haemoglobin, increased body weight and increased ghrelin concentration (Luvuno *et al.,* 2017, Gamede *et al.,* 2018, Akinnuga *et al.,* 2019).

Therefore, in this study, this prediabetes model was adopted to mimic the diet combination and long-term consumption of high caloric diet which exposes individuals to prediabetes in developing and

developed countries. Also, this high fat high carbohydrate prediabetic model was adopted to induce complications associated with glucose homeostasis, liver, cardiovascular and renal functions during prediabetes.

1.7 Justification of the study

Prediabetes is an asymptomatic and risk state of developing T2DM and its complications unknowingly. The prevalence of prediabetes is increasing rapidly in developed and developing countries such as South Africa due to high consumption of high caloric diets, urbanization and lifestyle of physical inactivity. Several kinds of literature have shown that various metabolic disorders and complications in the liver, heart, blood vessels and kidney begin at the prediabetic stage and become aggravated in the overt diabetic stage. However, pharmacological and diet interventions are the current therapeutic approaches to manage prediabetes. Unfortunately, the combination of pharmacological and diet interventions has not yielded adequate results as patients do not comply to their diet modifications due to the cost of the diet, inconvenience of change of diet and other shortfalls. Despite the combination of these two interventions, prediabetic patients still progress to T2DM as well as other complications in various body systems. Hence, the efficacy of pharmacological intervention is reduced. Therefore, a need for an alternative anti-diabetic compound that can possibly regulate glucose metabolism and ameliorate liver, cardiovascular and renal complications in the prediabetic state regardless of diet intervention is necessary. Several pentacyclic triterpenes (such as oleanolic acid, maslinic acid, ursolic acid) have been reported to have anti-diabetic properties and low pharmacokinetic activity without any undesirable effects. Therefore, due to the anti-diabetic properties and low pharmacokinetic activity of triterpenes, triterpenes pharmacological activity may sustainably become active and ameliorate several complications of prediabetes despite consumption of high caloric diet by the prediabetic patient. Bredemolic acid is an isomer of maslinic acid that has been reported to have more biological activity than maslinic acid in terms of regulation of blood glucose via inhibition of glycogen phosphorylase in the skeletal muscle. However, the biological effects of bredemolic acid remain unknown on glucose homeostasis and other associated complications of prediabetes in the liver, blood vessels, heart and the kidney.

1.8 Aim of the study

This study aims to determine the effects of bredemolic acid on selected markers of some prediabetes-associated dysfunctions such as dysregulation of glucose homeostasis, hepatic, cardiovascular and renal functions in a diet-induced prediabetic rat model.

1.9 Objectives

The objectives of this study are:

1. To investigate the effects of bredemolic acid on glucose homeostasis by determination of

selected glucose homeostasis parameters (such as caloric intake, fasting blood glucose, glycated haemoglobin, glucose tolerance, insulin resistance, insulin concentration, ghrelin concentration and expression of GLUT4) in prediabetic rats with or without diet intervention.

2. To evaluate the effects of bredemolic acid on liver functions in prediabetic rat model with or without diet intervention by determination of selected liver function parameters such as liver enzymes (AST and ALT), liver triglycerides, SREBP1c concentration, liver glycogen, liver oxidative stress and antioxidant system biomarkers (MDA, SOD and GPx) .
3. To demonstrate the effects of bredemolic acid on cardiovascular functions in prediabetic rats with or without diet intervention by evaluation of cardiovascular function indices such as the BMI, waist circumference, lipid profile, blood pressure (systolic and diastolic), heart rate, endothelial dysfunction (eNOS), heart oxidative stress markers (MDA, SOD and GPx) and proinflammatory cytokines (hs-CRP, IL-6 and TNF-α).
4. To determine the effects of bredemolic acid on renal function in diet-induced prediabetic rats with or without diet intervention by assessment of fluid intake, urine output and renal function markers (such as the GFR, creatinine, urea, total protein, albumin, uric acid and electrolytes (Na^+ and K^+) concentrations in the plasma and urine), oxidative stress and antioxidant system markers, and urinary gene expression of podocin mRNA.

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PROLOGUE

Studies indicate that chronic consumption of a high caloric diet results in the dysregulation of glucose homeostasis and the development of prediabetes. Management of prediabetes involves a combination of dietary and pharmacological intervention. However, the efficacy of the pharmacological intervention is often compromised due to a lack of compliance from patients when it comes to dietary modifications. There is therefore a need to develop effective and alternative treatment strategies in the absence or presence of dietary intervention. Pentacyclic triterpenes have been promising alternative agents since they have been shown to possess hypoglycaemic effects. In Chapter 2 of this study, we sought to investigate the effects of bredemolic acid, a pentacyclic triterpene on markers associated with glucose homeostasis in diet-induced prediabetic rats. This was done in both the presence and absence of dietary intervention. The authors of this manuscript are Akinnuga AM, Siboto A, Khumalo B, Sibiya NH, Ngubane P and Khathi A. This manuscript has been published in the journal: Archives of Physiology and Biochemistry. See Appendix III

CHAPTER 2

Evaluation of the effects of bredemolic acid on selected markers of glucose homeostasis in diet-induced prediabetic rats

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Abstract

Context: Pentacyclic triterpenes (such as maslinic acid) are natural anti-diabetic agents which ameliorate glucose metabolism in diet-induced prediabetes. However, the effects of bredemolic acid (BA), maslinic acid isomer, is yet unknown in prediabetic condition.

Objectives: To investigate the effects of BA on some glucose homeostasis parameters in high-fat high-carbohydrate (HFHC) diet-induced prediabetic rats.

Methods: Thirty-six (36) male rats (150 - 180 g) were divided into two groups, the normal diet (ND) non-prediabetic (n=6) and the HFHC diet prediabetic groups (n=30). The prediabetic animals were further sub-divided into five groups (n=6) where they were treated with BA for 12 weeks while monitoring changes in blood glucose, caloric intake and body weight.

Results: Diet-induced prediabetes resulted in increased body weight, caloric intake, glycated haemoglobin and glucose tolerance. BA treatment ameliorated glucose tolerance, lowered plasma insulin and increased expression of glucose transporter 4 (GLUT 4) in rats.

Conclusion: BA administration restored glucose homeostasis in diet-induced prediabetes regardless of diet intervention.

Keywords: bredemolic acid; glucose homeostasis; high-fat diet; high-carbohydrate diet; prediabetes

Introduction

Prediabetes is a state of abnormal glucose homeostasis that is characterized by intermediate hyperglycaemia, elevated glycated haemoglobin and impaired glucose tolerance (Huang *et al.* 2016, Brannick and Dagogo-Jack 2018). It is said to be caused by chronic consumption of a diet that consists of food rich in carbohydrates and saturated fats coupled with a lifestyle of physical inactivity (Lam and LeRoith 2012, Edwards and Cusi 2016). The prevalence of prediabetes is markedly increasing in developed and developing countries especially in Africa, and the International Diabetes Federation (IDF) has predicted that the number of prediabetic individuals is expected to rise from 280 million to about 398 million in 2030 (Lam and LeRoith 2012, Roglic 2014, Edwards and Cusi 2016). In addition, prediabetes is a great precursor for type 2 diabetes mellitus (T2DM) and its complications if left untreated (Tabák *et al.* 2012, Brannick *et al.* 2016).

While abnormal glucose metabolism is often associated with overt T2DM, studies have shown that these abnormalities begin in the prediabetic state (Brannick *et al.* 2016, Luvuno *et al.* 2016). In the prediabetic condition, hyperinsulinaemia results as a compensatory mechanism to regulate insulin resistance and impaired glucose tolerance (Tabák *et al.* 2012). Impaired glucose tolerance is associated with decreased insulin sensitivity and sustained intermediate hyperglycaemia (Brannick *et al.* 2016). Subsequently, glucose uptake decreases gradually and the insulin-dependent peripheral tissues such as skeletal muscles are gradually starved of glucose, thus causing a decrease in glycogen level in the muscles (Brannick *et al.* 2016). Supposedly, due to decreased glucose uptake, the peripheral cells are depleted of energy. Therefore, a compensatory mechanism of ghrelin hormone release is initiated to stimulate the hypothalamus via the orexigenic signalling pathway and increase food intake (hyperphagia) to circumvent hypoglycaemia (Chabot *et al.* 2014).

However, the combination of dietary and pharmacological intervention has been explored to manage prediabetes and prevent the progression to T2DM (Ley *et al.* 2014, Salas-Salvadó *et al.* 2014). There is low compliance in the combination of the two interventions as patients only use pharmacological intervention without change of diet, hence reduce the efficacy of the drugs (Gamede *et al.* 2018). Therefore, management of prediabetes by natural anti-diabetic agents that can remain effective regardless of a change in diet is necessary.

Maslinic acid, a pentacyclic triterpene and anti-diabetic agent has been reported to improve glucose homeostasis in diabetic rodents through inhibition of intestinal carbohydrate hydrolysing enzymes and glucose transporters, and also by increasing glycogen synthesis in the liver and skeletal muscle via inhibition of glycogen phosphorylase (Mkhwanazi *et al.* 2014, Nazaruk and Borzym-Kluczyk 2015, Luvuno *et al.* 2016, Liou *et al*. 2019). An isomer of maslinic acid, bredemolic acid (BA), was discovered to have increased biological activity in regulating glucose homeostasis by inhibition of glycogen phosphorylase enzyme in rabbit skeletal muscle, however, its effects on glucose homeostasis in prediabetes are yet to be determined (Wen *et al.* 2006, Cheng *et al.* 2008). Therefore, to establish the effects of bredemolic acid on glucose homeostasis in prediabetic condition, we sought to investigate the

effects of bredemolic acid administration on selected glucose homeostasis parameters in high-fat high-carbohydrate diet-induced prediabetic rats.

Materials and methods

Animals

In this study, thirty-six (36) male Sprague Dawley rats that weighed 150 g – 180 g were obtained from the Biomedical Research Unit, University of KwaZulu-Natal (UKZN). The animals were kept and maintained under laboratory conditions of constant humidity (55±5%), temperature (22±2°C), and 12 h day: 12 h night cycle. They were acclimatized to their new environment for 2 weeks while consuming standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* before exposure to the experimental high-fat high-carbohydrate (HFHC) diet. The HFHC diet was formulated to consist of carbohydrate (55% Kcal/g), fats (30% Kcal/g), and proteins (15% Kcal/g). All experimental designs and procedures were according to the approved ethics (Ethics number: AREC/024/018D) and guidelines of the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa.

Experimental design

After the two weeks of acclimatization, the animals were initially divided into two different groups, the normal diet (ND) non-prediabetic (n=6) and HFHC diet prediabetic groups (n=30). All the animals in the prediabetic group were given an HFHC diet and drinking water that was supplemented with 15% fructose for 20 weeks to induce prediabetes. The non-prediabetic control group (NPD, Group 1) was fed on ND and water *ad libitum* for 20 weeks. At the 20th week, prediabetes was confirmed by determination of fasting blood glucose (FBG) and oral glucose tolerance test (OGTT) in animals in non-prediabetic and prediabetic groups.

Treatment of animals

The treatment period lasted for 12 weeks, i.e. 21st – 32nd week. After the 20th week, the prediabetic animals were either continuously fed on HFHC or changed to ND and treated with either oral administration of BA (80 mg/kg) or MET (Metformin, 500 mg/kg) at every third day. The non-prediabetic control (Group 1) animals continuously fed on ND and received as the vehicle, 3 mL/kg of diluted dimethyl sulphoxide, DMSO (2 mL DMSO: 19 mL normal saline, p.o.) for 12 weeks. The animals in the prediabetic group were further divided into 5 groups (Group 2 to Group 6) of six animals each. The prediabetes control group (PD, Group 2) were fed on the HFHC diet and received 3 mL/kg of diluted DMSO orally. Group 3 (ND+MET) changed the diet to ND (from HFHC to ND) and treated with MET while Group 4 (HFHC+MET) was continuously given the HFHC diet and treated with MET. The Group 5 (ND+BA) animals changed the diet to ND and treated with BA while Group 6 animals (HFHC+BA) were continuously given the HFHC diet and treated with BA.

Caloric intake

At every 4 weeks of treatment, the caloric intake of all the animals was determined by measuring food and water intakes via metabolic cages (Techniplats, Labotec, South Africa).

Blood glucose concentration

The blood glucose concentration was determined by using the tail-prick method and measured via One-Touch select glucometer (Lifescan, Mosta, Malta, United Kingdom) at every 4 weeks of treatment.

Oral glucose tolerance (OGT) response

At the 12th week of the treatment period, the oral glucose tolerance test (OGTT) was conducted following glucose loading. The OGTT responses were monitored in all the animal groups through established laboratory protocol (Ngubane et al. 2011, Khathi et al. 2013, Gamede et al. 2018). In brief, after a 12-hour fasting period, blood glucose concentrations (FBG) were measured (time, 0 minutes) in all the animals. Then, the animals were loaded with glucose (0.86 g/kg, p.o.) via oral gavage (18-gauge gavage needle, 38 mm long curved with 21/4mm ball end). The glucose concentrations were measured at 30, 60, and 120 minutes following glucose loading.

Blood collection and tissue harvesting

All animals were anaesthetised with Isofor (100 mg/kg, Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) for 3 minutes. When the animals were unconscious, blood samples were collected from the animals through cardiac puncture into different pre-cooled heparinised containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes to obtain plasma. Then, each of the plasma was aspirated into plain sample bottles and stored at -80°C in a Bio Ultra freezer (Snijers Scientific, Tilburg, Holland) until ready for biochemical analysis. Also, the skeletal muscle (gastrocnemius) were removed, rinsed with cold normal saline solution and snapped frozen in liquid nitrogen before storage in Bio Ultra freezer at -80°C for biochemical analysis of selected metabolic parameters. The caloric intake, body weight gain and fasting blood glucose (FBG) were assessed at 20th, 24th, 28th and 32nd week in all the animals while OGTT was assessed at 20th and 32nd week only. The other selected parameters such as HOMA2-IR (Homeostasis model assessment) index, glycated haemoglobin, muscle glycogen, insulin and ghrelin concentrations were only determined at 32nd week, i.e. 12th week of the treatment period

Biochemical analysis

Ghrelin and glycated haemoglobin concentrations were determined by using their respective ELISA kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) as instructed by the manufacturer. Insulin concentration was measured via an ultrasensitive rat insulin ELISA kit (Mercodia AB, Sylveniusgatan 8A, SE-754 50, Uppsala, Sweden) as directed in the manufacturer's instruction manual. HOMA2-IR index was calculated from the insulin concentrations and fasting blood glucose.

Glycogen assay

Glycogen assay was determined in skeletal muscle by following previously established protocols (Gamede *et al.* 2018, Musabayane *et al.* 2005, Mukundwa *et al.* 2016). The harvested tissues were weighed (50 mg) and heated with potassium hydroxide (KOH) (30%, 2 mL) for 30 minutes at 100^{o}C. Immediately, 0.194 mL of 10% of sodium tetraoxosulphate VI (Na_2SO_4) was added into the mixture to

stop the reaction. When the mixture was allowed to cool, the glycogen precipitate was formed. 200 µL of the cooled mixture with the precipitate was aspirated and mixed with ethanol (95%, 200 µL). The precipitated glycogen was pelleted, washed and resolubilized in H_2O (1 mL). Thereafter, 4 ml of anthrone (0.5 g dissolved in 250 mL of 95% sulphuric acid) was added and boiled for 10 minutes. After cooling, the absorbance was determined by using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW Germany) at 620 nm.

Western blot analysis of GLUT 4

GLUT 4 was analysed in skeletal muscle (gastrocnemius) as established in the previous protocol (Mkhwanazi *et al.* 2014). The skeletal muscle tissues (0.1 g) were homogenized on ice in isolation buffer and centrifuged for 10 min at 400 X g (4^0C). The protein content was quantified via the Lowry method. All the samples were standardized to one concentration (1 mg/mL), and the proteins were denatured by boiling in Laemmli sample buffer for 5 minutes. Then, 25 µL of the denatured proteins were loaded on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with 5 µL of molecular weight marker. The gel was electrophoresed at 150 V for 1 hour in running buffer. After the electrophoresis, the resolved proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer for 1 hour. After the transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS). The membrane was then immuno-probed with GLUT 4 antibodies (1:1000 in 1% BSA, Neogen, USA) for 1 hour at room temperature. The PVDF membrane was subjected to 5 washes (10 min each with gentle agitation) with TTBS. The membranes were then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:1000; Bio-Rad) for 1 hour at room temperature. After further washing, antigen-antibody complexes were detected by chemiluminescence through the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). The chemiluminescence signals were determined through the Chemi-doc XRS gel documentation system and analysed via the quantity one software (Bio-Rad, Johannesburg, South Africa).

Statistical analysis

The statistical data were presented in mean ± SEM. The data were analysed by using a two-way Analysis of Variance (ANOVA) with the Bonferroni test (post hoc test) via GraphPad Prism 5 software. The level of statistical significance was considered from $p<0.05$ and above.

Results

Caloric intake

The caloric intake of all the experimental groups were determined every fourth week from the start of the treatment period (week 0) to the 12^{th} week of treatment (Figure 1). The result showed that PD and HFHC+MET groups had significantly higher caloric intake in comparison to the NPD group throughout the treatment period except for the 12^{th} week of treatment at $p<0.05$. However, the administration of MET and BA with dietary intervention significantly decrease caloric intake throughout the treatment period in comparison to PD and HFHC+MET ($p<0.05$). On the other hand, the administration of BA

without dietary intervention resulted in decreased caloric intake but insignificant when compared to PD and HFHC+MET.

Body weight

The body weights of the animals were monitored throughout the experiment as shown in Table 1. The result showed that the percentage changes in body weight of the PD group increased throughout the experimental period when compared to that of the NPD group ($p<0.05$). The administration of BA with or without dietary intervention showed a significant decrease in the percentage changes of body weight in comparison to the PD group throughout the treatment periods.

Oral glucose tolerance test (OGTT)

As shown in Figure 2, the OGTT and Area under curve (AUC) were measured at the end of the treatment period (12^{th} Week) in all the groups. At time 0, the fasting blood glucose concentration increased in PD and other prediabetic treated groups but insignificant when compared to the NPD group. At 120 minutes post-load of glucose, the blood glucose concentrations of PD were significantly different from the NPD group ($p<0.05$). Conversely, at the same time (120 minutes), the blood glucose concentration of both BA treated groups significantly decreased in comparison to the PD group.

HOMA2-IR index

The HOMA2-IR index of all the animals was calculated from the product of plasma glucose and insulin at the end of the treatment period (12^{th} week). The results showed that the HOMA2-IR index in PD and HFHC+MET groups was significantly different when compared to NPD and other experimental groups ($p<0.05$). However, both BA treated groups and ND+MET group had a significantly decreased HOMA2-IR index in comparison to PD and HFHC+MET groups at $p<0.001$ as shown in Table 2.

Glycated haemoglobin concentration (HbA1c)

At 12^{th} week, all the experimental groups were analysed for HbA1cconcentration (Figure 3). The HbA_{1c} concentration of the PD group was significantly higher when compared to the NPD group. However, the HbA1cconcentrations of both BA treated with or without diet intervention (ND+BA or HFHC+BA) as well as metformin treated with diet intervention (ND+MET) decreased significantly in comparison to PD ($p<0.05$). Conversely, there was no significant difference between the HbA1cconcentration of metformin-treated rats without diet intervention (HFHC+MET) and PD group.

Ghrelin concentration

The plasma concentration of ghrelin was measured in all the experimental groups at the end of the treatment period. The results showed that the ghrelin concentration of PD, HFHC+MET and ND+MET groups were significantly higher in comparison to the NPD group (Figure 4). However, the BA treated animals with or without dietary intervention had a significantly lowered ghrelin concentration when compared to PD ($p<0.05$).

Skeletal muscle glycogen concentration

The skeletal muscle glycogen concentrations were measured at the end of the treatment period. The results showed that the skeletal muscle glycogen concentrations in the PD group and all the treated

experimental groups were significantly increased in comparison to the NPD group. Moreover, the skeletal muscle glycogen of BA treated animals with or without dietary intervention increased significantly when compared to PD groups ($p<0.05$) as shown in Figure 5.

Skeletal muscle GLUT 4 expression

As shown in Figure 6, GLUT 4 expression was increased significantly ($p<0.05$) in BA treated rats with or without dietary intervention when compared to NPD and PD groups. However, the administration of metformin with diet intervention (ND+MET) significantly increased GLUT 4 expression when compared to non-prediabetic group (NPD).

Discussion

In several studies, triterpenes have been reported to have anti-diabetic properties which cause reduction of fasting blood glucose and glycated haemoglobin concentrations as well as ameliorating insulin sensitivity in diet-induced prediabetes (Musabayane *et al.* 2005, Jung *et al.* 2007). Maslinic acid is a pentacyclic triterpene that has been reported to regulate glucose metabolism in diabetic rats (Jung *et al.* 2007, Mkhwanazi *et al.* 2014). Bredemolic acid, an isomer of maslinic acid, has been reported to have more increased biological activity due to differences in the structural arrangement of their hydroxyl groups (Wen *et al.* 2006, Cheng *et al.* 2008). However, the effects of this compound on glucose homeostasis in the prediabetic state have not been explored. Therefore, in this study, we sought to investigate the effects of bredemolic acid on some glucose homeostasis parameters in prediabetic rats.

It has been established that excessive consumption of high caloric diets drives animals toward a positive energy balance with resultant weight gain (Burchfield *et al.* 2018). This transition causes a decline in insulin sensitivity which ultimately results in compensatory hyperinsulinaemia and a state that resembles intermediate hyperglycaemia known as prediabetes (Barclay *et al.* 2013, Samuel and Shulman 2016). Similarly, in this study, chronic consumption of an HFHC diet increased food intake which caused an increase in body weight in the untreated prediabetic animals. Ghrelin is a hormone that plays a key role in the regulation of caloric intake and energy balance that leads to weight gain (Barazzoni 2014, Chabot *et al.* 2014). Under normal physiological conditions, there exists an inverse relationship in the plasma concentrations of ghrelin and insulin (Barazzoni 2014, Chabot *et al.* 2014). In the pre-prandial state, ghrelin plasma level increases and suppresses insulin release from pancreatic beta-cells via Ca^{2+}mediated pathway while under postprandial state ghrelin plasma level reduces, thus, insulin release is enhanced (Alamri *et al.* 2016). In the postprandial state, when ghrelin level decreases, insulin is released and facilitates the uptake of glucose into the insulin-dependent peripheral cells to reduce blood glucose levels (Barazzoni *et al.* 2014, Alamri *et al.* 2016). However, when the blood glucose level reduces, ghrelin plasma concentration increases to stimulate the hypothalamus to increase food intake via the orexigenic signalling pathway (Chabot *et al.* 2014, Alamri *et al.* 2016). In contrast, under diabetic conditions, the pancreatic beta-cell releases more insulin to compensate for the abnormal glucose metabolism and this leads to hyperinsulinaemia and decreased insulin sensitivity. The blood glucose level is increased, and the insulin-dependent peripheral cells are starved of glucose. In addition,

since the peripheral cells are starved of glucose, ghrelin plasma concentration increases and stimulates the hypothalamus to increase food intake (Barazzoni *et al.* 2014, Alamri *et al.* 2016). Therefore, in diabetic conditions, both the ghrelin and insulin plasma concentrations are sustainably high (Barazzoni *et al.* 2014, Alamri *et al.* 2016, Luvuno *et al.* 2016). Similarly, in this study, we observed that the food intake, percentage changes in body weight and plasma concentration of ghrelin were higher in untreated prediabetic animals compared to other experimental groups from 0 week to 12th week in the treatment period. This suggested that the increased plasma ghrelin concentration caused the increased caloric intake which resulted in increased percentage changes in body weight in untreated prediabetic rats. We observed that the administration of BA with or without dietary intervention significantly decreased the percentage changes in body weight and caloric intake by a decrease in plasma ghrelin concentration. We further observed a significant increase in HOMA2-IR index as well as elevated postprandial glucose at 120 min in the OGTT of untreated prediabetic (PD) animals. The administration of BA also enhanced glucose tolerance, and this was proven in HOMA2-IR index and OGTT results. These results correlated with similar studies on other triterpenes such as maslinic and oleanolic acids (Musabayane *et al.* 2005, Mkhwanazi *et al.* 2014, Luvuno *et al.* 2016, Gamede *et al.* 2018).

Studies have shown that high-fat feeding in rodents led to transient muscle diacylglycerol (DAG) accumulation followed by muscle insulin resistance and impaired insulin signalling pathway (Szendroedi *et al.* 2014). Consequently, the muscle protein kinase C (PKCθ) is activated, and limited phosphorylation of IRS-1 (insulin receptor substrate) occurred (Samuel and Shulman 2016). Under this condition, glucose uptake decreases due to reduced translocation of glucose transporter 4 (GLUT4) containing storage vesicles to the plasma membrane and phosphorylation of glycogen synthase enzyme (Bogan 2012). Decreased glucose uptake leads to reduced glycogen synthesis in the muscle cell. However, the majority of postprandial glucose disposal drives toward muscle glycogen synthesis (Bogan 2012, Samuel and Shulman 2016). Therefore, the significant difference in skeletal muscle glycogen content in untreated prediabetic rats when compared to BA treated rats is eminent in this study. This demonstrated that there might be muscle insulin resistance and reduced glucose uptake as well as reduced glycogen synthesis in the untreated prediabetic rats when compared to BA treated rats. Hence, we suggest that the administration of BA with or without diet intervention caused the observed increased muscle glycogen synthesis in BA treated rats by an increment of the expression of GLUT 4 via GLUT 4 translocation and probably by inhibition of muscle glycogen phosphorylase or stimulation of glycogen synthase enzymes. Indeed, previous studies have shown that other triterpenes (maslinic acid, oleanolic acid and ursolic acid) inhibited glycogen phosphorylase and increased expression of GLUT 4 in skeletal muscle in prediabetic or diabetic condition, and this present study correlated with those studies (Cheng *et al.* 2008, Mkhwanazi *et al.* 2014, Pimentel *et al.* 2017).

Chronic consumption of high caloric diets leads to ectopic lipid accumulation which has been implicated in peripheral insulin resistance (Barclay *et al.* 2013, Samuel and Shulman 2016). The skeletal muscle and the liver are primary organs of glucose homeostasis which store surplus glucose as glycogen

and any insulin resistance in these organs alters glucose metabolism with consequent hyperglycaemia and impaired glucose tolerance. In this study, there was observed hyperinsulinaemia and impaired glucose tolerance in the untreated prediabetic rats. The hyperinsulinaemia depicted peripheral insulin resistance and this may be responsible for the impaired glucose tolerance observed in the untreated prediabetic rats. However, the administration of BA with or without diet intervention normalized impaired glucose tolerance as observed in the OGTT probably due to the decreased insulin resistance which was obvious in plasma insulin concentration and HOMA2-IR index results. Importantly, when there is a decrease in insulin sensitivity, glucose attaches to the haemoglobin in red blood cells resulting in high levels of glycated haemoglobin (Incani *et al.* 2015). Similarly, the high level of glycated haemoglobin was observed in untreated prediabetic rats in this study. However, the glycated haemoglobin of BA treated rats was reduced to within range of the non-prediabetic rats possibly due to increased skeletal muscle glucose uptake and decreased insulin resistance by BA administration. Also, previous studies have reported that reduced glycated haemoglobin is a sign of sustained regulation of glucose metabolism and this study is in agreement with those studies (Huang *et al.* 2016, Watson 2017, Weiss *et al.* 2017).

Taken together, the administration of bredemolic acid to diet-induced prediabetic rats resulted in improved insulin sensitivity leading to improved glucose homeostasis in both the presence and absence of dietary intervention. Furthermore, the effects of this triterpene are comparable to those shown by the administration of metformin which may suggest that BA may be a good alternative in the management of prediabetes. More studies are needed, however, to determine the effects of this compound on other physiological parameters.

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Disclosure statement

The authors declare no conflicts of interest.

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Table 1: Effects of BA on body weight and percentage changes in body weight from week 0 to week32 in rats with or without diet intervention. Values are presented as mean±SEM (n=6)

Body weight (g)

Groups / **Weeks**	NPD	PD	ND + MET	HFHC+MET	ND + BA	HFHC + BA
Week 0	167.00 ±2.35	187.20±5.24	187.17±8.13	172.00 ±2.52	168.17±5.24	166.83±5.40
Week 20	366.60±5.57 100%	429.50±12.50 100%	400.33±7.32 100%	426.67±14.08 100%	404.67±20.48 100%	404.20±15.73 100%
Week 24	412.33±6.24 ↑11.09%	498.83±15.45 ↑16.14% [a]	459.83±5.22 ↑14.86%[a]	479.00±13.15 ↑12.26%[a]	435.40±19.37 ↑7.59%[a b]	447.83±26.15 ↑10.78%[a b]
Week 28	437.83±20.37 ↑16.27%	523.50±16.00 ↑21.89%[a]	481.50±6.27 ↑20.27%[a]	506.17±12.51 ↑18.63%[a]	446.00±21.35 ↑10.21%[a b]	461.00±26.89 ↑14.05%[a b]
Week 32	462.50±17.44 ↑20.74%	540.50±16.05 ↑25.84%[a]	481.80±7.83 ↑20.35%	516.00±14.14 ↑20.94%	449.80±22.43 ↑11.15%[a b]	468.80±30.09 ↑15.98%[a b]

[a]$p<0.05$ in comparison to non-prediabetic (NPD) control, [b]$p<0.05$ in comparison to prediabetic (PD) control. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Table 2: Effects of BA on fasting blood glucose, fasting blood insulin and HOMA2-IR Index in rats with or without dietary intervention after 12 weeks of treatment period. Values are presented as mean±SEM (n=6)

Groups	**Fasting blood glucose (mmol/L)**	**Fasting blood Insulin (ng/mL)**	**HOMA2-IR Index Values**
NPD	4.68±0.19	3.42±0.33	0.71±0.09
PD	5.15±0.13	12.28±0.18[a]	2.81±0.05[a]
ND+MET	5.28±0.17	3.66±0.12[b]	0.86±0.04[b]
HFHC+MET	5.68±0.44	5.06±0.08[a b]	1.28±0.06 [a b]
ND+BA	4.63±0.46	3.14±0.09[b]	0.65±0.05[b]
HFHC+BA	4.75±0.46	3.91±0.48[b]	0.83±0.12[b]

[a]$p<0.001$ in comparison to non-prediabetic (NPD) control, [b]$p<0.05$ in comparison to prediabetic (PD) control. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid, HOMA2-IR: Homeostasis model assessment of insulin resistance

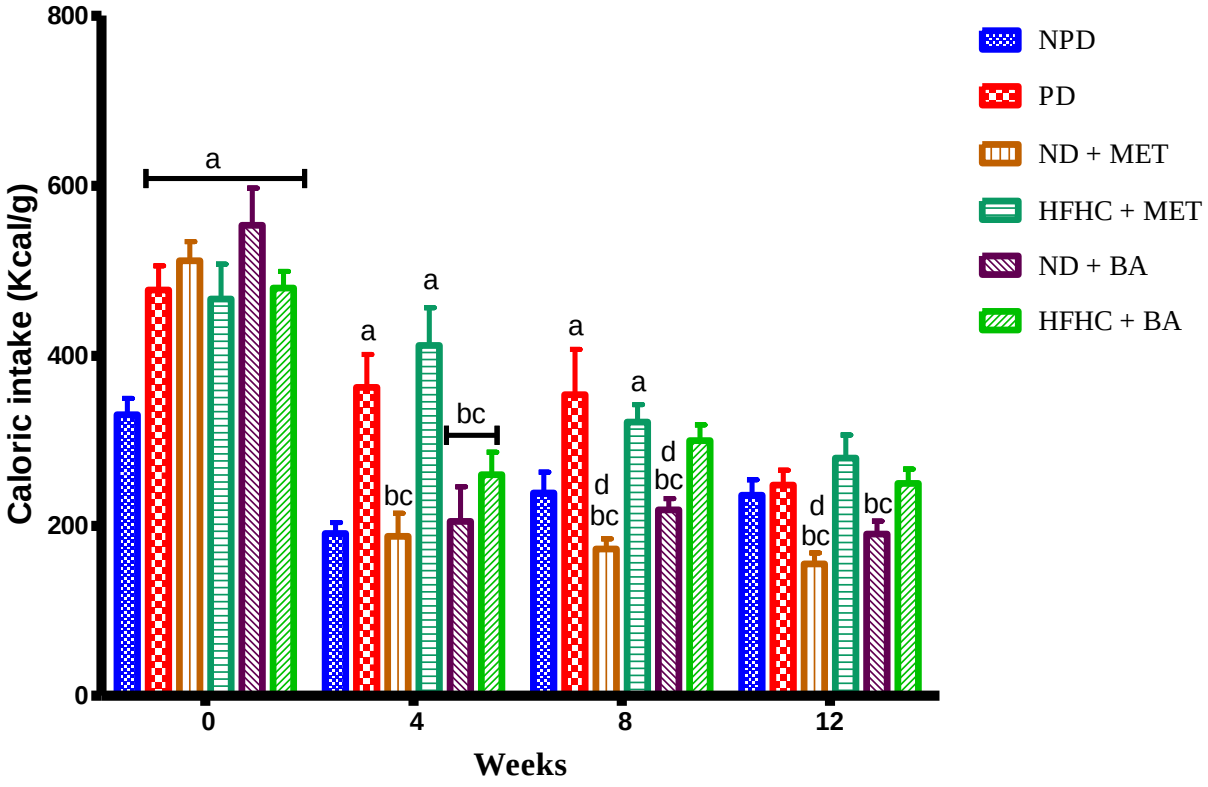

Figure 1: Effect of BA on caloric intake in rats with or without dietary intervention. Values are expressed as mean±SEM (n=6). [a]p<0.001 in comparison to non-prediabetic (NPD) control, [b]p<0.001 in comparison to prediabetic (PD) control, [c]p<0.001 in comparison to HFHC + MET, [d]p<0.05 in comparison to HFHC + BA. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

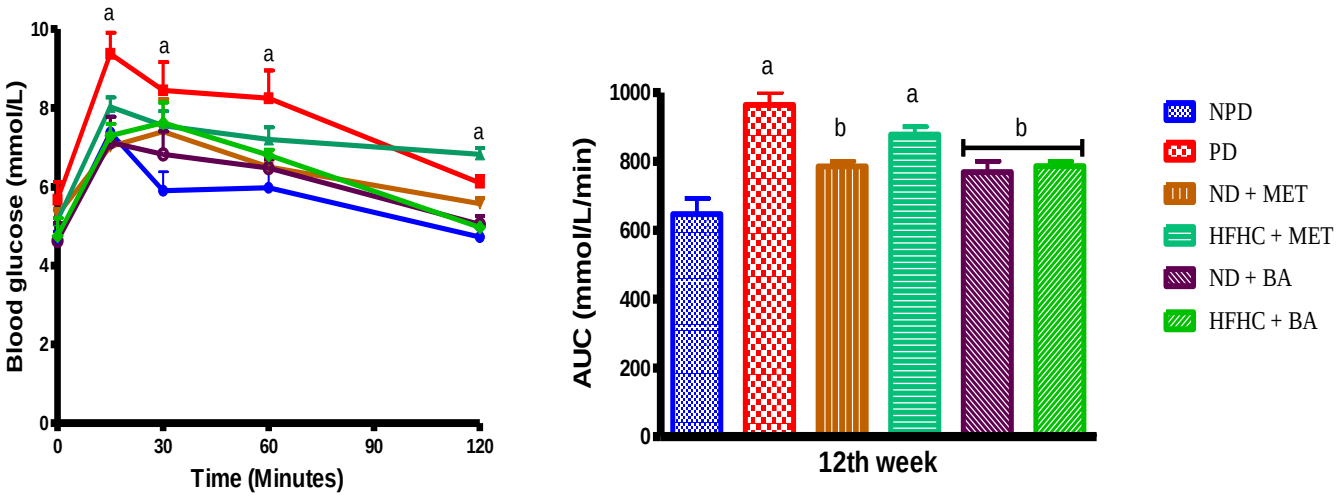

Figure 2: Effect of BA on OGTT (oral glucose tolerance test) and AUC (area under curve) in rats with or without dietary intervention. Values are expressed as mean±SEM (n=6). [a]p<0.001 in comparison to non-prediabetic (NPD) control, [b]p<0.05 in comparison to prediabetic (PD) control. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

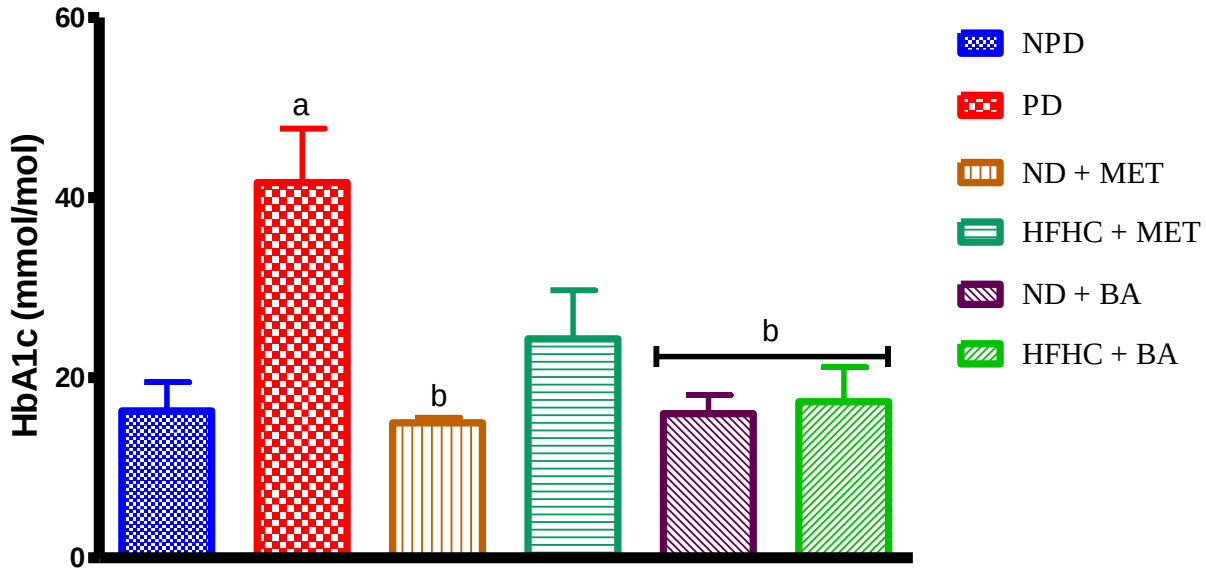

Figure 3: Effect of BA on glycated haemoglobin (HbA1c) in rats with or without dietary intervention. Values are expressed as mean±SEM (n=6). [a]p<0.001 in comparison to non-prediabetic (NPD) control, [b]p<0.001 in comparison to prediabetic (PD) control. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

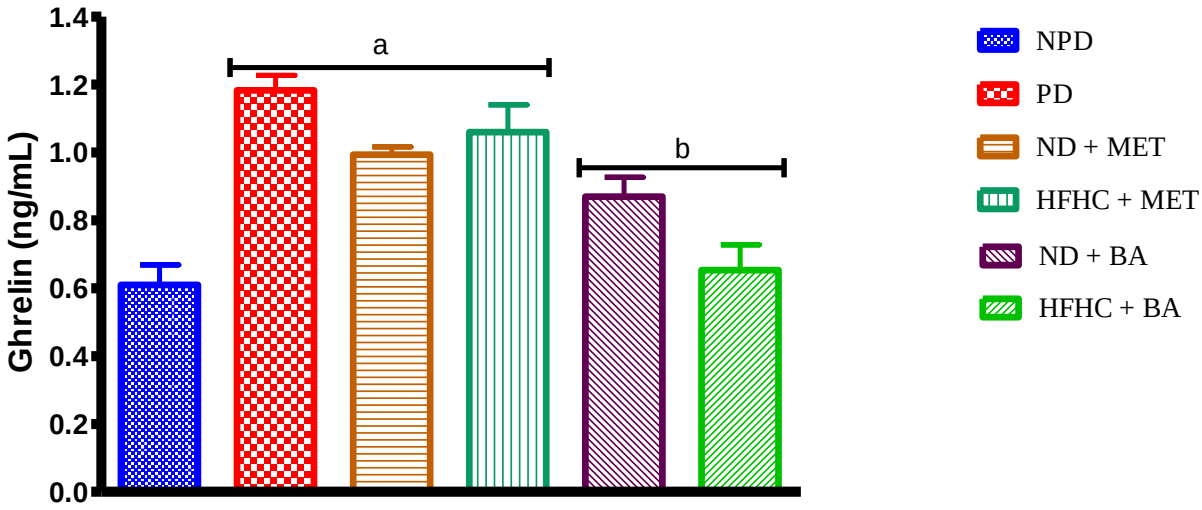

Figure 4: Effect of BA on Ghrelin in rats with or without dietary intervention. Values are expressed as mean±SEM (n=6). [a]p<0.001 in comparison to non-prediabetic (NPD) control, [b]p<0.05 in comparison to prediabetic (PD) control. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

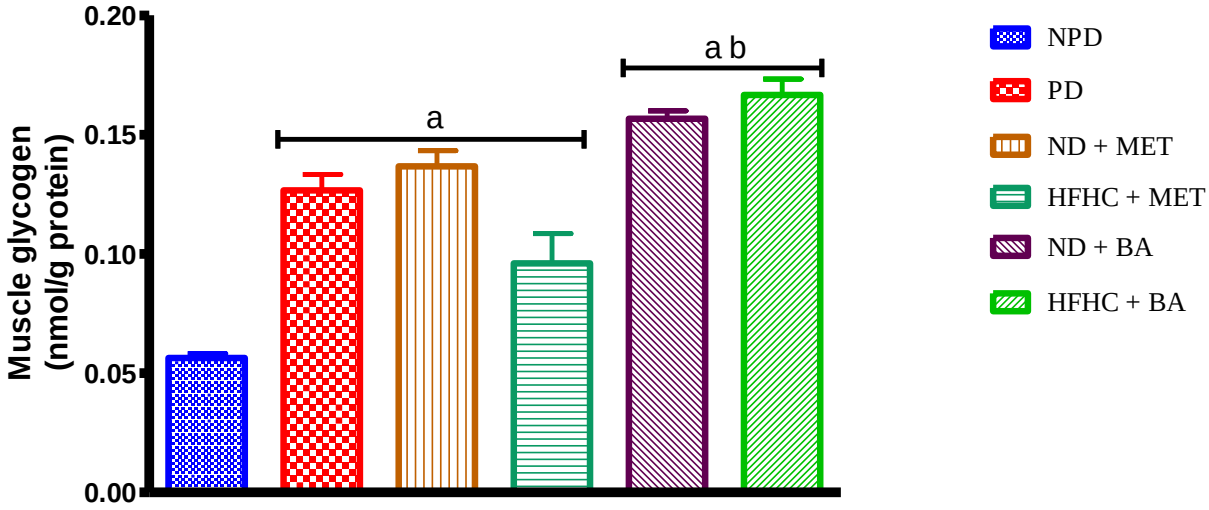

Figure 5: Effect of BA on muscle glycogen in rats with or without diet intervention. Values are expressed as mean±SEM (n=6). [a]p<0.001 in comparison to non-prediabetic (NPD) control, [b]p<0.05 in comparison to prediabetic (PD) control. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

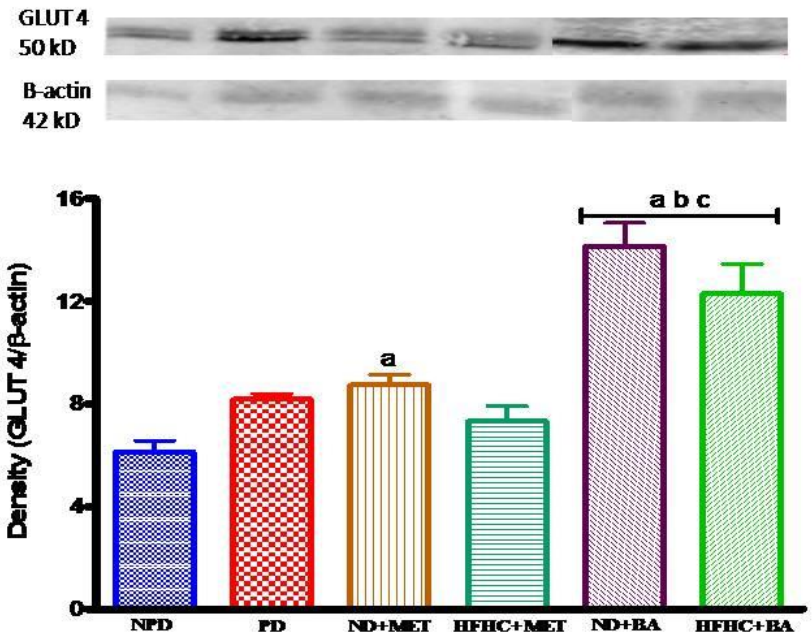

Figure 6: Effects of BA on GLUT (glucose transporter) 4 expression in rats with or without diet intervention after treatment period of 12 weeks. Values are expressed as mean±SEM (n=6). Values were obtained from Western blots for six preparations. [a]$p<0.01$ in comparison to non-prediabetic (NPD) control, [b]$p<0.05$ in comparison to prediabetic (PD) control, [c]$p<0.05$ in comparison to HFHC+MET. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

PROLOGUE

Studies have shown that chronic consumption of high caloric diets result into hepatic oxidative stress, hepatic fat accumulation and elevation of markers associated with NAFLD in prediabetes. Management of hepatic complications in prediabetes involves a combination of dietary and pharmacological intervention. However, the efficacy of the pharmacological intervention is low due to a lack of compliance from patients in following a recommended diet modification. In chapter 2 of this study, we observed that consumption of high caloric diet resulted in a significant change in body weight gain, hyperinsulinaemia and increased plasma concentration of ghrelin. Moreover, the induction of prediabetes resulted in selective muscle insulin resistance which was confirmed by decreased skeletal muscle glycogen concentration and decreased expression of GLUT 4 in the skeletal muscle. However, the administration of BA improved insulin sensitivity, decreased food intake and body weight due to decreased ghrelin plasma concentration and increased the expression of GLUT 4 in the skeletal muscle of prediabetic rats. Therefore, in Chapter 3 of this study, we investigated the effects of BA on selected markers associated with hepatic functions in prediabetic rats in both the presence and absence of dietary intervention. The chapter was written and prepared in manuscript format. The authors of this manuscript are Akinnuga AM, Siboto A, Khumalo B, Sibiya NH, Ngubane P and Khathi A. This manuscript has been published in the Canadian Journal of Gastroenterology and Hepatology. See Appendix IV.

CHAPTER 3

Bredemolic acid ameliorates selected liver function biomarkers in a diet-induced prediabetic rat model

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Abstract

Background. Prediabetes is an intermediary hyperglycaemic state that precedes type 2 diabetes mellitus (T2DM) in which abnormal metabolism of glucose and lipids occurs in organs such as the liver. Evidence has shown that about 70% of T2DM patients develop hepatic dysfunction which is found to begin during the prediabetic stage. Bredemolic acid, a pentacyclic triterpene, has been found to improve insulin sensitivity in diet-induced prediabetic rats. The effects of this compound on liver function, however, are unknown. This study was therefore designed to investigate the effects of BA on liver function in high fat high carbohydrate (HFHC) diet-induced prediabetic rats. *Methods.* Thirty six (36) male rats that weigh 150g-180g were divided into two groups, the non-prediabetic (n=6) and the prediabetic groups (n=30) that were fed a normal diet (ND) and HFHC diet respectively. The prediabetic rats were further sub-divided into five groups (n=6) and treated with either BA (80 mg/kg) or metformin (MET, 500mg/kg) every third day for 12 weeks. After 12 weeks, blood samples and the liver were collected for biochemical analysis. *Results.* The induction of prediabetes resulted in increased release of liver enzymes (AST and ALT), increased liver glycogen and triglyceride, lipid peroxidation, decreased sterol regulatory element binding protein (SREBP1c) and antioxidant enzymes. However, the administration of BA decreased liver enzyme concentrations, decreased hepatic oxidative stress, and improved antioxidant enzymes such as SOD and GPx. *Conclusion.*BA administration improved liver function in diet-induced prediabetic rats in the presence or absence of dietary intervention.

1. Introduction

Prediabetes is a state of intermediate hyperglycaemia that causes abnormal changes in intracellular metabolism of most body tissues including the liver [1]. Presently, the observed increase in the prevalence of prediabetes and type 2 diabetes mellitus (T2DM) in developed and developing countries is reported to be due to sedentary lifestyles coupled with high caloric diets [1-3]. However, studies have shown that excessive intake of high caloric diets induces skeletal muscle insulin resistance which results into the shunting of glucose from the skeletal muscle to the liver thereby leading to increased hepatic glycogen production and storage [4-6]. Several studies have shown that continuous intake of high quantities of fats and carbohydrates alters liver function by accumulation of ectopic fats as a result of *de novo* lipogenesis which is mediated by transcription factors such as sterol regulatory element binding protein (SREBP1c) under insulin action [7, 8]. Moreover, excessive hepatic accumulation of free fatty acid or triglyceride leads to hepatic insulin resistance, hepatic dysfunction and non-alcoholic fatty liver disease (NAFLD) that is characterized by fat infiltration into the hepatocytes [9-14]. Consequently, the infiltration of fat into the hepatocytes triggers oxidative stress, reduced antioxidant enzymes production and an inflammatory cascade of reactions that produce progressive fibrotic hepatic damage known as non-alcoholic steatohepatitis (NASH). Cross-sectional studies have demonstrated that liver function markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are altered due to oxidative stress and hepatic dysfunction [15-18]. However, it has been established that approximately 70% of T2DM patients have liver dysfunction and complications [19-21]. There is also evidence from other studies that suggested that liver dysfunction and complications can also begin during the prediabetic stage [21, 22, 23].

Current treatment focuses on a combination of dietary and pharmacological interventions but there have been reports of low compliance as patients merely use pharmacological intervention without diet modification thus reducing the efficacy of the pharmacological intervention [24-27]. Therefore, novel compounds that can ameliorate liver dysfunction in the prediabetic condition even in the absence of dietary intervention are necessary. Oleanolic acid and maslinic acid are pentacyclic triterpenes that have been found to have anti-diabetic and antioxidant properties [28-30]. In our laboratory, we have shown that chronic ingestion of a high fat high carbohydrate diet leads to the development of prediabetes which is accompanied by liver complications. We have further shown that bredemolic acid (BA), a structural isomer of maslinic acid, is able to restore glucose homeostasis in diet-induced prediabetes by improving insulin sensitivity in both the presence and absence of dietary intervention [31]. However, the effects of BA on liver function in diet-induced prediabetes have not been established. Hence, the aim of this study is to investigate the effects of bredemolic acid on selected biomarkers of liver function in a diet-induced prediabetic rat model.

2. Materials and Methods

2.1. *Animals*

Thirty six (36) male Sprague Dawley rats (150g–180g) obtained from Biomedical Research Unit, University of KwaZulu-Natal (UKZN), were kept under standard environmental conditions i.e. constant humidity (55±5%), temperature (22±2°C), 12h day:12h night cycle. The animals were acclimatized for 2 weeks, and consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* before being fed on the experimental high fat–high carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, South Africa). The HFHC diet consist of carbohydrate (55% kcal/g), fats (30% kcal/g), and proteins (15% kcal/g). All the experimental designs and procedures were carried out according to the ethics and guidelines of the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa.

2.2. *Experimental Design*

After acclimatization, the animals were divided into two groups, the normal diet (ND) non-prediabetic control (n=6) and the HFHC diet prediabetic groups (n=30). All the animals in the prediabetic group consumed HFHC diet and drinking water that was supplemented with 15% fructose for 20 weeks to induce prediabetes while the non-prediabetic control group (NPD, Group 1) fed on ND and water *ad libitum* for 20 weeks as well. At the 20th week, prediabetes was confirmed by fasting blood glucose and oral glucose tolerance test which have been described in the previous research study [31].

2.3. *Treatment of Prediabetic Animals*

After the 20 weeks of prediabetes induction, the non-prediabetic control (NPD, Group 1) animals continuously fed on standard rat chow for 12 weeks. The thirty (30) prediabetic animals were randomly assigned into 5 different groups (Group 2 to Group 6, n=6). Group 2 (PD) served as the untreated prediabetic control group and continuously consumed the HFHC diet for 12 weeks; Group 3 (ND+MET) were prediabetic animals that switched to standard rat chow and received metformin (MET) for 12 weeks; Group 4 (HFHC+MET) were prediabetic animals that continuously consumed HFHC diet with MET treatment; Group 5 (ND+BA) were prediabetic animals that switched to standard rat chow and received BA for 12 weeks; Group 6 (HFHC+BA) were prediabetic animals that continuously consumed HFHC diet and received BA as treatment for 12 weeks. Treatment with either MET (500mg/kg) or BA (80mg/kg) was carried out every third day for 12 weeks.

2.4. *Blood Collection and Tissue Harvesting*

After the 12-week treatment period, the animals were sacrificed. The animals were placed in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) and anaesthetised with Isofor (100 mg/kg, Safeline Pharmaceuticals, Roodeport, South Africa) for 3 minutes. Blood samples were collected from the animals using cardiac puncture and put into different pre-cooled EDTA containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes to obtain plasma. Each of the plasma was aspirated into plain sample bottles and stored

at -80 °C in a Bio Ultra freezer (Snijers Scientific, Tilburg, Holland) until ready for biochemical analysis. Also, the liver tissue samples were excised, weighed and rinsed in cold normal saline solution, and snapped frozen in liquid nitrogen before storage in the Bio Ultra freezer for biochemical analysis of selected metabolic parameters.

2.5. *Relative Liver Weight.* The relative liver weights of all the animals in each experimental group were determined from the percentage of the ratio of liver weight to the body weight

i.e. relative liver weight = liver weight / body weight × 100.

2.6. *Biochemical Analysis*

Liver enzymes (AST and ALT) were analysed with IDEXX Catalyst One Chemistry Analyzer (IDEXX Laboratories Inc. Westbrook, USA) while SREBP1c was analysed by following specific ELISA kit procedures using manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

2.7. *Liver Triglycerides*

The preparation of liver tissue samples and the homogenate medium used for determination of hepatic triglyceride was according to the manufacturer instruction in triglyceride assay kit (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). 50mg of liver tissue were homogenized on ice in 500µl phosphate buffer saline (PBS) and centrifuged at 8000 rpm for 10 minutes, 4^{o}C. The supernatant was then aspirated into eppendorf tubes, and triglycerides were determined using the triglyceride assay kit as instructed in the manufacturer's manual. The absorbance of the samples was measured at 510 nm by using Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW Germany).

2.8. *Liver Glycogen Assay*

Glycogen assay was determined in the liver by following a previous established protocol [27, 28, 32]. The absorbance was determined by using the Spectrostar Nano spectrophotometer at 620nm.

2.9. *Lipid Peroxidation and Antioxidant Profile*

The concentration of malondialdehyde in the liver was determined to estimate the amount of lipid peroxidation, according to a previously described protocol [29, 32]. Furthermore, the antioxidant profile of the liver was determined by measuring the concentration of SOD and GPx according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

2.10. *Statistical Analysis*

The statistical data were presented in mean ± SEM. The data were analysed by two-way Analysis of Variance (ANOVA) with Bonferroni test (post hoc test) via GraphPad Prism 5 software. The level of statistical significance was determined at $p<0.05$.

3. Results

3.1. Relative L*iver Weight*

The effects of BA treatment on relative liver weights in non-prediabetic and prediabetic rats with or without diet intervention were determined. The relative liver weights of untreated prediabetic (PD) rats was significantly increased by comparison to the non-prediabetic control (NPD) rats ($p<0.05$).

However, the administration of BA with diet intervention (ND+BA) significantly decreased relative liver weight when compared to PD ($p<0.05$). Similarly, the relative liver weight of metformin-treated rats with diet intervention (ND+MET) significantly decreased in comparison to PD. See Figure 1.

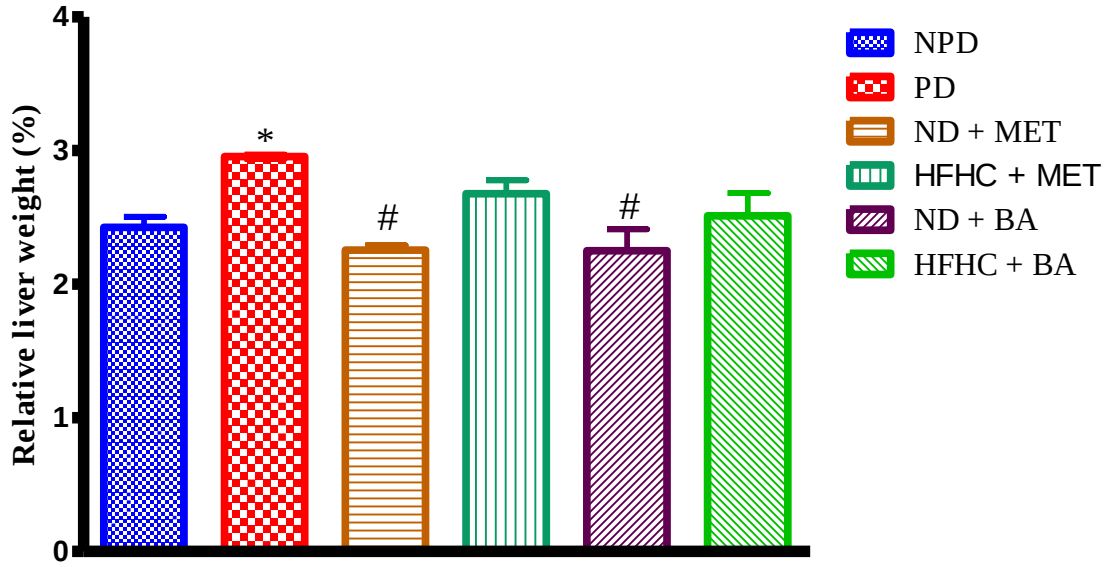

Figure 1: Effects of BA on the relative liver weight in non-prediabetic and prediabetic rats with or without diet intervention. *$p<0.05$ in comparison to NPD, #$p<0.001$ in comparison to PD. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.2. *Liver Enzymes*

Plasma AST and ALT concentrations in the PD group were significantly increased ($p<0.01$) compared to the NPD group. However, the administration of BA with or without diet intervention significantly decreased the plasma AST and ALT concentrations when compared to PD. The plasma ALT levels of metformin-treated rats with diet intervention (ND+MET) was significantly decreased when compared to PD, while the plasma AST of ND+MET was insignificantly different when compared to PD ($p<0.05$). See Figure 2

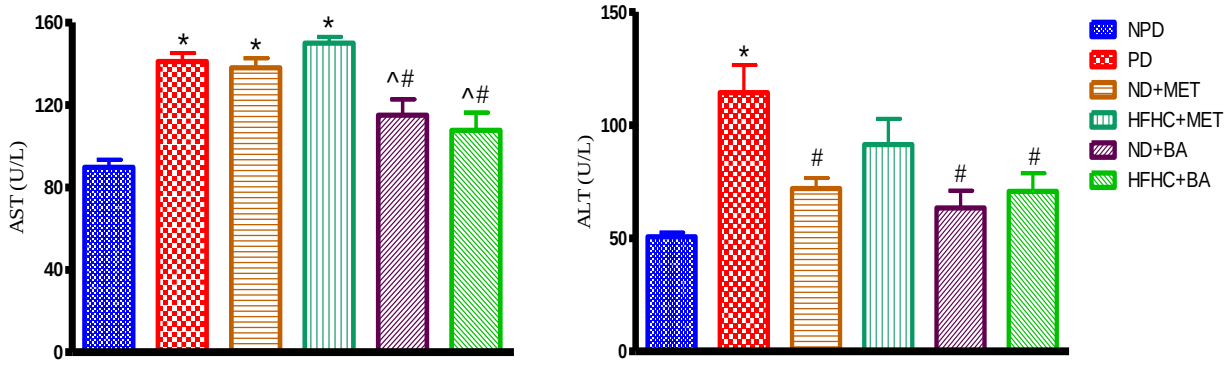

Figure 2: Effects of BA on aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.01 in comparison to NPD, #p<0.05 in comparison to PD, ^p<0.05 in comparison to HFHC + MET. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.3. *SREBP1c*

The SREBP1c concentration was determined in non-prediabetic and prediabetic rats. SREBP1c levels were significantly decreased in PD groups when compared to NPD group (p<0.001). The administration of BA with or without diet intervention significantly increased the liver SREBP1c concentration in comparison to the PD group (p<0.001). Interestingly, the administration of metformin with diet intervention (ND+MET) significantly increased the SREBP1c concentration when compared to the PD group (p<0.05). The administration of metformin in the absence of dietary intervention did not have any significant effects when compared to PD control. See Figure 3

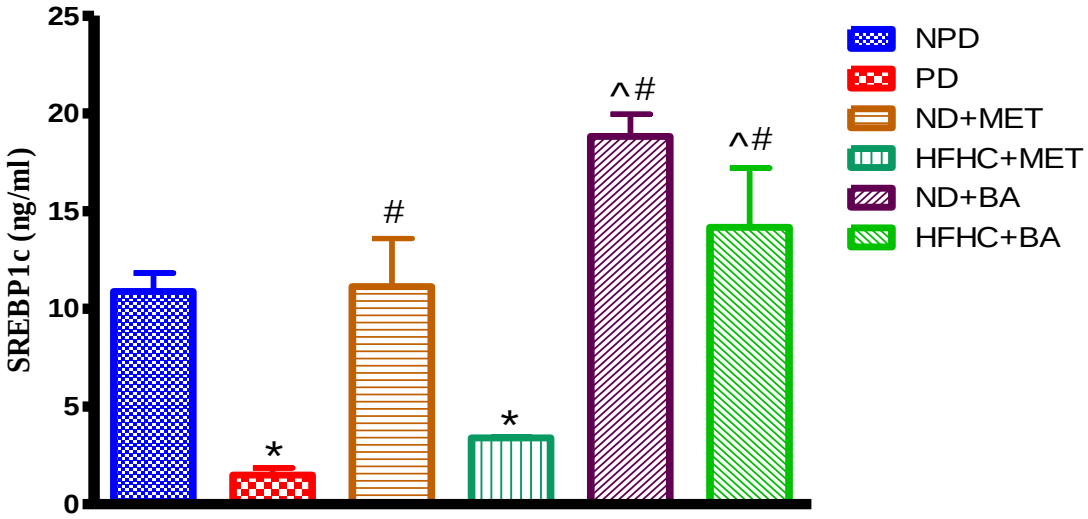

Figure 3: Effects of BA on SREBP1c (sterol regulatory element binding protein) in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.001 in comparison to NPD, #p<0.001 in comparison to PD, ^p<0.01 in comparison to HFHC+MET. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.4. *Liver Triglycerides*

Liver triglyceride concentrations were significantly increased in the PD group by comparison to the NPD group ($p<0.001$). The liver triglyceride concentration of BA treated rats with or without diet intervention significantly decreased when compared to the PD group ($p<0.001$). Similar results were observed with the use of metformin. There was no significant difference in the liver triglyceride of prediabetic rats that fed on ND or HFHC and received MET or BA treatment. See Figure 4

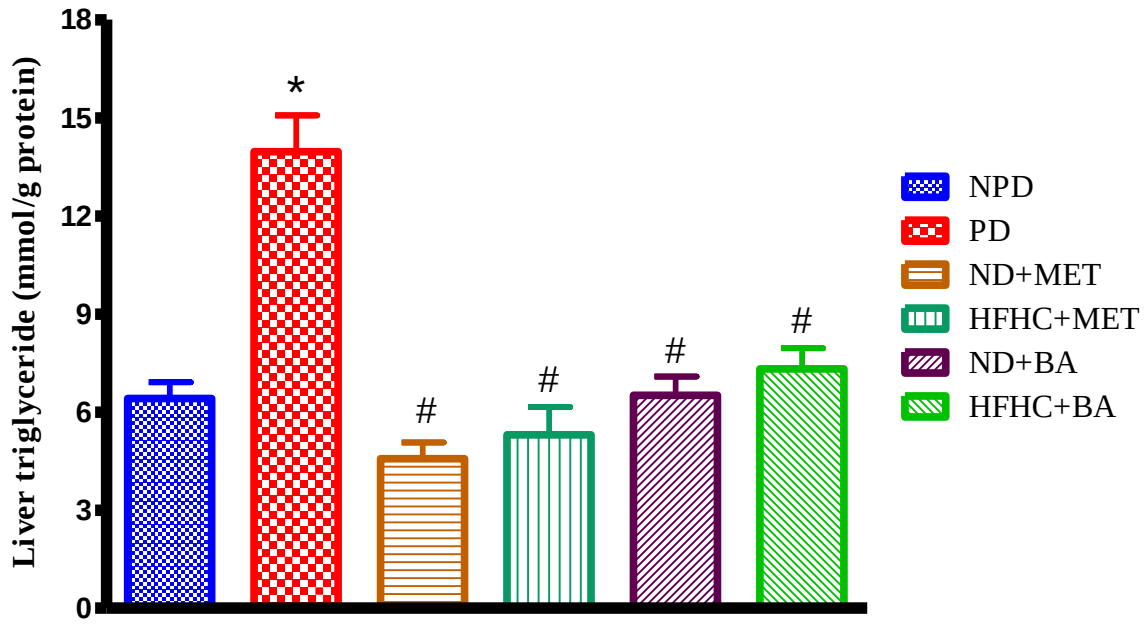

Figure 4: Effects of BA on liver triglyceride in non-prediabetic and prediabetic rats with or without diet intervention. *$p<0.001$ in comparison to NPD, #$p<0.001$ in comparison to PD. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.5. *Liver Glycogen*

Liver glycogen concentrations of PD group were significantly increased by comparison to the NPD group ($p<0.001$). The administration of BA with or without diet intervention significantly decreased liver glycogen concentrations by comparison to PD ($p<0.001$). Similarly, the administration of metformin-treated with or without diet intervention significantly decreased the liver glycogen concentration when compared to PD. See Figure 5

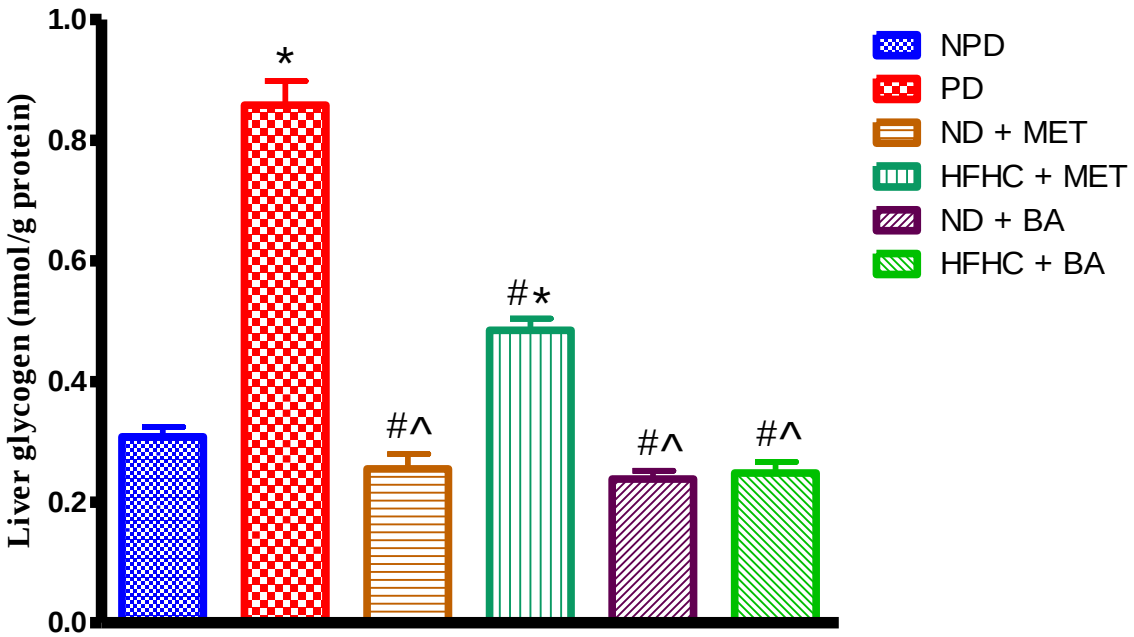

Figure 5: Effects of BA on liver glycogen in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.001 in comparison to NPD, #p<0.001 in comparison to PD, ^p<0.001in comparison to HFHC + MET. NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.6. *Lipid Peroxidation and Antioxidant Enzyme Concentration*

As shown in Table 1, liver MDA concentrations in the untreated PD group were significantly increased by comparison to the NPD group ($p<0.001$). The administration of BA and metformin with or without diet intervention significantly decreased the liver MDA concentration when compared to the PD group ($p<0.05$). Liver SOD and GPx concentrations of the untreated PD group were significantly decreased when compared to the NPD group ($p<0.05$). The SOD and GPx concentrations in the liver of BA treated rats with or without diet intervention were significantly increased in comparison to that of the PD group ($p<0.05$).

Table 1: Effects of BA on the liver lipid peroxidation and antioxidant enzyme concentrations in non-prediabetic and prediabetic rats with or without diet intervention. Values are presented as mean±SEM (n=6)

Groups	**MDA (nmol/g protein)**	**SOD (nmol $min^{-1}mL\ mg^{-1}protein$)**	**GPx (nmol $min^{-1}mL\ mg^{-1}protein$)**
NPD	4.11±0.51	2.99±0.06	1.67±0.09
PD	12.34±1.31*	1.66±0.22*	1.08±0.06*
ND+MET	5.00±0.26$^{\#}$	2.14±0.02$^{\#}$	1.79±0.07$^{\#\wedge}$
HFHC+MET	6.41±0.27$^{\#}$	1.83±0.13*	1.05±0.05*
ND+BA	4.89±0.44$^{\#}$	2.47±0.06$^{\#}$	1.87±0.10$^{\#\wedge}$
HFHC+BA	6.68±0.65$^{\#}$	2.59±0.02$^{\#}$	1.89±0.04$^{\#\wedge}$

*$p<0.05$ in comparison to non-prediabetic (NPD) control, $^{\#}p<0.05$ in comparison to prediabetic (PD) control, $\wedge p<0.05$ in comparison to HFHC+MET group.

ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid, SOD: superoxide dismutase, MDA: malondialdehyde, GPx: glutathione peroxidase

4. Discussion

This study examined the effects of BA on selected markers of liver function in diet-induced prediabetic rats. Triterpenes such as maslinic acid and oleanolic acid have been reported to ameliorate oxidative stress in the liver via the increased release of antioxidant enzymes and improved liver function via increased activity of glycogenic enzymes to decrease hepatic glucose production in diabetic rats [29, 32]. In a previous study, BA was shown to improve insulin sensitivity in skeletal muscle by increasing the expression of GLUT 4 however, the effects of this triterpene on liver function in the prediabetic state were not determined. Hence, this study sought to evaluate the effects of BA on selected markers of liver function in a diet-induced prediabetic rat model [31]. The liver plays a key role in maintaining glucose homeostasis as it balances the production of glucose and the conversion of glucose to glycogen [33]. In a postprandial state, blood glucose increases and insulin is secreted to enhance glycogenesis and inhibit glycogenolysis [34]. However, studies in our laboratory have shown that chronic consumption of high fat high carbohydrate diet results in the induction of prediabetes which is characterized by hyperinsulinaemia, impaired glucose tolerance, peripheral and hepatic insulin resistance as well as liver damage [1, 35, 36]. In the prediabetic state, due to hyperinsulinaemia and selective muscle insulin resistance, most ingested glucose is shunted to the liver leading to increased hepatic glycogenesis [6, 37]. In addition, since the liver is insulin-independent, excess glucose in the blood can diffuse into the hepatic cells through facilitated diffusion which is mediated by glucose

transporter 2 (GLUT 2) [14, 34, 38]. Similarly, the elevated liver glycogen concentration observed in untreated prediabetic rats in this study can be attributed to the increased diversion of excess glucose to the liver. This showed that the consumption of high fat high carbohydrate diet could result in the diversion of glucose to the liver as a compensatory mechanism in the presence of selective muscle resistance in a prediabetic state [34]. However, the administration of BA with or without diet intervention significantly reduced liver glycogen concentrations. Previous studies have shown that administration of BA in the prediabetic state improves insulin sensitivity in skeletal muscle through increased GLUT 4 expression [31]. We suggest that this improved insulin sensitivity in the periphery led to decreased amounts of glucose being shunted to the liver thus resulting in the observed decrease in liver glycogen concentrations.

In non-diabetic subjects, metabolism of glucose is largely carried out in the skeletal muscle [39, 40]. In the prediabetic state, as glucose delivery to the liver increases, *de novo* lipogenesis and hepatic lipid accumulation increase under the influence of transcription factors such as SREBP1c [6, 14, 37, 40]. The SREBP1c is a major transcription factor which regulates *de novo* lipogenesis through direct activation from AKT (protein kinase B) in the insulin signalling pathway [8, 41, 42]. In the prediabetic state, when insulin signalling is impaired, the direct activation of SREBP1c by AKT is altered, and the SREBP1c expression decreases [6-8]. On the contrary, the hepatic *de novo* lipogenesis is not solely dependent on insulin signalling through activation of SREBP1c but the activation of SREBP1c to stimulate *de novo* lipogenesis depends on insulin signalling [6, 43]. However, when the insulin signalling pathway is impaired in prediabetes, the *de novo* lipogenesis is still elevated due to substrate push mechanism in which there is increased substrates delivery to the liver followed by increased esterification of fatty acids into triglycerides [6]. In this study, we observed that the concentration of SREBP1c in the liver was significantly lowered in untreated prediabetic rats by comparison to the non-prediabetic rats. The decreased SREBP1c in untreated prediabetic rats may be due to the alteration of insulin signalling in the prediabetic state since SREBP1c expression is insulin dependent. This observation is in correlation with previous studies which reported that insulin signalling is not totally required for hepatic lipogenesis and that availability of substrate can facilitate delivery of substrates into the liver for lipogenesis [6, 44]. The BA treated rats had a significantly increased SREBP1c, thus suggesting that BA ameliorated insulin signalling which may have resulted in the increased SREBP1c concentration in the liver. Furthermore, high fructose consumption has been reported to increase hepatic lipogenesis and glycogenesis [1]. Fructose, unlike glucose, is solely metabolized in the liver, thereby providing additional substrates for *de novo* lipogenesis and ectopic fat accumulation in the liver, thus leading to NAFLD [1, 10]. In this study, we observed that the liver triglyceride in untreated prediabetic rats significantly increased when compared to non-prediabetic rats. The increased liver triglyceride in untreated prediabetic rats can be attributed to increased substrates delivery to the liver or decreased hepatocellular triglyceride disposal as well as decreased fatty acid oxidation [45]. However, the administration of BA significantly decreased hepatic triglycerides, and this suggests that BA may

decrease substrate delivery to the liver by a divergence of the substrates to other organs for metabolism, increased β oxidation of fat or increased triglyceride disposal via very-low-density lipoprotein (VLDL) exportation from the liver.

Moreover, due to the increased hepatic lipogenesis and glycogenesis, the production of free radicals is elevated, and this results in oxidative stress [46]. Oxidative stress is due to an imbalance between oxidants and antioxidants enzymes [46]. Antioxidants are stable molecules that donate electrons to rampaging free radicals in order to neutralise the free radical capacity to damage tissues or organs [48]. In this study, we observed that the lipid peroxidation (MDA) in the liver was significantly increased, and the antioxidant enzymes (SOD and GPx) production in the liver was significantly decreased in the untreated prediabetic rats when compared to non-prediabetic rats. The increased lipid peroxidation was due to increased production of free radicals while the decreased antioxidant capacity of the liver was as a result of decreased production of antioxidant enzymes (SOD and GPx) in the mitochondria of hepatocytes during prediabetes. On the other hand, BA administration with or without diet intervention significantly lowered lipid peroxidation and significantly increased the liver antioxidant enzymes. This may be due to the fact that BA neutralises the free radicals in the mitochondria of hepatocytes by donation of electron through hydroxyl radical scavenging activity which has been reported in other triterpenes [49]. This is in line with similar observations made on earlier studies using other triterpenes [28, 32, 49].

Furthermore, studies have shown that elevated liver enzymes (AST and ALT) in the plasma can be due to necrosis of the hepatocyte during liver damage [18]. AST and ALT are released into the bloodstream whenever hepatocytes are damaged, and this has been reported to occur during prediabetes [18]. In this study, these enzymes were significantly elevated in untreated prediabetic rats by comparison to non-prediabetic rats. The increased liver enzymes in the plasma suggested that liver cells are damaged through oxidative stress and increased hepatic lipogenesis or glycogenesis. However, BA administration caused a decrease in the concentration of liver enzymes suggesting that BA may improve hepatic function via its antioxidant and antilipidemic effects in the liver as observed in this study. Triterpenes are non-toxic antioxidants and have low pharmacokinetics of three days; therefore, the ameliorative effects of BA in the absence of dietary intervention on liver function markers compared to metformin in this study may be attributed to this low pharmacokinetic feature. In conclusion, the administration of BA, in both the presence and absence of dietary modification can potentially be one of the therapeutic approaches to attenuate hepatic dysfunction or improve hepatic functions in the prediabetic state.

Data Availability

The data used to support the findings in this study are available upon request from the corresponding author. However, the data on body weight as well as fasting blood glucose and oral glucose tolerance test for confirmation of prediabetes are reported in our previous study.

Conflicts of Interest

The authors declare no conflicts of interest

Funding Statement

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PROLOGUE

Literature has indicated that chronic consumption of high caloric diet results in cardiovascular and vascular endothelial complications during the prediabetic stage. Management of cardiovascular complications during the prediabetic stage involves the combination of dietary and pharmacological interventions. However, due to lack of compliance from patients in adhering to the combination of the two interventions, the efficacy of the pharmacological intervention is reduced. In chapter 3, we demonstrated the effects of BA on hepatic functions; however, the effects of BA on cardiovascular functions are yet to be established. In Chapter 4 of this study, we investigated the effects of BA with or without diet intervention on markers associated with cardiovascular and endothelial functions in diet-induced prediabetic rats. The chapter was prepared in manuscript format. The authors of this manuscript are Akinnuga AM, Siboto A, Khumalo B, Sibiya NH, Ngubane P and Khathi A. This manuscript has been published in the journal: Cardiovascular Therapeutics. See Appendix V.

CHAPTER 4

Bredemolic acid improves cardiovascular function and attenuates endothelial dysfunction in diet-induced prediabetes: effects on selected markers

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Abstract

Prediabetes is an intermediate hyperglycaemic state which has been associated with cardiovascular dysfunction. However, cardiovascular dysfunction is not only caused by intermediate hyperglycaemia, but also endothelial dysfunction, inflammation and oxidative stress associated with prediabetes. Bredemolic acid (BA), an isomer of maslinic acid, has been reported to ameliorate the intermediate hyperglycaemia found in prediabetes; however, the effects of this triterpene on cardiovascular function have not yet been determined. Therefore, this study investigated the effects of BA on cardiovascular function in diet-induced prediabetic rats. Thirty six (36) male rats that weighed 150g-180g were divided into two groups, the non-prediabetic (n=6) and the prediabetic groups (n=30) that were fed a normal diet (ND) and HFHC diet respectively. The prediabetic rats were further sub-divided into five groups (n=6) and treated with either BA (80mg/kg) or metformin (MET, 500mg/kg) every third day for 12 weeks. After 12 weeks, blood samples and the heart were collected for biochemical analysis. The untreated prediabetic rats showed a significant increase in body mass index (BMI), waist circumference (WC), blood pressure, heart rate, lipid profile, lipid peroxidation and inflammatory markers with a significant decrease in endothelial function and antioxidant biomarkers by comparison to the non-prediabetic animals. The administration of BA significantly improved cardiovascular functions such as blood pressure, heart rate and endothelial function. There was also a significant decrease in BMI, WC, lipid profile, lipid peroxidation and inflammation with a concomitant increase in antioxidant capacity. BA administration improved cardiovascular function by attenuation of oxidative stress, inflammatory and endothelial dysfunction markers.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a heterogeneous metabolic disorder which is associated with cardiovascular diseases (CVD), that is often preceded by the onset of prediabetes [1]. One of the identified causes of this disorder is chronic consumption of high caloric diets which are rich in carbohydrates as well as saturated and polyunsaturated fats coupled with sedentary lifestyles [2,3]. Consequently, this leads to inefficient metabolism of carbohydrate and fats, resulting in accumulation of intracellular and extracellular glucose and lipids known as glucolipotoxicity [4].

However, glucolipotoxicity is associated with insulin resistance which subsequently causes high body mass index (BMI), high waist circumference, hyperlipidaemia, oxidative stress, the release of inflammatory cytokines such as high sensitive C-reactive protein, hs-CRP, interleukin 6, IL-6 and tumour necrotic factor, TNFα [3-6]. Glucolipotoxicity is also associated with endothelial dysfunction, hypertension, arteriosclerosis, coronary heart disease and stroke [5-8]. In addition, insulin resistance is associated with decreased nitric oxide (NO) production due to inhibition of endothelial nitric oxide synthase (eNOS) via impaired phosphatidylinositol 3 kinase (PI3K) – AKT (protein kinase B) pathway [9]. The decreased NO production causes an imbalance in the vascular endothelial tone which triggers vasoconstriction followed by increased heart rate and high blood pressure [9,10]. Prediabetes is an asymptomatic and intermediate hyperglycaemic stage that has been reported to precede the onset of cardiovascular complications observed in T2DM [8,11,12]. Additionally, previous studies have shown that intermediate hyperglycaemia below the level used to define diabetes mellitus is a risk factor for CVD development [13,14].

The combination of dietary modification with pharmacotherapy is the main approach in preventing the development of CVD in prediabetic or diabetic individuals [6,15]. However, there has been reported low compliance to this combination therapy as most patients only observe pharmacological intervention without changing their diet [16]. This inadvertently reduces the efficacy of the pharmacological interventions [17]. Therefore, anti-diabetic agents that could restore glucose homeostasis and prevent the risk of CVD development regardless of diet intervention are necessary.

Pentacyclic triterpenes such as oleanolic acid, maslinic acid are anti-diabetic and antioxidant agents with proofs and literature evidence [18,19]. More importantly, bredemolic acid (BA), an isomer of maslinic acid, has been shown in previous study to have anti-diabetic effects by reduction of blood glucose through increased expression of GLUT 4 in skeletal muscle of prediabetic rats [20]. However, the effects of this triterpene on the cardiovascular system in prediabetes have not been established. Therefore, the aim of this study was to investigate the effect of BA on selected markers of cardiovascular function in a diet-induced prediabetic rat model.

2. Materials and Methods

2.1. *Animals*

In this study, thirty six (36) male Sprague Dawley rats with body weight 150–180g were used. The rats were obtained and bred at the Biomedical Research Unit (BRU), University of KwaZulu-Natal (UKZN). The animals were kept and maintained in a standard experimental condition at room temperature (22±2°C), humidity (55±5%) and 12h day:12h night cycle. The animals consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* for 2 weeks to acclimatize before being exposed to the experimental diet (high fat high carbohydrate). The high fat high carbohydrate (HFHC) diet composed of carbohydrate (55% Kcal/g), fats (30% Kcal/g) and proteins (15% Kcal/g). All experimental procedures were according to the ethics and animal care guidelines of the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa (AREC/024/018D)

2.2. *Experimental Design*

After 2 weeks of acclimatization, the animals were distributed into two main groups: the non-prediabetic control group (n=6) and the prediabetic group (n=30). The non-prediabetic control (NPC) animals served as the negative control and were given normal diet (ND) and water *ad libitum* while the prediabetic animals were given HFHC diet and drinking water supplemented with fructose (15%) for 20 weeks to induce prediabetes. After 20 weeks, prediabetes was confirmed via fasting blood glucose and oral glucose tolerance test using criteria of the American Diabetes Association, as described in our previous study [20].

2.3. *Treatment of Animals*

After the 20 weeks of prediabetes induction, the non-prediabetic control (Group 1) continuously fed on ND for a further 12 weeks while the prediabetic animals (n=30) were divided into 5 groups (Group 2 – Group 6, n=6 in each group). Group 2 (PD) served as the untreated prediabetic control group and continuously consumed the HFHC diet for 12 weeks; Group 3 (ND+MET) were prediabetic animals that switched to standard rat chow and received MET for 12 weeks; Group 4 (HFHC+MET) were prediabetic animals that continuously consumed HFHC diet with MET treatment; Group 5 (ND+BA) were prediabetic animals that switched to standard rat chow and received BA for 12 weeks; Group 6 (HFHC+BA) were prediabetic animals that continuously consumed HFHC diet and received BA as treatment for 12 weeks. Treatment with either MET (500mg/kg) or BA (80mg/kg) was carried out twice every third day for 12 weeks. The body mass index (BMI), waist circumference (WC), blood pressure and heart rate were assessed in all animals at week 20 and every 4 weeks (24^{th}, 28^{th} and 32^{nd} week).

2.4. *Blood Collection and Tissue Harvesting*

After the 12 weeks of treatment, the animals were sacrificed. The animals were placed in a gas chamber (BRU, UKZN, South Africa) and anaesthetised with 100 mg/kg of Isofor (Safeline Pharmaceuticals Ltd, Roodeport, South Africa) for 3 minutes to collect blood samples. In an unconscious state, blood samples were collected by cardiac puncture into pre-cooled heparinized containers. The blood samples

were centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes for plasma collection. The plasma was collected and stored at -80°C in a Bio Ultra freezer (Snijers Scientific, Tilburg, Holland). The hearts of all the animals were excised, rinsed with cold normal saline solution, weighed and snapped frozen in liquid nitrogen before storage in Bio Ultra freezer at -80°C for biochemical analysis.

2.5. *Determination of BMI and WC*

The determination of BMI was measured from the ratio of the weight to the square of the length of the animals as described in the established protocol [21]. Also, the waist circumference of the animals was determined according to the previous protocol [22].

2.6. *Determination of Blood Pressure and Heart Rate*

The blood pressure and heart rate were measured as described in the established protocol [19]. Briefly, at every 4 weeks of treatment, the non-invasive MRBP IITC Model 31, Life Sciences multichannel tail-cuff blood pressure system (Life Sciences, Woodland Hills, CA) was used to monitor the blood pressure and the heart rate by placing the animals in a restrainer (3" ID (75 mm) - 12" Length) while the tail of the animals is attached to the tail-cuff. All the rats in the restrainer were placed in a warming chamber (IITC Model 303sc Animal Test Chamber, Life Sciences, Woodland Hills, CA) maintained at 32^{o}C, and the blood pressure, as well as the heart rate, was measured by occlusion or deflation of the tail-cuff which detect alteration of blood flow in the tail artery. An average of three measured sessions consisting of 15 cycles was used for statistical analysis.

2.7. *Biochemical Analysis*

The lipid profile, antioxidant, inflammatory and endothelial markers were measured at 32nd week only.

2.8. *Lipid Profile Analysis*

The plasma total cholesterol (TC), high-density lipoprotein (HDL) cholesterol and triglycerides (TG) were analysed via Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW Germany) by using specialized commercial kits according to the instruction from the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The other lipid profiles such as very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol were calculated according to Friedewald's formula [23]. VLDL cholesterol = TG x 0.2, and LDL cholesterol = TC – (VLDL cholesterol + HDL cholesterol).

2.9. *MDA and Antioxidant Status*

The lipid peroxidation was determined by estimation of the amount of malondialdehyde (MDA) in the heart tissue homogenate according to previously described protocols [19,24]. However, the antioxidant status of the heart homogenates was determined by using specific ELISA kit to analyse the concentration of superoxide dismutase (SOD) and glutathione peroxidase (GPx) according to the instruction manual of the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

2.10. *Determination of Endothelial Function and Inflammatory Markers*

The endothelial function and inflammation were evaluated from the plasma by determination of the endothelial nitric oxide synthase (eNOS) through the commercialized ELISA kit in accordance to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The inflammatory markers (TNF-α, IL-6 and hs-CRP) were measured in the plasma via specific ELISA kits in accordance to the manufacturer's instruction (Elabscience Biotechnology Co., Ltd., Houston, TX, USA), and the absorbance was measured via the microplate reader, Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW, Germany).

2.11. *Statistical Analysis*

The data were presented as mean ± standard error of mean (SEM). Statistical analysis was determined by two-way Analysis of Variance (ANOVA) followed by Bonferroni test as post hoc via GraphPad Prism 5 software. The level of statistically significant difference was considered from $p<0.05$ and above.

3. Results

3.1. *Body Mass Index (BMI) and Waist Circumference (WC)*

The effects of BA treatment on BMI and WC in non-prediabetic and prediabetic rats with or without diet intervention were determined as indicated in Figure 1 and Figure 2. The BMI and WC of the untreated prediabetic (PD) rats were significantly increased by comparison to the non-prediabetic (NPD) control rats throughout the treatment period ($p<0.001$). However, the administration of BA with or without diet intervention significantly decreased both BMI and WC when compared to the PD group, as shown in Figure 1 and Figure 2, respectively ($p<0.01$).

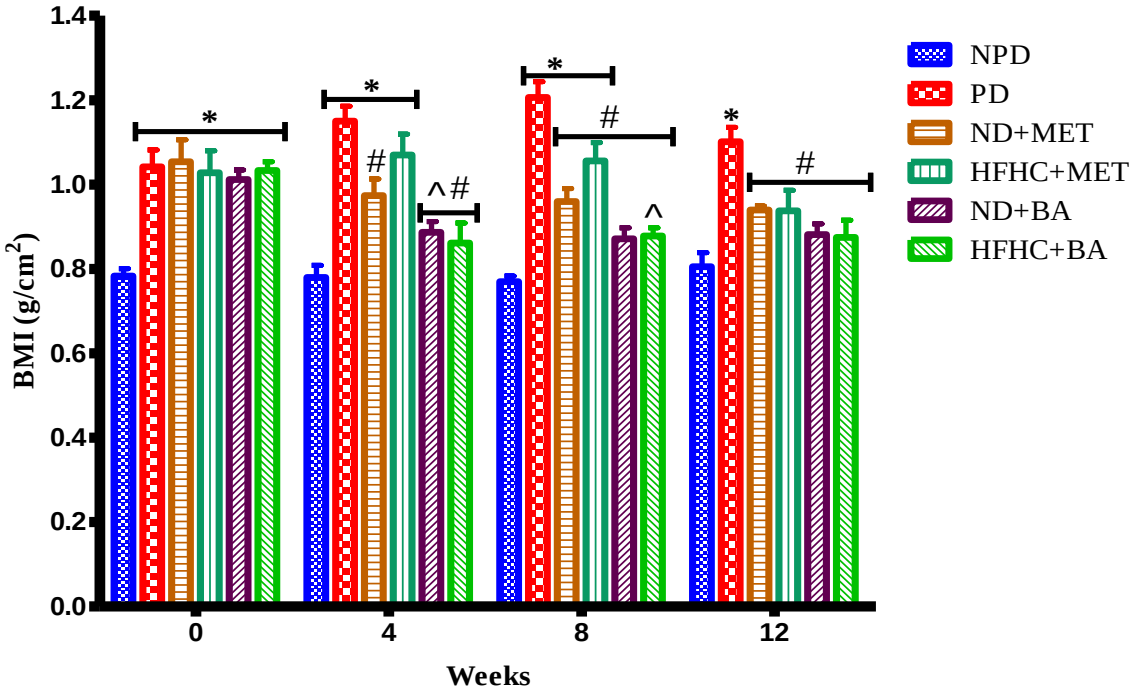

Figure 1: Effects of BA on BMI in non-prediabetic (NPD) and prediabetic rats with or without diet intervention

*p<0.001 in comparison to NPD, #p<0.01 in comparison to PD, ^p<0.01 in comparison to HFHC + MET

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

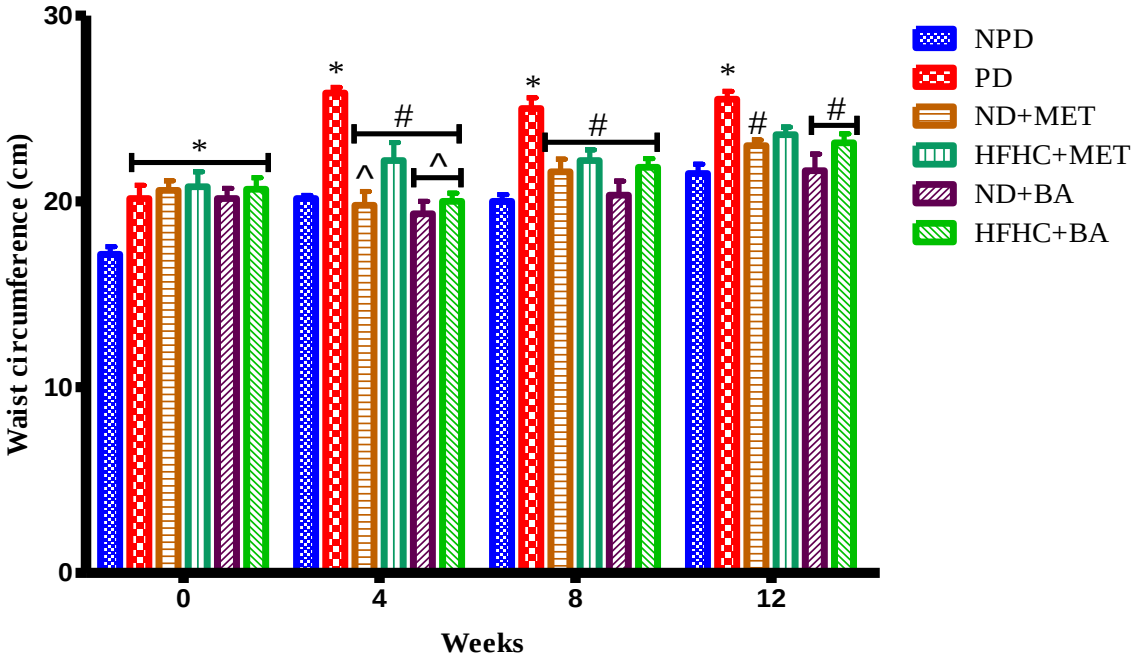

Figure 2: Effects of BA on waist circumference in non-prediabetic and prediabetic rats with or without diet intervention

*p<0.001 in comparison to NPD, #p<0.05 in comparison to PD, ^p<0.05 in comparison to HFHC + MET

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.2. *Blood Pressure and Heart Rate*

As shown in Figure 3, the systolic blood pressure of PD control rats was significantly increased throughout the treatment period when compared to the NPD control rats ($p<0.001$). However, the systolic blood pressure of BA treated rats with or without diet intervention significantly decreased when compared to that of PD control rats. As demonstrated in Figure 4, the diastolic blood pressure of PD control rats was significantly increased when compared to NPD control rats ($p<0.001$). The administration of BA with or without diet intervention significantly decreased the diastolic blood pressure when compared to the PD group ($p<0.05$). The same results were observed with the ND+MET group. A significant increase in heart rate was observed in the PD rats throughout the period of treatment when compared to the NPD control rats as indicated in Figure 5 ($p<0.01$). However, the heart rate of BA treated rats with or without diet intervention and MET treated rats with diet intervention (ND+MET) were significantly lowered by comparison to the PD control rats ($p<0.01$).

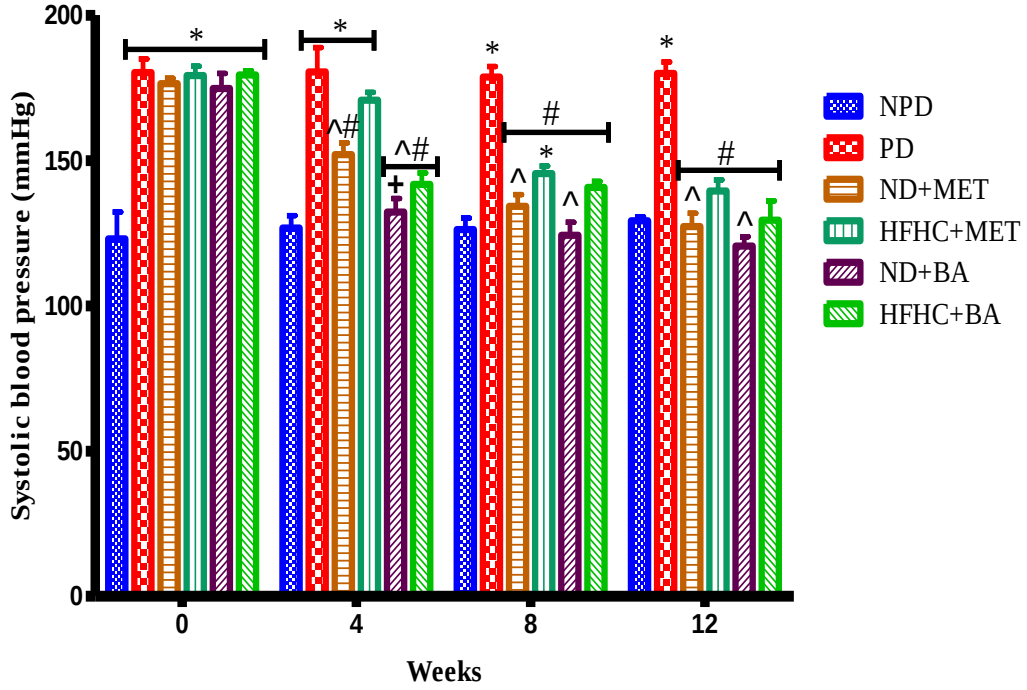

Figure 3: Effects of BA on systolic blood pressure in non-prediabetic and prediabetic rats with or without diet intervention

*$p<0.001$ in comparison to NPD, $^{\#}p<0.001$ in comparison to PD, ^$p<0.01$ in comparison to HFHC + MET, $^{+}p<0.01$ in comparison to ND + MET

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

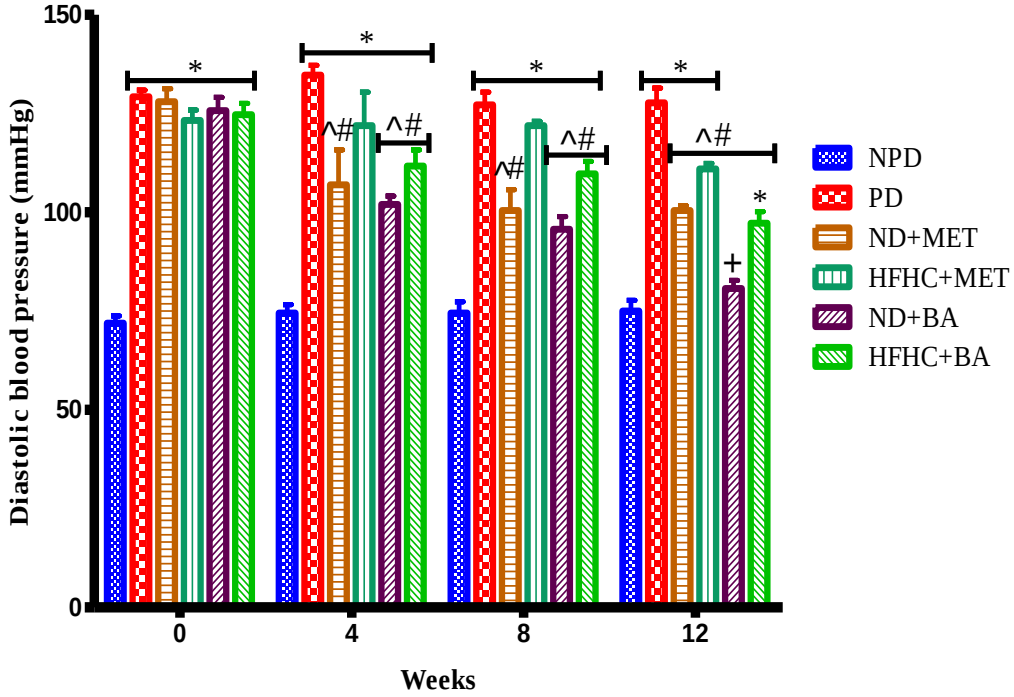

Figure 4: Effects of BA on diastolic blood pressure in non-prediabetic (NPD) and prediabetic rats with or without diet intervention

*p<0.001 in comparison to NPD, #p<0.01 in comparison to PD, ^p<0.01 in comparison to HFHC + MET, +p<0.01 in comparison to ND + MET

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

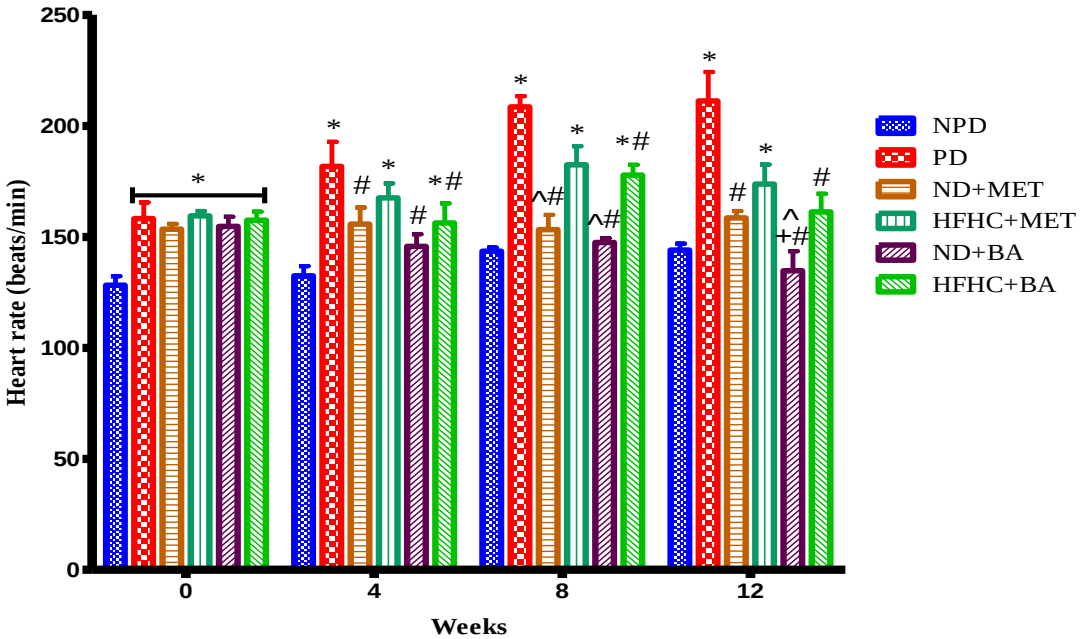

Figure 5: Effects of BA on heart rate in non-prediabetic (NPD) and prediabetic rats with or without diet intervention

*p<0.001 in comparison to NPD, #p<0.01 in comparison to PD, ^p<0.01 in comparison to HFHC + MET, +p<0.05 in comparison to ND + MET

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.3. *Lipid Profile*

As shown in Table 1, the TC, TG, LDL and VLDL of the untreated PD group significantly increased in comparison to the NPD group ($p<0.001$). The TC and LDL of BA-treated rats with or without diet intervention were significantly decreased when compared to the PD control rats ($p<0.01$). Similar results were obtained for the ND+MET group. Additionally, only the ND+BA and ND+MET groups had significantly lowered TG and VLDL when compared to the PD control rats ($p<0.05$).

Table 1: The effects of BA on lipid profile in non-prediabetic and prediabetic rats with or without diet intervention. Values are presented as mean±SEM (n=6)

Groups / **Parameters**	**NPD**	**PD**	**ND+MET**	**HFHC+MET**	**ND+BA**	**HFHC+BA**
TC (mmol/L)	2.00±0.04	2.88±0.03***	2.06±0.03$^{\#\#\#}$	2.43±0.16	2.10±0.09$^{\#\#\#}$	2.25±0.13$^{\#\#}$
TG (mmol/L)	1.12±0.10	1.75±0.02**	1.13±0.03$^{\#\#}$	1.58±0.22	1.18±0.02$^{\#}$	1.45±0.02
HDL (mmol/L)	1.11±0.03	1.04±0.04	1.13±0.04	1.08±0.09	1.16±0.06	1.10±0.05
LDL (mmol/L)	0.67±0.04	1.49±0.05***	0.70±0.06$^{\#\#\#}$	1.03±0.05*$^{\#\#}$	0.71±0.07$^{\#\#\#}$	0.86±0.12$^{\#\#\#}$
VLDL (mmol/L)	0.22±0.02	0.35±0.01**	0.23±0.01$^{\#\#}$	0.32±0.05	0.24±0.01$^{\#}$	0.29±0.01

*$p<0.05$, **$p<0.01$, ***$p<0.001$ (vs. NPD), $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ (vs. PD).

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid, TC: total cholesterol, TG: triglyceride, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low-density lipoprotein

3.4. *Endothelial Function Marker*

The plasma concentration of eNOS in PD control rats significantly decreased when compared to NPD control rats as indicated in Figure 6 ($p<0.001$). However, the plasma concentration of eNOS in BA treated rats with or without diet intervention significantly increased by comparison to the PD control rats ($p<0.01$).

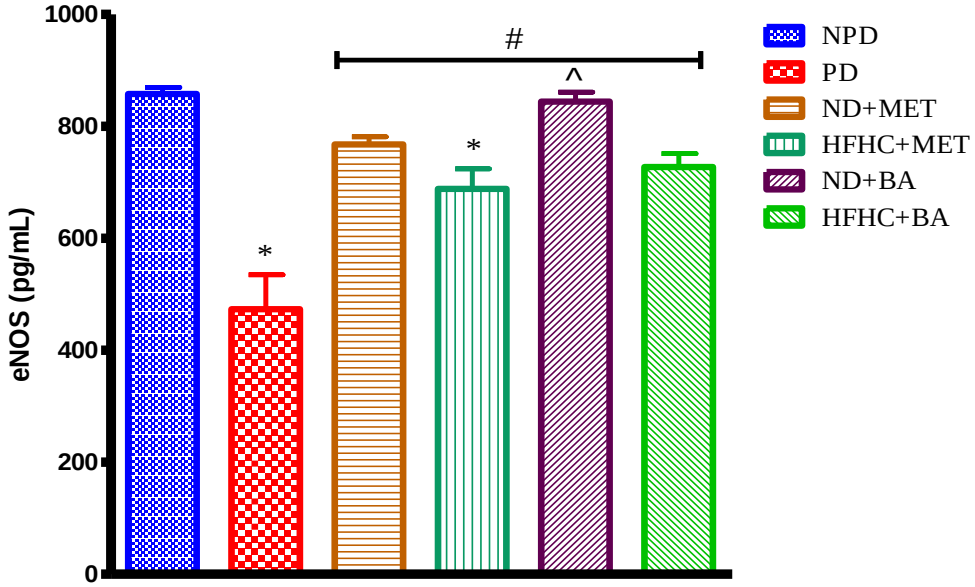

Figure 6: Effects of BA on eNOS concentration in non-prediabetic (NPD) and prediabetic rats with or without diet intervention

*p<0.001 in comparison to NPD, #p<0.001 in comparison to PD, ^p<0.05 in comparison to HFHC + MET

eNOS: endothelial nitric oxide synthase, NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

3.5. *Lipid Peroxidation and Antioxidant Status*

As indicated in Table 2, a significant increase in the heart MDA concentration was observed in the PD groups by comparison to the NPD group ($p<0.01$). Rats treated with BA in the presence and absence of diet intervention had a significantly decreased MDA concentration by comparison to untreated PD rats. However, there was no significant difference in heart MDA concentrations in the HFHC+MET group when compared to PD control rats. The heart SOD and GPx concentration of the PD control rats significantly decreased in comparison to NPD control rats ($p<0.01$). On the other hand, administration of BA with or without diet intervention significantly increased both SOD and GPx concentration in the heart tissue by comparison to the untreated PD group ($p<0.05$).

Table 2: The effects of BA on oxidative stress and inflammatory biomarkers in non-prediabetic and prediabetic rats with or without diet intervention. Values are presented as mean±SEM (n=6)

Groups / Parameters	NPD	PD	ND+MET	HFHC+MET	ND+BA	HFHC+BA
MDA (nmol/g protein)	4.35±0.16	5.78±0.43 **	4.62±0.09 #	5.11±0.24	4.14±0.20 ###	4.53±0.12 #
SOD (ng/mL)	7.00±0.90	1.78±0.23 **	6.74±0.66 ##	6.11±0.88 #	11.43±1.14 *###	10.56±0.90 *###
GPx (pg/mL)	847.52±53.56	245.43±12.29 ***	989.72±129.55 ###	517.99±78.53 *	1001.20±62.37 ###	669.51±40.59 ##
hs-CRP (ng/mL)	1.35±0.06	2.22±0.01 ***	1.53±0.16 ###	1.74±0.15	1.71±0.07 #	1.73±0.07 #
TNF-α (pg/mL)	948.42±30.79	1296.97±7.98 ***	1005.49±19.17 ##	1108±96.11	945.63±13.49 ###	1011.33±17.83 ##
IL-6(pg/mL)	22.20±2.71	37.13±1.14 ***	30.02±1.30	33.95±2.10 **	23.46±2.50 ##	24.06±1.71 ##

*p<0.05, **p<0.01, ***p<0.001 (vs. NPD), #p<0.05, ##p<0.01, ###p<0.001 (vs. PD).

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid, SOD: superoxide dismutase, MDA: malondialdehyde, GPx: glutathione peroxidase, hs-CRP: high sensitive C-reactive protein, TNF-α: tumour necrotic factor alpha, IL-6: interleukin 6

3.6. *Inflammatory Markers*

As shown in Table 2, the plasma concentrations of hs-CRP, TNF-α and IL-6 in the untreated PD group was significantly increased by comparison to the NPD control group ($p<0.001$). However, the administration of BA with or without diet intervention significantly decreased the concentration of these markers by comparison to the PD group. Similar results were obtained for the ND+MET group.

4. Discussion

This study was designed to investigate the effects of bredemolic acid on cardiovascular function risk factors, endothelial function, oxidative stress and proinflammatory markers in diet-induced prediabetes. High caloric diets have been implicated with prediabetes which has been associated with endothelial dysfunction, reactive oxygen species (ROS) and inflammatory cytokine production [5, 6]. Studies indicate that chronic consumption of high caloric diets promote excess adiposity which results in high BMI, high waist circumference and hyperlipidaemia [3, 25]. These have all been identified as risk factors for developing insulin resistance, impaired glucose metabolism and cardiovascular diseases during the prediabetic stage [26-28]. In addition, previous researchers have also shown that the risk of

developing diabetes and its associated cardiovascular diseases rises as body fat, BMI and waist circumference increase [25, 27]. Our results showed that induction of prediabetes through chronic ingestion of a high fat high carbohydrate diet significantly increased BMI and waist circumference in the untreated prediabetic rats. We suggest that the increased BMI and waist circumference can be attributed to increased caloric intake as we have reported in our previous study [20]. Conversely, the administrations of BA significantly reduced the BMI and waist circumference in BA treated prediabetic rats with or without diet intervention. In a previous study, we reported that BA administration significantly decreased food intake through reduced plasma ghrelin concentrations and improved insulin sensitivity [20]. Therefore, in this study, we suggest that the decreased BMI and waist circumference in BA treated prediabetic rats may be due to the decreased food intake and decreased body weight gain.

Moreover, consumption of high caloric diet has been associated with increased delivery of free fatty acid (FFA) to the liver [29]. The increased delivery of FFA leads to increased hepatic and plasma TG concentrations as well as increased export of TG as VLDL from the liver [29, 30]. The VLDL is, in turn, converted into atherogenic LDL with low clearance. Consequently, due to the increased conversion of TG to VLDL, HDL clearance increases and results in decreased plasma HDL concentration [31, 32].

Similarly, in this study, consumption of high caloric diet probably caused increased delivery of FFA to the liver with a subsequent significant increase in plasma concentrations of TC, TG, LDL and VLDL as well as a slight decrease in the HDL concentration in the untreated prediabetic rats. However, we suggest that even though the HDL concentration slightly decreased, the clearance of HDL as a result of increased VLDL formation remains unaffected in this study. Hence, this abnormal lipid profile showed that the risk of developing dyslipidaemia and other cardiovascular complications begins during the prediabetic stage [11]. On the other hand, the administration of BA significantly normalized the TC, TG, LDL and VLDL levels in BA treated prediabetic rats with or without diet intervention. In our previous study, BA was reported to inhibit caloric intake and decrease body weight gain, and this may contribute to the observed normal lipid profile in the BA treated rats [20].

High caloric diets have also been reported to result in glucolipotoxicity which in turn triggers mitochondrial overproduction of reactive oxygen species (ROS) due to impairment of mitochondrial electron transport chain activity [4, 33]. The mitochondrial overproduction of ROS leads to oxidative stress which further leads to an impaired balance between the production of ROS and antioxidant enzymes [3, 34]. MDA and antioxidant enzymes (SOD and GPx) are markers for lipid peroxidation and antioxidant capacity in the cells or tissues, respectively. Indeed, in this study, MDA concentrations significantly increased while SOD and GPx concentrations significantly decreased in the hearts of untreated prediabetic rats. These results correlated with research done by Lozano et al. [3] which showed a positive correlation between the consumption of high caloric diets and increased lipid peroxidation. On the other hand, we observed that the administration of BA significantly reduced the

heart lipid peroxidation activity and significantly increased the heart antioxidant capacity of BA treated prediabetic rats. This biological effect of BA on the oxidative stress markers correlated with the earlier reports that triterpenes are antioxidant agents which neutralize free radicals in the mitochondria by donation of electrons due to the presence of hydroxyl radical in their structures [19]. Similarly, we speculate that BA attenuated oxidative stress by neutralizing free radicals through electron donation capacity of its hydroxyl radicals and improved antioxidant activity by the promotion of antioxidant enzymes production. This antioxidant property of BA has also been reported in other triterpenes such as maslinic acid, oleanolic acid and ursolic acid [19, 35].

Studies indicate that intermediate hyperglycaemia and oxidative stress alter endothelial cell function and contribute to cardiovascular diseases during prediabetic stage [33, 36, 37]. Intermediate hyperglycaemia has been linked to oxidative stress through the activation of protein kinase C (PKC) which in turn enhances nicotinamide adenine dinucleotide phosphate (NADPH) oxidase action [8, 38]. Activation of PKC alters vascular homeostasis and decreases nitric oxide (NO) production via inhibition of eNOS [1, 12]. As a result of the decreased NO production, vascular changes that result in vasoconstriction with subsequent increase in blood pressure, heart rate and arteriosclerotic processes occur [1]. In this study, we observed that the eNOS concentration significantly decreased with concomitant increases in heart rate, systolic and diastolic blood pressure in the untreated prediabetic rats when compared to non-prediabetic control rats. The increased heart rate, systolic and diastolic blood pressure can be attributed to vasoconstriction of vascular endothelium due to decreased eNOS activity that results in decreased NO production which has been reported in prediabetes [6]. The results of this study further showed that the administration of BA significantly increased the eNOS concentration and ameliorated heart rate, systolic and diastolic blood pressure in both the presence and absence of diet intervention. In accordance with a similar study, we suggest that the administration of BA which ameliorated oxidative stress, contributed to the increased eNOS concentration in the BA treated rats [39]. Increased eNOS concentration in turn leads to increase in NO production which further leads to vasodilation with subsequent significant decrease in heart rate and blood pressure when compared to untreated prediabetic rats.

Furthermore, increased blood glucose has been reported to result in the formation of advanced glycation product (AGE) [40]. Formation of AGEs increases the expression of adhesion molecules on vascular endothelial cells and subsequently promotes migration of monocytes to form macrophages [1, 41]. Stimulation of the monocytes by AGEs leads to low-grade inflammation with increased production of cytokines (such as IL-6, TNF-α and hs-CRP) [4, 41]. However, literature has reported that increased levels of proinflammatory cytokines are associated with prediabetes [12, 14]. Similarly, in this study, the plasma concentration of IL-6, TNF-α and hs-CRP significantly increased in untreated prediabetic rats. The elevated proinflammatory cytokines are inflammatory responses that alter vascular endothelium and result in endothelial dysfunction during prediabetic stage [12, 42]. The hs-CRP is not

just a pro-inflammatory cytokine but a biomarker for injured heart caused by coronary heart disease or ischemic heart disease [43].

The observed increase in plasma hs-CRP concentrations in untreated prediabetic rats in this study indicated the risk of developing cardiovascular diseases during the prediabetic stage. These results correlated with other studies which reported that plasma hs-CRP concentration and other pro-inflammatory cytokines were significantly increased in prediabetic condition [44, 45] Additionally, BA administration significantly decreased the pro-inflammatory cytokines such as hs-CRP, IL-6 and TNF-α in prediabetic rats with or without diet intervention. The decreased in the plasma proinflammatory cytokines concentration can be suggested to be due to the anti-inflammatory property that has been previously attributed to pentacyclic triterpenes [19, 35]. Pentacyclic triterpenes (such as maslinic acid, oleanolic acid) have been reported to have low pharmacokinetic activity of 3 days without any side effects [35, 46, 47]. Therefore, as a result of the low pharmacokinetic activity exhibited by the pentacyclic triterpenes, the biological effects of BA last longer and sustainably remain active than synthetic drugs. However, we suggest that the sustained biological activities of BA probably compensated for the ameliorated cardiovascular functions in the prediabetic rats even in the absence of diet intervention.

5. Conclusion

The findings of this study suggest that the administration of BA in both the presence and absence of diet intervention attenuated inflammation and oxidative stress, as well as improved cardiovascular and endothelial functions which are impaired in diet-induced prediabetes. More studies are, however, required to investigate the molecular mechanisms by which this triterpene exerts its biological effects.

Data Availability

The data used in this study to support our findings are available upon request from the corresponding author. However, the data on body weight, food intake, as well as fasting blood glucose and oral glucose tolerance test for confirmation of prediabetes are reported in our previous study.

Conflicts of Interest

The authors declare no conflicts of interest

Funding Statement

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PROLOGUE

It has been demonstrated in several studies that chronic consumption of high caloric diets results in renal complications not only in overt diabetes but in the prediabetic stage. Currently, the management of renal complications or dysfunctions during the prediabetes stage is via the combination of dietary and pharmacological interventions. However, the efficacy of the pharmacological intervention has been reported to be reduced due to low compliance from patients in following a recommended diet modification. In chapter 4 of this study, we observed dyslipidaemia, increased blood pressure, increased heart rate and decreased eNOS plasma concentration which depicted cardiovascular dysfunction in the untreated prediabetic rats. Also, the results showed that oxidative stress and plasma concentration of inflammatory cytokines are significantly increased in the insulin-resistant state. The administration of BA significantly ameliorated the aforementioned markers of cardiovascular dysfunctions, however, the effects of BA on renal functions have not been established in a prediabetic state. Therefore, in Chapter 5 of this study, we investigated the effects of BA on selected markers of renal dysfunction in a prediabetic rat model in both the presence and absence of dietary intervention. The chapter was written and prepared in manuscript format. The authors of this manuscript are Akinnuga AM, Siboto A, Khumalo B, Sibiya NH, Ngubane P and Khathi A. This manuscript is in press in the journal, Oxidative Medicine and Cellular Longevity. See Appendix VI.

CHAPTER 5

Ameliorative effects of bredemolic acid on renal dysfunction in a prediabetic rat model

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Abstract

Studies have shown that renal dysfunction does not only occur in overt diabetes but also the preceding stage known as prediabetes. Lifestyle and pharmacological interventions are the approaches to managing prediabetes but the compliance in combining the two interventions is low. Hence, pharmacological intervention efficacy is reduced. In our previous study, bredemolic acid ameliorated glucose homeostasis via increased expression of GLUT 4 in the skeletal muscle of prediabetic rats. However, bredemolic acid effects on renal dysfunction are unknown. Therefore, this study was aimed at investigating the ameliorative effects of bredemolic acid on renal dysfunction in a diet-induced prediabetic rat model. Thirty-six (36) Sprague Dawley male rats (150 – 180g) were divided into two groups: the non-prediabetic (n=6) and the prediabetic groups (n=30) which were fed a normal diet (ND) and high fat high carbohydrate (HFHC) diet respectively for 20 weeks. The prediabetic rats were subdivided into five groups (n=6) and treated with either BA (80 mg/kg) or metformin (MET, 500 mg/kg) for 12 weeks. Blood, urine and kidney samples were collected for biochemical analysis. The untreated prediabetic (PD) rats presented albuminuria, proteinuria, sodium retention, potassium loss, increased aldosterone concentration, increased kidney injury molecule (KIM-1) and increased urinary podocin expression. Also, the PD rats had a significantly increased creatinine, urea and uric acid plasma concentrations, fluid intake and urine output. However, BA administration attenuated the aforementioned renal dysfunction, oxidative stress and decreased podocin mRNA expression in the urine. Conclusively, BA administration regardless of diet modification attenuates renal dysfunction in a prediabetic state.

Introduction

Uncontrolled diabetes mellitus leads to diabetic nephropathy which accounts for about 50% of all end-stage renal disease worldwide (Kowalski *et al.* 2015). More than 25% of type 1 and type 2 diabetes mellitus patients develop diabetic nephropathy (DN) with altered renal function markers such as reduced glomerular filtration rate (GFR), increased serum creatinine and urea, albuminuria as well as increased excretion of kidney injury molecule (KIM-1) (Powell *et al.* 2013, Lopez-Giacoman & Madero, 2015). Studies have shown that the risk of developing structural and functional changes in diabetic nephropathy does not only occur in overt diabetes but in the early stages of impaired glucose metabolism (Mac-Moune Lai *et al.* 2004, Melsom *et al.* 2016, De Nicola *et al.* 2016). Moreover, previous studies have demonstrated that persistent hyperglycaemia activates oxidative stress and the renin-angiotensin-aldosterone system (RAAS) which leads to stimulation of renal cell proliferation, expression of growth factors such as transforming growth factor (TGFβ) as well as inflammatory cytokines such as tumour necrosis factor (TNFα) and interleukin-6 (IL-6) (Schrijvers *et al.* 2004, Chawla *et al.* 2010, Chou & Fang, 2010, Luther & Brown, 2011, Jaikumkao *et al.* 2017). Furthermore, the activation of RAAS triggers the release of aldosterone which stimulates serum/glucocorticoid-regulated kinase 1 (SGK1) that regulate epithelial sodium channel (ENaC) and consequently lead to increased sodium retention and potassium loss in type 2 diabetes mellitus (Lang *et al.* 2009, Artunc *et al.* 2016). Literature evidence showed that about one-third of individuals with newly diagnosed diabetes mellitus have varying degrees of renal dysfunction (Echouffo-Tcheugui *et al.* 2016). This can be attributed to the abnormal changes that occur during prediabetes. The prediabetic stage often precedes the onset of type 2 diabetes mellitus and it is characterized by fasting blood glucose concentration that is higher than normal but below the threshold for diagnosis of diabetes mellitus (Tabák *et al.* 2012). Cross-sectional clinical studies have confirmed that prediabetes is associated with glomerular hyperfiltration as well as the onset of chronic kidney disease (CKD) (Okada *et al.* 2012, De Nicola *et al.* 2016). Therefore, the screening of markers of kidney function during the prediabetic state offers an early window of opportunity of preventing and managing CKD (Echouffo-Tcheugui *et al.* 2016). More importantly, lifestyle modification such as diet intervention and increased physical activity as well as a pharmacological intervention have been reported as the two major ways of managing prediabetes (Ramachandran *et al.* 2006, Salas-Salvadó *et al.* 2014, Ley *et al.* 2014). However, the compliance of combining the two interventions is low as several patients only make use of pharmacological intervention without diet intervention, and consequently, the efficacy of the pharmacological intervention is reduced (Ramachandran *et al.* 2009, Gamede *et al.* 2018). Therefore, anti-diabetic agents that ameliorate CKD or DN regardless of diet intervention are considered necessary.

Studies in our laboratory have indicated that pentacyclic triterpenes such as oleanolic acid, maslinic acid and ursolic acid ameliorate renal dysfunction in streptozotocin-induced diabetes mellitus (Ngubane *et al.* 2011, Mkhwanazi *et al.* 2014). Recently, in our study, bredemolic acid, was reported to regulate blood glucose concentration in the prediabetic state via increased expression of GLUT 4 in the skeletal

muscle (Akinnuga *et al.* 2019). However, the biological effects of bredemolic acid on renal dysfunction associated with prediabetes are yet to be known. Therefore, this study sought to investigate the effects of bredemolic acid on selected markers for renal function in a high fat high carbohydrate diet-induced prediabetic rat model.

Materials and Methods

Animals

Thirty-six (36) male Sprague Dawley rats with body weight 150–180g were used for this study. The rats were obtained from the Biomedical Research Unit (BRU), University of KwaZulu-Natal (UKZN). The animals were kept and maintained in a standard animal facility under controlled environmental conditions at room temperature (22±2°C), humidity (55±5%) and 12h day:12h night cycle. The animals consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* for 2 weeks to acclimatize before being exposed to the experimental diet (high–fat high–carbohydrate). The components of the high–fat high–carbohydrate (HFHC) diet are carbohydrate (55% Kcal/g), fats (30% Kcal/g) and proteins (15% Kcal/g). All experimental procedures in this study were carried out in absolute compliance with the ethics and animal care guidelines of the Animal Research Ethics Committee (AREC, ethics no: AREC/024/018D) of the UKZN, Durban, South Africa.

Experimental design

After acclimatization, the animals were divided into 2 main groups: the non-prediabetic control group (n=6) and the prediabetic group (n=30). The non-prediabetic (NPD) control animals (negative control) were given normal diet (ND) and water *ad libitum* for 20 weeks while the prediabetic animals were given HFHC diet and drinking water supplemented with fructose (15%) for 20 weeks to induce prediabetes. At 20^{th} week, prediabetes was confirmed via assessment of fasting blood glucose and oral glucose tolerance test (OGTT) as described in our previous study (Akinnuga *et al.* 2019).

Treatment of animals

The treatment period lasted for 12 weeks (21^{st} – 32^{nd}). After prediabetes induction, the non-prediabetic control group (Group 1) continuously fed on ND and received diluted dimethyl sulphoxide, DMSO (2 ml DMSO: 19 ml normal saline) as a vehicle for 12 weeks while the prediabetic animals (n=30) were further divided into 5 groups (n=6). All the prediabetic animals continuously fed on either HFHC or ND and were treated with either oral administration of BA (80mg/kg) or metformin (MET, 500mg/kg) every third day for 12 weeks. The prediabetes control group, PD (Group 2) rats were continuously fed on the HFHC diet and received the diluted DMSO (vehicle) for 12 weeks. The ND+MET (Group 3) rats changed the diet to ND and received MET orally, whereas the HFHC+MET (Group 4) rats were continuously fed on the HFHC diet and received MET orally. The ND+BA (Group 5) rats changed the diet to ND and received BA orally while HFHC+BA (Group 6) rats continuously fed on the HFHC diet and were treated with BA. After the 12 weeks of treatment, the animals were sacrificed, blood samples and the kidneys were collected from all the animals for biochemical analysis. The fluid intake and urine volumes were assessed in all the animals at 20^{th} week and every 4 weeks (24^{th}, 28^{th} and 32^{nd} week). The

renal function parameters and other biochemical parameters were measured at the end of the experiment.

Determination of fluid intake and urine output

At 20th week and every 4 weeks thereafter, all the animals in each group were placed in different metabolic cages for 24 hours to measure fluid intake and urine output. The urine samples were measured, centrifuged at 13000 rpm for 5 minutes at 4^{o}C, and the supernatants were stored at -80°C in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) until ready for kidney function parameters analysis.

Blood collection and tissue harvesting

All the animals were placed in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) and anaesthetised with 100 mg/kg of Isofor (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa). In an unconscious state, blood samples were collected from all the animals via cardiac puncture into different pre-cooled EDTA containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany), 503 g for 15 minutes at 4°C to obtain plasma. Thereafter, the plasma samples were aspirated into plain sample bottles and stored in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) at -80°C until ready for biochemical analysis. Also, the kidneys were removed, rinsed with cold normal saline solution, weighed on the weighing balance, snapped frozen in liquid nitrogen and stored at -80°C in a Bio Ultra freezer for biochemical analysis of selected parameters.

Relative kidney weight

The relative kidney weight of all the animals was determined from the ratio of kidney weight to the body weight. Relative kidney weight = $\frac{\text{Kidney weight}}{\text{Body weight}} \times 100$

Biochemical analysis

The biochemical analysis of kidney function parameters (such as creatinine, urea, uric acid, albumin and total protein) and electrolytes (Na^+ and K^+) were determined at 32nd week in the plasma and urine samples by using their respective assay kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) as instructed by the manufacturer. However, the kidney injury molecule (KIM-1) and aldosterone plasma concentrations were determined from their specific ELISA kits as instructed by the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) via the microplate reader, Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW, Germany)

Determination of GFR

The GFR of all the animals were determined at 32nd week of the experiment from the estimation of creatinine in the plasma and urine (creatinine clearance) as follows:

$$\text{GFR [mL/min]} = \frac{\text{Urine creatinine (mg/dl)} \times \text{24 hrs urine volume}}{\text{Plasma creatinine (mg/dl)} \times \text{60 min} \times \text{24 hrs}}$$

Lipid peroxidation and antioxidant status

The lipid peroxidation was assessed by determination of the concentration of malondialdehyde (MDA) in the kidney homogenized tissue according to the previously established protocol (Mkhwanazi *et al.* 2014). However, the antioxidant status of the kidney homogenate was assessed by determination of the concentration of superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant capacity (TOAC) by using their specific ELISA kits according to the instruction of the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

Urine RNA isolation

RNA was isolated from urine (4 ml) by using ZR Urine RNA Isolation Kit™ (Zymo Research Corp, Irvine, USA) according to the manufacturer's protocol. The purity of the RNA was confirmed by the relative absorbance of ratio 260/280 nm via Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Urine RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) by using iScript™ cDNA Synthesis Kit (Bio Rad, California, USA) through incubation in a thermal cycler (SimpliAmp Thermal Cycler, Applied biosystems, Life technologies).

Urine complementary DNA (cDNA) synthesis

For cDNA synthesis, urine RNA (2 µL) was mixed with 5X iScript reaction (4 µL), iScript reverse transcriptase enzyme (1µL) (Bio Rad, USA) and nuclease-free water to a final volume of 20 µL. The mixture was incubated in the thermal cycler (SimpliAmp Thermal Cycler, Applied biosystems, Life technologies) at 25ºC for 5 minutes, 42ºC for 30 minutes and finally at 85ºC for 5 minutes. Thereafter, the synthesized cDNA was stored at -80°C until use for real-time PCR (Polymerase chain reaction).

Real-time PCR

The urinary mRNA level of podocin was quantified by real-time PCR lightcycler (Roche LightCycler 96, USA). cDNA template (2 µL), SYBR Green PCR master mix (5 µL) (Bio Rad, USA), podocin forward primer (1 µL), podocin reverse primer (1 µL) and nuclease-free water were mixed to a final volume of 10 µL. Thereafter, the sample mixtures were cycled 40 times at 95ºC for 10 seconds, 60ºC for 20 seconds and 72ºC for 20 seconds in the lightcycler (Roche LightCycler 96, USA). All the samples were run in duplicate, and β-actin mRNA levels were used as a housekeeping gene to normalize the podocin mRNA level. The sequences of the used oligonucleotide primers (Metabion International AG, Planegg, Germany) were as followed: *podocin forward 5`-TGG AAG CTG AGG CAC AAA GA-3`, podocin reverse 5`-AGA ATC TCA GCC GCC ATC CT-3`*.

Statistical analysis

The data were presented in mean ± SEM and analysed via a two-way Analysis of Variance (ANOVA) with the Bonferroni test as a post hoc test by using GraphPad Prism 5 software. The results were considered to be statistically significant from $p<0.05$ and above.

Results

Kidney weight

As indicated in Table 1, the relative kidney weight of untreated prediabetes (PD) rats was significantly decreased in comparison to non-prediabetic (NPD) control rats ($p<0.001$). However, with the administration of BA in the presence of diet intervention, there was a significant difference in kidney weight when compared to PD rats ($p<0.05$).

Table 1: The effects of BA on relative kidney weight, lipid peroxidation and antioxidant status in non-prediabetic and prediabetic rats with or without diet intervention. Values are presented as mean±SEM (n=6)

Groups / Parameters	NPD	PD	ND+MET	HFHC+MET	ND+BA	HFHC+BA
Relative kidney weight (%)	0.38±0.01	0.25±0. 01 ***	0.28±0.01	0.29±0.01	0.31±0.01 #	0.28±0.01
MDA (nmol/g protein)	5.10±0.13	7.72±0.41 ***	5.69±0.19 ##	6.75±0.40 **	5.07±0.08 ###	5.63±0.25 ###
SOD (ng/mL)	8.66±0.27	3.14±0.38 ***	9.92±0.52 ###	6.62±0.12 ###	11.45±0.63 *###	8.08±0.81 ###
GPx (pg/mL)	1793.00±42.38	849.27±24.69 ***	1820.11±25.88 ###	1274.50±36.14 ***	1914.21±37.18 ###	1698.61±33.17 ###
TOAC (U/mL)	44.40±2.57	14.80±1.03 ***	31.45±1. 02 * ###	22.14±3. 03 ***	41.31±1.65 ###	24.17±3.10 #***

*p<0.05, **p<0.01, ***p<0.001 (vs. NPD), #p<0.05, ##p<0.01, ###p<0.001 (vs. PD).

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid, SOD: superoxide dismutase, MDA: malondialdehyde, GPx: glutathione peroxidase, TOAC: total antioxidant capacity

Fluid intake and Urine output

As shown in Figure 1, the effects of BA administration on fluid intake and urine output were determined in non-prediabetic and prediabetic rats. The fluid intake and urine output of PD rats were significantly increased in comparison to the NPD control rats throughout the treatment period (0 – 12 weeks) ($p<0.001$). However, administration of BA with or without dietary intervention as well as metformin with diet intervention (ND+MET) significantly decreased the fluid intake and urine output in comparison to the PD rats, especially at 12th week period of treatment as shown in Figure 1 ($p<0.05$).

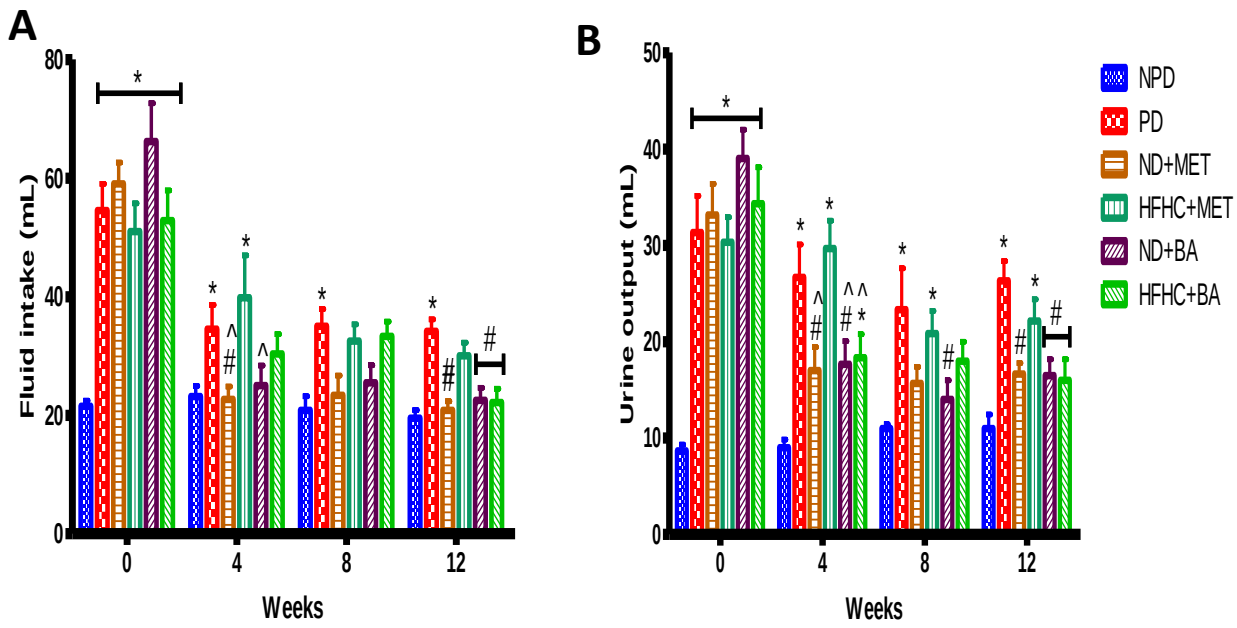

Figure 1: Effects of BA on fluid intake (A) and urine output (B) in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.001 (vs. NPD), #p<0.05 (vs. PD), ^p<0.05 (vs. HFHC+MET).
NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Creatinine and GFR

The plasma concentration of creatinine was significantly increased in PD rats and metformin-treated rats without diet intervention (HFHC+MET) by comparison to the NPD control rats at p<0.05 (Figure 2). However, there was no significant difference between the plasma concentrations of BA treated rats with or without dietary intervention as well as metformin-treated rats with diet intervention in comparison to the NPD control rats. Moreover, the PD rats had significantly decreased urine creatinine when compared to the NPD control rats (Figure 2). Conversely, administration of BA in the absence or presence of diet intervention and metformin in the presence of diet intervention significantly increased the urine creatinine by comparison to the PD rats (p<0.001). The GFR of PD rats, as well as BA and metformin, treated rats without diet intervention significantly decreased when compared to the NPD control rats. On the other hand, the GFR of BA and metformin-treated rats with diet intervention significantly increased by comparison to the PD rats (p<0.05).

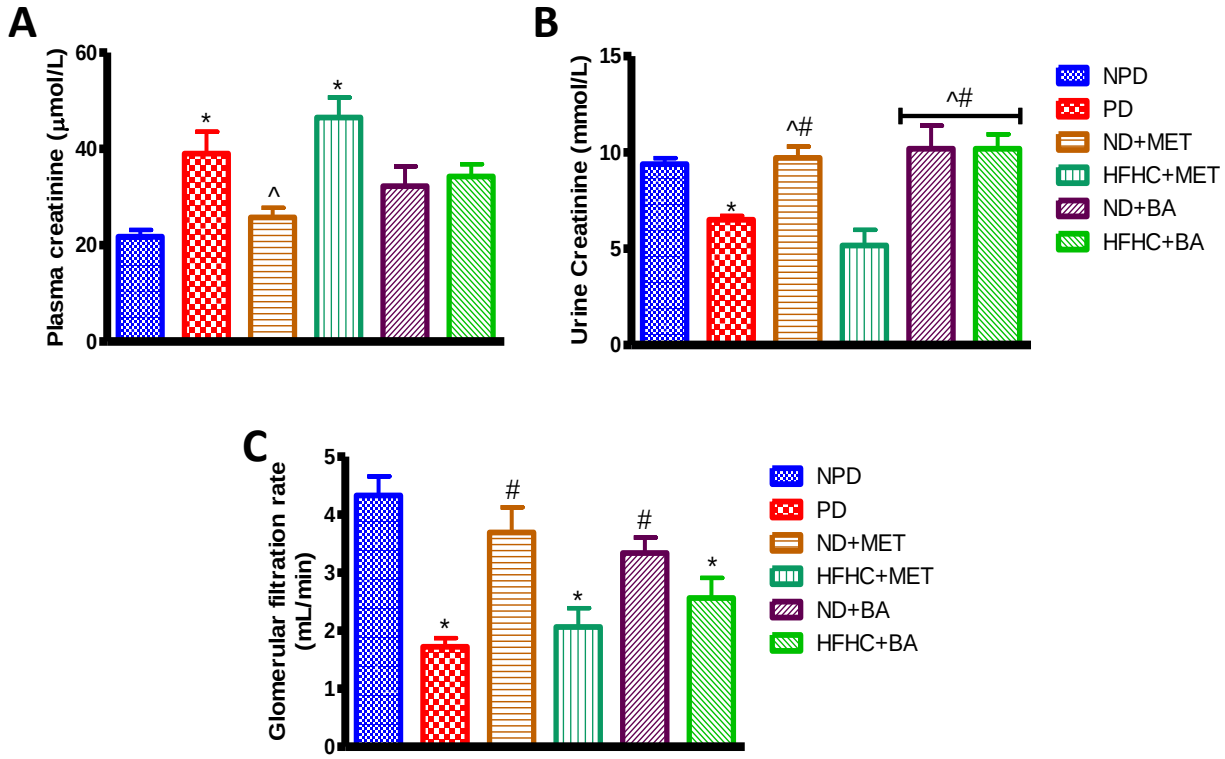

Figure 2: Effects of BA on plasma creatinine (A), urine creatinine (B) and GFR(C) in non-prediabetic and prediabetic rats with or without diet intervention.
*p<0.001 (vs. NPD), #p<0.001 (vs. PD). ^p<0.01(vs. HFHC+MET**).**
NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Urea and Uric acid

As depicted in Figure 3, the plasma concentration of urea and uric acid significantly increased in PD rats when compared to the NPD control rats (p<0.001). Moreover, administration of BA and metformin in the presence of diet intervention significantly decreased the plasma concentration of urea and uric acid by comparison to the PD rats (p<0.001). On the other hand, the urine concentration of urea decreased significantly while the urine concentration of uric acid increased significantly in PD rats by comparison to the NPD control rats as shown in Figure 3. However, there were significant differences in the concentration of urea and uric acid in the urine of BA and metformin-treated rats with diet intervention when compared to the PD rats.

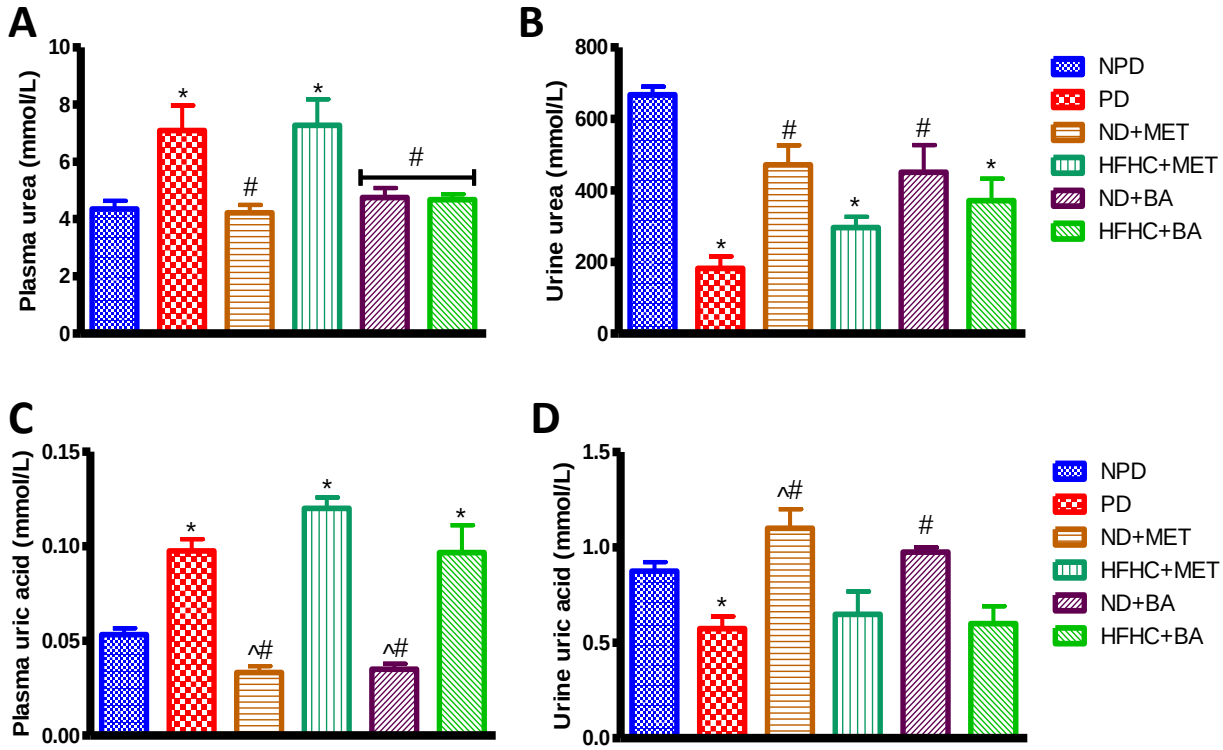

Figure 3: Effects of BA on plasma urea (A), urine urea (B), plasma uric acid (C) and urine uric acid (D) in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.001 (vs. NPD), #p<0.001 (vs. PD), ^p<0.001 (vs. HFHC+MET).
NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Albumin and Total protein

As indicated in Figure 4, the plasma albumin and plasma total protein concentrations of PD rats were significantly decreased by comparison to the NPD control rats ($p<0.001$). The administration of metformin with diet intervention as well as BA with or without dietary intervention significantly increased the plasma albumin and the plasma total protein concentrations when compared to the PD rats. As shown in Figure 4, the urinary albumin and total protein concentrations were significantly increased in PD rats when compared to the NPD control rats ($p<0.05$).In comparison to the PD rats, the BA and metformin-treated rats with diet intervention had a significantly decreased urinary albumin and total protein while the BA treated rats without diet intervention only had a significantly decreased urinary albumin.

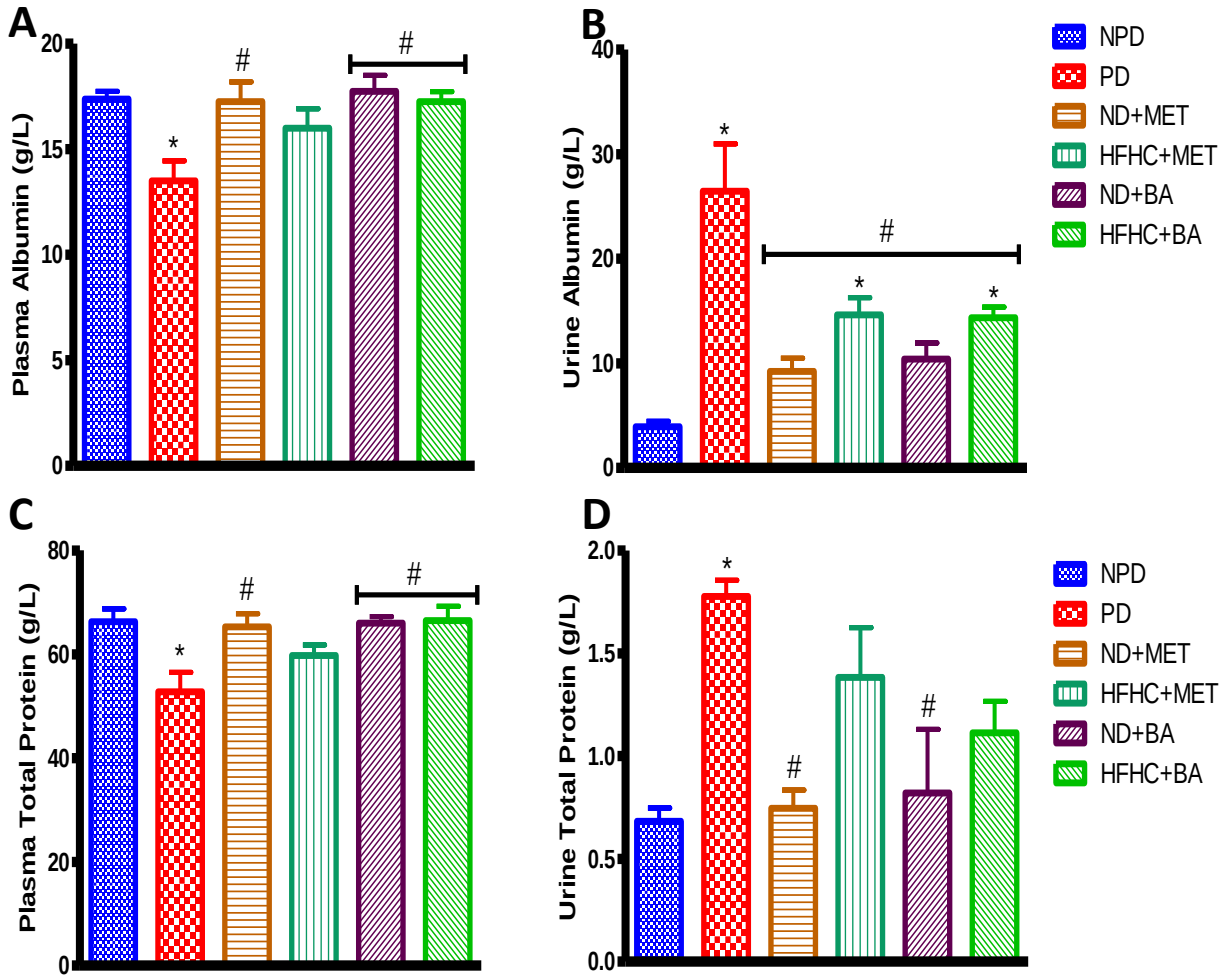

Figure 4: Effects of BA on plasma albumin (A), urine albumin (B), plasma total protein (C) and urine total protein (D) in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.001 (vs. NPD), #p<0.05 (vs. PD).
NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Electrolytes concentration (Sodium and Potassium)

The sodium and potassium plasma concentrations of PD rats were significantly different by comparison to that of the NPD control rats (Figure 5). However, the administration of BA and metformin with diet intervention significantly decreased the plasma sodium concentration and increased the plasma potassium concentration when compared to the PD rats ($p<0.05$). Moreover, the urine sodium and potassium concentrations of PD rats were significantly decreased and increased respectively when compared to the PD rats as indicated in Figure 5. The BA and metformin-treated rats with diet intervention had significantly increased urine sodium and decreased urine potassium by comparison to the PD rats.

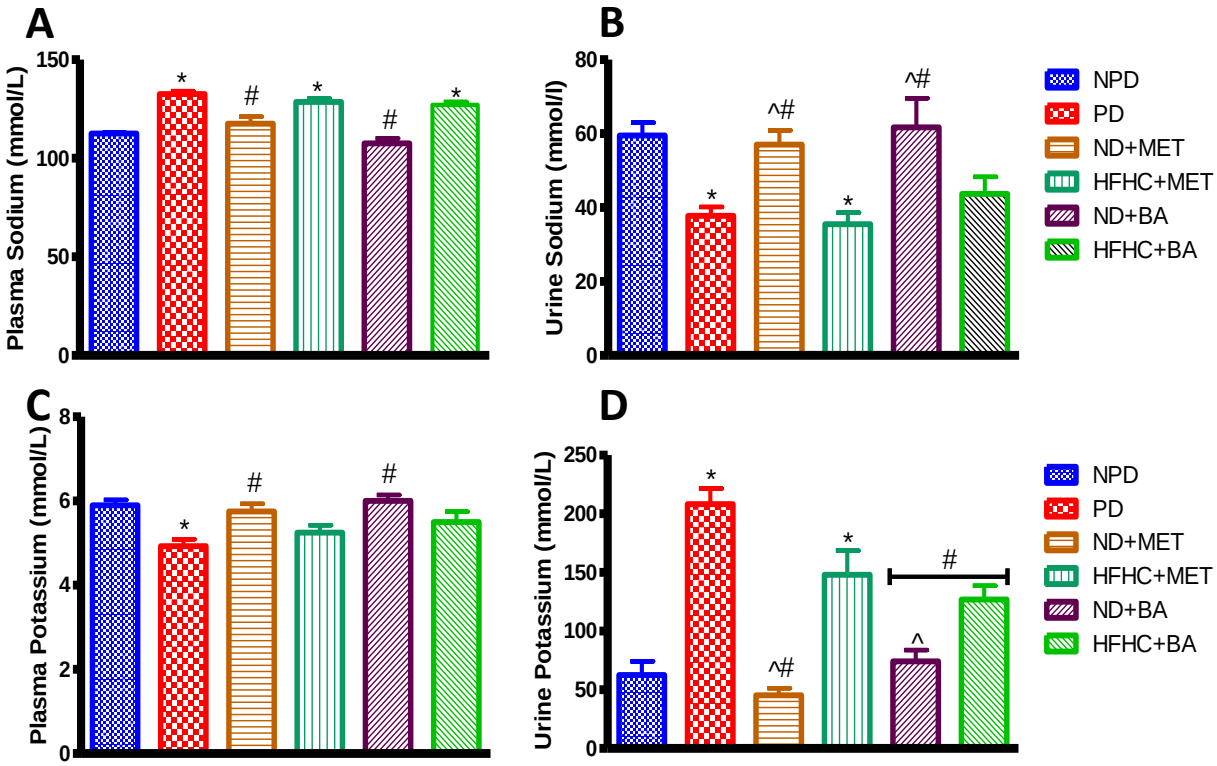

Figure 5: Effects of BA on plasma sodium(A), urine sodium (B), plasma potassium (C) and urine potassium (D) in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.001 (vs. NPD), #p<0.05 (vs. PD), ^p<0.001 (vs. HFHC+MET).
NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Plasma aldosterone

The plasma aldosterone concentration of PD rats was significantly increased when compared to the NPD rats at p<0.001 (Figure 6). Conversely, the aldosterone concentration of BA and metformin-treated rats with or without diet intervention was significantly decreased by comparison to the PD rats (p<0.001).

KIM-1

The KIM-1 plasma concentrations of the untreated PD rats and metformin-treated rats without diet intervention (HFHC+MET) were significantly increased when compared to the NPD control group, as shown in Figure 6 (p<0.001). Also, the KIM-1 plasma concentration of BA treated rats with or without dietary intervention as well as metformin-treated rats with diet intervention (ND+MET) was significantly decreased in comparison to the PD rats.

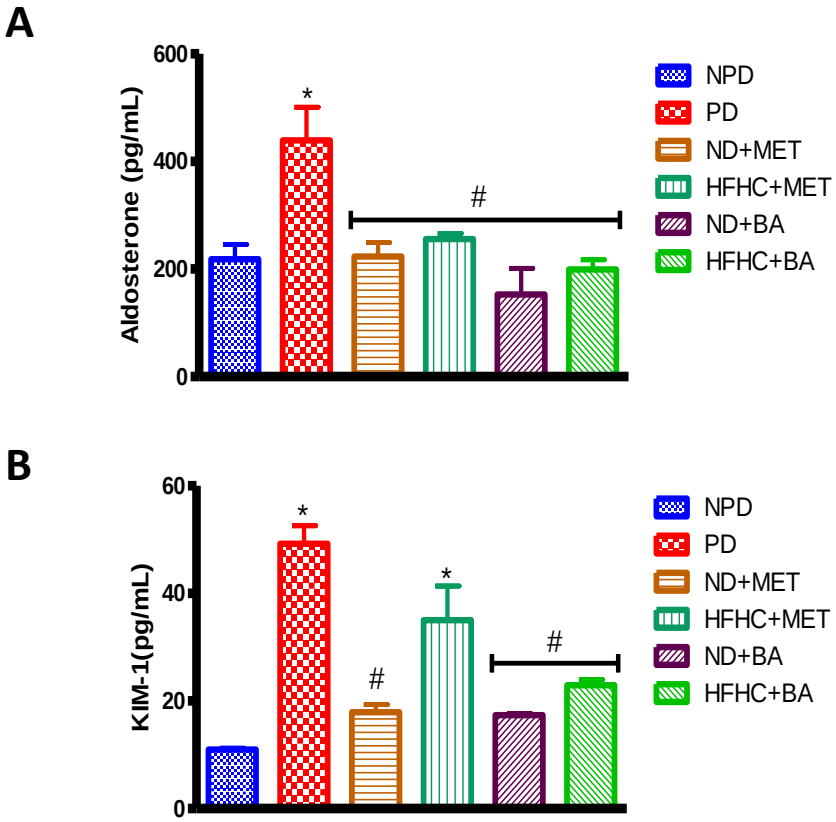

Figure 6: Effects of BA on aldosterone (A) and kidney injury molecule, KIM-1 (B) in non-prediabetic and prediabetic rats with or without diet intervention.

*p<0.001 (vs. NPD), #p<0.001(vs. PD).

NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Lipid peroxidation

The kidney MDA concentrations of the PD rats and metformin-treated rats without diet intervention were significantly increased in comparison to the NPD control rats as shown in Table 1 ($p<0.001$).Conversely, the MDA concentration of BA treated rats with or without dietary intervention as well as metformin-treated rats with diet intervention were significantly decreased when compared to PD rats.

Antioxidant status

The kidney SOD, GPx and TOAC concentrations of the PD rats significantly decreased in comparison to NPD control rats as indicated in Table 1 ($p<0.001$). On the other hand, administration of BA with or without diet intervention and metformin with diet intervention significantly increased the SOD, GPx and TOAC concentrations in the kidney tissue when compared to the PD rats. Moreover, except for the kidney SOD concentration, there was a significant difference in the kidney GPx and TOAC concentrations in the HFHC+MET group when compared to PD control rats.

Urine podocin mRNA

The podocin mRNA in the urine of PD rats was significantly increased by 12.04-fold when compared to the NPD control rats (Figure 7). The podocin mRNA levels in the urine of BA and metformin-treated rats in the presence or absence of diet intervention were significantly decreased in comparison to the PD rats.

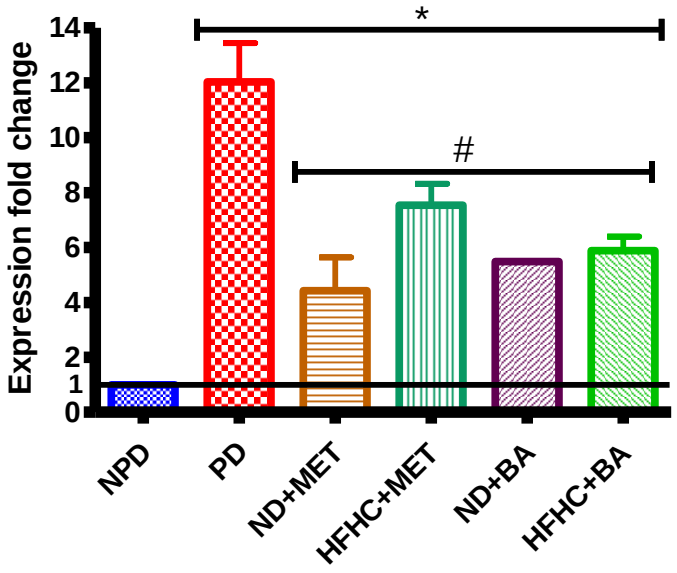

Figure 7: Effects of BA on urinary podocin mRNA expression in non-prediabetic and prediabetic rats with or without diet intervention. *p<0.001 (vs. NPD), #p<0.001(vs. PD).
NPD: non-prediabetic, PD: prediabetic, ND: normal diet, HFHC: high fat high carbohydrate diet, MET: metformin, BA: bredemolic acid

Discussion

This study examined the ameliorative effects of bredemolic acid on parameters associated with renal dysfunction in diet-induced prediabetic rats. In our previous study, we have established that bredemolic acid, a pentacyclic triterpene and isomer of maslinic acid, ameliorated markers of glucose homeostasis, improved insulin sensitivity and increased the expression of GLUT 4 in the skeletal muscle of prediabetic rats as a compensatory mechanism to regulate glucose metabolism in the prediabetic state (Akinnuga *et al.* 2019). Furthermore, this study is a continuation of our previous research and the data on body weight, food intake, fasting blood glucose, oral glucose tolerance test, fasting insulin concentration and insulin resistance were presented in the previous study (Akinnuga *et al.* 2019).

In this study, the kidney weight to body weight ratio significantly decreased in untreated prediabetic rats. The decrease in the relative kidney weight can be suggested to be due to an increase in the body weight without a proportional increase in the visceral kidney weight. However, administration of BA caused a significant increase in the relative kidney weight in the BA treated rats. This may be due to decreased body weight as a result of diet intervention and BA administration (Akinnuga *et al.* 2019).

High fat or high fructose diets have been associated with non-diabetic range hyperglycaemia and insulin resistance which in turn leads to metabolic disturbances with complications that results in renal dysfunction (Chou & Fang, 2010, Odermatt, 2011). Literature evidence has established a relationship between insulin-resistant states (such as obesity, prediabetes and T2DM) and the occurrence of renal dysfunction (Sarafidis & Ruilope, 2006, Wang *et al.* 2008, Echouffo-Tcheugui, 2016). Insulin resistance is one of the physiological linkages between prediabetes and renal dysfunction. It has been associated with pathological changes (such as decreased GFR, albuminuria, proteinuria and diffuse thickening of glomerular capillary basement membrane) which are similar to those observed in diabetic nephropathy (Mac-Moune Lai *et al.* 2004, Ritz *et al.* 2011, Echouffo-Tcheugui, 2016). Therefore, prediabetes is a risk factor for albuminuria or proteinuria as well as other renal dysfunction independent of the occurrence of T2DM (Mac-Moune Lai *et al.* 2004, Sun *et al.* 2010, Chou & Fang, 2010, Markus *et al.* 2018).

Similarly, in this study, we observed a decreased GFR, albuminuria and proteinuria in the prediabetic control rats in comparison to the non-prediabetic control rats. The decreased GFR, albuminuria and proteinuria observed in the prediabetic control rats are an apparent indication of glomerular damage (Sarafidis & Ruilope, 2006, Artunc et al, 2016, Markus *et al.* 2018). Additionally, KIM-1 is an expressed biomarker on the apical membrane of proximal tubular cells, and an established indicator of acute kidney injury (Bonventre & Yang, 2010). The increased concentration of KIM-1 in the urine of the prediabetic rats indicated kidney injury which further confirmed the observed albuminuria or proteinuria (Peralta *et al.* 2012). Furthermore, the plasma concentrations of albumin and total protein are decreased due to the albuminuria and proteinuria respectively, therefore, this suggests that the prediabetes might have resulted in impaired filtration barrier with consequent loss of plasma albumin and protein (Mac-Moune Lai *et al.* 2004, Sun *et al.* 2010, Markus *et al.* 2018). Therefore, early diagnosis of these structural changes during the prediabetic stage and intervention of an anti-diabetic agent may prevent the occurrence of overt diabetic kidney disease.

Moreover, the increased plasma concentrations of creatinine and urea, as well as decreased urine concentrations of the same parameters in the prediabetic control rats, were in accordance to the results of previous studies (Mkhwanazi *et al.* 2014, Ngubane, 2014). The observed significant changes in these parameters can be suggested to be due to impaired excretory or regulatory function of the kidney to maintain constant homeostasis of these parameters in the untreated prediabetic rats by comparison to the BA treated prediabetic rats. Additionally, the impaired regulation of the plasma and urine creatinine altered the creatinine clearance which further contributed to the decreased GFR in the untreated prediabetic rats. However, literature evidence suggests that insulin resistance triggers oxidative stress which in turn leads to renal dysfunction or kidney injury (Sarafidis & Ruilope, 2006, Chou & Fang, 2010, Markus *et al.* 2018). Therefore, we suggest that the aforementioned renal dysfunction parameters may be due to insulin resistance which further triggered oxidative stress which correlated with the

observed increase in the lipid peroxidation (MDA) and decrease in the antioxidant status (SOD, GPx and TOAC) concentrations in the prediabetic control rats (Mahat *et al.* 2019).

On the other hand, administration of BA in the presence or absence of diet intervention normalized the GFR and attenuated the albuminuria, proteinuria as well as KIM-1 concentration in the urine. Also, we suggest that BA attenuated these renal dysfunctions by the improvement of insulin sensitivity which we have earlier reported in our previous study (Akinnuga *et al.* 2019). Also, like other triterpenes, BA ameliorates oxidative stress by donation of an electron through its hydroxyl radical scavenging activity. Therefore, our findings suggest that the combination of the improved insulin sensitivity and the antioxidant property of BA probably lead to the attenuation of the aforementioned renal dysfunction indicators in BA treated rats with or without diet modifications.

High fructose diet has been reported to result in ATP depletion due to the utilization of two molecules of ATP for each fructose molecule metabolized (Abdelmalek *et al.* 2010, Softic *et al.* 2016). Therefore, the resultant ADP is further degraded to AMP. In insulin-resistant state (prediabetes), xanthine dehydrogenase enzyme is activated and triggered the conversion of the AMP to uric acid, hence resulting in hyperuricaemia and elevated uric acid excretion (Johnson *et al.* 2013, Elizalde-Barrera *et al.* 2017, Kawada, 2018). Similarly, in this study, the plasma and urine concentrations of uric acid significantly increased in the untreated prediabetic rats. The significant increase may be due to the consumption of high amounts of fructose which triggered insulin resistance followed by hyperuricaemia and significant urinary excretion of uric acid. However, administration of BA with diet intervention significantly ameliorated the hyperuricaemia probably due to improved insulin sensitivity in BA treated rats. High fat feeding has been reported to activate the renin-angiotensin-aldosterone system (RAAS) via insulin resistance or hyperinsulinaemia (Luther & Brown, 2011). Hyperinsulinaemia induces production of aldosterone which in turn triggered sodium retention and potassium loss in the insulin-resistant state (Brands & Manhiani, 2012). However, hyperinsulinaemia has been reported to activate the aldosterone-induced SGK1 signalling pathway which in turn leads to sodium retention (Lang *et al.* 2009, Artunc *et al.* 2016). In our study, a similar elevated plasma concentration of aldosterone was observed, and this suggested that the high fat diet probably activated increased production of aldosterone in the prediabetic rats. Consequently, due to the elevated aldosterone concentration, a significantly increased sodium reabsorption and potassium secretion which subsequently led to sodium retention, hypokalaemia, increased fluid intake and increased urine output was observed in the prediabetic control rats. Studies have shown that elevated aldosterone concentration induced proteinuria and glomerular podocyte injury with decreased gene expression of podocin in the kidney tissues and increased gene expression of podocin mRNA in the urine (Shibata *et al.* 2007, Shrestha *et al.* 2019). Podocin is an exclusive integral membrane protein in the podocytes, localizes to the slit diaphragm and directly interact with nephrin and CD2-associated protein (Fan *et al.* 2006). A previous study by Fan *et al* (2006) showed that knockdown of podocin in podocytes decreased the expression of nephrin. Therefore, this showed that the existence of podocin might be very important for anchoring nephrin to

the membrane surface of the slit diaphragm and alteration in podocin expression mechanically affect nephrin expression (Fan *et al.* 2006, Markus *et al.* 2018). However, urinary podocin is a marker of podocyte injury which is associated with albuminuria and proteinuria (Shankland, 2006, Lioudaki *et al.* 2015). Also, studies have shown that podocytes express mineralocorticoid receptors (MR), hence, podocytes are targeted cells for aldosterone (Shibata *et al.* 2007, Kiyomoto *et al.* 2008). Therefore, when aldosterone concentration is increased, oxidative stress is induced in the podocytes and this subsequently promoted apoptosis of the podocytes by increased ROS production in the mitochondria (Zhu *et al.* 2011, Su *et al.* 2013). Consequently, the aldosterone-induced oxidative stress resulted in podocyte injury which confirmed the increased urinary gene expression of podocin mRNA in this study (Lioudaki *et al.* 2015, Shrestha *et al.* 2019). The increased urinary gene expression of podocin can be suggested to be due to the aldosterone-induced podocyte injury or podocyte detachment. Also, it has been established that podocytes are insulin-responsive cells that similarly respond to insulin in the same manner as the skeletal muscle (Coward *et al*. 2005). Therefore, podocytes survival is modulated by insulin signalling, thus, insulin resistance has been implicated with podocytes loss which in turn leads to proteinuria. Hence, administration of BA which increased insulin sensitivity may improve insulin signalling in podocytes and further contribute to the observed attenuated proteinuria in BA treated rats. Pentacyclic triterpenes have been reported to selectively inhibit 11β-Hydroxysteroid dehydrogenase type I enzyme which converts inactive cortisone into active cortisol, thus preventing glucocorticoid activation of MR in aldosterone tissue such as the kidney (Lipson *et al.* 2011, Nazaruk & Borzym-Kluczyk, 2015). Therefore, administration of BA can be suggested to inhibit 11β-Hydroxysteroid dehydrogenase type I enzyme which prevent activation of MR, hence inhibiting the activities of aldosterone on MR which in turn ameliorates sodium and potassium regulations as well as the fluid intake and urine output in the BA treated rats. Also, we suggest that the same enzymatic inhibition prevented aldosterone biological actions on podocyte MR, and this subsequently led to the reduced urinary gene expression of podocin mRNA in the BA treated rats with or without diet modification.

In summary, with the aforementioned renal dysfunction, administration of BA with or without diet modification has been shown in this study to ameliorate renal dysfunction by attenuation of oxidative stress and renal dysfunction markers in a prediabetic state. However, because prediabetes is an early stage of diabetes, early screening for renal dysfunction prevents the occurrence of end-stage renal disease. More studies, however, are still needed to investigate the molecular mechanisms by which BA ameliorates renal dysfunction.

Declaration of Interest

The authors declare no conflict of interest.

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CHAPTER 6

Synthesis and Conclusion

6.0 Synthesis

Prediabetes is an asymptomatic state which is associated with moderate hyperglycaemia and if left untreated can lead to the development of hepatic, cardiovascular, and renal dysfunction (Brannick *et al.,* 2016). The prevalence of prediabetes is currently observed in developing and developed countries of the world due to urbanization that promotes increased consumption of high caloric diets as well as sedentary lifestyles (Hostalek, 2019). The combination of dietary and pharmacological interventions is the current therapeutic approach in managing prediabetes but the compliance of patients to combine the two interventions is low as patients merely adhere to pharmacological intervention without diet modifications (Glechner *et al.,* 2018). This reduces the efficacy of the pharmacological interventions with increased risk of progression of prediabetes to T2DM (Ramachandran *et al.,* 2009, Glechner *et al.,* 2018). Therefore, there is a need for alternative anti-diabetic agents that could ameliorate prediabetes and its complications without the use of diet modification. Bredemolic acid is a pentacyclic triterpene which has been reported to have enhanced biological activity compared to other triterpenes (Wen *et al.,* 2006, Cheng *et al.,* 2008). Therefore, in this study, we sought to investigate the effects of bredemolic acid on glucose homeostasis in a diet-induced prediabetic rat model in the presence and absence of dietary modification. We further investigated the effects of this triterpene on markers associated with hepatic, cardiovascular and renal function.

Impaired glucose tolerance and impaired fasting blood glucose are indicators of prediabetes and have been identified as important factors in the development of several complications that are associated with prediabetes (Tabák *et al.,* 2012, Edwards & Cusi, 2016). Therefore, attenuation of impaired glucose tolerance and impaired fasting blood glucose is of therapeutic importance in not only ameliorating glucose homeostasis but also preventing the onset and progression of hepatic, cardiovascular and renal complications in prediabetes. Impaired glucose tolerance is due to skeletal muscle insulin resistance which is the major onset of the decreased skeletal muscle glycogen concentration in prediabetes (Samuel & Shulman, 2016). Indeed, the prediabetic animals in this study had a significantly impaired glucose tolerance, decreased skeletal muscle glycogen concentration and GLUT 4 expression.

However, as one of the novelties of this study, the administration of BA attenuated the impaired glucose tolerance, impaired fasting blood glucose and skeletal muscle insulin resistance observed in prediabetes in both the presence and absence of dietary intervention. These observations suggest that BA is an anti-diabetic agent that ameliorates the aforementioned glucose metabolic disturbances, in part, by increasing insulin sensitivity and glucose uptake in skeletal muscle via improved GLUT 4 expression. Studies have shown that hyperinsulinaemia, increased food intake, increased body weight, increased glycated haemoglobin and increased ghrelin plasma concentration are not only associated with T2DM but also prediabetes (Briggs & Andrews, 2011, Barclay *et al.,* 2013, Punthakee *et al.,* 2018).

Hyperinsulinaema is a product of compensatory secretion of insulin from pancreatic β-cells to ameliorate impaired fasting blood glucose in the insulin-resistant state (Samuel & Shulman, 2016). Impaired fasting blood glucose is the cause of increased glycated haemoglobin and since red blood cells are insulin-independent, excessive plasma glucose becomes glycated with haemoglobin (Punthakee *et al.,* 2018). Increased food intake and increased body weight have been associated with consumption of high caloric diet due to increased ghrelin secretion in prediabetic condition (Castañeda *et al.,* 2010). The prediabetic animals in this study presented increased body weight, increased food intake and increased plasma concentration of ghrelin.

As a contribution to knowledge, we suggest that the attenuation of the aforementioned markers of abnormal glucose homeostasis by the administration of BA can be attributed to glycaemic control via decreased ghrelin secretion, improved insulin sensitivity and increased expression of GLUT 4 in the skeletal muscle. These observations further contribute to the findings of this study which suggest that BA administration decreases the risk of developing abnormal glucose metabolism in the prediabetic state even in the absence of dietary intervention.

Apart from abnormal glucose homeostasis in the skeletal muscle, increased diversion of glucose to the liver leading to increased hepatic *de novo* lipogenesis and glycogenesis is another complication associated with prediabetes. Hepatic *de novo* lipogenesis is the main cause of hepatic fat accumulation (Lambert *et al.,* 2014). Estimation of hepatic triglyceride is used to indicate hepatic fat accumulation (Kawano & Cohen, 2013). Therefore, attenuation of hepatic *de novo* lipogenesis is a crucial approach in preventing liver complications such as non-alcoholic liver disease and non-alcoholic steatohepatitis that are prevalent in the prediabetic condition. The prediabetic control animals in this study had increased hepatic fat accumulation due to increased concentrations of hepatic triglyceride and hepatic *de novo* lipogenesis.

As part of the novelties of this study, we observed that the administration of BA attenuated the hepatic *de novo* lipogenesis and hepatic triglyceride concentrations. Besides hepatic lipogenesis, accumulation of hepatic glycogen has been associated with prediabetes (Samuel & Shulman, 2018). Increased hepatic glycogenesis leads to accumulation of glycogen in the liver due to increased stimulation of hepatic glycogen synthase or increased inhibition of hepatic glycogen phosphorylase enzymes. In addition, the previous study has shown that administration of BA inhibited glycogen phosphorylase enzyme in the skeletal muscles (Cheng *et al.,* 2008). This observation, therefore, suggests that the administration of BA, in both the presence and absence of dietary intervention, may also inhibit glycogen synthase or stimulate glycogen phosphorylase in the liver in order to ameliorate hepatic glycogen accumulation in the prediabetic condition. Hepatic fat and glycogen accumulations are possibly the cause of lipid peroxidation and decreased antioxidant enzyme concentrations in the liver in the insulin-resistant state (Takaki *et al.,* 2013). Increased AST and ALT plasma concentrations have been reported to be associated with hepatic oxidative stress in prediabetes (Takaki *et al.,* 2013, Huang *et al.,* 2015). Lipid

peroxidation, decreased plasma concentration of antioxidant enzymes and increased plasma concentration of liver enzymes (ALT and AST) are markers of liver damage (Takaki *et al.*, 2013, Huang *et al.*, 2015). As an addition to knowledge, we observed that the administration of BA, in both the presence and absence of dietary intervention, attenuated oxidative stress and liver damage enzyme marker concentrations via improved hepatic lipogenesis and glycogenesis. Taken together, the results of this study suggest that administration of BA improves insulin sensitivity in skeletal muscle, thus reducing the amount of glucose shunted towards the liver and thereby significantly reducing the risk of developing liver complications in the prediabetic state.

Studies show that the cardiovascular system is also affected in prediabetes (Wasserman *et al.*, 2018, Brannick & Dagogo-Jack, 2018). Cardiovascular dysfunction can be indicated by changes such as increased blood pressure, increased heart rate, decreased eNOS plasma concentration and lipid profile disturbances (Eringa *et al.*, 2013, Wasserman *et al.*, 2018). Indeed, these changes were observed in the untreated prediabetic rats in this study and these observations correlated with other studies on prediabetes (Eringa *et al.*, 2013, Brannick & Dagogo-Jack, 2018). Abnormal lipid profile is a common anomaly in prediabetes due to high caloric diet consumption which is associated with increased body mass index and increased waist circumference (Zaman *et al.*, 2011). The eNOS is an enzyme that catalyses the production of nitric oxide (NO) in the vascular endothelium and depicts the state of vascular endothelial function (Huang *et al.*, 2016). Vascular endothelial dysfunction has been reported as one of the causes of increased blood pressure and heart rate in prediabetes (Eringa *et al.*, 2013, Huang *et al.*, 2016). Apart from vascular endothelial dysfunction due to decreased eNOS activity, intermediate hyperglycaemia has also been associated with the aforementioned cardiovascular dysfunction via oxidative stress (Tabák *et al.*, 2012). The administration of BA, in both the presence and absence of dietary intervention, ameliorated cardiovascular dysfunctions in the prediabetic rats as there was observed decreases in intermediate hyperglycaemia, lipid profile and eNOS concentrations. The results suggest that BA administration improved cardiovascular function via the attenuation of the intermediate hyperglycaemia observed in prediabetes thus leading to amelioration of oxidative stress. Vascular endothelial dysfunction, oxidative stress and intermediate hyperglycaemia has been linked with increased inflammatory responses that caused increased release of inflammatory cytokines (such as TNF-α, IL-6 and hs-CRP) in prediabetes (Huang *et al.*, 2016, Mahat *et al.*, 2019). TNF-α and IL-6 are cytokines that mediate cardiac injury by transmigration of white blood cells to cardiac tissue. CRP is a low-grade inflammation cytokine which is associated with myocardial infarction, stroke and other cardiovascular dysfunctions (Grossmann *et al.*, 2015). The administration of BA, in both the presence and absence of dietary intervention, ameliorated the plasma concentration of inflammatory cytokines via its anti-inflammatory effect. Taken together, this study, for the first time showed that BA administration ameliorated markers of cardiovascular dysfunction and prevents the risk of developing

cardiovascular complications via the attenuation of oxidative stress, inflammation and vascular endothelial dysfunctions.

Renal dysfunction is a complication that has been associated with prediabetes (Echouffo-Tcheugui *et al.,* 2016). Reduced glomerular filtration rate (GFR), increased urinary KIM-1 concentration, albuminuria and proteinuria are apparent indicators of renal dysfunction and glomerular filtration barrier damage which has been reported not only in overt diabetes but also prediabetes (Mac-Moune Lai *et al.,* 2004, Echouffo-Tcheugui *et al.,* 2016, Nowak *et al.,* 2016). The podocytes are the main component of the filtration barrier in which their loss leads to loss of integral membrane protein called podocin. However, all these alterations in renal function have been found to sequel hyperinsulinaemia and hyperglycaemia in the prediabetic condition (Sarafidis & Ruilope, 2006, Artunc *et al.,* 2016). Therefore, attenuation of hyperinsulinaemia and hyperglycaemia is crucial in delaying the onset of renal dysfunction in prediabetes. We have shown in this study that the administration of BA restores glucose homeostasis when given to prediabetic rats. Indeed, the administration of BA, in both the presence and absence of dietary intervention, improved renal function as shown by attenuation of abnormal creatinine, albumin, total protein, urea and uric acid plasma concentrations. Hyperinsulinaemia has also been reported to activate the renin-angiotensin-aldosterone system (RAAS) in prediabetes (Chou & Fang, 2010). Activation of RAAS triggers elevated plasma concentration of aldosterone that promotes increased sodium reabsorption and potassium loss which subsequently alters fluid intake and urine output (Brands & Manhiani, 2012). Elevated aldosterone concentration has been reported to induce oxidative stress in podocytes and promotes apoptosis of podocytes due to increased activity of aldosterone on the mineralocorticoid receptors that are present on the podocytes (Shibata *et al.*, 2007, Su *et al.,* 2013). Therefore, podocyte injury is, in part, caused by aldosterone-induced oxidative stress (Zhu *et al.*,2011). As one of the novelties of this study, the administration of BA attenuated podocyte injury which was assessed by urinary podocin mRNA expression. All these observations suggest that BA administration in both the presence and absence of dietary intervention improved glucose homeostasis and further prevented the risk of developing renal dysfunction in the prediabetic state.

6.1 Conclusions

The consumption of high caloric diets coupled with sedentary lifestyles is increasing in developing and developed countries due to increased urbanization, therefore, the risk of developing abnormal glucose metabolism that will lead to hepatic, cardiovascular and renal complications is increased. From this study, we observed that markers of these complications are present during the prediabetic state. The overall observations in this study suggest that the administration of BA, in both the presence and absence of dietary intervention, improved glucose homeostasis through increased sensitivity to insulin in diet-induced prediabetic rats. This further resulted in ameliorated markers of hepatic, cardiovascular and renal complications in the prediabetic rats.

6.2 Limitations and Future studies

In this study, some shortfalls that provide opportunities for more future studies have been identified. Assessment of the effect of BA on leptin plasma and adipose tissue concentrations as well as adipose tissue GLUT 4 expression in relation to the glucose homeostasis in the prediabetic state is a shortfall in this study. This shortfall is due to limited funding, and in future studies, the effects of BA on these parameters in prediabetic condition will be examined.

In addition, the effect of BA on immunohistochemistry of the liver, heart and kidney in relation to the complications of these organs is another shortfall of this study that was not examined due to limited funding. However, in the future studies, the immunohistochemistry of these organs will be examined to validate the selected markers of hepatic, cardiovascular and renal dysfunctions in this study.

Furthermore, electrocardiogram (ECG) is a cardiovascular parameter that further validates cardiovascular dysfunction apart from the observed cardiovascular dysfunction parameters but due to non-availability of animal electrocardiograph in our laboratory, this parameter was not measured. Determination of diastolic function via assessment of left ventricular end-diastolic volume by echocardiography, in addition to the observed blood pressure and heart rate, is also parts of the limitations of this study. Also, the effects of BA on more markers of vascular endothelial dysfunction such as nitric oxide (NO) and endothelin1 (ET1) should have been determined but due to limited funding, these parameters were not measured in this study. Therefore, to eliminate all these shortfalls, more studies on the effects of BA on these parameters in relations to glucose homeostasis, hepatic, cardiovascular and renal dysfunctions are needed.

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CHAPTER 7

Appendix 1

Composition of the high fats high carbohydrates (HFHC) diet

Ingredient	Incl(%)	Mix(kg)
Maize	38.98	390.000
Palm Oil	20.99	210.000
Soya Full Fat	14.99	150.000
Wheat Gluten	6.50	65.000
Flour	6.00	60.000
Monodex	5.00	50.000
Sugar – White	5.00	50.000
Limestone	1.00	10.000
Dicalcium Phosphate	0.50	5.000
Vitamin Premix	0.35	3.500
Salt – Fine	0.30	3.000
Amino Acid - DL Methionine	0.30	3.000
Mineral Premix	0.10	1.000
	100.01	**1000.50**

Appendix I1

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 May 2018

Mr Akinjide Akinnuga (217081429)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Mr Akinnuga,

Protocol reference number: AREC/024/018D
Project title: Investigating the effects of bredemolic acid on pre-diabetic rats model

Full Approval – Research Application

With regards to your revised application received on 16 April 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

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

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

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 04 May 2019.

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

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

Yours faithfully

.....

Professor Shahidul Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Andile Khathi
Cc Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Anita Engelbrecht
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Appendix III

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Evaluation of the effects of bredemolic acid on selected markers of glucose homeostasis in diet-induced prediabetic rats

Akinjide Moses Akinnuga, Angezwa Siboto, Bongiwe Khumalo, Ntethelelo Hopewell Sibiya, Phikelelani Ngubane & Andile Khathi

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ORIGINAL ARTICLE

Evaluation of the effects of bredemolic acid on selected markers of glucose homeostasis in diet-induced prediabetic rats

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ABSTRACT

Context: Pentacyclic triterpenes (such as maslinic acid) are natural anti-diabetic agents that ameliorate glucose metabolism in diet-induced prediabetes. However, the effects of bredemolic acid (BA), maslinic acid isomer, is yet unknown in prediabetic (PD) conditions.

Objectives: To investigate the effects of BA on some glucose homeostasis parameters in high-fat high-carbohydrate (HFHC) diet-induced PD rats.

Methods: Thirty-six (36) male rats (150–180 g) were divided into two groups, the normal diet (ND) non-prediabetic, NPD ($n = 6$) and the HFHC diet PD groups ($n = 30$). The PD animals were further sub-divided into five groups ($n = 6$) where they were treated with BA for 12 weeks while monitoring changes in blood glucose, caloric intake, and body weight.

Results: Diet-induced prediabetes resulted in increased body weight, caloric intake, glycated haemoglobin, and glucose tolerance. BA treatment ameliorated glucose tolerance, lowered plasma insulin and increased expression of glucose transporter 4 (GLUT 4) in rats.

Conclusions: BA administration restored glucose homeostasis in diet-induced prediabetes regardless of diet intervention.

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KEYWORDS
Bredemolic acid; glucose homeostasis; high-fat diet; high-carbohydrate diet; prediabetes

Introduction

Prediabetes is a state of abnormal glucose homeostasis that is characterised by intermediate hyperglycaemia, elevated glycated haemoglobin and impaired glucose tolerance (Huang *et al.* 2016, Brannick and Dagogo-Jack 2018). It is said to be caused by chronic consumption of a diet that consists of food rich in carbohydrates and saturated fats coupled with a lifestyle of physical inactivity (Lam and LeRoith 2012, Edwards and Cusi 2016). The prevalence of prediabetes is markedly increasing in developed and developing countries especially in Africa, and the International Diabetes Federation (IDF) has predicted that the number of prediabetic (PD) individuals is expected to rise from 280 million to about 398 million in 2030 (Lam and LeRoith 2012, Roglic 2014, Edwards and Cusi 2016). In addition, prediabetes is a great precursor for type 2 diabetes mellitus (T2DM) and its complications if left untreated (Tabák *et al.* 2012, Brannick *et al.* 2016).

While abnormal glucose metabolism is often associated with overt T2DM, studies have shown that these abnormalities begin in the PD state (Brannick *et al.* 2016, Luvuno *et al.* 2016). In the PD condition, hyperinsulinaemia results as a compensatory mechanism to regulate insulin resistance and impaired glucose tolerance (Tabák *et al.* 2012). Impaired glucose tolerance is associated with decreased insulin sensitivity and sustained intermediate hyperglycaemia (Brannick *et al.* 2016). Subsequently, glucose uptake decreases gradually and the insulin-dependent peripheral tissues such as skeletal muscles are gradually starved of glucose, thus causing a decrease in glycogen level in the muscles (Brannick *et al.* 2016). Supposedly, due to decreased glucose uptake, the peripheral cells are depleted of energy. Therefore, a compensatory mechanism of ghrelin hormone release is initiated to stimulate the hypothalamus *via* the orexigenic signalling pathway and increase food intake (hyperphagia) to circumvent hypoglycaemia (Chabot *et al.* 2014).

However, the combination of dietary and pharmacological intervention has been explored to manage prediabetes and prevent the progression of T2DM (Ley *et al.* 2014, Salas-Salvadó *et al.* 2014). Of note, there is low compliance in the combination of the two interventions as patients only use pharmacological intervention without change of diet, hence reduce the efficacy of the drugs (Gamede *et al.* 2018). Therefore, management of prediabetes by natural anti-diabetic agents that can remain effective regardless of a change in diet is necessary.

Maslinic acid, a pentacyclic triterpene and anti-diabetic agent, has been reported to improve glucose homeostasis in diabetic rodents through inhibition of intestinal carbohydrate hydrolysing enzymes and glucose transporters, and also by increasing glycogen synthesis in the liver and skeletal muscle *via* inhibition of glycogen phosphorylase (Mkhwanazi *et al.*

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2014, Nazaruk and Borzym-Kluczyk 2015, Luvuno *et al*. 2016, Liou *et al*. 2019). An isomer of maslinic acid, bredemolic acid (BA), was discovered to have increased biological activity in regulating glucose homeostasis by inhibition of glycogen phosphorylase enzyme in rabbit skeletal muscle, however, its effects on glucose homeostasis in prediabetes are yet to be determined (Wen *et al*. 2006, Cheng *et al*. 2008). Therefore, to establish the effects of BA on glucose homeostasis in PD condition, we sought to investigate the effects of BA administration on selected glucose homeostasis parameters in high-fat high-carbohydrate (HFHC) diet-induced PD rats.

Materials and methods

Animals

In this study, thirty-six (36) male Sprague Dawley rats that weighed 150–180 g were obtained from the Biomedical Research Unit, University of KwaZulu-Natal (UKZN). The animals were kept and maintained under laboratory conditions of constant humidity (55 ± 5%), temperature (22 ± 2°C), and 12 h day : 12 h night cycle. They were acclimatised to their new environment for 2 weeks while consuming standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* before exposure to the experimental HFHC diet. The HFHC diet was formulated to consist of carbohydrates (55% Kcal/g), fats (30% Kcal/g), and proteins (15% Kcal/g). All experimental designs and procedures were according to the approved ethics (Ethics number: AREC/024/018D) and guidelines of the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa.

Experimental design

After the 2 weeks of acclimatisation, the animals were initially divided into two different groups, the normal diet (ND) NPD ($n = 6$) and HFHC diet PD groups ($n = 30$). All the animals in the PD group were given an HFHC diet and drinking water that was supplemented with 15% fructose for 20 weeks to induce prediabetes. The NPD control group (Group 1) was fed on ND and water *ad libitum* for 20 weeks. In the 20th week, prediabetes was confirmed by determination of fasting blood glucose (FBG) and oral glucose tolerance test (OGTT) in animals in NPD and PD groups.

Treatment of animals

The treatment period lasted for 12 weeks i.e. 21st–32nd week. After the 20th week, the PD animals were either continuously fed on HFHC or changed to ND, and treated with either oral administration of BA (80 mg/kg) or MET (Metformin, 500 mg/kg) at every third day. The NPD control (Group 1) animals continuously fed on ND and received as the vehicle, 3 ml/kg of diluted dimethyl sulphoxide, DMSO (2 ml DMSO: 19 ml normal saline, p.o.) for 12 weeks. The animals in the PD group were further divided into five groups (Group 2–Group 6) of six animals each. The prediabetes control group (PD, Group 2) were fed on the HFHC diet and received 3 ml/kg of diluted DMSO orally. Group 3 (ND + MET) changed the diet to ND (from HFHC to ND) and treated with MET while Group 4 (HFHC + MET) was continuously given the HFHC diet and treated with MET. The Group 5 (ND + BA) animals changed the diet to ND and treated with BA while Group 6 animals (HFHC + BA) were continuously given the HFHC diet and treated with BA.

Caloric intake

At every 4 weeks of treatment, the caloric intake of all the animals was determined by measuring food and water intakes *via* metabolic cages (Tecniplast; Labotec, Cape Town, South Africa).

Blood glucose concentration

The blood glucose concentration (FBG) was determined by using the tail-prick method and measured *via* One-Touch select glucometer (Lifescan, Malta, United Kingdom) at every 4 weeks of treatment.

Oral glucose tolerance (OGT) response

In the 12th week of treatment period, the OGTT was conducted following glucose loading. The OGTT responses were monitored in all the animal groups through established laboratory protocol (Ngubane *et al*. 2011, Khathi *et al*. 2013, Gamede *et al*. 2018). In brief, after a 12 h fasting period, FBG was measured (time, 0 min) in all the animals. Then, the animals were loaded with glucose (0.86 g/kg, p.o.) *via* oral gavage (18-gauge gavage needle, 38 mm long curved with 21/4 mm ball end). The glucose concentrations were measured at 30, 60, and 120 min following glucose loading.

Blood collection and tissue harvesting

All animals were anaesthetised with Isofor (100 mg/kg, Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) for 3 min. When the animals were unconscious, blood samples were collected from the animals through cardiac puncture into different pre-cooled heparinised containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Hamburg, Germany) at 4°C, 503 g for 15 min to obtain plasma. Then, each of the plasma was aspirated into plain sample bottles and stored at −80°C in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) until ready for biochemical analysis. Also, the skeletal muscle (gastrocnemius) was removed, rinsed with cold normal saline solution and snapped frozen in liquid nitrogen before storage in Bio Ultra freezer at −80°C for biochemical analysis of selected metabolic parameters. The caloric intake, body weight gain, and FBG were assessed at 20th, 24th, 28th, and 32nd week in all the animals while OGTT was assessed at 20th and 32nd week only. The other selected parameters such as Homeostasis model assessment (HOMA2-IR) index, glycated haemoglobin, muscle glycogen, insulin, and ghrelin

concentrations were only determined at 32nd week i.e. 12th week of treatment period

Biochemical analysis

Ghrelin and glycated haemoglobin concentrations were determined by using their respective ELISA kits (Elabscience Biotechnology Co., Ltd., Houston, TX) as instructed by the manufacturer. Insulin concentration was measured *via* an ultrasensitive rat insulin ELISA kit (Mercodia AB, Sylveniusgatan 8A, SE-754 50, Uppsala, Sweden) as directed in the manufacturer's instruction manual. HOMA2-IR index was calculated from the insulin concentrations and FBG.

Glycogen assay

Glycogen assay was determined in skeletal muscle by following previously established protocols (Musabayane *et al*. 2005, Mukundwa *et al*. 2016, Gamede *et al*. 2018). The harvested tissues were weighed (50 mg) and heated with potassium hydroxide (KOH) (30%, 2 ml) for 30 min at 100 °C. Immediately, 0.194 ml of 10% of sodium tetraoxosulphate VI (Na_2SO_4) was added into the mixture to stop the reaction. When the mixture was allowed to cool, the glycogen precipitate was formed. 200 µl of the cooled mixture with the precipitate was aspirated and mixed with ethanol (95%, 200 µl). The precipitated glycogen was pelleted, washed and resolubilised in H_2O (1 ml). Thereafter, 4 ml of anthrone (0.5 g dissolved in 250 ml of 95% sulphuric acid) was added and boiled for 10 min. After cooling, the absorbance was determined by using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Germany) at 620 nm.

Western blot analysis of GLUT 4

GLUT 4 was analysed in skeletal muscle (gastrocnemius) as established in the previous protocol (Mkhwanazi *et al*. 2014). The skeletal muscle tissues (0.1 g) were homogenised on ice in isolation buffer and centrifuged for 10 min at 400 × g (4 °C). The protein content was quantified *via* the Lowry method. All the samples were standardised to one concentration (1 mg/ml) and the proteins were denatured by boiling in Laemmli sample buffer for 5 min. Then, 25 µl of the denatured proteins were loaded on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with 5 µl of molecular weight marker. The gel was electrophoresed at 150 V for 1 h in running buffer. After the electrophoresis, the resolved proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane in transfer buffer for 1 h. After the transfer, the membrane was blocked with 5% non-fat dry milk in tris-buffered saline with 0.1% Tween 20 (TTBS). The membrane was then immuno-probed with GLUT 4 antibodies (1:1000 in 1% BSA; Neogen, Lansing, MI) for 1 h at room temperature. The PVDF membrane was subjected to five washes (10 min each with gentle agitation) with TTBS. The membranes were then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:1000; Bio-Rad, Johannesburg, South Africa) for 1 h at room temperature. After further washing, antigen-antibody complexes were detected by chemiluminescence through the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). The chemiluminescence signals were determined through the Chemi-doc XRS gel documentation system and analysed *via* the quantity one software (Bio-Rad, Johannesburg, South Africa).

Statistical analysis

The statistical data were presented in mean ± SEM. The data were analysed by using a two-way analysis of variance (ANOVA) with the Bonferroni test (post-hoc test) *via* GraphPad Prism version 5 software (GraphPad Software, La Jolla, CA). The level of statistical significance was determined at $p < .05$.

Results

Caloric intake

The caloric intake of all the experimental groups was determined every fourth week from the start of the treatment period (week 0) to the 12th week of treatment (Figure 1). The result showed that PD and HFHC + MET groups had significantly higher caloric intake in comparison to the NPD group throughout the treatment period except for 12th week of treatment at $p < .05$. However, the administration of MET and BA with dietary intervention significantly decrease caloric intake throughout the treatment period in comparison to PD and HFHC + MET ($p < .05$). On the other hand, the administration of BA without dietary intervention resulted in decreased caloric intake but insignificant when compared to PD and HFHC + MET.

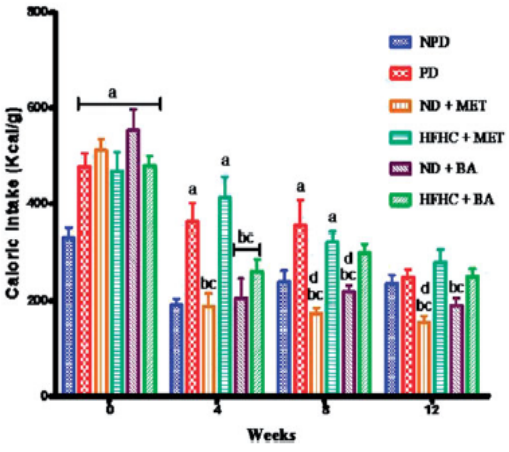

Figure 1. Effect of BA on caloric intake in rats with or without dietary intervention. Values are expressed as mean ± SEM ($n = 6$). [a]$p<.001$ in comparison to non-prediabetic (NPD) control, [b]$p<.001$ in comparison to prediabetic (PD) control, [c]$p<.001$ in comparison to HFHC + MET, and [d]$p<.05$ in comparison to HFHC + BA.

Table 1. Effects of BA on body weight and percentage changes in body weight from week 0 to 12 in prediabetic rats with or without diet intervention.

Body weight (g)						
Groups Weeks	NPD	PD	ND + MET	HFHC + MET	ND + BA	HFHC + BA
Week 0	410.33 ± 13.20 100%	429.50 ± 12.50 100%	400.33 ± 7.32 100%	426.67 ± 14.08 100%	404.67 ± 20.48 100%	404.20 ± 15.73 100%
Week 4	412.33 ± 6.24 ↑0.49%	498.83 ± 15.45 ↑16.14%[a]	459.83 ± 5.22 ↑14.86%[a]	479.00 ± 13.15 ↑12.26%[a]	435.40 ± 19.37 ↑7.59%[a,b]	447.83 ± 26.15 ↑10.78%[a,b]
Week 8	437.83 ± 20.37 ↑6.18%	523.50 ± 16.00 ↑21.89%[a]	481.50 ± 6.27 ↑20.27%[a]	506.17 ± 12.51 ↑18.63%[a]	446.00 ± 21.35 ↑10.21%[a,b]	461.00 ± 26.89 ↑14.05%[a,b]
Week 12	462.50 ± 17.44 ↑12.17%	540.50 ± 16.05 ↑25.84%[a]	481.80 ± 7.83 ↑20.35%[a]	516.00 ± 14.14 ↑20.94%[a]	449.80 ± 22.43 ↑11.15%[b]	468.80 ± 30.09 ↑15.98%[b]

Values are presented as mean ± SEM ($n = 6$).
[a]$p < .05$ in comparison to non-prediabetic (NPD) control.
[b]$p < .05$ in comparison to prediabetic (PD) control.

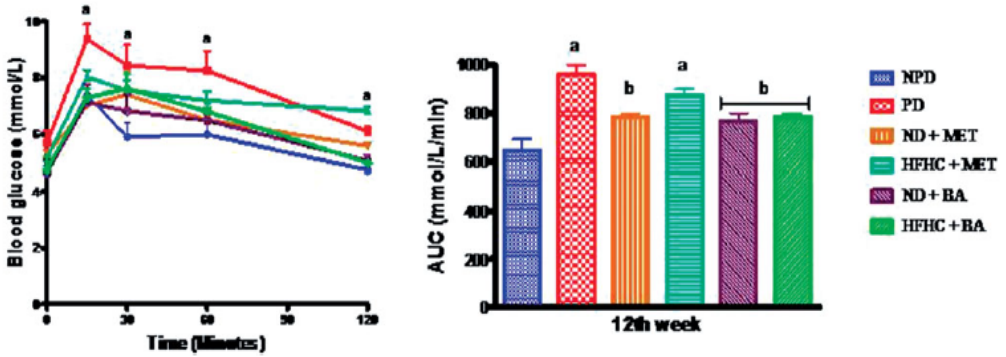

Figure 2. Effect of BA on OGTT and AUC in rats with or without dietary intervention. Values are expressed as mean ± SEM ($n = 6$). [a]$p<.001$ in comparison to non-prediabetic (NPD) control and [b]$p<.05$ in comparison to prediabetic (PD) control.

Body weight

The body weights of the animals were monitored throughout the experiment as shown in Table 1. The result showed that the percentage changes in body weight of the PD group increased throughout the experimental period when compared to that of the NPD group ($p < .05$). The administration of BA with or without dietary intervention showed a significant decrease in the percentage changes of body weight in comparison to the PD group throughout the treatment periods.

Oral glucose tolerance test (OGTT)

As shown in Figure 2, the OGTT and Area under curve (AUC) were measured at the end of the treatment period (12th Week) in all the groups. At time 0, the FBG concentration increased in PD and other PD treated groups but insignificant when compared to the NPD group. At 120 min post-load of glucose, the FBGs of PD was significantly different from the NPD group ($p < .05$). Conversely, at the same time (120 min), the blood glucose concentration of both BA treated groups significantly decreased in comparison to the PD group.

HOMA2-IR index

The HOMA2-IR index of all the animals was calculated from the product of plasma glucose and insulin at the end of the treatment period (12th week). The results showed that the HOMA2-IR index in PD and HFHC + MET groups was significantly different when compared to NPD and other experimental groups ($p < .05$). However, both BA treated groups and ND + MET groups had a significantly decreased HOMA2-IR index in comparison to PD and HFHC + MET groups at $p < .001$ as shown in Table 2.

Table 2. Effects of BA on fasting blood glucose, fasting blood insulin and HOMA2-IR Index in rats with or without dietary intervention after 12 weeks of treatment period.

Groups	Fasting blood glucose (mmol/l)	Fasting blood insulin (ng/ml)	HOMA2-IR index values
NPD	4.68 ± 0.19	3.42 ± 0.33	0.71 ± 0.09
PD	5.15 ± 0.13	12.28 ± 0.18[a]	2.81 ± 0.05[a]
ND + MET	5.28 ± 0.17	3.66 ± 0.12[b]	0.86 ± 0.04[b]
HFHC + MET	5.68 ± 0.44	5.06 ± 0.08[a,b]	1.28 ± 0.06[a,b]
ND + BA	4.63 ± 0.46	3.14 ± 0.09[b]	0.65 ± 0.05[b]
HFHC + BA	4.75 ± 0.46	3.91 ± 0.48[b]	0.83 ± 0.12[b]

Values are presented as mean ± SEM ($n = 6$).
[a]$p < .001$ in comparison to non-prediabetic (NPD) control.
[b]$p < .05$ in comparison to prediabetic (PD) control.

Glycated haemoglobin concentration (HbA1c)

In 12th week, all the experimental groups were analysed for HbA1c concentration (Figure 3). The HbA1c concentration of the PD group was significantly higher when compared to the NPD group. However, the HbA1c concentrations of both BA treated with or without diet intervention (ND + BA or HFHC

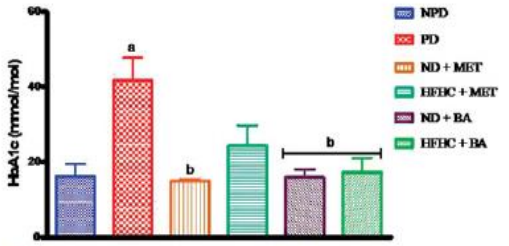

Figure 3. Effect of BA on glycated haemoglobin in rats with or without dietary intervention. Values are expressed as mean ± SEM ($n = 6$). [a]$p<.001$ in comparison to non-prediabetic (NPD) control and [b]$p<.001$ in comparison to prediabetic (PD) control.

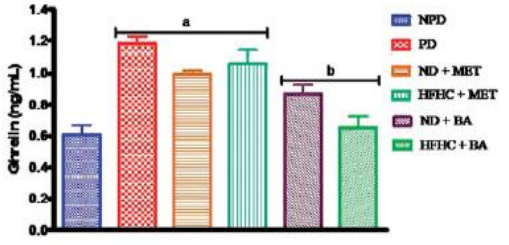

Figure 4. Effect of BA on Ghrelin in rats with or without dietary intervention. Values are expressed as mean ± SEM ($n = 6$). [a]$p<.001$ in comparison to non-prediabetic (NPD) control and [b]$p<.05$ in comparison to prediabetic (PD) control.

+ BA) as well as metformin treated with diet intervention (ND + MET) decreased significantly in comparison to PD ($p < .05$). Conversely, there was no significant difference between the HbA1c concentration of metformin-treated rats without diet intervention (HFHC + MET) and PD group.

Ghrelin concentration

The plasma concentration of ghrelin was measured in all the experimental groups at the end of the treatment period. The results showed that the ghrelin concentration of PD, HFHC + MET, and ND + MET groups was significantly higher in comparison to the NPD group (Figure 4). However, the BA treated animals with or without dietary intervention had a significantly lowered ghrelin concentration when compared to PD ($p < .05$).

Skeletal muscle glycogen concentration

The skeletal muscle glycogen concentrations were measured at the end of the treatment period. The results showed that the skeletal muscle glycogen concentrations in the PD group and all the treated experimental groups were significantly increased in comparison to the NPD group. Moreover, the skeletal muscle glycogen of BA treated animals with or without dietary intervention increased significantly when compared to PD groups ($p < .05$) as shown in Figure 5.

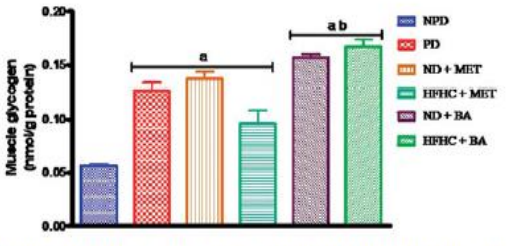

Figure 5. Effect of BA on muscle glycogen in rats with or without diet intervention. Values are expressed as mean ± SEM ($n = 6$). [a]$p<.001$ in comparison to non-prediabetic (NPD) control and [b]$p<.05$ in comparison to prediabetic (PD) control.

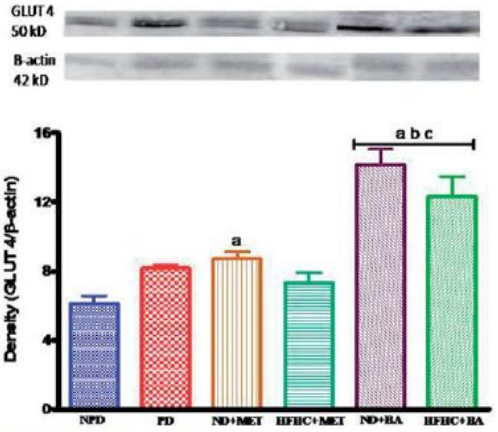

Figure 6. Effects of BA on GLUT 4 expression in rats with or without diet intervention after treatment period of 12 weeks. Values are expressed as mean ± SEM ($n = 6$). Values were obtained from Western blots for six preparations. [a]$p < .01$ in comparison to non-prediabetic (NPD) control, [b]$p < .05$ in comparison to prediabetic (PD) control, and [c]$p < .05$ in comparison to HFHC + MET.

Skeletal muscle GLUT 4 expression

As shown in Figure 6, GLUT 4 expression was increased significantly ($p < .05$) in BA treated rats with or without dietary intervention when compared to NPD and PD groups. However, the administration of metformin with diet intervention (ND + MET) significantly increased GLUT 4 expression when compared to NPD group.

Discussion

In several studies, triterpenes have been reported to have anti-diabetic properties which cause reduction of FBG and glycated haemoglobin concentrations as well as ameliorating insulin sensitivity in diet-induced prediabetes (Musabayane *et al.* 2005, Jung *et al.* 2007). Maslinic acid is a pentacyclic triterpene that has been reported to regulate glucose metabolism in diabetic rats (Jung *et al.* 2007, Mkhwanazi *et al.* 2014). BA, an isomer of maslinic acid, has been reported to have more increased biological activity due to differences in the structural arrangement of their hydroxyl groups (Wen *et al.* 2006, Cheng *et al.* 2008). However, the effects of this compound on glucose homeostasis in the PD state have not

been explored. Therefore, in this study, we sought to investigate the effects of BA on some glucose homeostasis parameters in PD rats.

It has been established that excessive consumption of high caloric diets drives animals towards a positive energy balance with resultant weight gain (Burchfield *et al.* 2018). This transition causes a decline in insulin sensitivity which ultimately results in compensatory hyperinsulinaemia and a state that resembles intermediate hyperglycaemia known as prediabetes (Barclay *et al.* 2013, Samuel and Shulman 2016). Similarly, in this study, chronic consumption of an HFHC diet increased food intake which caused an increase in body weight in the untreated PD animals. Ghrelin is a hormone that plays a key role in the regulation of caloric intake and energy balance that leads to weight gain (Barazzoni 2014, Chabot *et al.* 2014). Under normal physiological conditions, there exists an inverse relationship in the plasma concentrations of ghrelin and insulin (Barazzoni 2014, Chabot *et al.* 2014). In the preprandial state, ghrelin plasma level increases and suppresses insulin release from pancreatic beta-cells *via* Ca^{2+}mediated pathway while under postprandial state ghrelin plasma level reduces, thus, insulin release is enhanced (Alamri *et al.* 2016). In the postprandial state, when ghrelin level decreases, insulin is released and facilitates the uptake of glucose into the insulin-dependent peripheral cells to reduce blood glucose levels (Barazzoni 2014, Alamri *et al.* 2016). However, when the blood glucose level reduces, ghrelin plasma concentration increases to stimulate the hypothalamus to increase food intake *via* the orexigenic signalling pathway (Chabot *et al.* 2014, Alamri *et al.* 2016). In contrast, under diabetic conditions, the pancreatic beta-cell releases more insulin to compensate for the abnormal glucose metabolism and this leads to hyperinsulinaemia and decreased insulin sensitivity. The blood glucose level is increased and the insulin-dependent peripheral cells are starved of glucose. In addition, since the peripheral cells are starved of glucose, ghrelin plasma concentration increases and stimulates the hypothalamus to increase food intake (Barazzoni 2014, Alamri *et al.* 2016). Therefore, in diabetic conditions, both the ghrelin and insulin plasma concentrations are sustainably high (Barazzoni 2014, Alamri *et al.* 2016, Luvuno *et al.* 2016). Similarly, in this study, we observed that the food intake, percentage changes in body weight and plasma concentration of ghrelin were higher in untreated PD animals compared to other experimental groups from 0 to 12th week in the treatment period. This suggested that the increased plasma ghrelin concentration caused the increased caloric intake which resulted in increased percentage changes in body weight in untreated PD rats. Of note, we observed that the administration of BA with or without dietary intervention significantly decreased the percentage changes in body weight and caloric intake by a decrease in plasma ghrelin concentration. We further observed a significant increase in HOMA2-IR index as well as elevated postprandial glucose at 120 min in the OGTT of untreated PD animals. The administration of BA also enhanced glucose tolerance and this was proven in HOMA2-IR index and OGTT results. These results correlated with similar studies on other triterpenes such as maslinic and oleanolic acids (Musabayane *et al.* 2005, Mkhwanazi *et al.* 2014, Luvuno *et al.* 2016, Gamede *et al.* 2018).

Studies have shown that high-fat feeding in rodents led to transient muscle diacylglycerol (DAG) accumulation followed by muscle insulin resistance and impaired insulin signalling pathway (Szendroedi *et al.* 2014). Consequently, the muscle protein kinase C (PKCθ) is activated and limited phosphorylation of insulin receptor substrate-1 (IRS-1) occurred (Samuel and Shulman 2016). Under this condition, glucose uptake decreases due to reduced translocation of glucose transporter 4 (GLUT4) containing storage vesicles to the plasma membrane and phosphorylation of glycogen synthase enzyme (Bogan 2012). Decreased glucose uptake leads to reduced glycogen synthesis in the muscle cell. However, the majority of postprandial glucose disposal drives towards muscle glycogen synthesis (Bogan 2012, Samuel and Shulman 2016). Therefore, the significant difference in skeletal muscle glycogen content in untreated PD rats when compared to BA treated rats is eminent in this study. This demonstrated that there may be muscle insulin resistance and reduced glucose uptake as well as reduced glycogen synthesis in the untreated PD rats when compared to BA treated rats. Hence, we suggest that the administration of BA with or without diet intervention caused the observed increased muscle glycogen synthesis in BA treated rats by an increment of the expression of GLUT 4 *via* GLUT 4 translocation and probably by inhibition of muscle glycogen phosphorylase or stimulation of glycogen synthase enzymes. Indeed, previous studies have shown that other triterpenes (maslinic acid, oleanolic acid, and ursolic acid) inhibited glycogen phosphorylase and increased expression of GLUT 4 in skeletal muscle in PD or diabetic condition, and this study correlated with those studies (Cheng *et al.* 2008, Mkhwanazi *et al.* 2014, Pimentel *et al.* 2017).

Of note, chronic consumption of high caloric diets leads to ectopic lipid accumulation which has been implicated in peripheral insulin resistance (Barclay *et al.* 2013, Samuel and Shulman 2016). The skeletal muscle and the liver are primary organs of glucose homeostasis which store surplus glucose as glycogen and any insulin resistance in these organs alters glucose metabolism with consequent hyperglycaemia and impaired glucose tolerance. In this study, there was observed hyperinsulinaemia and impaired glucose tolerance in the untreated PD rats. The hyperinsulinaemia depicted peripheral insulin resistance and this may be responsible for the impaired glucose tolerance observed in the untreated PD rats. However, the administration of BA with or without diet intervention normalised impaired glucose tolerance as observed in the OGTT probably due to the decreased insulin resistance which was obvious in plasma insulin concentration and HOMA2-IR index results. Importantly, when there is a decrease in insulin sensitivity, glucose attaches to the haemoglobin in red blood cells resulting in high levels of glycated haemoglobin (Incani *et al.* 2015). Similarly, the high level of glycated haemoglobin was observed in untreated PD rats in this study. However, the glycated haemoglobin of BA treated rats was reduced to within range of the NPD rats possibly due to increased skeletal muscle glucose uptake and decreased insulin resistance by BA administration. Also, previous studies have reported that reduced glycated haemoglobin is a sign of sustained regulation of glucose

metabolism and this study is in agreement with those studies (Huang *et al.* 2016, Watson *et al.* 2017, Weiss *et al.* 2017).

Taken together, the administration of BA to diet-induced PD rats resulted in improved insulin sensitivity leading to improved glucose homeostasis in both the presence and absence of dietary intervention. Furthermore, the effects of this triterpene are comparable to those shown by the administration of metformin which may suggest that BA may be a good alternative in the management of prediabetes. More studies are needed, however, to determine the effects of this compound on other physiological parameters.

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Disclosure statement

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Appendix IV

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Research Article

Bredemolic Acid Ameliorates Selected Liver Function Biomarkers in a Diet-Induced Prediabetic Rat Model

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Background. Prediabetes is an intermediary hyperglycaemic state that precedes type 2 diabetes mellitus (T2DM) in which abnormal metabolism of glucose and lipids occurs in organs such as the liver. Evidence has shown that, about 70% of T2DM patients develop hepatic dysfunction which is found to begin during the prediabetic stage. Bredemolic acid, a pentacyclic triterpene, has been found to improve insulin sensitivity in diet-induced prediabetic rats. The effects of this compound on liver function, however, are unknown. This study was therefore designed to investigate the effects of BA on liver function in high fat-high carbohydrate (HFHC) diet-induced prediabetic rats. *Methods*. Thirty-six (36) male rats that weigh 150 g–180 g were divided into two groups, the non-prediabetic ($n = 6$) and the prediabetic groups ($n = 30$) that were fed normal diet (ND) and HFHC diet, respectively. The prediabetic rats were further subdivided into five groups ($n = 6$) and treated with either BA (80 mg/kg) or metformin (MET, 500 mg/kg) every third day for 12 weeks. After 12 weeks, blood samples and the liver were collected for biochemical analysis. *Results*. The induction of prediabetes resulted in increased release of liver enzymes (AST and ALT), increased liver glycogen and triglyceride, lipid peroxidation, and decreased sterol regulatory element-binding protein (SREBP1c) and antioxidant enzymes. However, the administration of BA decreased liver enzyme concentrations, decreased hepatic oxidative stress, and improved antioxidant enzymes such as SOD and GPx. *Conclusion*. BA administration improved liver function in diet-induced prediabetic rats in the presence or absence of dietary intervention.

1. Introduction

Prediabetes is a state of intermediate hyperglycaemia that causes abnormal changes in intracellular metabolism of most body tissues including the liver [1]. Presently, the observed increase in the prevalence of prediabetes and type 2 diabetes mellitus (T2DM) in developed and developing countries is reported to be due to sedentary lifestyles coupled with high-caloric diets [1–3]. However, studies have shown that excessive intake of high-caloric diets induces skeletal muscle insulin resistance which results into the shunting of glucose from the skeletal muscle to the liver thereby leading to increased hepatic glycogen production and storage [4–6]. Several studies have shown that continuous intake of high quantities of fats and carbohydrates alters liver function by accumulation of ectopic fats as a result of *de novo* lipogenesis which is mediated by transcription factors such as sterol regulatory element-binding protein (SREBP1c) under insulin action [7, 8]. Moreover, excessive hepatic accumulation of free fatty acid or triglyceride leads to hepatic insulin resistance, hepatic dysfunction, and nonalcoholic fatty liver disease (NAFLD) that is characterized by fat infiltration into the hepatocytes [9–14]. Consequently, the infiltration of fat into the hepatocytes triggers oxidative stress, and reduces antioxidant enzymes production and caused an inflammatory cascade of reactions that produce progressive fibrotic

hepatic damage known as nonalcoholic steatohepatitis (NASH). Cross-sectional studies have demonstrated that liver function markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are altered due to oxidative stress and hepatic dysfunction [15–18]. However, it has been established that approximately 70% of T2DM patients have liver dysfunction and complications [19–21]. There is also evidence from other studies that suggested that liver dysfunction and complications can also begin during the prediabetic stage [21–23].

Current treatment focuses on a combination of dietary and pharmacological interventions, but there has been reports of low compliance as patients merely use pharmacological intervention without diet modification thus reducing the efficacy of the pharmacological intervention [24–27]. Therefore, novel compounds that can ameliorate liver dysfunction in the prediabetic condition even in the absence of dietary intervention are necessary. Oleanolic acid and maslinic acid are pentacyclic triterpenes that have been found to have antidiabetic and antioxidant properties [28–30]. In our laboratory, we have shown that chronic ingestion of a high fat-high carbohydrate diet leads to the development of prediabetes which is accompanied by liver complications. We have further shown that bredemolic acid (BA), a structural isomer of maslinic acid, is able to restore glucose homeostasis in diet-induced prediabetes by improving insulin sensitivity both in presence and absence of dietary intervention [31]. However, the effects of BA on liver function in diet-induced prediabetes have not been established. Hence, the aim of this study is to investigate the effects of bredemolic acid on selected biomarkers of liver function in a diet-induced prediabetic rat model.

2. Materials and Methods

2.1. Animals. Thirty-six (36) male Sprague Dawley rats (150–180 g) obtained from Biomedical Research Unit, University of KwaZulu-Natal (UKZN), were kept under standard environmental conditions i.e., constant humidity (55 ± 5%), temperature (22 ± 2°C), 12 h day : 12 h night cycle. The animals were acclimatized for 2 weeks and consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* before being fed on the experimental high fat-high carbohydrate (HFHC) diet (AVI Products (Pty) Ltd., Waterfall, South Africa) to induce prediabetes. The HFHC diet consists of carbohydrate (55% kcal/g), fats (30% kcal/g), and proteins (15% kcal/g) as described in our previous study [27, 31]. All the experimental designs and procedures were carried out according to the ethics and guidelines of the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa.

2.2. Experimental Design. After acclimatization, the animals were divided into two groups, the normal diet (ND) non-prediabetic control ($n = 6$) and the HFHC diet prediabetic groups ($n = 30$). All the animals in the prediabetic group consumed HFHC diet and drinking water that was supplemented with 15% fructose for 20 weeks to induce prediabetes while the non-prediabetic control group (NPD, Group 1) fed on ND and water *ad libitum* for 20 weeks as well. At the 20th week, prediabetes was confirmed by fasting blood glucose and oral glucose tolerance test which have been described in the previous research study [31].

2.3. Treatment of Prediabetic Animals. After 20 weeks of prediabetes induction, the non-prediabetic control (NPD, Group 1) animals were continuously fed on standard rat chow for 12 weeks. Thirty (30) prediabetic animals were randomly assigned into 5 different groups (Group 2 to Group 6, $n = 6$). Group 2 (PD) served as the untreated prediabetic control group and continuously consumed the HFHC diet for 12 weeks; Group 3 (ND + MET) were prediabetic animals that switched to standard rat chow and received metformin (MET) for 12 weeks; Group 4 (HFHC + MET) were prediabetic animals that continuously consumed HFHC diet with MET treatment; Group 5 (ND + BA) were prediabetic animals that switched to standard rat chow and received BA for 12 weeks; and Group 6 (HFHC + BA) were prediabetic animals that continuously consumed HFHC diet and received BA as treatment for 12 weeks. Treatment via oral administration of MET (7.2 mg/kg, extrapolated from 500 mg/70 kg human dose) or BA (80 mg/kg) was carried out every third day for 12 weeks as described in our previous study [31].

2.4. Blood Collection and Tissue Harvesting. After the 12th week treatment period, the animals were sacrificed. The animals were placed in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) and anaesthetised with Isofor (100 mg/kg, Safeline Pharmaceuticals, Roodepoort, South Africa) for 3 minutes. Blood samples were collected from the animals using cardiac puncture and put into different precooled EDTA containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503g for 15 minutes to obtain plasma. Each of the plasma was aspirated into plain sample bottles and stored at −80°C in a BioUltra freezer (Snijers Scientific, Tilburg, Holland) until ready for biochemical analysis. Also, the liver tissue samples were excised, weighed, and rinsed in cold normal saline solution and snapped frozen in liquid nitrogen before storage in the BioUltra freezer for biochemical analysis of selected metabolic parameters.

2.5. Relative Liver Weight. The relative liver weights of all the animals in each experimental group were determined from the percentage of the ratio of liver weight to the body weight i.e.,

$$\text{relative liver weight} = \frac{\text{liver weight}}{\text{body weight}} \times 100. \quad (1)$$

2.6. Biochemical Analysis. Liver enzymes (AST and ALT) in the plasma were analysed with IDEXX Catalyst One Chemistry Analyzer (IDEXX Laboratories Inc., Westbrook,

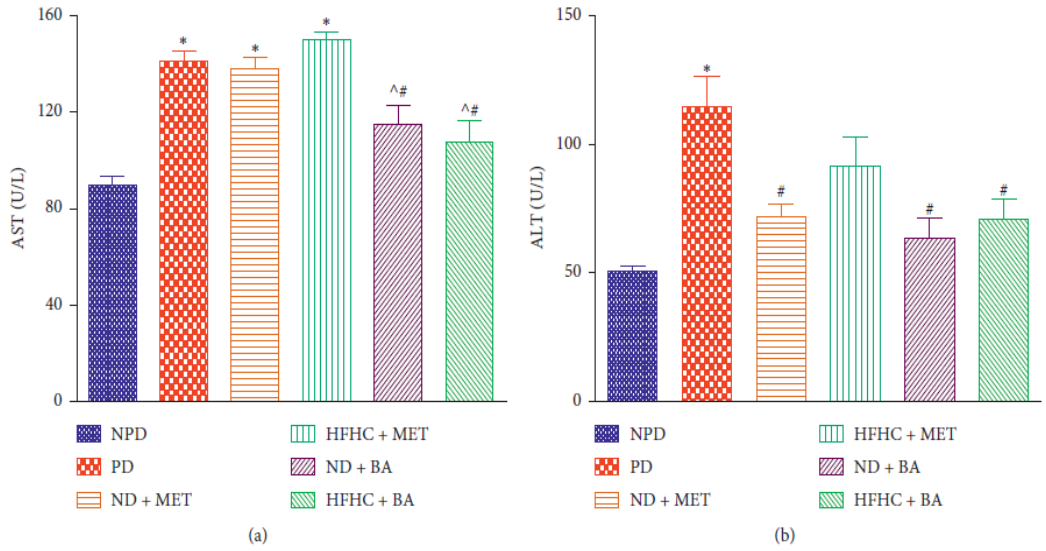

FIGURE 2: Effects of BA with the presence or absence of dietary intervention on the plasma AST and ALT in prediabetic rats. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.05$ in comparison with PD.

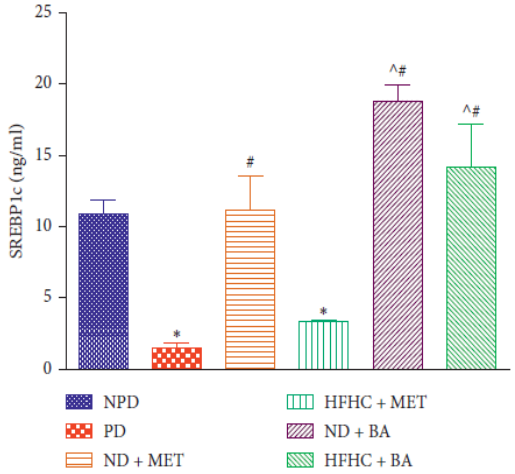

FIGURE 3: Effects of BA with the presence or absence of dietary intervention on the liver SREBP1c in prediabetic rats. $^{*}p < 0.001$ in comparison with NPD, $^{\#}p < 0.001$ in comparison with PD, and $\hat{}p < 0.01$ in comparison with HFHC + MET.

treated with BA in the absence or presence of dietary intervention.

3.5. Liver Triglycerides. Liver triglyceride concentrations were significantly increased in the PD group by comparison with the NPD group ($p < 0.001$). The liver triglyceride concentration of BA-treated rats with or without diet intervention significantly decreased when compared with the PD group ($p < 0.001$). Similar results were observed with the use of metformin, see Figure 4.

3.6. Liver Glycogen. Liver glycogen concentrations of the PD group were significantly increased by comparison with the NPD group ($p < 0.001$). The administration of BA with or without diet intervention significantly decreased liver glycogen concentrations by comparison with PD ($p < 0.001$). Similarly, the administration of metformin treated with or without diet intervention significantly decreased the liver glycogen concentration when compared with PD, see Figure 5.

3.7. Lipid Peroxidation and Antioxidant Enzyme Activity. As shown in Table 2, liver MDA concentration in the untreated PD group was significantly increased by comparison with the NPD group ($p < 0.001$). The administration of BA and metformin with or without diet intervention significantly decreased the liver MDA concentration when compared with the PD group ($p < 0.05$). Liver SOD and GPx activities of the untreated PD group were significantly decreased when compared with the NPD group ($p < 0.05$). The SOD and GPx activities in the liver of BA-treated rats with or without diet intervention were significantly increased in comparison with those in PD group ($p < 0.05$).

4. Discussion

This study examined the effects of BA on selected markers of liver function in diet-induced prediabetic rats. Triterpenes such as maslinic acid and oleanolic acid have been reported to ameliorate oxidative stress in the liver via increased release of antioxidant enzymes and improved liver function via increased activity of glycogenic enzymes to decrease hepatic glucose production in diabetic rats [29, 32]. In a previous study, BA was shown to improve insulin sensitivity in the skeletal muscle by increasing the expression of GLUT 4;

Table 1: Correlation between fasting blood insulin (FBI) and hepatic sterol regulatory element-binding protein (SREBP1c) in non-prediabetic (NPD) rats, prediabetic control (PD), and prediabetic rats treated with BA in the presence or absence of dietary intervention. r = Pearson's correlation coefficient, R^2 = coefficient of determination, and n = sample size.

Groups	Correlation analysis	Independent variable: FBI	Dependent variable: hepatic SREBP1c
NPD	r	0.8068	0.8068
	R^2	0.6510	0.6510
	n	6	6
	p value	0.0524^{NS}	0.0524^{NS}
PD	r	−0.9144	−0.9144
	R^2	0.8361	0.8361
	n	6	6
	p value	0.0107*	0.0107*
ND + MET	r	−0.8691	−0.8691
	R^2	0.7552	0.7552
	n	6	6
	p value	0.0246*	0.0246*
HFHC + MET	r	−0.8869	−0.8869
	R^2	0.7866	0.7866
	n	6	6
	p value	0.0185*	0.0185*
ND + BA	r	0.4651	0.4651
	R^2	0.2164	0.2164
	n	6	6
	p value	0.3526^{NS}	0.3526^{NS}
HFHC + BA	r	−0.7381	0.7381
	R^2	0.5448	0.5448
	n	6	6
	p value	0.0939^{NS}	0.0939^{NS}

^{NS}Not significant; $^*p < 0.05$.

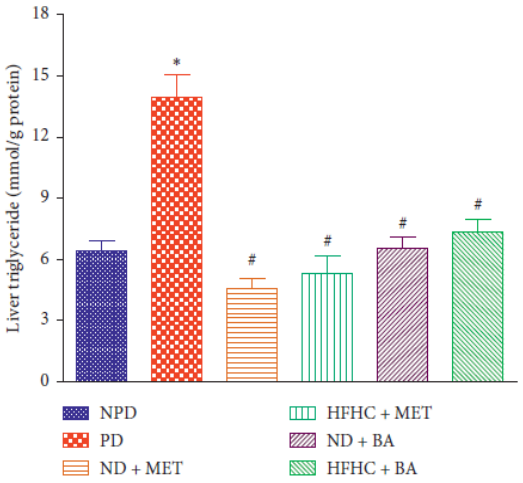

Figure 4: Effects of BA with the presence or absence of dietary intervention on the liver triglyceride in prediabetic rats. $^*p < 0.001$ in comparison with NPD; $^{\#}p < 0.001$ in comparison with PD.

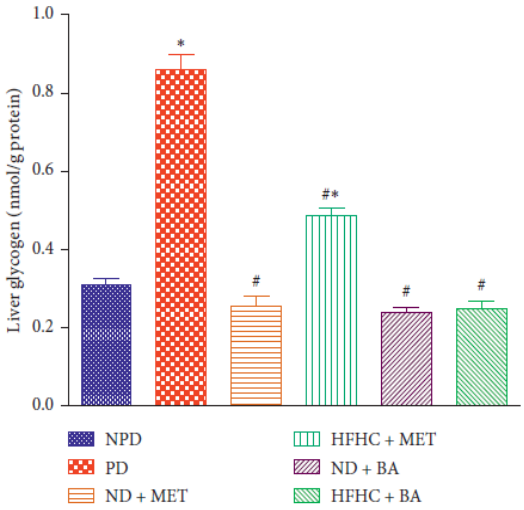

Figure 5: Effects of BA with the presence or absence of dietary intervention on the liver glycogen in prediabetic rats. $^*p < 0.001$ in comparison with NPD; $^{\#}p < 0.001$ in comparison with PD.

however, the effects of this triterpene on liver function in the prediabetic state were not determined [31]. Hence, this study is a continuation of the previous study [31] and sought to evaluate the effects of BA on selected markers of liver function in a diet-induced prediabetic rat model. The liver plays a key role in maintaining glucose homeostasis as it balances the production of glucose and the conversion of glucose to glycogen [33]. In a postprandial state, blood glucose increases, and insulin is secreted to enhance glycogenesis and inhibit glycogenolysis [34]. However, studies have shown that chronic consumption of high fat-high carbohydrate diet results in the induction of prediabetes which is characterized by hyperinsulinaemia, impaired

Table 2: Effects of BA with the presence or absence of dietary intervention on the liver lipid peroxidation and antioxidant enzyme activities in prediabetic rats. Values are presented as mean ± SEM ($n = 6$).

Groups	Malondialdehyde (MDA) (nmol/g protein)	Superoxide dismutase (SOD) ($nmol \cdot min^{-1} \cdot mL \cdot mg^{-1}$protein)	Glutathione peroxidase (GPx) ($nmol \cdot min^{-1} \cdot mL \cdot mg^{-1}$protein)
NPD	4.11 ± 0.51	2.99 ± 0.06	1.67 ± 0.09
PD	12.34 ± 1.31*	1.66 ± 0.22*	1.08 ± 0.06*
ND + MET	5.00 ± 0.26#	2.14 ± 0.02#	1.79 ± 0.07#^
HFHC + MET	6.41 ± 0.27#	1.83 ± 0.13*	1.05 ± 0.05*
ND + BA	4.89 ± 0.44#	2.47 ± 0.06#	1.87 ± 0.10#^
HFHC + BA	6.68 ± 0.65#	2.59 ± 0.02#	1.89 ± 0.04#^

*$p < 0.05$ in comparison with the non-prediabetic (NPD) control, #$p < 0.05$ in comparison with the prediabetic (PD) control, and ^$p < 0.05$ in comparison with the HFHC + MET group.

glucose tolerance, and peripheral and hepatic insulin resistance, as well as liver damage [1, 35, 36]. In the prediabetic state, due to hyperinsulinaemia and selective muscle insulin resistance, most ingested glucose is shunted to the liver leading to increased hepatic glycogenesis [6, 37]. In addition, since the liver is insulin-independent, excess glucose in the blood can diffuse into the hepatic cells through facilitated diffusion which is mediated by glucose transporter 2 (GLUT (2)) [14, 34, 38]. Similarly, the elevated liver glycogen concentration observed in untreated prediabetic rats in this study can be attributed to the increased diversion of excess glucose to the liver. This showed that consumption of high fat-high carbohydrate diet can result into diversion of glucose to the liver as a compensatory mechanism in the presence of selective muscle resistance in the prediabetic state [34]. However, the administration of BA with or without diet intervention significantly reduced liver glycogen concentrations. Previous studies have shown that administration of BA in the prediabetic state improves insulin sensitivity in the skeletal muscle through increased GLUT 4 expression [31]. We suggest that this improved insulin sensitivity in the periphery leads to decreased amounts of glucose being shunted to the liver thus resulting in the observed decrease in liver glycogen concentrations.

In nondiabetic subjects, metabolism of glucose is largely carried out in the skeletal muscle [39, 40]. In the prediabetic state, as glucose delivery to the liver increases, *de novo* lipogenesis and hepatic lipid accumulation increase under the influence of transcription factors such as SREBP1c [6, 14, 37, 40]. SREBP1c is a major transcription factor which regulates *de novo* lipogenesis through direct activation from AKT (protein kinase B) in the insulin signaling pathway [8, 41, 42]. In the prediabetic state, when insulin signaling is impaired, the direct activation of SREBP1c by AKT is altered, and the SREBP1c expression decreases [6–8]. On the contrary, the hepatic *de novo* lipogenesis is not solely dependent on insulin signaling through activation of SREBP1c, but the activation of SREBP1c to stimulate *de novo* lipogenesis depends on insulin signaling [6, 43]. However, when the insulin signaling pathway is impaired in prediabetes, *de novo* lipogenesis is still elevated due to the substrate push mechanism in which there is increased substrate delivery to the liver followed by increased esterification of fatty acids into triglycerides [6]. In this study, we observed that the concentration of SREBP1c in the liver was significantly lowered in untreated prediabetic rats by comparison with the non-prediabetic rats. According to our correlation analysis between fasting blood insulin and hepatic SREBP1c, the decreased hepatic SREBP1c in untreated prediabetic rats may be due to the alteration of insulin signaling in the prediabetic state since SREBP1c expression is insulin-dependent. In addition, the correlation analysis showed that there was an inverse relationship between the increased fasting blood insulin and the hepatic SREBP1c concentration under the insulin-resistant condition. This observation is in correlation with previous studies which reported that insulin signaling is not totally required for hepatic lipogenesis, and that availability of the substrate can facilitate delivery of substrates into the liver for lipogenesis [6, 44]. Of note, the BA-treated rats had a significantly increased SREBP1c thus suggesting that BA ameliorated insulin signaling which may have resulted into the increased SREBP1c concentration in the liver. Furthermore, high fructose consumption has been reported to increase hepatic lipogenesis and glycogenesis [1]. Fructose, unlike glucose, is solely metabolized in the liver thereby providing additional substrates for *de novo* lipogenesis and ectopic fat accumulation in the liver, thus leading to NAFLD [1, 10]. In this study, we observed that the liver triglyceride in untreated prediabetic rats significantly increased when compared with non-prediabetic rats. The increased liver triglyceride in untreated prediabetic rats can be attributed to increased substrate delivery to the liver or decreased hepatocellular triglyceride disposal, as well as decreased fatty acid oxidation [45]. However, the administration of BA significantly decreased hepatic triglycerides, and this suggests that BA may decrease substrate delivery to the liver by divergence of the substrates to other organs for metabolism, increased β oxidation of fat, or increased triglyceride disposal via very low-density lipoprotein (VLDL) exportation from the liver.

Moreover, due to the increased hepatic lipogenesis and glycogenesis, the production of free radicals is elevated, and this results into oxidative stress [46]. Oxidative stress is due to an imbalance between oxidant and antioxidant enzymes [46]. Antioxidants are stable molecules that donate electrons to rampaging free radicals in order to neutralise the free radical capacity to damage tissues or organs [47, 48]. In this study, we observed that lipid peroxidation (MDA) in the liver was significantly increased, and antioxidant enzyme (SOD and GPx) production in the liver was significantly decreased in the

untreated prediabetic rats when compared with non-prediabetic rats. The increased lipid peroxidation was due to increased production of free radicals while the decreased antioxidant capacity of the liver was as a result of decreased production of antioxidant enzymes (SOD and GPx) in the mitochondria of hepatocytes during prediabetes. On the contrary, BA administration with or without diet intervention significantly lowered lipid peroxidation and significantly increased the liver antioxidant enzymes. This may be due to the fact that BA neutralises the free radicals in the mitochondria of hepatocytes by donation of electron through hydroxyl radical scavenging activity which has been reported in other triterpenes [49]. This is in line with similar observations made on earlier studies using other triterpenes [28, 32, 49].

Furthermore, studies have shown that elevated liver enzymes (AST and ALT) in the plasma can be due to necrosis of the hepatocyte during liver damage [18]. AST and ALT are released into the blood stream whenever hepatocytes are damaged, and this has been reported to occur during prediabetes [18]. In this study, these enzymes were significantly elevated in untreated prediabetic rats by comparison with non-prediabetic rats. The increased liver enzymes in the plasma suggested that liver cells are damaged through oxidative stress and increased hepatic lipogenesis or glycogenesis. However, BA administration caused a decrease in the concentration of liver enzymes suggesting that BA may improve hepatic function via its antioxidant and antilipidemic effects in the liver as observed in this study. Of note, triterpenes are nontoxic antioxidants and have low pharmacokinetics of three days; therefore, the ameliorative effects of BA in the absence of dietary intervention on liver function markers compared with metformin in this study may be attributed to this low pharmacokinetic feature. In conclusion, the administration of BA in both the presence and absence of dietary modification can potentially be one of the therapeutic approaches to attenuate hepatic dysfunction or improve hepatic functions in the prediabetic state.

Data Availability

The data used to support the findings of this study are available upon request from the corresponding author. However, the data on body weight, fasting blood insulin (FBI), fasting blood glucose, and oral glucose tolerance test which are relevant for this study have been reported in our previous study.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix V

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Research Article

Bredemolic Acid Improves Cardiovascular Function and Attenuates Endothelial Dysfunction in Diet-Induced Prediabetes: Effects on Selected Markers

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Prediabetes is an intermediate hyperglycaemic state which has been associated with cardiovascular dysfunction. However, cardiovascular dysfunction is not only caused by intermediate hyperglycaemia but also endothelial dysfunction, inflammation, and oxidative stress associated with prediabetes. Bredemolic acid (BA), an isomer of maslinic acid, has been reported to ameliorate the intermediate hyperglycaemia found in prediabetes; however, the effects of this triterpene on cardiovascular function have not yet been determined. Therefore, this study investigated the effects of BA on cardiovascular function in diet-induced prediabetic rats. Thirty-six male rats that weighed 150–180 g were divided into two groups, the non-prediabetic ($n = 6$) and the prediabetic groups ($n = 30$), which were fed normal diet (ND) and HFHC diet, respectively. The prediabetic rats were further subdivided into five groups ($n = 6$) and treated with either BA (80 mg/kg) or metformin (MET, 500 mg/kg) every third day for 12 weeks. After 12 weeks, blood samples and the heart were collected for biochemical analysis. The untreated prediabetic rats showed a significant increase in body mass index (BMI), waist circumference (WC), blood pressure, heart rate, lipid profile, lipid peroxidation, and inflammatory markers with significant decrease in endothelial function and antioxidant biomarkers by comparison with the non-prediabetic animals. The administration of BA significantly improved cardiovascular functions such as blood pressure, heart rate, and endothelial function. There was also a significant decrease in BMI, WC, lipid profile, lipid peroxidation, and inflammation with a concomitant increase in antioxidant capacity. BA administration improved cardiovascular function by attenuation of oxidative stress, inflammatory, and endothelial dysfunction markers.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a heterogeneous metabolic disorder which is associated with cardiovascular diseases (CVDs), that is often preceded by the onset of prediabetes [1]. One of the identified causes of this disorder is chronic consumption of high caloric diets which are rich in carbohydrates as well as saturated and polyunsaturated fats coupled with sedentary lifestyles [2, 3]. Consequently, this leads to inefficient metabolism of carbohydrates and fats resulting in accumulation of intracellular and extracellular glucose and lipids known as glucolipotoxicity [4].

However, glucolipotoxicity is associated with insulin resistance which subsequently causes high body mass index (BMI), high waist circumference, hyperlipidaemia, oxidative stress, and release of inflammatory cytokines such as high sensitive C-reactive protein, hs-CRP, interleukin 6, IL-6, and tumour necrotic factor alpha (TNF-α) [3–6]. Glucolipotoxicity is also associated with endothelial dysfunction, hypertension, arteriosclerosis, coronary heart disease, and

stroke [5–8]. In addition, insulin resistance is associated with decreased nitric oxide (NO) production due to inhibition of endothelial nitric oxide synthase (eNOS) via impaired phosphatidylinositol 3 kinase (PI3K)–AKT (protein kinase B) pathway [9]. The decreased NO production causes an imbalance in the vascular endothelial tone which triggers vasoconstriction followed by increased heart rate and high blood pressure [9, 10]. Prediabetes is an asymptomatic and intermediate hyperglycaemic stage that has been reported to precede the onset of cardiovascular complications observed in T2DM [8, 11, 12]. Additionally, previous studies have shown that intermediate hyperglycaemia below the level used to define diabetes mellitus is a risk factor for CVD development [13, 14].

Of note, the combination of dietary modification with pharmacotherapy is the main approach in preventing the development of CVDs in prediabetic or diabetic individuals [6, 15]. However, there has been reported low compliance to this combination therapy as most patients only observe pharmacological intervention without changing their diet [16]. This inadvertently reduces the efficacy of the pharmacological interventions [17]. Therefore, antidiabetic agents that have the ability to restore glucose homeostasis and prevent the risk of CVD development regardless of diet intervention are necessary.

Pentacyclic triterpenes such as oleanolic acid and maslinic acid are antidiabetic and antioxidant agents with proofs and literature evidence [18, 19]. More importantly, bredemolic acid (BA), an isomer of maslinic acid, has been shown in the previous study to have antidiabetic effects by reduction of blood glucose through increased expression of GLUT 4 in the skeletal muscle of prediabetic rats [20]. However, the effects of this triterpene on cardiovascular system in prediabetes have not been established. Therefore, the aim of this study was to investigate the effect of BA on selected markers of cardiovascular function in a diet-induced prediabetic rat model.

2. Materials and Methods

2.1. Animals. In this study, thirty-six male Sprague Dawley rats with body weight 150–180 g were used. The rats were obtained and bred at the Biomedical Research Unit (BRU), University of KwaZulu-Natal (UKZN). The animals were kept and maintained in standard experimental conditions at room temperature (22 ± 2°C), humidity (55 ± 5%), and 12 h day : 12 h night cycle. The animals consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* for 2 weeks to acclimatize before being exposed to the experimental diet (high fat high carbohydrate). The high fat high carbohydrate (HFHC) diet was composed of carbohydrates (55% kcal/g), fats (30% kcal/g), and proteins (15% kcal/g). All experimental procedures were according to the ethics and animal care guidelines of the Animal Research Ethics Committee (AREC) of UKZN, Durban, South Africa (AREC/024/018D).

2.2. Experimental Design. After 2 weeks of acclimatization, the animals were distributed into two main groups: the non-prediabetic control group ($n = 6$) and the prediabetic group ($n = 30$). The non-prediabetic control (NPDC) animals served as the negative control and were given normal diet (ND) and water *ad libitum* while the prediabetic animals were given HFHC diet and drinking water supplemented with fructose (15%) for 20 weeks to induce prediabetes. After 20 weeks, prediabetes was confirmed via fasting blood glucose and oral glucose tolerance test using criteria of the American Diabetes Association as described in our previous study [20].

2.3. Treatment of Animals. After the 20 weeks of prediabetes induction, the non-prediabetic control (Group 1) continuously fed on ND for a further 12 weeks while the prediabetic animals ($n = 30$) were divided into 5 groups (Group 2–Group 6, $n = 6$ in each group). Group 2 (PD) served as the untreated prediabetic control group and continuously consumed the HFHC diet for 12 weeks; Group 3 (ND + MET) were prediabetic animals that switched to standard rat chow and received MET for 12 weeks; Group 4 (HFHC + MET) were prediabetic animals that continuously consumed HFHC diet with MET treatment; Group 5 (ND + BA) were prediabetic animals that switched to standard rat chow and received BA for 12 weeks; Group 6 (HFHC + BA) were prediabetic animals that continuously consumed HFHC diet and received BA as treatment for 12 weeks. Treatment with either MET (500 mg/kg) or BA (80 mg/kg) was carried out twice every third day for 12 weeks. The body mass index (BMI), waist circumference (WC), blood pressure, and heart rate were assessed in all animals at week 20 and every 4 weeks (24^{th}, 28^{th}, and 32^{nd} week).

2.4. Blood Collection and Tissue Harvesting. After the 12 weeks of treatment, the animals were sacrificed. The animals were placed in a gas chamber (BRU, UKZN, South Africa) and anaesthetised with 100 mg/kg of Isofor (Safeline Pharmaceuticals Ltd, Roodeport, South Africa) for 3 minutes to collect blood samples. In an unconscious state, blood samples were collected by cardiac puncture into precooled heparinized containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes for plasma collection. The plasma were collected and stored at −80°C in a Bio Ultra freezer (Snijers Scientific, Tilburg, Holland). The hearts of all the animals were excised, rinsed with cold normal saline solution, weighed, and snapped frozen in liquid nitrogen before storage in Bio Ultra freezer at −80°C for biochemical analysis.

2.5. Determination of BMI and WC. The determination of BMI was measured from the ratio of the weight to the square of the length of the animals as described in the established protocol [21]. Also, the waist circumference of the animals was determined according to the previous protocol [22].

2.6. Determination of Blood Pressure and Heart Rate. The blood pressure and heart rate were measured as described in the established protocol [19]. Briefly, at every 4 weeks of

treatment, the noninvasive MRBP IITC Model 31, Life Sciences multichannel tail cuff blood pressure system (Life Sciences, Woodland Hills, CA) was used to monitor the blood pressure and the heart rate by placing the animals in a restrainer (3″ ID (75 mm)–12″ length) while the tail of the animals is attached to the tail cuff. All the rats in the restrainer were placed in a warming chamber (IITC Model 303sc Animal Test Chamber, Life Sciences, Woodland Hills, CA) maintained at 32°C, and the blood pressure as well as the heart rate was measured by occlusion or deflation of the tail cuff which detects alteration of blood flow in the tail artery. An average of three measured sessions consisting of 15 cycles was used for statistical analysis.

2.7. Biochemical Analysis. The lipid profile, antioxidant, inflammatory, and endothelial markers were measured at 32nd week only.

2.8. Lipid Profile Analysis. The plasma total cholesterol (TC), high density lipoprotein (HDL) cholesterol, and triglycerides (TG) were analysed via a Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW Germany) by using commercial specialized kits according to the instruction from the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The other lipid profiles such as very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol were calculated according to Friedewald's formula [23]. VLDL cholesterol = TG × 0.2, and LDL cholesterol = TC – (VLDL cholesterol + HDL cholesterol).

2.9. MDA and Antioxidant Status. The lipid peroxidation was determined by estimation of the amount of malondialdehyde (MDA) in the heart tissue homogenate according to previously described protocols [19, 24]. However, the antioxidant status of the heart homogenates was determined by using a specific ELISA kit to analyse the concentration of superoxide dismutase (SOD) and glutathione peroxidase (GPx) according to the instruction manual of the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

2.10. Determination of Endothelial Function and Inflammatory Markers. The endothelial function and inflammation were evaluated from the plasma by determination of the endothelial nitric oxide synthase (eNOS) through the commercialized ELISA kit in accordance with the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Houston, TX, USA). The inflammatory markers (TNF-α, IL-6, and hs-CRP) were measured in the plasma via specific ELISA kits in accordance with the manufacturer's instruction (Elabscience Biotechnology Co., Ltd., Houston, TX, USA), and the absorbance was measured via the microplate reader, Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW, Germany).

2.11. Statistical Analysis. The data were presented as mean ± standard error of mean (SEM). Statistical analysis was determined by two-way analysis of variance (ANOVA) followed by the Bonferroni test as post hoc via GraphPad Prism 5 software. The level of statistical significant difference was considered at $p < 0.05$.

3. Results

3.1. Body Mass Index (BMI) and Waist Circumference (WC). The effects of BA treatment on BMI and WC in non-prediabetic and prediabetic rats with or without diet intervention were determined as indicated in Figures 1 and 2. The BMI and WC of the untreated prediabetic (PD) rats were significantly increased by comparison with the non-prediabetic (NPD) control rats throughout the treatment period ($p < 0.001$). However, the administration of BA with or without diet intervention significantly decreased both BMI and WC when compared to the PD group as shown in Figures 1 and 2, respectively ($p < 0.01$).

3.2. Blood Pressure and Heart Rate. As shown in Figure 3, the systolic blood pressure of PD control rats was significantly increased throughout the treatment period when compared to the NPD control rats ($p < 0.001$). However, the systolic blood pressure of BA-treated rats with or without diet intervention significantly decreased when compared to that of PD control rats. As demonstrated in Figure 4, the diastolic blood pressure of PD control rats were significantly increased when compared to NPD control rats ($p < 0.001$). The administration of BA with or without diet intervention significantly decreased the diastolic blood pressure when compared to the PD group ($p < 0.05$). The same results were observed with the ND + MET group. A significant increase in heart rate was observed in the PD rats throughout the period of treatment when compared to the NPD control rats as indicated in Figure 5 ($p < 0.01$). However, the heart rate of BA-treated rats with or without diet intervention and MET-treated rats with diet intervention (ND + MET) were significantly lowered by comparison with the PD control rats ($p < 0.01$).

3.3. Lipid Profile. As shown in Table 1, the TC, TG, LDL, and VLDL of the untreated PD group significantly increased in comparison with the NPD group ($p < 0.001$). The TC and LDL of BA-treated rats with or without diet intervention were significantly decreased when compared to the PD control rats ($p < 0.01$). Similar results were obtained for the ND + MET group. Additionally, only the ND + BA and ND + MET groups had significantly lowered TG and VLDL when compared to the PD control rats ($p < 0.05$).

3.4. Endothelial Function Marker. The plasma concentration of eNOS in PD control rats significantly decreased when compared to NPD control rats as indicated in Figure 6 ($p < 0.001$). However, the plasma concentration of eNOS in BA-treated rats with or without diet intervention significantly increased by comparison with the PD control rats ($p < 0.01$).

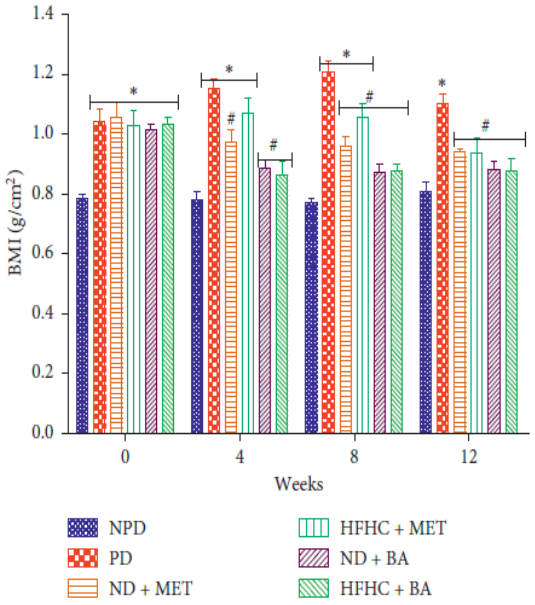

FIGURE 1: Effects of BA on BMI in non-prediabetic (NPD) and prediabetic rats with or without diet intervention. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.01$ in comparison with PD.

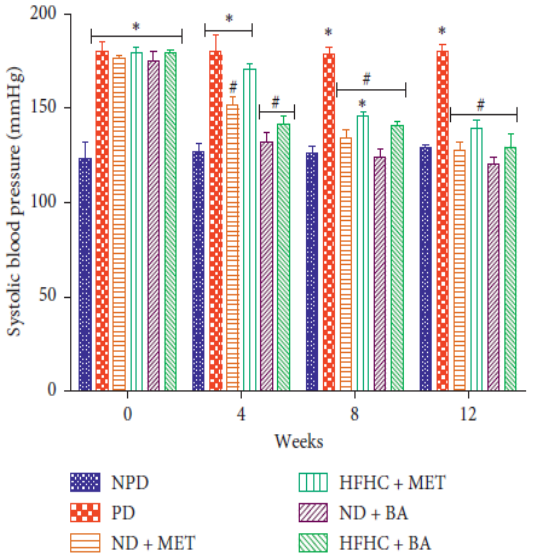

FIGURE 3: Effects of BA on systolic blood pressure in non-prediabetic and prediabetic rats with or without diet intervention. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.001$ in comparison with PD.

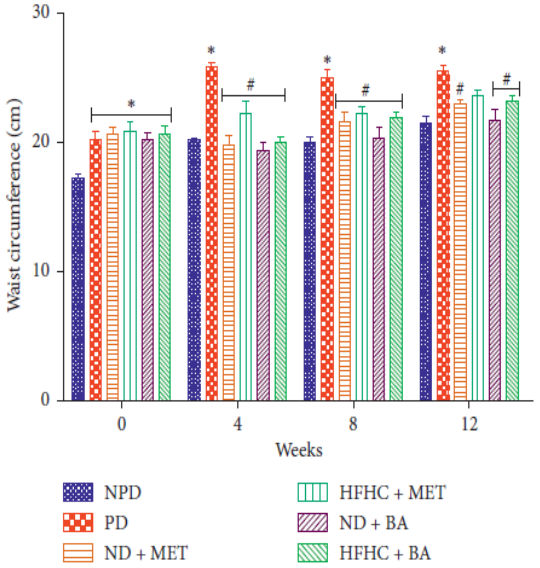

FIGURE 2: Effects of BA on waist circumference in non-prediabetic and prediabetic rats with or without diet intervention. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.05$ in comparison with PD.

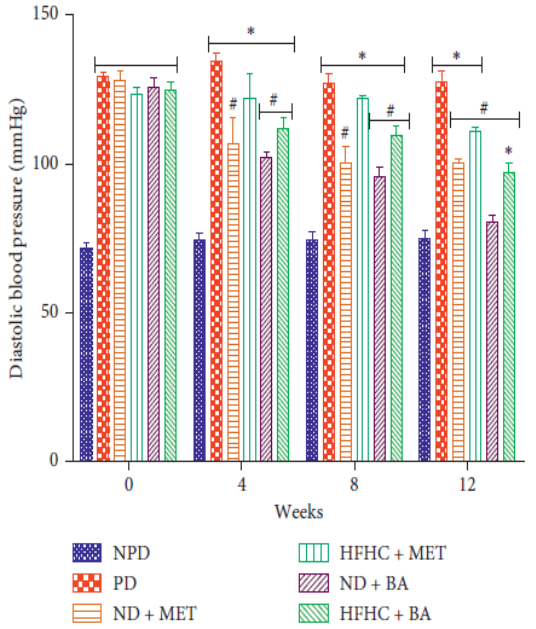

FIGURE 4: Effects of BA on diastolic blood pressure in non-prediabetic (NPD) and prediabetic rats with or without diet intervention. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.01$ in comparison with PD.

3.5. Lipid Peroxidation and Antioxidant Status. As indicated in Table 2, a significant increase in the heart MDA concentration was observed in the PD groups by comparison with the NPD group ($p < 0.01$). Rats treated with BA in the presence and absence of diet intervention had a significantly decreased MDA concentration by comparison with untreated PD rats. However, there was no significant difference in heart MDA concentrations in the HFHC + MET group when compared to PD control rats. The heart SOD and GPx concentration of the PD control rats significantly decreased in comparison with NPD control rats ($p < 0.01$). On the other hand, administration of BA with or without diet intervention significantly increased both SOD and GPx concentration in the heart tissue by comparison with the untreated PD group ($p < 0.05$).

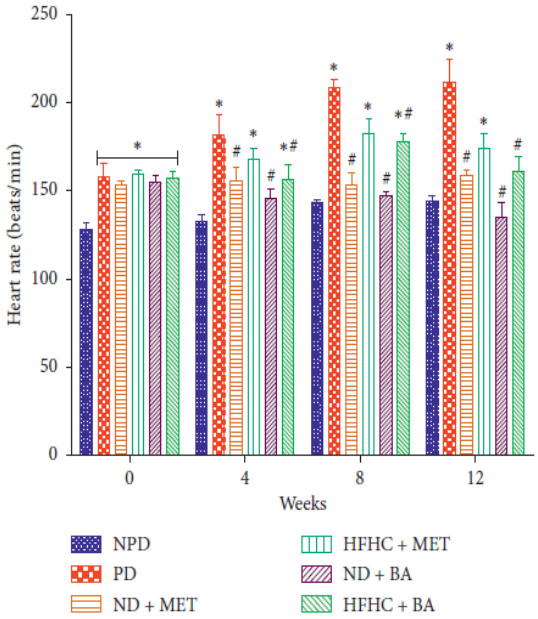

FIGURE 5: Effects of BA on heart rate in non-prediabetic (NPD) and prediabetic rats with or without diet intervention. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.01$ in comparison with PD.

TABLE 1: The effects of BA on lipid profile in non-prediabetic and prediabetic rats with or without diet intervention.

Parameters	Groups					
	NPD	PD	ND + MET	HFHC + MET	ND + BA	HFHC + BA
TC (mmol/L)	2.00 ± 0.04	2.88 ± 0.03***	2.06 ± 0.03$^{\#\#\#}$	2.43 ± 0.16	2.10 ± 0.09$^{\#\#\#}$	2.25 ± 0.13$^{\#\#}$
TG (mmol/L)	1.12 ± 0.10	1.75 ± 0.02**	1.13 ± 0.03$^{\#\#}$	1.58 ± 0.22	1.18 ± 0.02$^{\#}$	1.45 ± 0.02
HDL (mmol/L)	1.11 ± 0.03	1.04 ± 0.04	1.13 ± 0.04	1.08 ± 0.09	1.16 ± 0.06	1.10 ± 0.05
LDL (mmol/L)	0.67 ± 0.04	1.49 ± 0.05***	0.70 ± 0.06$^{\#\#\#}$	1.03 ± 0.05$^{*\#\#}$	0.71 ± 0.07$^{\#\#\#}$	0.86 ± 0.12$^{\#\#\#}$
VLDL (mmol/L)	0.22 ± 0.02	0.35 ± 0.01**	0.23 ± 0.01$^{\#\#}$	0.32 ± 0.05	0.24 ± 0.01$^{\#}$	0.29 ± 0.01

Values are presented as mean ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (vs. NPD). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (vs. PD).

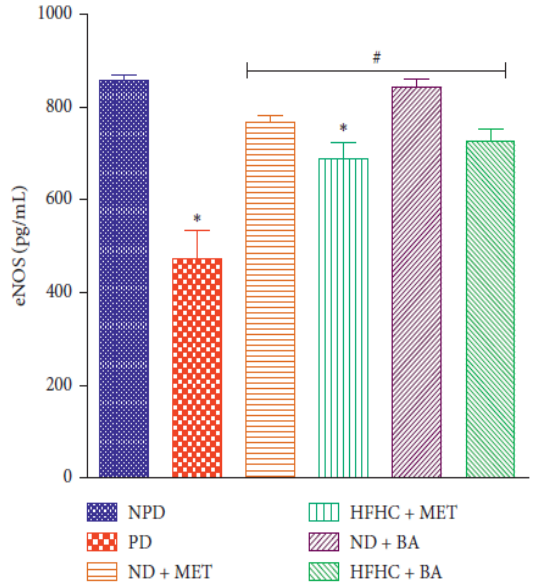

FIGURE 6: Effects of BA on eNOS concentration in non-prediabetic (NPD) and prediabetic rats. with or without diet intervention. $^{*}p < 0.001$ in comparison with NPD; $^{\#}p < 0.001$ in comparison with PD.

Table 2: The effects of BA on oxidative stress and inflammatory biomarkers in non-prediabetic and prediabetic rats with or without diet intervention.

Parameters	Groups					
	NPD	PD	ND + MET	HFHC + MET	ND + BA	HFHC + BA
MDA (nmol/g protein)	4.35 ± 0.16	5.78 ± 0.43**	4.62 ± 0.09#	5.11 ± 0.24	4.14 ± 0.20###	4.53 ± 0.12#
SOD (ng/mL)	7.00 ± 0.90	1.78 ± 0.23**	6.74 ± 0.66##	6.11 ± 0.88#	11.43 ± 1.14*###	10.56 ± 0.90*###
GPx (pg/mL)	847.52 ± 53.56	245.43 ± 12.29***	989.72 ± 129.55###	517.99 ± 78.53*	1001.20 ± 62.37###	669.51 ± 40.59##
hs-CRP (ng/mL)	1.35 ± 0.06	2.22 ± 0.01***	1.53 ± 0.16###	1.74 ± 0.15	1.71 ± 0.07#	1.73 ± 0.07#
TNF-α (pg/mL)	948.42 ± 30.79	1296.97 ± 7.98***	1005.49 ± 19.17##	1108 ± 96.11	945.63 ± 13.49###	1011.33 ± 17.83##
IL-6 (pg/mL)	22.20 ± 2.71	37.13 ± 1.14***	30.02 ± 1.30	33.95 ± 2.10**	23.46 ± 2.50##	24.06 ± 1.71##

Values are presented as mean ± SEM ($n = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (vs. NPD). #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (vs. PD).

3.6. Inflammatory Markers. As shown in Table 2, the plasma concentrations of hs-CRP, TNF-α, and IL-6 in the untreated PD group was significantly increased by comparison with the NPD control group ($p < 0.001$). However, the administration of BA with or without diet intervention significantly decreased the concentration of these markers by comparison with the PD group. Similar results were obtained for the ND + MET group.

4. Discussion

This study was designed to investigate the effects of bredemolic acid on cardiovascular function risk factors, endothelial function, oxidative stress, and proinflammatory markers in diet-induced prediabetes. High caloric diets have been implicated with prediabetes which has been associated with endothelial dysfunction, reactive oxygen species (ROS), and inflammatory cytokine production [5, 6]. Studies indicate that chronic consumption of high caloric diets promotes excess adiposity which results in high BMI, high waist circumference and hyperlipidaemia [3, 25]. These have all been identified as risk factors for developing insulin resistance, impaired glucose metabolism, and cardiovascular diseases during the prediabetic stage [26–28]. In addition, previous researchers have also shown that the risk of developing diabetes and its associated cardiovascular diseases rises as body fat, BMI, and waist circumference increase [25, 27]. Our results showed that induction of prediabetes through chronic ingestion of a high fat high carbohydrate diet significantly increased BMI and waist circumference in the untreated prediabetic rats. We suggest that the increased BMI and waist circumference can be attributed to increased caloric intake as we have reported in our previous study [20]. Conversely, the administrations of BA significantly reduced the BMI and waist circumference in BA-treated prediabetic rats with or without diet intervention. In a previous study, we reported that BA administration significantly decreased food intake through reduced plasma ghrelin concentrations and improved insulin sensitivity [20]. Therefore, in this study, we suggest that the decreased BMI and waist circumference in BA-treated prediabetic rats may be due to the decreased food intake and decreased body weight gain.

Moreover, consumption of high caloric diet has been associated with increased delivery of free fatty acid (FFA) to the liver [29]. The increased delivery of FFA leads to increased hepatic and plasma TG concentrations as well as increased export of TG as VLDL from the liver [29, 30]. The VLDL is in turn converted into atherogenic LDL with low clearance. Consequently, due to the increased conversion of TG to VLDL, HDL clearance increases and results in decreased plasma HDL concentration [31, 32].

Similarly, in this study, consumption of high caloric diet probably caused increased delivery of FFA to the liver with subsequent significant increase in plasma concentrations of TC, TG, LDL, and VLDL as well as a slight decrease in the HDL concentration in the untreated prediabetic rats. However, we suggest that even though the HDL concentration slightly decreased, the clearance of HDL as a result of increased VLDL formation remains unaffected in this study. Hence, this abnormal lipid profile showed that the risk of developing dyslipidaemia and other cardiovascular complications begins during the prediabetic stage [11]. On the other hand, the administration of BA significantly normalized the TC, TG, LDL, and VLDL levels in BA-treated prediabetic rats with or without diet intervention. In our previous study, BA was reported to inhibit caloric intake and decrease body weight gain, and this may contribute to the observed normal lipid profile in the BA-treated rats [20].

High caloric diets have also been reported to result in glucolipotoxicity which in turn triggers mitochondrial overproduction of reactive oxygen species (ROS) due to impairment of mitochondrial electron transport chain activity [4, 33]. The mitochondrial overproduction of ROS leads to oxidative stress which further leads to impaired balance between production of ROS and antioxidant enzymes [3, 34]. MDA and antioxidant enzymes (SOD and GPx) are markers for lipid peroxidation and antioxidant capacity in the cells or tissue, respectively. Indeed, in this study, MDA concentrations significantly increased while SOD and GPx concentrations significantly decreased in the hearts of untreated prediabetic rats. These results correlated with a research done by Lozano et al. [3] which showed a positive correlation between the consumption of high caloric diets and increased lipid peroxidation. On the other hand, we observed that the administration of BA significantly reduced the heart lipid peroxidation activity and significantly increased the heart antioxidant capacity of BA-treated prediabetic rats. This biological effect of BA on the oxidative stress markers correlated with the earlier reports that triterpenes are antioxidant agents which neutralize free radicals in the mitochondria by donation of electrons due to the presence of hydroxyl radical in their structures [19].

Similarly, we speculate that BA attenuated oxidative stress by neutralizing free radicals through electron donation capacity of its hydroxyl radicals and improved antioxidant activity by promotion of antioxidant enzyme production. This antioxidant property of BA has also been reported in other triterpenes such as maslinic acid, oleanolic acid, and ursolic acid [19, 35].

Studies indicate that intermediate hyperglycaemia and oxidative stress alter endothelial cell function and contribute to cardiovascular diseases during the prediabetic stage [33, 36, 37]. Intermediate hyperglycaemia has been linked to oxidative stress through the activation of protein kinase C (PKC) which in turn enhances the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [8, 38]. Activation of PKC alters vascular homeostasis and decreases nitric oxide (NO) production via inhibition of eNOS [1, 12]. As a result of the decreased NO production, vascular changes that result in vasoconstriction with subsequent increase in blood pressure, heart rate, and arteriosclerotic processes occur [1]. In this study, we observed that the eNOS concentration significantly decreased with concomitant increases in heart rate and systolic and diastolic blood pressure in the untreated prediabetic rats when compared to non-prediabetic control rats. The increased heart rate and systolic and diastolic blood pressure can be attributed to vasoconstriction of vascular endothelium due to decreased eNOS activity that result in decreased NO production which has been reported in prediabetes [6]. The results of this study further showed that the administration of BA significantly increased the eNOS concentration and ameliorated heart rate and systolic and diastolic blood pressure in both the presence and absence of diet intervention. In accordance to similar study, we suggest that the administration of BA which ameliorated oxidative stress contributed to the increased eNOS concentration in the BA-treated rats [39]. Increased eNOS concentration in turn leads to increase in NO production which further leads to vasodilation with subsequent significant decrease in heart rate and blood pressure when compared to untreated prediabetic rats.

Furthermore, increased blood glucose has been reported to result in formation of advanced glycation product (AGE) [40]. Formation of AGEs increases expression of adhesion molecules on vascular endothelial cells and subsequently promotes migration of monocytes to form macrophages [1, 41]. Stimulation of the monocytes by AGEs leads to low grade inflammation with increased production of cytokines (such as IL-6, TNF-α, and hs-CRP) [4, 41]. However, literatures have reported that increased levels of proinflammatory cytokines are associated with prediabetes [12, 14]. Similarly, in this study, the plasma concentration of IL-6, TNF-α, and hs-CRP significantly increased in untreated prediabetic rats. The elevated proinflammatory cytokines are inflammatory responses that alter vascular endothelium and result in endothelial dysfunction during prediabetic stage [12, 42]. Of notes, hs-CRP is not just a proinflammatory cytokine but a biomarker for injured heart caused by coronary heart disease or ischemic heart disease [43].

The observed increase in plasma hs-CRP concentrations in untreated prediabetic rats in this study indicated the risk of developing cardiovascular diseases during the prediabetic stage. These results correlated with other studies which reported that plasma hs-CRP concentration and other proinflammatory cytokines were significantly increased in prediabetic condition [44, 45]. Additionally, BA administration significantly decreased the proinflammatory cytokines such as hs-CRP, IL-6, and TNF-α in prediabetic rats with or without diet intervention. The decrease in the plasma proinflammatory cytokines concentration can be suggested to be due to the anti-inflammatory property that has been previously attributed to pentacyclic triterpenes [19, 35]. Pentacyclic triterpenes (such as maslinic acid and oleanolic acid) have been reported to have low pharmacokinetic activity of 3 days without any side effects [35, 46, 47]. Therefore, as a result of the low pharmacokinetic activity exhibited by the pentacyclic triterpenes, the biological effects of BA last longer and sustainably remain active than synthetic drugs. However, we suggest that the sustained biological activities of BA probably compensated for the ameliorated cardiovascular functions in the prediabetic rats even in the absence of diet intervention.

5. Conclusion

The findings of this study suggest that the administration of BA in both the presence and absence of diet intervention attenuated inflammation and oxidative stress, as well as improved cardiovascular and endothelial functions which are impaired in diet-induced prediabetes. More studies are, however, required to investigate the molecular mechanisms by which this triterpene exerts its biological effects.

Data Availability

The data used in this study to support our findings are available upon request from the corresponding author. However, the data on body weight, food intake, as well as fasting blood glucose and oral glucose tolerance test for confirmation of prediabetes are reported in our previous study.

Conflicts of Interest

The authors declare no conflicts of interest.

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Appendix VI

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Research Article

Ameliorative Effects of Bredemolic Acid on Markers Associated with Renal Dysfunction in a Diet-Induced Prediabetic Rat Model

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Recently, studies have shown that renal dysfunction is associated not only with overt diabetes but also with the preceding stage known as prediabetes. Diet and pharmacological interventions are the therapeutic approaches to managing prediabetes, but the compliance in combining the two interventions is low. Hence, the efficacy of pharmacological intervention is reduced without diet modification. In our previous study, we established that bredemolic acid (BA) ameliorated glucose homeostasis via increased GLUT 4 expression in the skeletal muscle of prediabetic rats in the absence of diet intervention. However, the effects of bredemolic acid on renal function in prediabetic condition are unknown. Therefore, this study was aimed at investigating the ameliorative effects of bredemolic acid on renal dysfunction in a diet-induced prediabetic rat model. Thirty-six Sprague-Dawley male rats (150–180 g) were divided into two groups: the nonprediabetic ($n = 6$) and prediabetic ($n = 30$) groups which were fed normal diet (ND) and high-fat high-carbohydrate (HFHC) diet, respectively, for 20 weeks. After the 20th week, the prediabetic groups were subdivided into prediabetic control (PD) and 4 other prediabetic groups which were treated with either BA (80 mg/kg) or metformin (MET, 500 mg/kg) for further 12 weeks (21st to 32nd). Plasma, urine, and kidney samples were collected for biochemical analysis. The untreated prediabetic (PD) rats presented increased fluid intake and urine output; increased creatinine, urea, and uric acid plasma concentrations; albuminuria; proteinuria; sodium retention; potassium loss; increased aldosterone and kidney injury molecule (KIM-1) concentration; and increased urinary podocin mRNA expression. However, BA administration attenuated the renal markers and oxidative stress and decreased the urinary podocin mRNA expression. In conclusion, BA administration, regardless of diet modification, attenuates renal dysfunction in an experimentally induced prediabetic state.

1. Introduction

More than 25% of type 1 and type 2 diabetes mellitus patients have been reported to develop renal dysfunction [1, 2]. However, the renal dysfunction does occur not only in overt diabetes but also in the early stages of impaired glucose metabolism [3, 4]. Renal dysfunction is defined by the appearance of abnormal kidney functional changes such as a reduced glomerular filtration rate (GFR), increased serum creatinine and urea, albuminuria, increased excretion of kidney injury molecule (KIM-1), and glomerular podocyte injury with urinary loss of podocin. Podocin is an exclusive integral membrane protein in the podocytes that directly interact with nephrin and CD2-associated protein [5]. Hence, urinary loss of podocin is an apparent indication of podocyte injury and renal dysfunction [6–8].

Moreover, literatures have shown that impaired glucose metabolism promotes renal dysfunction via activation of

oxidative stress and renin-angiotensin-aldosterone system (RAAS) [9–11]. The activation of RAAS triggers the release of aldosterone which stimulates serum/glucocorticoid-regulated kinase 1 (SGK1) that regulate epithelial sodium channel (ENaC) and consequently lead to sodium retention and potassium loss in diabetic conditions [12–14]. Of note, literature evidence showed that about one-third of individuals with newly diagnosed diabetes mellitus have varying degrees of renal dysfunction [15]. This can only be attributed to the abnormal changes that occur during prediabetes. The prediabetic stage often precedes the onset of type 2 diabetes mellitus and is said to be caused by chronic consumption of high-caloric diets coupled with a sedentary lifestyle [15, 16]. Cross-sectional clinical studies have confirmed that prediabetes is associated with the onset of chronic kidney disease (CKD) [4, 17]. Therefore, screening of markers of renal function during the prediabetic state offers an early window of opportunity of preventing and managing CKD [15]. More importantly, diet modification and pharmacological intervention have been reported as the therapeutic approaches to managing prediabetes [18–20]. However, the compliance of combining the two interventions is low as patients adhere to pharmacological intervention without diet modification, and consequently, the efficacy of the pharmacological intervention is reduced [21, 22]. Hence, antidiabetic agents that can possibly ameliorate CKD regardless of diet intervention are considered necessary.

Studies in our laboratory have demonstrated that pentacyclic triterpenes, such as oleanolic acid, ursolic acid, and maslinic acid, are antidiabetic agents which attenuate renal dysfunction in streptozotocin-induced diabetes mellitus [23, 24]. Similarly, we have previously demonstrated that a maslinic acid isomer, bredemolic acid, is an antihyperglycaemic agent that regulated blood glucose concentration via increased expression of GLUT 4 in the gastrocnemius muscle of the prediabetic rat model without diet intervention [25]. However, the biological effects of bredemolic acid on renal dysfunction in the prediabetic state are unknown. Therefore, this study sought to investigate the effects of bredemolic acid on selected markers of renal function in a diet-induced prediabetic rat model, and we also treated the prediabetic rats with metformin, a common first-line drug in the therapy of type 2 diabetes and obesity [26].

2. Materials and Methods

2.1. Animals. Thirty-six (36) male Sprague-Dawley rats with body weight 150–180 g were used for this study as described in previous research [25]. The rats were obtained from the Biomedical Research Unit (BRU), University of KwaZulu-Natal (UKZN). The animals were kept and maintained in a standard animal facility under controlled environmental conditions at room temperature (22 ± 2°C), humidity (55 ± 5%), and 12 h day : 12 h night cycle. The animals consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* for 2 weeks to acclimatize before being exposed to the experimental diet (high-fat high-carbohydrate). The components of the high-fat high-carbohydrate (HFHC) diet are carbohydrate (55%%kcal/g), fats (30% kcal/g), and proteins (15% kcal/g) as described in the previous research [22]. All experimental procedures in this study were carried out in absolute compliance with the animal care guidelines and approved with ethical number (AREC/024/018D) by the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa.

2.2. Experimental Design. After the acclimatization, the animals were divided into 2 main groups: the nonprediabetic control group ($n = 6$) and the prediabetic group ($n = 30$). The nonprediabetic (NPD) control animals (negative control) were given standard rat chow (ND) and water *ad libitum* for 20 weeks while the prediabetic animals were given HFHC diet and drinking water supplemented with fructose (15%) for 20 weeks to induce prediabetes. At the 20th week, prediabetes was confirmed via assessment of fasting blood glucose and oral glucose tolerance test (OGTT) as described by the American Diabetes Association and our previous study [25, 27]. Of notes, this study is a continuation of the previous study, and the data on body weight, food intake, fasting blood glucose, oral glucose tolerance test, fasting insulin concentration, and insulin resistance in the previous study are relevant for this present study.

2.3. Treatment of Animals. The treatment period lasted for 12 weeks (21st–32nd). The nonprediabetic control group (Group 1) fed on standard rat chow (ND) without treatment for 12 weeks while the prediabetic animals ($n = 30$) were further divided into the 5 groups (Group 2–Group 6, $n = 6$) and fed on HFHC or ND for 12 weeks as well. Group 2 served as the prediabetes control group (PD) and continuously fed on the HFHC diet without treatment for 12 weeks. The other 4 groups of the prediabetic animals continuously fed on HFHC diet or switched to ND and were treated with either oral administration of BA (80 mg/kg) or metformin (MET, 500 mg/kg) every third day for 12 weeks due to the three-day pharmacokinetic activity of pentacyclic triterpenes as previously described [28, 29]. The switch of diet from HFHC to ND is the dietary intervention while the continuous feeding on HFHC diet is the absence of dietary intervention. The ND+MET (Group 3) rats changed diet from HFHC to ND and received MET orally whereas the HFHC+MET (Group 4) rats were continuously fed on the HFHC diet and received MET orally. The ND+BA (Group 5) rats changed diet from HFHC to ND and received BA orally while HFHC +BA (Group 6) rats continuously fed on the HFHC diet and were treated with BA. After the 12 weeks of treatment, the animals were sacrificed; blood samples and the kidneys were collected from all the animals for biochemical analysis. The fluid intake and urine volumes were assessed in all the animals at the 20th week and every 4 weeks (24th, 28th, and 32nd week). The renal function parameters and other biochemical parameters were measured at the end of the experiment.

2.4. Determination of Fluid Intake and Urine Output. At the 20th week and every 4 weeks thereafter, all the animals in each group were placed in different metabolic cages for 24

hours to measure fluid intake and urine output. The urine samples were measured and centrifuged at 13000 rpm for 5 minutes at 4°C, and the supernatants were stored at -80°C in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) until ready for kidney function parameter analysis.

2.5. Blood Collection and Tissue Harvesting. All the animals were placed in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) and anaesthetised with 100 mg/kg of Isofor (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa). In an unconscious state, blood samples were collected from all the animals via a cardiac puncture into different precooled EDTA containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) 503 g for 15 minutes at 4°C to obtain plasma. Thereafter, the plasma samples were aspirated into plain sample bottles and stored in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) at -80°C until ready for biochemical analysis. Also, the kidneys were removed, rinsed with cold normal saline solution, weighed on the weighing balance, snapped frozen in liquid nitrogen, and stored at -80°C in a Bio Ultra freezer for biochemical analysis of selected parameters.

2.6. Biochemical Analysis. The biochemical analysis of kidney function parameters (such as creatinine, urea, uric acid, albumin, and total protein) and electrolytes (Na^+ and K^+) was determined at the 32nd week in the plasma and urine samples by using their respective assay kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) as instructed by the manufacturer. However, the kidney injury molecule (KIM-1) and aldosterone plasma concentrations were determined from their specific ELISA kits as instructed by the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) via the microplate reader, SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenburg, LGBW, Germany).

2.7. Determination of GFR. The GFR of all the animals were determined at the 32nd week of the experiment from the estimation of creatinine in the plasma and urine (creatinine clearance) as follows:

$$\mathrm{GFR[mL/min]} = \frac{\text{Urine creatinine (mg/dL)} \times \text{24hrs urine volume (mL)}}{\text{Plasma creatinine (mg/dL)} \times 60\ \text{min} \times \text{24hrs}}. \quad (1)$$

2.8. Lipid Peroxidation and Antioxidant Status. The lipid peroxidation was assessed by determination of the concentration of malondialdehyde (MDA) in the kidney homogenized tissue according to the previously established protocol [24]. However, the antioxidant status of the kidney homogenate was assessed by determination of the concentration of superoxide dismutase (SOD), glutathione peroxidase (GPx), and total antioxidant capacity (TOAC) by using their specific ELISA kits according to the instruction of the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

2.9. Urine RNA Isolation. RNA was isolated from urine (4 mL) by using a ZR Urine RNA Isolation Kit™ (Zymo Research Corp., Irvine, USA) according to the manufacturer's protocol. The purity of the RNA was confirmed by the relative absorbance of ratio 260/280 nm via a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Urine RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) by using the iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA) through incubation in a thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems, Life Technologies).

2.10. Urine Complementary DNA (cDNA) Synthesis. For cDNA synthesis, urine RNA (2 μL) was mixed with 5x iScript reaction (4 μL), iScript reverse transcriptase enzyme (1 μL) (Bio-Rad, USA), and nuclease-free water to a final volume of 20 μL. The mixture was incubated in the thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems, Life Technologies) at 25°C for 5 minutes, 42°C for 30 minutes, and finally at 85°C for 5 minutes. Thereafter, the synthesized cDNA was stored at -80°C until use for real-time PCR (polymerase chain reaction).

2.11. Real-Time PCR. The urinary mRNA level of podocin was quantified by real-time PCR LightCycler (Roche LightCycler 96, USA). cDNA template (2 μL), SYBR Green PCR master mix (5 μL) (Bio-Rad, USA), podocin forward primer (1 μL), podocin reverse primer (1 μL), and nuclease-free water were mixed to a final volume of 10 μL. Thereafter, the sample mixtures were cycled 40 times at 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 20 seconds in the LightCycler (Roche LightCycler 96, USA). All the samples were run in duplicate, and β-actin mRNA levels were used as a housekeeping gene to normalize the podocin mRNA level. The sequences of the used oligonucleotide primers (Metabion International AG, Planegg, Germany) were as follows: *podocin forward* $5'$*-TGG AAG CTG AGG CAC AAA GA-*$3'$ and *podocin reverse* $5'$*-AGA ATC TCA GCC GCC ATC CT-*$3'$.

2.12. Statistical Analysis. The data were normally distributed and presented as the mean ± SEM. Multiple intergroup differences were analysed by one-way ANOVA with the Bonferroni test as a post hoc test through GraphPad Prism 7 software. The results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Effects of BA Administration with or without Diet Intervention on Plasma and Urinary Albumin and Total Protein. High-fat or high-fructose diet has been associated with impaired glucose metabolism and insulin resistance which in turn leads to metabolic disturbances with complications that result in renal dysfunction such as decreased plasma concentration of albumin and total protein, albuminuria, proteinuria, and diffuse thickening of the glomerular capillary basement membrane [30–33]. In this study, a significant decrease in plasma concentrations of albumin (Figure 1(a)) and total protein (Figure 1(b)) was observed in the prediabetic control rats when compared to nonprediabetic control rats. In addition, albuminuria (Figure 1(c)) and proteinuria (Figure 1(d)) which are apparent indicators of

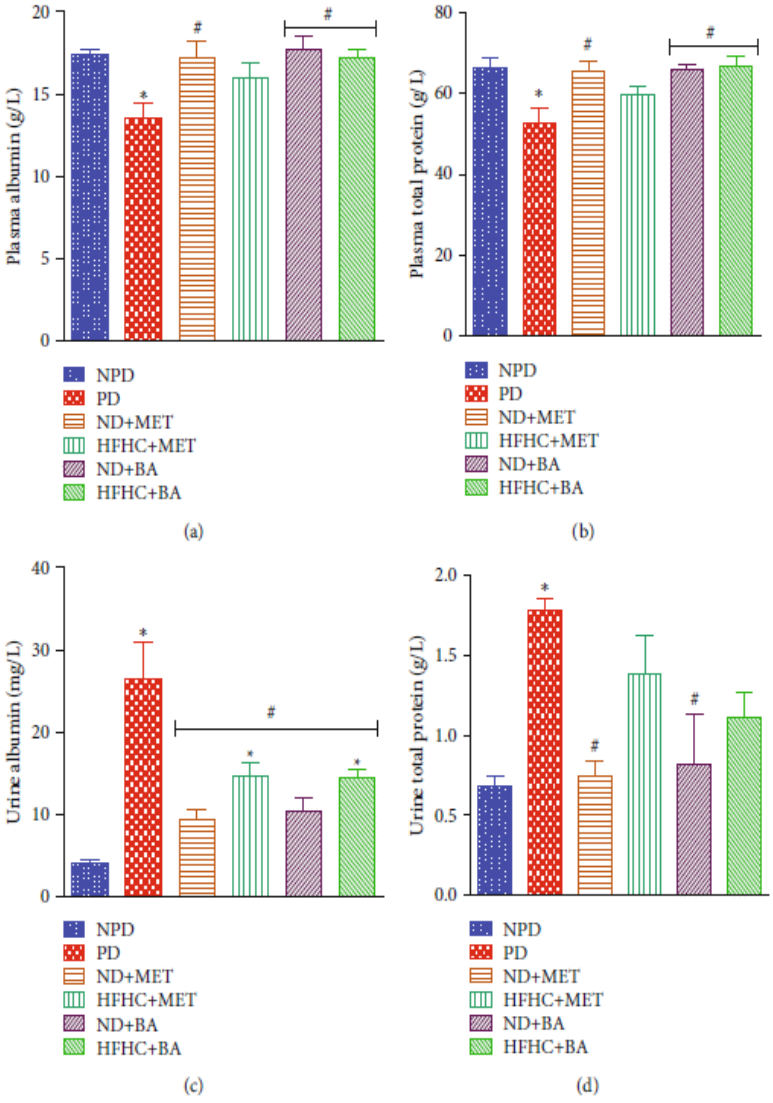

FIGURE 1: Effects of BA on plasma albumin (a), plasma total protein (b), urine albumin (c), and urine total protein (d) in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.05$ (vs. PD).

renal damage were observed in the prediabetic control rats compared to nonprediabetic rats. These observations may be attributed to the impaired filtration barrier which has been reported in prediabetic condition in other studies [30, 34]. Therefore, we suggest that the abnormal glucose homeostasis and insulin resistance that are associated with prediabetes due to chronic consumption of high-caloric diet might have resulted into the impaired filtration barrier with consequent loss of plasma albumin and protein, thus resulting into significant albuminuria and proteinuria [3, 35]. However, the administration of BA in the presence or absence of diet intervention as well as metformin administration with diet intervention attenuated albuminuria and proteinuria in the BA- and metformin-treated prediabetic rats, and this in turn contributed to the improved plasma concentrations of albumin (Figure 1(a)) and total protein (Figure 1(b)). We therefore suggest that BA attenuated these renal dysfunction markers by its antihyperglycaemic property and the improved insulin sensitivity which we have earlier reported in our study [25].

3.2. Effects of BA Administration with or without Diet Intervention on Plasma KIM-1. Apart from albuminuria or proteinuria, another indicator of renal damage is the KIM-1, which is an expressed biomarker on the apical membrane of proximal tubular cells [36]. The observed significant increase in the plasma concentration of KIM-1 in the prediabetic control rats compared to nonprediabetic control rats in this study (Figure 2) was also an indication of decline in renal function, and this observation on KIM-1 correlated with other studies in insulin-resistant states [37, 38]. However, the KIM-1 plasma concentration of BA-treated prediabetic rats with or without dietary intervention as well as metformin-treated prediabetic rats with diet intervention

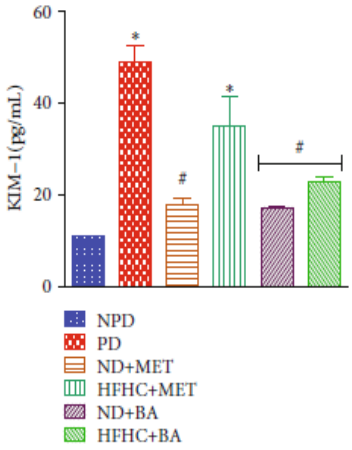

FIGURE 2: Effects of BA on plasma kidney injury molecule (KIM-1) concentrations in prediabetic rats in the presence or absence of dietary intervention.

was significantly decreased in comparison to the prediabetic control rats.

3.3. Effects of BA Administration with or without Diet Intervention on Plasma and Urinary Uric Acid and Urea. In this study, the plasma concentrations of urea and uric acid (Figure 3(a) and Figure 3(b), respectively) significantly increased in the prediabetic control rats when compared to the nonprediabetic control rats. The alterations in the plasma or urinary concentrations of urea may be suggested to be due to impaired excretory or regulatory function of the kidney in maintaining constant homeostasis in the prediabetic or diabetic state [39]. Moreover, decreased urinary concentration of urea (Figure 3(c)) in the prediabetic control rats in comparison to the nonprediabetic control rats was observed in this study. This observation was in accordance with the results of previous studies [24, 40]. Administration of BA in the absence or presence of dietary intervention as well as metformin in the presence of dietary intervention significantly decreased the plasma and increased the urinary concentrations of urea.

Of note, high fructose diet has been reported to result in ATP depletion due to utilization of two molecules of ATP for each fructose molecule metabolized [41, 42]. Therefore, the resultant ADP is further degraded to AMP. In the insulin-resistant state (prediabetes), xanthine dehydrogenase enzyme is activated and triggered the conversion of the AMP to uric acid, hence resulting into the observed hyperuricaemia and elevated uric acid excretion in this study [43, 44]. Therefore, we suggest that the significant increase in uric acid levels in the plasma may be due to the chronic consumption of fructose diet which triggered insulin resistance and further leads to the observed hyperuricaemia and significant urinary excretion of uric acid in prediabetic control rats (Figure 3(d)). However, we hypothesized that the administration of BA and metformin in the presence of dietary intervention significantly ameliorated the hyperuricaemia probably due to the improved insulin sensitivity in the BA- and metformin-treated prediabetic rats.

3.4. Effects of BA Administration with or without Diet Intervention on Lipid Peroxidation and Antioxidant Status in the Kidney. The observed increase in the lipid peroxidation (MDA) and decrease in the concentration of antioxidant enzymes (SOD, GPx, and TOAC) in the prediabetic control rats in comparison to the nonprediabetic control rats are apparent indicators of oxidative stress (Table 1). Increased glucose influx into the cells (due to consumption of high-caloric diet) which results into increased glucose catabolism through the Krebs cycle and production of electron donors (NADH and FADH2) at quantities that overwhelm the capacity of oxidative phosphorylation electron transport chain triggers oxidative stress under hyperglycaemic conditions [45]. This process occurs in microvascular endothelial cells such as the glomerular endothelial cells which are unable to decrease glucose influx during a hyperglycaemic state [46]. The glomerular endothelium plays a significant role in the pathogenesis of diabetic nephropathy directly and through its interaction with podocytes [45]. Therefore, we suggest that another mechanism for the antioxidant effect of BA may probably be due to the decreased postmeal glucose in BA-treated prediabetic animals.

3.5. Effects of BA Administration with or without Diet Intervention on Plasma, Urine Creatinine, and GFR. The plasma concentrations of creatinine significantly increased (Figure 4(a)) while the urinary concentration of the same parameter (Figure 4(b)) in the prediabetic control rats was significantly decreased in comparison to the nonprediabetic control rats. These observations were correlated with the results of other studies [24, 40]. The impaired creatinine clearance altered the plasma and urine creatinine concentrations and further contributed to the decreased GFR in the prediabetic control rats (Figure 4(c)) [2]. Studies have shown that insulin resistance triggers oxidative stress in renal tissues [47, 48]. Therefore, we suggest that the impaired creatinine clearance which resulted into the decreased GFR may be due to insulin resistance which further triggered oxidative stress as reported in other studies [49, 50]. However, the administration of BA in the absence or presence of diet intervention and metformin administration in the presence of diet intervention significantly increased the urine creatinine by comparison to the prediabetic control rats. Also, the GFR of BA and metformin-treated prediabetic rats with diet intervention significantly increased by comparison to the PD control rats (Figure 4(c)). Therefore, we suggest that the improved creatinine clearance in BA-treated prediabetic rats is due to the antioxidant activity of the pentacyclic triterpene.

3.6. Effects of BA Administration with or without Diet Intervention on Plasma Aldosterone. A high-fat diet has been reported to activate the renin-angiotensin-aldosterone system (RAAS) in insulin-resistant states [14, 20, 30]. Also, literatures have shown that due to hyperinsulinaemia in insulin-resistant states, aldosterone production increases, and this in turn activates the aldosterone-induced SGK1 signaling

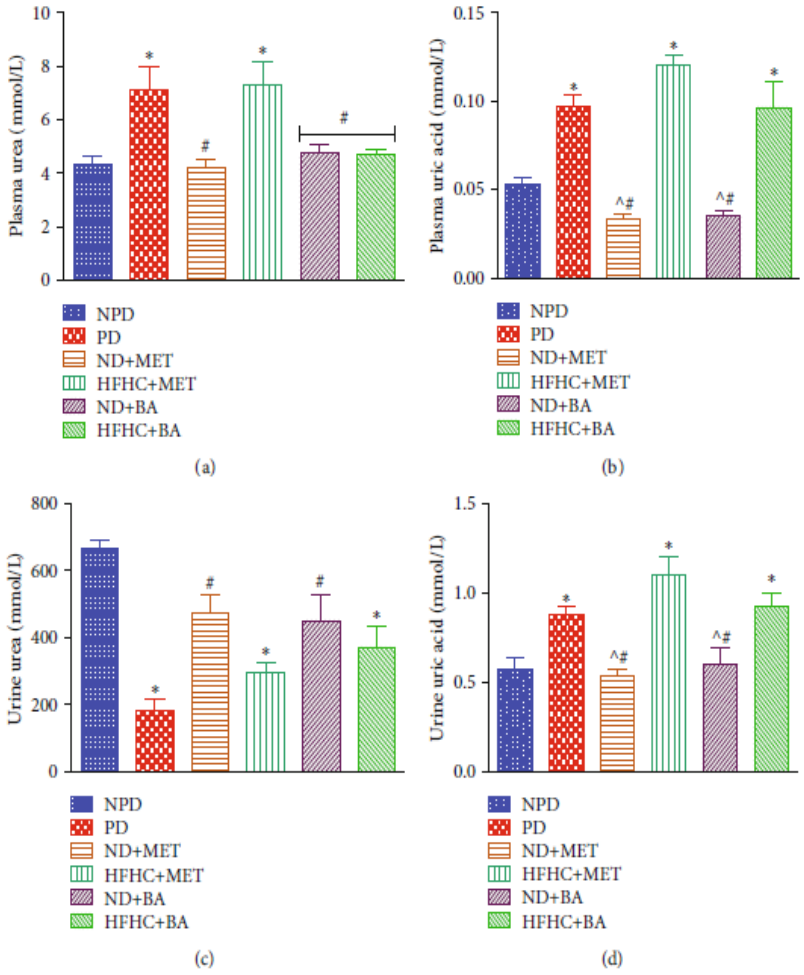

FIGURE 3: Effects of BA on plasma urea (a), plasma uric acid (b), urine urea (c) and urine uric acid (d) in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD), and $^{\wedge}p < 0.001$ (vs. HFHC+MET).

TABLE 1: The effects of BA on lipid peroxidation and antioxidant status in prediabetic rats in the presence or absence of dietary intervention. Values are presented as the mean ± SEM ($n = 6$).

Groups Parameters	NPD	PD	ND+MET	HFHC+MET	ND+BA	HFHC+BA
MDA (nmol/g protein)	5.10 ± 0.13	7.72±0.41***	5.69 ± 0.19###	6.75±0.40**	5.07 ± 0.08###	5.63 ± 0.25###
SOD (ng/mL)	8.66 ± 0.27	3.14±0.38***	9.92 ± 0.52###	6.62 ± 0.12###	11.45 ± 0.63*###	8.08 ± 0.81###
GPx (pg/mL)	1793.00 ± 42.38	849.27±24.69***	1820.11 ± 25.88###	1274.50±36.14***	1914.21 ± 37.18###	1698.61 ± 33.17###
TOAC (U/mL)	44.40 ± 2.57	14.80±1.03***	31.45 ± 1.02*###	22.14±3.03***	41.31 ± 1.65###	24.17±3.10***#

$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (vs. NPD); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ (vs. PD).

pathway [13, 51]. In correlation with other studies [40, 52], significantly elevated plasma concentration of aldosterone was also observed in the prediabetic control rats when compared to the nonprediabetic control rats (Figure 5). Therefore, we suggest that the consumption of the high-fat diet contributed to the elevated aldosterone concentration through the activation of RAAS in the prediabetic control rats. In this study, the administration of BA and metformin in the absence or presence of diet intervention significantly decreased the plasma aldosterone concentration in the BA- and metformin-treated prediabetic rats. Therefore, we suggest that the administration of BA probably improved insulin sensitivity which in turn reduced the activation of RAAS and consequently leads to the significantly decreased plasma

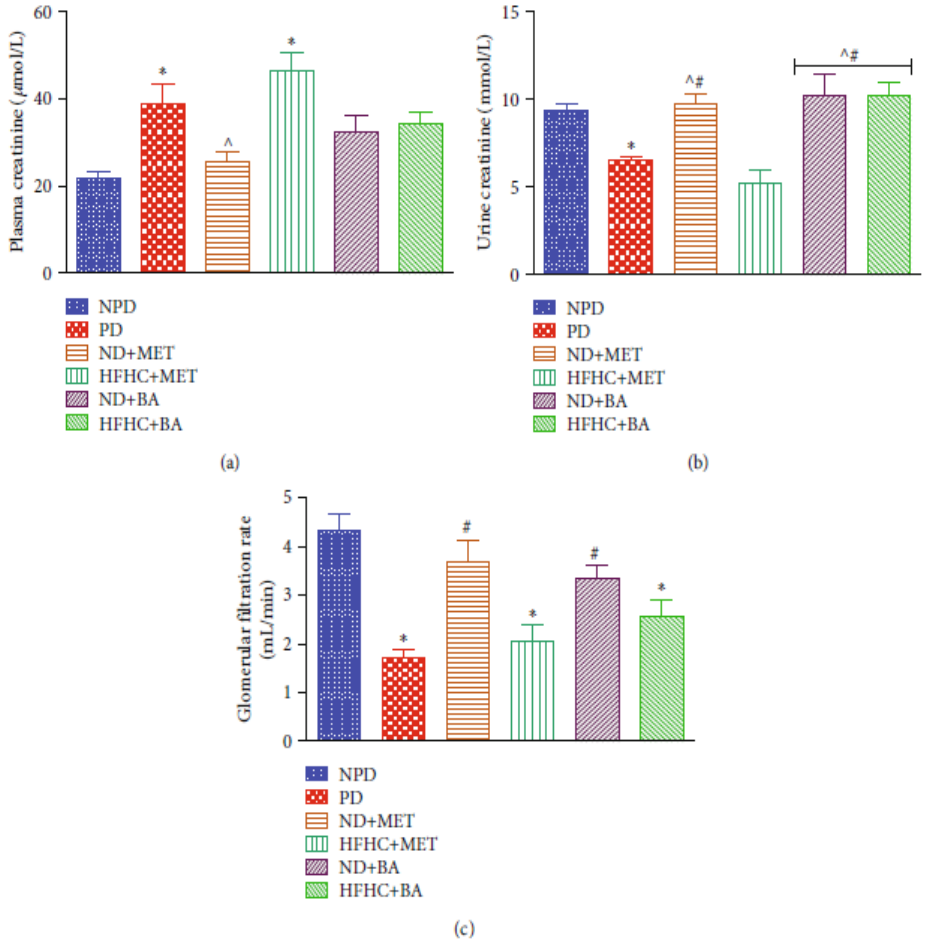

FIGURE 4: Effects of BA on plasma creatinine (a), urine creatinine (b), and GFR (c) in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD), and $^{\wedge}p < 0.01$ (vs. HFHC+MET).

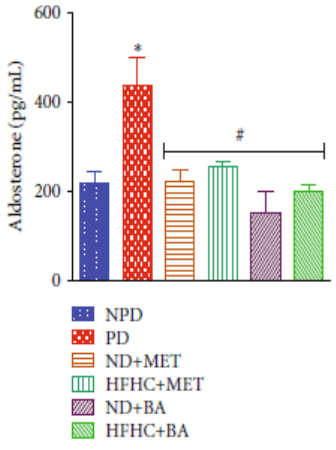

FIGURE 5: Effects of BA on plasma aldosterone concentrations in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD).

aldosterone concentration in BA-treated prediabetic rats even in the absence of diet intervention.

3.7. Effect of BA Administration with or without Diet Intervention on Plasma and Urinary Sodium and Potassium, Fluid Intake, and Urine Output. Due to the aforementioned RAAS activation and elevated plasma concentration of aldosterone in insulin-resistant states, the fluid intake, urine output, sodium reabsorption, and potassium loss significantly increased in the prediabetic control rats in this study. Literature has shown that the activation of RAAS subsequently activates the serum/glucocorticoid-regulated kinase 1 (SGK1) which further triggers the stimulation of the epithelial sodium channel (ENaC) to cause sodium retention, hypokalemia, and increased fluid intake [13, 51]. In this study, the fluid intake and urine output of the prediabetic control rats were significantly increased in comparison to the nonprediabetic control rats throughout the treatment period (Table 2). However, in the presence or absence of dietary intervention with BA administration as well as metformin administration with diet intervention, the fluid intake and urine output significantly decreased when compared to

Table 2: Effects of BA on fluid intake and urine output in prediabetic rats in the presence or absence of dietary intervention. Values are presented as the mean ± SEM ($n = 6$).

Parameters	Groups					
	NPD	PD	ND+MET	HFHC+MET	ND+BA	HFHC+BA
Fluid intake (mL)						
0 week	21.50 ± 0.96	54.50 ± 4.54*	59.00 ± 3.63*	51.00 ± 4.73*	66.17 ± 6.43*	52.83 ± 5.10*
4 weeks	23.17 ± 1.76	34.50 ± 4.07*	22.67 ± 2.16#^	39.83 ± 7.10*	25.00 ± 3.37^	30.33 ± 3.33
8 weeks	20.83 ± 2.39	35.00 ± 2.89*	23.33 ± 3.33	32.5 ± 2.81	25.50 ± 2.93	33.33 ± 2.47
12 weeks	19.50 ± 1.38	34.17 ± 2.01*	20.83 ± 1.54#	30.00 ± 2.24	22.50 ± 2.14#	22.17 ± 2.32#
Urine output (mL)						
0 week	8.67 ± 0.67	31.33 ± 3.82*	33.17 ± 3.21*	30.33 ± 2.60*	39.00 ± 3.00*	34.33 ± 3.77*
4 weeks	9.00 ± 0.86	26.67 ± 3.41*	17.00 ± 2.46#^	29.67 ± 2.89*	17.67 ± 2.39#^	18.33 ± 2.45*^
8 weeks	11.00 ± 0.45	23.33 ± 4.28*	15.67 ± 1.75	20.83 ± 2.34*	14.00 ± 2.00#	18.00 ± 2.00
12 weeks	11.00 ± 1.44	26.33 ± 2.03*	16.67 ± 1.12#	22.17 ± 2.23*	16.50 ± 1.67#	16.00 ± 2.19#

*$p < 0.001$ (vs. NPD), #$p < 0.05$ (vs. PD), and ^$p < 0.05$ (vs. HFHC+MET).

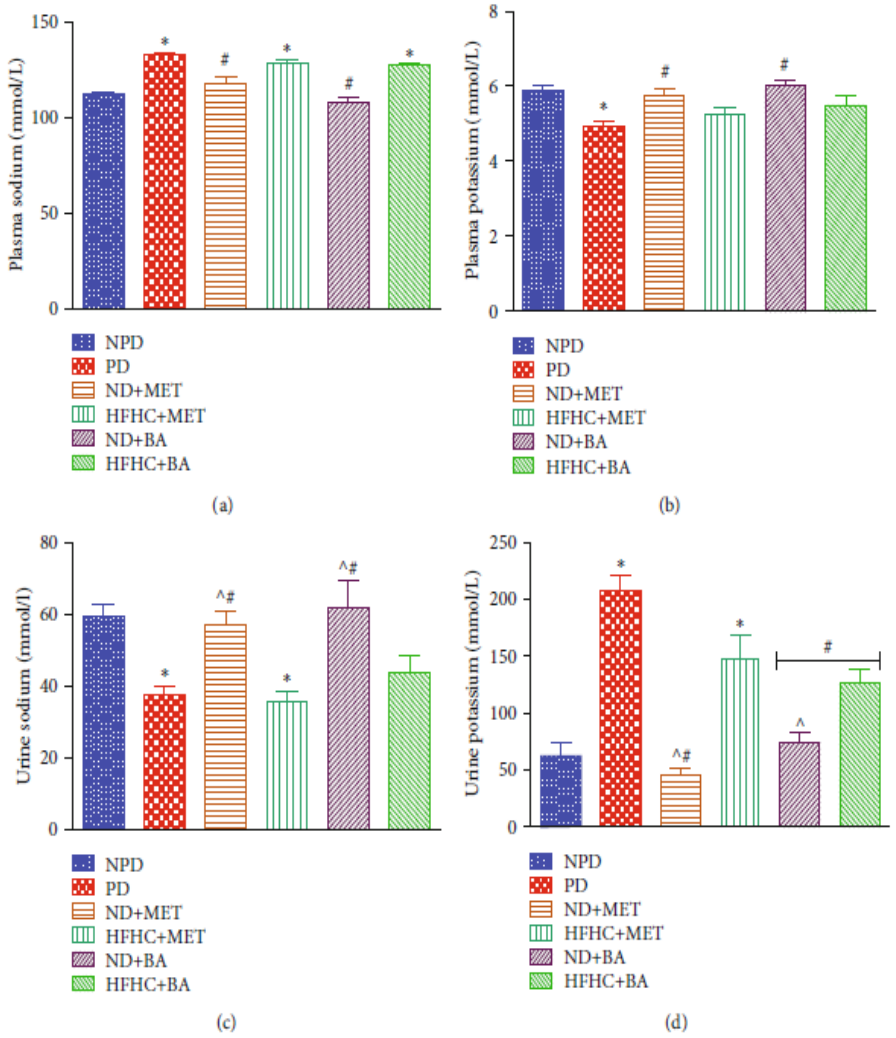

Figure 6: Effects of BA on plasma sodium (a), plasma potassium (b), urine sodium (c), and urine potassium (d) in prediabetic rats in the presence or absence of dietary intervention. *$p < 0.001$ (vs. NPD), #$p < 0.05$ (vs. PD), and ^$p < 0.001$ (vs. HFHC+MET).

the prediabetic control rats, especially at the 12th week period of treatment ($p < 0.05$).

Moreover, the administration of BA or metformin with diet intervention significantly decreased the plasma sodium concentration (Figure 6(a)) and increased the plasma potassium concentration (Figure 6(b)) when compared to the prediabetic control rats ($p < 0.05$). On the other hand, the BA- or metformin-treated prediabetic rats with diet intervention had significantly increased urinary sodium (Figure 6(c)) and decreased urinary potassium (Figure 6(d)) by comparison to the prediabetic control rats. Apart from RAAS, other mechanisms that can possibly be associated with the increased fluid intake, urine output, and electrolyte imbalance in the prediabetic control rats are hyperglycaemia and glycosuria. Therefore, we suggest that the amelioration of fluid intake, urine output, and the electrolytes by administration of BA may be attributed to the improved hyperglycaemia and glycosuria in the BA-treated prediabetic rats as reported in the previous study [25].

3.8. Effect of BA Administration with or without Diet Intervention on Urinary Podocin mRNA Expression. Literature evidences revealed that elevated aldosterone concentration induced proteinuria and glomerular podocyte injury with decreased gene expression of podocin in the kidney tissues and increased gene expression of podocin mRNA in the urine [53, 54]. Also, it has been established that podocytes express mineralocorticoid receptors (MR); hence, podocytes are targeted cells for aldosterone hormone [53, 55]. Therefore, when aldosterone concentration is increased, oxidative stress is induced in the podocytes, and this subsequently promotes podocyte injury by increased reactive oxygen species (ROS) production in the mitochondria [56]. In addition, it has been demonstrated that podocytes are insulin-responsive cells that similarly respond to insulin in the same manner as the skeletal muscle [57]. This showed that podocyte survival is modulated by insulin signaling [57]. Similarly, in this study, the aforementioned increase in urinary podocin mRNA expression was observed in prediabetic control rats, and this correlated with other similar studies [8, 58]. The podocin mRNA expression in the urine of prediabetic control rats was significantly increased by 12.04-fold when compared to the nonprediabetic control rats (Figure 7). The podocin mRNA expressions in the urine of BA and metformin-treated prediabetic rats in the presence or absence of diet intervention were significantly decreased when compared to the prediabetic control rats.

However, we suggest that the administration of BA probably improved insulin sensitivity and ameliorated the insulin signaling in podocytes, and this further contributed to the observed decreased gene expression of urinary podocin mRNA in BA-treated prediabetic rats in this study. Moreover, pentacyclic triterpenes have been reported to selectively inhibit 11β-hydroxysteroid dehydrogenase type I enzyme, an enzyme that converts inactive cortisone into active cortisol, thus preventing activation of mineralocorticoid receptors in aldosterone tissue such as the kidney [59, 60]. Therefore, we hypothesized that the same enzymatic inhibition may probably prevent aldosterone biological actions on podocyte

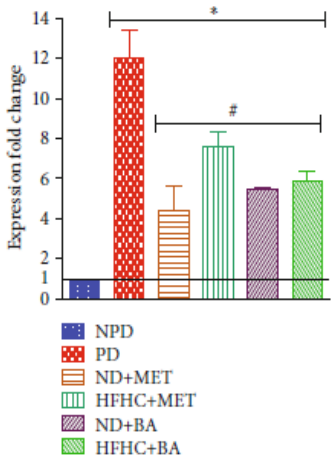

Figure 7: Effects of BA on urinary podocin mRNA expression in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD).

mineralocorticoid receptors and this subsequently led to reduced podocyte injury which in turn contributed to the decreased urinary gene expression of podocin mRNA in the BA-treated prediabetic rats with or without diet modification.

4. Conclusion

Administration of BA with or without diet modification has been shown in this study to attenuate renal dysfunction markers and urinary expression of podocin mRNA in the prediabetic state. These biological actions of BA may be due to the combination of the improved insulin sensitivity and antihyperglycaemic and antioxidant properties of the pentacyclic triterpene (BA). Pentacyclic triterpenes have been reported as nontoxic antioxidants that have low pharmacokinetic activity of three days without side effects [28, 29]. Therefore, we suggest that the ameliorative effects of BA on renal function markers compared to metformin in this study may be attributed to the low pharmacokinetic feature of BA even in the absence of dietary intervention. However, this is a preliminary study, more structural and molecular findings are still needed to clarify the mechanisms by which BA ameliorates renal function.

Data Availability

The data used in this study to support our findings are available upon reasonable request from the corresponding author. However, the data on body weight, food intake, fasting blood glucose and oral glucose tolerance test for confirmation of pre-diabetes are reported in our previous study.

Conflicts of Interest

The authors declare no conflicts of interest.

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Appendix VII

PSSA
Physiology Society of Southern Africa

Certificate of Attendance

PRESENTED TO

Akinjide Akinnuga

FOR ATTENDING THE

47 TH CONFERENCE OF THE PHYSIOLOGY SOCIETY OF SOUTHERN AFRICA

ORGANIZED BY

WALTER SISULU UNIVERSITY

18-21ST AUGUST 2019

INTERNATIONAL CONVENTION CENTRE, EAST LONDON, SOUTH AFRICA

PROF CONSTANCE RUSIKE
LOC Chairperson

AUGUST 27, 2019

Appendix VIII

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

LMMS RESEARCH SYMPOSIUM 2019

CERTIFICATE OF PRESENTATION

Awarded to

AKINJIDE AKINNUGA

University of KwaZulu-Natal

For presenting the oral:

Bredemolic Acid, A Pentacyclic Triterpene, Ameliorates Selected Liver Function Biomarkers in High Fat High Carbohydrate Diet-Induced Pre-diabetic Rat Model

At the

Annual Laboratory Medicine and Medical Sciences Research Symposium 2019

Which took place in Westville, Durban, South Africa

On the 6th of September 2019

Yours sincerely,

Dr De Gama

Academic Leader Research SLMMS