The effects of *Zingiber officinale* (ginger) extract on inflammation, innate immune system, haematological factors and cardiovascular diseases in diet-induced prediabetic rats

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PREFACE

High fat high carbohydrate diet had been shown to lead to the development of prediabetes which precedes the onset of type 2 diabetes (T2DM). Once a patient is diagnosed with prediabetes, lifestyle intervention and pharmacological interventions is employed. The primary aim of this intervention is to prevent or delay the development of T2DM and its complications. However, there is reported poor patients regarding lifestyle intervention which reduces the efficacy of the pharmacological treatments. Therefore, there is a need to find alternative treatment which can work in both presence and the absence of lifestyle intervention. Medicinal plants have been identified as a promising future treatment for the management of health care in the twenty-first century. *Zingiber officinale* has been shown to possessed multiple biological activities especially antioxidant, anti-thrombotic, anti-inflammatory and immune modulatory capacities. In our laboratory, we used high fat high carbohydrate rats that accurately mimic the prediabetic human condition. However, no studies have investigated the effects of *Zingiber officinale* on these parameters during prediabetic state. Therefore, this study sought to the effect of *Zingiber officinale* (ginger) on inflammation, haematological factors, immune system and cardiovascular disease in diet-induced prediabetic rats. The experimental work described in this dissertation was conducted at the University of KwaZulu Natal, Westville Campus, Durban, South Africa. All work was conducted by Bonakele Langa under the supervision of Dr Lindokuhle Mabuza and Prof Andile Khathi.

DECLARATION

I, **Bonakele Langa** hereby declare that the dissertation entitled:

"The effects of *Zingiber officinale* (ginger) extract on inflammation, immune system, haematological factors and cardiovascular diseases in diet-induced prediabetic rats" is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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DEDICATION

This work is dedicated to me, seeing my capabilities and making my dreams come true.

PLAGIARISM DECLARATION

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MASTER OF MEDICAL SCIENCES

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
2. I have used the Vancouver convention for citation and referencing. Each contribution to, and quotation in, this thesis from the works of other people has been attributed and has been cited and referenced.
3. This thesis is my own work.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature 

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First and foremost, I would like to thank myself for not giving up, even when things felt overwhelming. For pushing through sleepless nights, for working without breaks, and for believing in my ability to finish this journey. I am proud that I didn't quit when it got hard, and I'm grateful to the version of me who kept going despite all challenges.

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List of symbols and abbreviations

α	Alpha
ADA	American diabetes association
AGE's	Advance glycation end products
AIC	Glycated haemoglobin
AMPK	Activated protein kinase
ANOVA	Analysis of variance
β	Beta
BHT	Butylated hydroxytoluene
BMI	Body max index
BRU	Biomedical research unit
cDNA	Complementary deoxyribonucleic acid
CHS	College of health science
CRP	C-reactive protein

CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DI	Dietary intervention
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
FBG	Fasting blood glucose
FBG	Fasting plasma glucose
G	Gram
GLP	Glucagon-like peptide
GLUT4	Glucose transporter type 4
Hb	Haemoglobin
HbAIC	Glycated haemoglobin
HCI	Hydrochloric acid
Hct	Haematocrit
HFHC	High fat, high carbohydrate
HOMA-IR	Homeostasis model assessment of insulin resistance
IFG	Impaired fasting glucose
IFT	Impaired fasting tolerance
IGT	Impaired glucose tolerance
IL	Interleukin
IR	Insulin resistance

L	Litre
LDL	Low-density lipoprotein
MCH	Mean cellular haemoglobin
MCHC	Mean cellular haemoglobin concentration
MCV	Mean cellular volume
MDA	Malondialdehyde
MPV	Mean platelet volume
MT	Metformin
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
ND	Normal diet
NFE2L2	Nuclear factor erythroid 2 related factor 2
NK	Natural killer
NLR	Neutrophil lymphocyte ratio
NPD	Non-prediabetic
OGTT	Oral glucose tolerance test
PCT	Procalcitonin
PCR	Polymerase chain reaction
PD	Prediabetic
P-LCC	Platelet large cell coefficient
P-LCR	Platelet large cell ratio
PLT	Platelet
RBC	Red blood cell

RDW	Red blood cell distribution width
RDW-CV	Red blood cell distribution width- coefficient of variation
RDW-SD	Red blood cell distribution width- standard deviation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SEM	Standard error of means
SOD	Superoxide dismutase
TAOC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TCR	T cell receptor
TG	Triglycerides
TNF	Tumour necrosis factor
T2DM	Type 2 diabetes mellitus
UKZN	University of KwaZulu Natal
WBC	White blood cell
WHO	World health organisation
ZO	*Zingiber officinale*
µ	Micro

Study outline

The current dissertation is presented in manuscript format, consisting of 6 sections viz. chapter 1: Introduction, chapter 2: literature review, chapter 3: abstract and manuscript 1, chapter 4: abstract and manuscript 2, and chapter 5: synthesis and conclusion. Chapter 1 is the introduction of this study; chapter 2 lay a foundation of the literature and the information that has been discovered in order to link the area of interest of the study and cover a gab that is the debatable issue. Chapter 2 contains the abstract of the manuscript 1 and the manuscript that covers the effects of *Zingiber officinale* on inflammation and innate immune system in diet-induced prediabetic rats. Chapter 4 contains abstract of manuscript 2 and manuscript 2 which covers the effect of *Zingiber officinale* on haematological factors and cardiovascular complications in diet-induced prediabetics rats. Chapter 5 discusses the synthesis of the study and the conclusion.

Abstract

Prediabetes, characterized by intermediate hyperglycemia, is frequently accompanied by chronic inflammation, disrupted haematological profiles, and an elevated risk of progressing to type 2 diabetes mellitus (T2DM) and cardiovascular disease. This study examines the effects of *Zingiber officinale* (ginger) extract on inflammation, innate immune system, haematological parameters and cardiovascular complications in diet-induced prediabetic rats. Thirty-six (36) male Sprague Dawley rats were divided into six groups (n=6 per group) and were induced with prediabetes using high carbohydrate for a period of 20 weeks. After induction, the groups were treated as follows for a period of 12 weeks: a prediabetic (PD) control; a dietary intervention (DI) control; *Zingiber officinale* treatment groups in the presence (ZO+DI) and absence [ZO+HFHC] of DI; as well as metformin in the presence (MT+DI) and absence (MT+HFHC) of DI. An additional six animals were kept on a standard diet and served as a non-prediabetic control (NPD). At the end of the treatment period, parameters measured included homeostatic model assessment index (HOMA-IR), body weight, anti-inflammatory cytokine (adiponectin), pro-inflammatory cytokines (TNF α, IL-6 and CRP), neutrophils cell count, MDA levels, nuclear factor erythroid 2-related factor 2 (NFE2L2). As well as fasting blood glucose (FBG), oral glucose tolerance test (OGTT), glycated haemoglobin (HbA1c), triglycerides (TG), heart-to-body weight ratio, RBC and platelet indices, as well as mTOR and erythropoietin (EPO) expression. Treatment with *Zingiber officinale* significantly improved glucose tolerance and decreased HbA1c and triglyceride levels. It normalized RBC and platelet counts, aligning with previous findings where ginger ameliorated haematological disturbances in diabetic rats. Expression of mTOR and EPO was modulated, suggesting reduced inflammation and attenuated cardiovascular risk. *Zingiber officinale* also demonstrated cardioprotective benefits, echoing earlier studies showing structural recovery and improvement in cardiac biomarkers in prediabetic rat models

Zingiber officinale extract enhances glycaemic control, restores normal haematological function, and mitigates early cardiovascular complications in diet-induced prediabetic rats. These findings support its potential as a nutraceutical therapeutic to prevent progression from prediabetes to T2DM and related cardiovascular conditions.

Chapter 1: Introduction

1.0 Background

Type 2 diabetes mellitus(T2DM) is a chronic, metabolic disorder characterized by hyperglycemia, whereby blood glucose levels are above normal but below the T2DM threshold. This condition may be caused by impaired insulin secretion, resistance to peripheral actions of insulin or both [1]. The chronic hyperglycemia seen in T2DM is linked to the development of complications that may lead to immune dysfunction and haematological disturbances [2, 3]. In patients with T2DM, haematological alteration is associated with the production of reactive oxygen species (ROS). Excessive ROS production causes oxidative stress, leading to tissue damage, haematological alterations and endothelial and red blood cell (RBC) dysfunction [4].

The management of T2DM involves a comprehensive approach that combines pharmacotherapy and lifestyle modification. The drug Metformin is a first line pharmacotherapy that had been used for several decades to treat T2DM. It primarily works by reducing hepatic glucose production and improving insulin sensitivity in peripheral tissues. This drug is used in conjunction with lifestyle intervention strategies that include increasing the level of physical activity as well as a reduction in dietary caloric intake [5, 6]. There have been reports of poor patient compliance where the patients neglect the lifestyle interventions leading to a reduction in the efficacy of Metformin [7]. A high proportion of patients do not take their medication as prescribed, and physicians may be unaware of the extent to which patients miss doses or stop treatment. Despite the importance of adherence to oral antidiabetic drugs, studies consistently report adherence levels that are suboptimal, thus reducing efficacy of the drug[8].

Prior to the onset of type 2 diabetes mellitus individuals may exhibit a condition known as prediabetes[9]. Prediabetes is a condition of intermediate hyperglycaemia where the glycaemic parameters are above the homeostatic range but below the threshold for a diagnosis of T2DM [10]. Prediabetes remains an under studied topic as it is clinically silent, which individuals unaware of their elevated blood glucose levels. In recent years, there has been growing attention to the importance of addressing prediabetes[11]. Prediabetes is a significant risk factor for the development of T2DM and early intervention has been shown to help prevent or delay the progression to overt T2DM [12]. The prevalence of prediabetes estimated from 164 studies

conducted in low-middle income countries was 13.1% based on the World Health Organization (WHO) criteria, and 27.0% based on the American Diabetes Association (ADA) criteria [13]. The management of prediabetes is similar to that of T2DM, however, the incidence of T2DM has only escalated in recent decades since, despite the success of early clinical trials primarily because of poor patient compliance. Thus, implementation of new intervention or treatments are needed that can work in both the absence or presence of lifestyle interventions [14].

The use of medicinal plants has a vital role in treatment of disease and had been used for thousands of years [15]. *Zingiber officinale Roscoe* (ginger) is an herbaceous rhizomatous plant that is a is member of the Zingiberaceae family [16]. Ginger is one of the most commonly consumed herbs with an array of applications in traditional medicines like Chinese medicine, Ayurveda, and Unani-Tibb[17]. Studies have shown that ginger includes pharmacologically potent natural antioxidant chemicals that can block free radicals[9]. Gingerols and shogaols are the phenolic chemicals in ginger that have antioxidant properties [18]. According to a study by Das et al, it was shown that ginger has been used for its wide array of medicinal properties such as being anti-inflammatory, immunomodulatory, anti-tumorigenic, anti-apoptotic, anti-lipidemic, anti-emetic, antimicrobial, antioxidant and anti-hyperglycaemic [19].

Experimental animal models have been essential tools to deepen human understanding of the occurrence and development of T2DM, providing valuable references for exploring the pathogenesis, aetiology and potential treatment of diabetes[9]. In our laboratory, we use a high fat high carbohydrate induced prediabetic rats which has shown to mimic the human condition in both terms of development and progression[20]. Accordingly, in this study, we used a diet induced prediabetic male Sprague Dawley rats to investigate the effect of ginger on immune system and haematological parameters.

Aim

To investigate the effects of *Zingiber officinale* (ginger) extract on haematological parameters and markers of immune function in diet-induced prediabetic rats.

1.1 Research questions

1.1.1 What are the effects of *Zingiber officinale* (ginger) extract on immune system response in diet-induced prediabetic male Sprague Dawley rats?

1.1.2 What are the effects of *Zingiber officinale* (ginger) extract on haematological parameters in diet-induced prediabetic male Sprague Dawley rats?

1.2 Objectives

The objectives for research question 1.1.1 are addressed under study 1 and the objectives for research question 1.1.2 are addressed under study 2.

Study 1

The objectives include examining its effects on change of body weight by measuring body weight, glycaemic control by measuring fasting blood glucose, oral glucose tolerance, glycated haemoglobin (HbA1c), and immune cell concentrations by assessing immune cells such as lymphocytes and neutrophils. Additionally, the study assesses the impact of ginger extract on inflammation by assessing inflammatory markers, including anti-inflammatory cytokines by measuring adiponectin and pro-inflammatory cytokines measuring TNF-α, IL-6, and CRP. The study also explores the effect on oxidative stress markers and antioxidant concentrations, including malondialdehyde (MDA) and Nrf2. Finally, the effect of ginger extract on insulin sensitivity is evaluated using the HOMA-IR index.

Study 2

The objectives include examining its effects on red blood cell indices by measuring mean cell volume (MCV), red blood cell distribution width (RDW), mean cell hemoglobin (MCH), mean cell hemoglobin content (MCHC), hemoglobin (HGB), and hematocrit (HCT)), assessing platelet indices by measuring including plateletcrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), platelet large cell count (P-LCC), and platelet large cell ratio (P-LCR), and lipid profile specifically assessing triglycerides. Additionally, the study evaluates the impact of ginger extract on cardiac pathology by assessing the heart-to-body weight ratio. The study also investigates changes in the insulin signalling pathway, focusing on the expression of mammalian target of rapamycin (mTOR) and glucose transporter type 4 (GLUT4).

1.3 Hypothesis

Zingiber officinale (ginger) extract will prevent the progression of immune dysfunction and haematological disturbances in diet induced prediabetic male Sprague Dawley rats.

1.4 Null hypothesis

Zingiber officinale (ginger) extract will not have any effect on immune dysfunction and haematological disturbances in diet induced prediabetic male Sprague Dawley rats.

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Chapter 2: Literature review

1. Type 2 diabetes mellitus

1.1.Epidemiology and diagnosis of T2DM.

Type 2 diabetes mellitus is a chronic, metabolic disorder characterized by hyperglycaemia [21]. Insulin resistance and beta cells dysfunction play central roles in the pathophysiology of T2DM [22]. According a study by Ismail et al, T2DM is thought to prevail in an individual from an interaction between several lifestyle, medical condition, hereditary, psychosocial and demographic risk factors [23]. The American Diabetes Association recommends screening for T2DM annually in patients 45 years and older, or in patients younger than 45 years with major risk factors [24].

They are two normal used diagnosis criteria organisations normally used which are world health organisation (WHO) and American diabetic association (ADA). WHO has suggested 'intermediate hyperglycaemia', whilst an ADA commissioned panel preferred 'high risk state of developing diabetes' [25]. There is overlap between these various parameters. The diagnosis can be made with a fasting plasma glucose level of 7 mmol/Lor greater or 2-hour post-load plasma glucose/ oral glucose tolerance test of greater or equal to 11.1 mmol/L by WHO [26]. And on ADA, the diagnosis criteria that is used is a glycated haemoglobin (HbA1c) level of 6.5% or greater; a fasting plasma glucose of 7 mmol/L or greater; or a 75-g two-hour oral glucose tolerance test with a plasma glucose level of 13 mmol/L or greater. Results should be confirmed with repeat testing on a subsequent day; however, a single random plasma glucose level of 13 mmol/L or greater with typical signs and symptoms of hyperglycaemia likely indicates T2DM [27].

T2DM has become a worldwide health burden due to its high incidence, morbidity and mortality, which is estimated to be the eighth leading cause of death combined with disability [28]. According to the International Diabetes Federation (IDF), approximately 415 million adults between the ages of 20 to 79 years had diabetes mellitus in 2015. T2DM is proving to be a global public health burden as this number is expected to rise to another 200 million by 2040 [29]. In 2019, 463 million of adults (20–79 years old) were living with T2DM and again only in 2019, T2DM caused 4.2 million deaths. The number of patients with T2DM is increasing at a very high rate, estimated to reach 700 million by 2045(see fig 1) [30]. According to the study by Pheiffer et al, they found out that 15.5 million adults had diabetes, with 69,2% of people not aware of their diabetic status. South Africa is ranked as an upper-middle-income

country and is the second largest economy in Africa and it prevalence of T2DM has almost doubled from 5.5% in 2000 to 9% in 2009 [31]. T2DM has been shown to lead to the development of several macro- and microvascular complications that leads to morbidity and mortality [3].

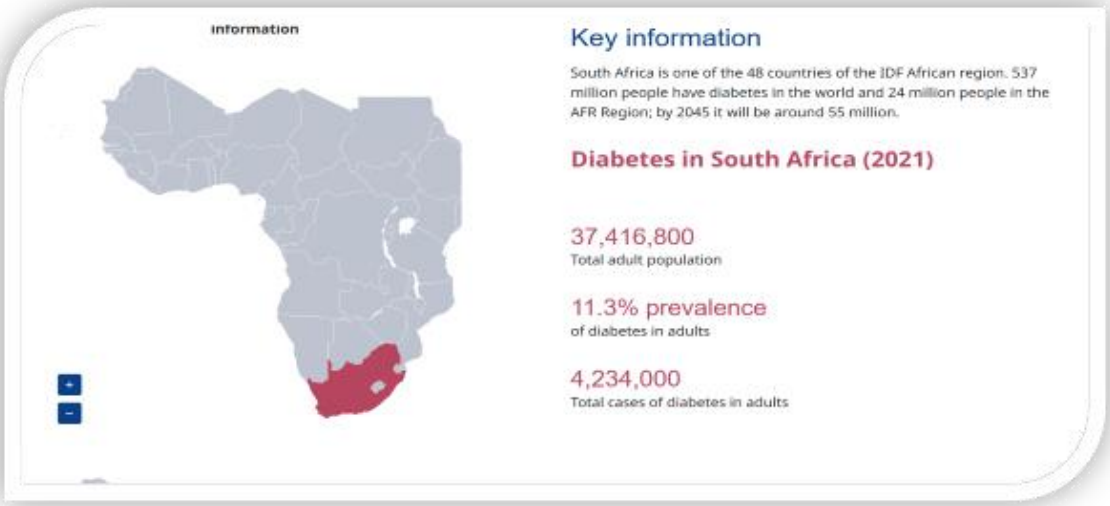

Figure 1: The prevalence of T2DM in South Africa (2021) according to the International Diabetes Federation. Adapted from Internal Diabetes Federation (2021).

1.2. Complications associated with T2DM

Patients with T2DM suffer a number of complications, which sometimes already present when it is diagnosed such as diabetic retinopathy, or develop in the later course of the disease [1]. These complications involve dysfunctions in many vital organs all over the body; mainly kidney, cardiovascular system, retina and the nervous system [1]. The chronic hyperglycaemia seen in T2DM is linked to the development of complications that may lead to immune dysfunction and haematological disturbances [2, 3].

1.2.1. Effect of T2DM on immune system.

There are two subsystems within the immune system [32], known as the innate/non-specific immune system and the adaptive/specific immune system. Both subsystems are intricately linked and work together whenever a harmful substance triggers an immune response [33].

The innate immune system comprises the human immune system's most ancient component [34]. Despite its age, the innate immune system is extraordinarily complex, consisting of infection barriers such as epithelia of the skin, gastrointestinal, respiratory, and genitourinary tracts, antimicrobial peptides and proteins, humoral components such as complement and opsonin's, and cellular components such as neutrophils, monocytes/macrophages, dendritic cells, and innate lymphoid cells [35]. Innate immunity acts as the first line of defence for the body, preventing infection while tolerating typical host flora [36]. Innate immune deficiencies are linked to invasive, life-threatening illness. Inappropriate innate immune system activation can result in autoinflammatory conditions. The innate immune system controls the development of adaptive immune responses. Its proper operation is therefore crucial for health[35].

Adaptive immunity is a complex branch of the immune system. Its "adaptability" refers to its ability to change in response to the various difficulties given by different infections. During an infection, the quantity and fitness of microbe-specific T and B cells increases [35]. Following disease resolution, the pathogen's immunological memory is preserved, allowing a stronger and more rapid immune defence to be mounted if the same pathogen is encountered again. Immunological memory is linked to antigen specificity, another key property of the adaptive immune system [37]. B and T lymphocytes are created with a large variety of specificities that are then selected by the specific antigens via a complex method of genetic recombination of genes coding for the variable portions of antigen receptors (i.e., antibodies and T cell receptors; TCR) [38]. B cell-released antibodies identify conformational antigen, either directly binding the pathogen or binding proteins secreted or produced on the surface of infected cells [39]. The TCR of the T lymphocyte is responsible for recognizing pathogen protein fragments known as epitopes that are expressed on the surface of cells in conjunction with MHC class I or class II molecules [40].

A) Immune cells

i) Lymphocytes

Lymphocytes are critical population of white blood counts and plays an essential role in both innate and adaptive immunity [41].For normal survival and functioning, lymphocytes require glucose uptake [42]. Lymphocytes such as B-cell mature to plasma cells and release more antibodies such as IgE antibodies in T2DM patients which then bind to recruited basophils to activate them and secrete pro inflammatory cytokines. There is increased production of

lymphocytes in the bone marrow secreting cytokines for inflammation [43]. In type 2 diabetes, human organism is more frequently exposed to infection than a healthy one, because of disturbances in the lymphocyte immunologic function [44]. The disorders are thought to be related to a pathologic glucose metabolism in these cells. Investigated in isolated lymphocytes cellular uptake of deoxy-d-glucose is impaired in patients with type 2 diabetes [45].

ii) Neutrophils

Neutrophils are typically the first immune cells to respond to inflammation and can exacerbate the chronic inflammatory state by helping to recruit macrophages and by interacting with antigen-presenting cells [46]. Neutrophils secrete several proteases, one of which is neutrophil elastase, which can promote inflammatory responses in several disease models. Neutrophils are the primary immune cells that respond to infection and are controlled under homeostatic conditions [47]. Neutrophils are highly effective in mediating local inflammation and eliminating pathogens, but hyperactive neutrophils can also induce substantial collateral damage to surrounding tissues. Therefore, even though they are superior cells for the elimination of pathogenic threats, they are unfavourable in cases of chronic sterile inflammation, such as T2DM and atherosclerosis [48]. In hyperglycaemic state, the neutrophils levels increases as they secrete the cytokines, which may increase infection episodes to patients with T2DM. Chronic hyperglycaemia associated with T2DM trigger neutrophils and monocytes to express a primed phenotype. Increased ROS and neutrophil extracellular trap generation by these cells lead to increased inflammation and tissue damage [49]. Hyperglycaemia in T2DM significantly reprograms neutrophil metabolism and reduces effector functions. As a consequence of elevated glucose concentrations in T2DM, molecular shunting of glucose metabolism from glycolysis to polyol pathways is observed [50].

B) Anti- and pro-inflammatory markers

i) Adiponectin

Adiponectin, as am indispensable regulator of immune system, is the most abundant adipokine and is mainly produced by white adipose tissue [51]. It reduces the proinflammatory mediator and increases anti-inflammatory mediator production [51]. And is the only adipokine that acts as anti-inflammatory cytokine (See Fig 2) and has the ability to ameliorate the deleterious effects of IL-2 6, TNF-α, MCP-1, TIMP-1, RBP-4, and leptin which are known to be produced in adipose tissues. It has also been found that the level of adiponectin is downregulated in

obesity and is positively associated with insulin sensitivity [52]. T2DM is associated with lower circulating adiponectin, especially in people with visceral obesity and this reduction is inversely related to insulin resistance [53]. Adiponectin has been recognised as a metabolically favourable adipokine, whose reduction plays a crucial role in both obesity and T2DM [54]. It has many beneficial metabolic effects, particularly lipid metabolism regulating insulin-sensitizing, and anti-inflammatory properties which might have potential roles [53].

ii) TNF-α

Tumor necrosis alpha (TNF α) is produced by adipocytes and inflammatory cells in response to chronic inflammation, and its serum levels are strictly related to obesity in T2DM [55]. TNF-α is a pro-inflammatory cytokine which increases insulin resistance via modulation of glucose transporter type 4 (GLUT 4) and phosphorylation of insulin receptor substrate-1(IRS-1) (Wu,Ruoyun et al,2021). Experimental studies conducted on obese animals indicate that the expression of TNF-α is increased in obese animals which modulates the insulin action. TNF-α binds with its receptor and triggers a broad-spectrum signalling cascade that results in the activation of various transcriptional pathways such as nuclear factor kappa-B cells (NF-κB) and Jun NH2-terminal kinase (JNK) [56]. It has pronounced proinflammatory properties and it is the one of the cytokines that initiate inflammatory cascade and is therefore, often referred as the primary cytokine [57]. It has been recently found that serum level of TNF-α is positively correlated with the pathophysiology of IR which exhibit that it is also a main causative factor that contributes the development of IR (See Fig 2) [58].

iii) Interleukin 6

Interleukins are known for their diverse biological functions and are pathological importance during the development of many diseases including T2DM [59]. Interleukin (IL)-6 is one of the main cytokines of chronic subclinical inflammation and is a cytokine with multidirectional effects [60]. Production of IL-6 is regulated by IL-1β via activation of interleukin-1 receptor type I (IL-1RI) [61], blocking the activity of IL-1RI with suitable anti-inflammatory agent like interleukin-1 receptor antagonist (IL-1Ra) antagonizes the agonistic effects of IL-1β that ultimately leads to the suppression of IL-6 production. IL-6 promotes the insulin resistance (IR) and impairs glucose homeostasis [62]. The mechanism by which IL-6 induces IR is complicated and versatile. It not only prevents the metabolism of non-oxidative glucose, but also suppresses the lipoprotein lipase that consecutively increases the plasma levels of triglycerides [63]. Moreover, IL-6 also activates the suppressor of cytokine signalling (SOCS)

proteins which may block the cytokine-mediated transcriptional factor activation of insulin receptor [64]. IL-6 can induce differentiation of naïve CD4+ T cells, further playing major roles in modulating acquired immune response. It protects beta cells against oxidative damage through effective modulation of autography and enhancing the antioxidant response [65].

iv) C-reactive protein (CRP)

CRP is a systemic inflammatory biomarker and has been considered as one of the major causative factors for development of T2DM [66]. It is the typical inflammatory biomarker produced in the liver, is regulated by adipocyte-derived proinflammatory cytokines, including interleukin 6 (IL-6) and tumor necrosis alpha (TNF α). CRP is chronically elevated in patients with T2DM [67]. Low grade inflammation is characterized by elevated inflammatory protein levels, including c-reactive protein (CRP), is linked with T2DM pathogenesis. In patients with T2DM, CRP levels range between 4.49 and 16.48 mg/L [66]. The production of CRP may be trigged by many metabolic and inflammatory factors associated with the development of T2DM, such as increased blood glucose, adipokines and free fatty acid levels. Increased levels of CRP represent a reliable predictor of vascular complications and progressions of cardiovascular disease T2DM patients [68]. It plays a pivotal role for pathogenesis of insulin resistance by inducing local and/or systemic inflammation [22].

v) Oxidative stress and antioxidants.

Oxidative stress is because of imbalance between the production of reactive oxygen species (ROS) and anti-oxidative defence mechanism against the production of ROS [69]. It is another factor that is consistently associated with beta cell destruction during the development of T2DM [70]. Oxidative stress normally arises due to the excessive production of free radicals, especially reactive oxygen species that severely affect the neutralising capacity of intracellular antioxidants [71]. Overnutrition increases the cellular overload of glucose and FFAs which in turn increases the oxidative stress [72]. Peripheral and adipose tissues protect themselves from the damaging effects of oxidative stress producing resistance to the action of insulin by preventing the penetration of glucose and FFAs into the cells. Generally, oxidative stress may induce its destructive effects through causing damage to DNA, proteins, and lipids [73]. And due to the dyslipidaemia features of most patients with T2DM, uncontrolled oxidative stress is associated with clustering interconnected plasma lipid and lipoprotein anomalies that may aggravate T2DM complications [70].

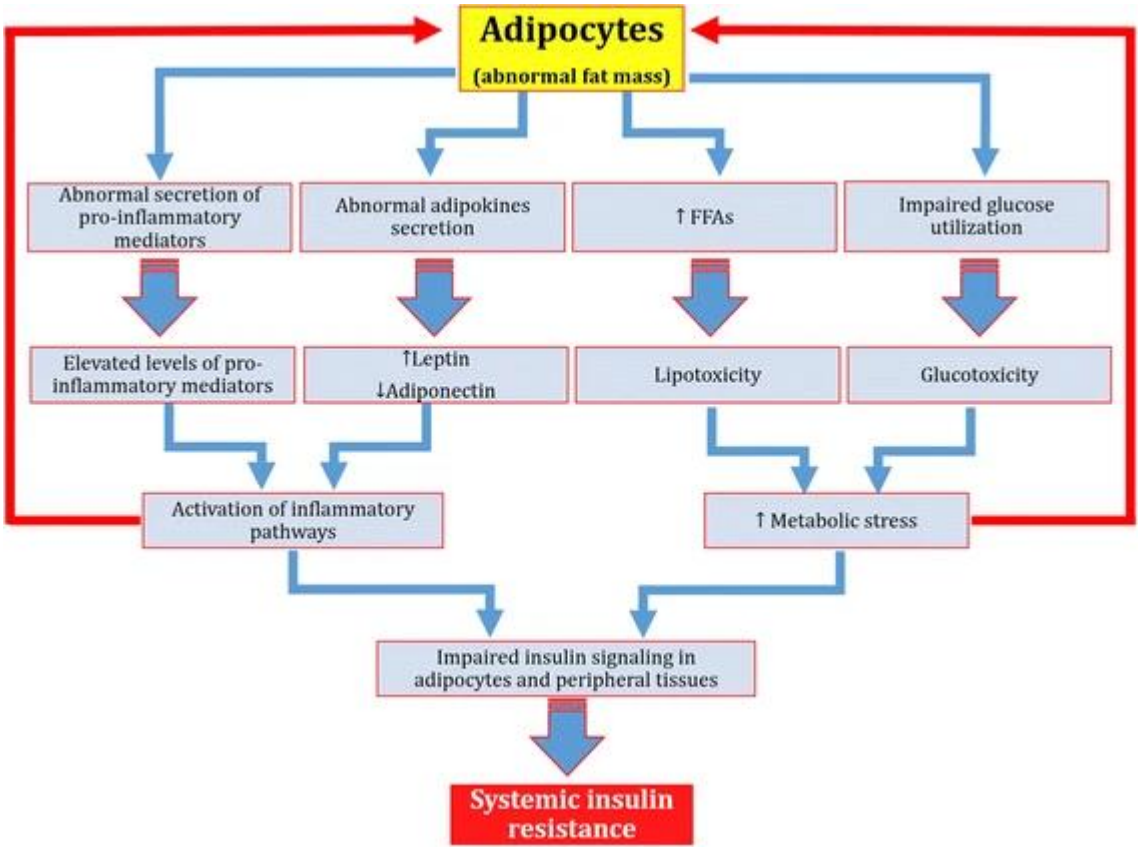

Figure 2: Showing the schematic representation of adipocytokines-induced IR adapted from Rehman and Akash (2016).

1.2.2. Effects of T2DM on haematological factors

Haematological parameters, including red and white blood cell counts and haemoglobin concentration, are widely used clinical indicators of health and disease. These traits are tightly regulated in healthy individuals and are under genetic control [74]. In haematological factors there haematological parameters which are haemoglobin (Hb), haematocrit (Hct), red blood cell (RBC), white blood cell (WBC), and haematological indices such as mean cellular volume (MCV), mean cellular haemoglobin (MCH), and mean cellular haemoglobin concentration (MCHC) [75]. Haematological changes have been reported in type 2 diabetes and play a major role in type 2 diabetes-associated complications. Chronic hyperglycaemia can lead to serious life-threatening complications and exacerbate damage done to various organs, including kidneys, nervous system and cardiovascular system [76].

A) Red blood cells indices

The persistent hyperglycaemia in diabetes is associated with metabolic, structural, and functional changes in RBCs due to the glycation of haemoglobin (Hgb) and membrane proteins

[77]. Pathological alterations in RBC morphology and functions related to chronic hyperglycaemia not only mechanistically underpin T2DM complications but can also be triggered by multisystemic changes such as the accumulation of toxins and altered cellular signalling. Anaemia is the most prominent clinical manifestation of RBC dysfunction in T2DM [78].

B) Platelet indices

Platelets play a vital role the integrity of normal homeostasis and mean platelet volume (MPV) is the marker for its function [79]. T2DM is complicated by accelerated atherosclerosis and platelet activation plays a role in inflammation and the atherothrombosis process contributes to the development of CVD in patients with T2DM [80]. Mean platelet volume reflects changes in platelet stimulation or rate of platelet production and increased MPV has been observed in T2DM patients coronary heart disease, nephropathy, and retinopathy [81].

C) Lipid profile

Patients with type 2 diabetes have increased risk of cardiovascular disease associated with atherogenic dyslipidaemia [82]. Persistent hyperglycaemia causes glycosylation of all protein, especially collagen cross linking and matrix proteins of arterial wall. This eventually causes endothelial dysfunction, contributing further to atherosclerosis. The prevalence of dyslipidaemia in T2DM is 95%. The dyslipidaemia is a major risk factor for coronary heart disease (CHD) [83]. The CV is cause of morbidity and mortality in patients with T2DM because of disturbance in lipoprotein i.e., serum triglycerides (TC) 69%, serum cholesterol 56.6%, low-density lipoprotein cholesterol (LDL) 77% and high-density lipoprotein (HDL) 71% [84].

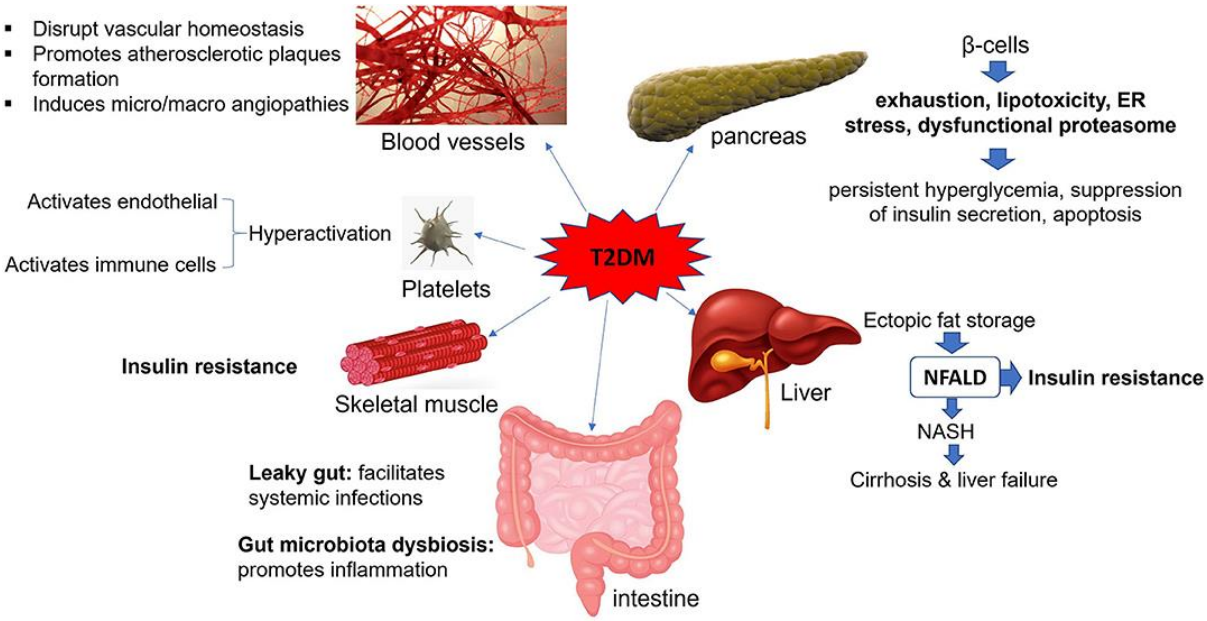

Figure 3: Showing the effects and complication of T2DM in the human body adapted from (Daryabor, Atashzar et al.2020).

2. Prediabetes

2.1. Definition and Epidemiology

Prior to the onset of type 2 diabetes mellitus (T2DM), there exists a condition known as prediabetes (see Fig 4)[9]. Prediabetes is a condition of intermediate hyperglycaemia where the glycaemic parameters are above the homeostatic range but below the threshold for a diagnosis of T2DM [10]. Prediabetes is frequently an asymptomatic disease however; it is often present before the onset of T2DM. Because blood glucose elevation is a continuum, prediabetes cannot be considered completely begin [10]. Prediabetes is a practical and convenient phrase that refers to impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or a glycated haemoglobin (HbA1C) of 6.0% to 6.4%, all of which put people at risk of developing diabetes and its complications [85]. In recent years, there has been growing attention to the importance of addressing prediabetes [11]. Prediabetes is a significant risk factor for the development of T2DM and early intervention has been shown to help prevent or delay the progression to overt T2DM [12]

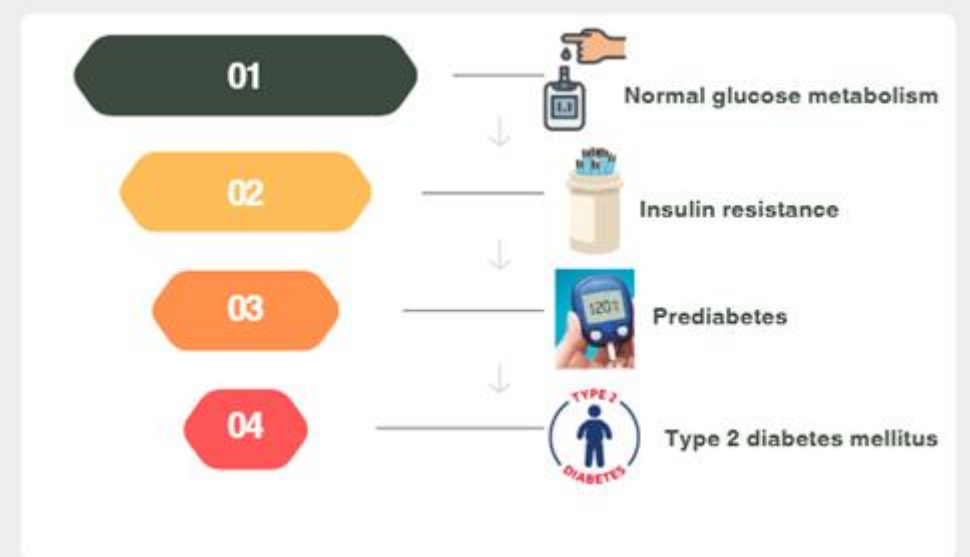

Figure 4: The various stages in the development of type 2 diabetes mellitus adapted from [9].

2.2. Prevalence of prediabetes.

There have been reports of increased mean FPG and prevalence of diabetes in developed as well as developing countries [86]. About 6% of the South African population, 35 million people suffer from diabetes and 5 million more are estimated to have prediabetes. Most cases of prediabetes in SA (South Africa) are undiagnosed [31]. The prevalence of prediabetes is expected to rise to 471 million people worldwide by 2035, according to the International Diabetes Federation [87]. These crucial discoveries must be considered in the context of the present obesity and diabetes epidemics. Studies showed that the prevalence of prediabetes is the highest in overweight and obese people, and it increased across all BMI (Body Mass Index) subgroups. As a result, the development of prediabetes is directly linked to the progression of obesity, and maybe even to weight within the normal BMI range [88].

2.3. Diagnosis of prediabetes

Due to the inability of the blood glucose cut points to capture pathology related to diabetes and the likelihood of developing diabetes in the future, the usefulness of diagnosing diabetes and prediabetes based on IFG and IGT has been questioned [89]. It is possible that the presence of IFG and IGT identifies subjects with different pathological abnormalities in their glucose metabolism and the presence of both of these signifies more advanced impairment in overall glucose homeostasis [90]. IFG is defined as fasting plasma glucose of 6.1-6.9 mmol/L and IGT

is defined as 2h plasma glucose of 7.8-11.0 mmol/Lafter ingestion of 75g of oral glucose [91]. The World Health Organization (WHO), and American Diabetes Association (ADA), use different FPG values to diagnose IFG (WHO: FPG 6.1–6.9 mmol/L and ADA: 5.6–6.9 mmol/L), and the WHO does not currently endorse the use of HbA1c for the diagnosis of prediabetes. The management of prediabetes is similar to that of T2DM, however, the incidence of T2DM has only escalated in recent decades since, despite the success of early clinical trials primarily because of poor patient compliance. Thus, implementation of new intervention or treatments are needed that can work in both the absence or presence of lifestyle interventions [14].

2.4. Complications of prediabetes

A) Complications of prediabetes on immune system.

According to the study by Mzimela, Sosibo et al, it was shown that the presence of prediabetes could lead to compromised immunity [92]. These reports indicated that during the progression from pre-diabetes to overt T2D, there are changes in immune cell concentrations in neutrophils, lymphocytes, monocytes, eosinophils, basophils and upregulation of inflammatory markers which are C-reactive protein (CRP), tumour necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), P-Selectin, cluster of differentiation 40 ligands (CD40L), and fibrinogen [92]. Together, these markers give us insight into whether there is immune activation as the changes in immune cells concentration may be an indicator of glucotoxicity. There is an alteration of the adipokine profile and cytokine production in prediabetic state, with an increased propensity to develop insulin resistance. The imbalance between pro and anti-inflammatory cytokines have been shown to impact immune response to infections [93]. In addition, neutrophils may have reduced chemotaxis, phagocytic ability, and (ROS) production which weakens the first line of defence against infections [94].

B) Complications of prediabetes on haematological parameters

Chronic hyperglycaemia disrupts various blood cell components, resulting in several microvascular issues. Disruptions in haematological parameters are followed by increased inflammatory marker levels, susceptibility to clot formation and microcirculatory abnormalities. Following the circulatory disturbance, advanced glycation end products were activated, generating oxidative stress systemically, altering membrane and vasculature structures and hemodynamic characteristics [95]. A few prior studies that surveyed the

association between haematological parameters and prediabetes showed increased red cell count and leukocyte count [2]. Also, increased platelet activation is related to prediabetic states and can lead to cardiovascular complications [96]. Prediabetes causes an increase in platelet reactivity through direct effects and by promoting glycation of platelet proteins on the platelet surface, this glycation decreases membrane fluidity and tends to activate the platelets [97]. It also leads to RBC dysfunction that may lead to deranged tissue oxygenation through a variety of mechanisms [78]. This includes impaired microcirculation and biochemical changes such as increased glycosylation of RBC 2,3-bisphosphoglycerate binding sites [78].

2.5. Management of prediabetes

A combination of lifestyle interventions and metformin is the most widely studied strategy for the prevention or delay of T2D in subjects with prediabetes [98]. The diabetes prevention program (DPP) showed that lifestyle intervention was more effective than metformin for diabetes prevention in certain subgroups, although other smaller randomized diabetes prevention trials produced variable results [98]. Metformin had been used for several decades and has been noted to have additional favourable outcomes such as body mass index (BMI) reduction and improved cholesterol profile [99]. It primarily works by reducing hepatic glucose production and improving insulin sensitivity in peripheral tissues and it has been shown to be less effective without lifestyle intervention. Furthermore metformin in higher doses has been reported to reduce the rate of prediabetes to diabetes progression [100].

The goal of lifestyle intervention programs is to improve the modifiable risk factors of prediabetes and diabetes by increasing physical activity and making dietary adjustments [101]. Exercise has been shown to possess anti-inflammatory and metabolic benefits [102]. Interleukin (IL)-1 has been linked to pancreatic-cell destruction, while tumour necrosis factor (TNF) appears to be a critical component in peripheral insulin resistance. Mechanistic studies in humans reveal that modest acute IL-6 increases caused by exercise have direct anti-inflammatory effects by inhibiting TNF and increasing IL-1ra (IL-1 receptor antagonist), hence restricting IL-1 signalling. IL-6 has a direct effect on glucose and lipid metabolism [103]. Furthermore, the indirect anti-inflammatory effects of exercise may be mediated through improvements in body composition [104]. However, prediabetic individual may still progress to develop T2DM due to poor patient compliance where by patients heavily rely on the pharmacological treatments and neglect the lifestyle interventions. This reduces the efficacy of the drugs. Therefore, there is a necessity to evaluate other alternative treatments such as

medicinal plants that may provide therapeutic benefits in both the presence and absence of lifestyle interventions.

3. Medicinal plants

Herbal plants have a vital role in disease prevention and treatment. Plants have been used as traditional medicine for thousands of years [15]. Plants have been linked to the advancement of human civilization all throughout the world. Plants are thought to be rich suppliers of phytochemical compounds, which enable them to have medical benefit. Medicinal plants have the potential to be a source of innovative herbal medications. The pharmacological effects of medicinal plants have been identified as a promising future drug/medicine for the management of health care in the twenty-first century [105]. There has been a renaissance of interest in rediscovering medicinal plants as a source of possible medication candidates in recent years. The plant of interest in this study is the medical plant *Zingiber officinale* popularly known as ginger.

A) Zingiber officinale Roscoe (ginger)

Zingiber officinale Roscoe (ginger) is an herbaceous rhizomatous plant that is a is member of the Zingiberaceae family and the rhizome of ginger has been used as an important dietary component and spice across the world [16]. The constituents of ginger are numerous and vary depending on the place of origin and form of rhizomes, e.g., fresh or dry. The ginger rhizome contains several components of interest, i.e., carbohydrates, minerals, phytochemicals, etc. It contains proximate components like moisture, proteins, fats, fiber, ash, and carbohydrates. It also contains appreciable amounts of vitamins and minerals as well as some enzymes, for example, a potent proteolytic enzyme called zingibain. Additionally, extractable oleoresins and waxes are its fundamental constituents or functional ingredients [17]. Studies have shown that ginger includes pharmacologically potent natural antioxidant chemicals that can block free radicals[9]. Gingerols and shogaols are the phenolic chemicals in ginger that have antioxidant properties [18]. According to a study by Das et al, it was shown that ginger has been used for its wide array of medicinal properties such as being anti-inflammatory, immunomodulatory, anti-tumorigenic, anti-apoptotic, anti-lipidemic, anti-emetic, antimicrobial, antioxidant and anti-hyperglycaemic (See Fig 5)[19]. Some studies reported reductive effects of ginger on fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and blood pressure (BP) in patients with T2DM. However, the effects of *Zingiber*

officinale (ginger) extract on haematological factors and immune system in prediabetes are yet to be investigated. Therefore, this study seeks to investigate the effects of Zingiber officinale extract on haematological factors and immune system in diet induced prediabetics rats.

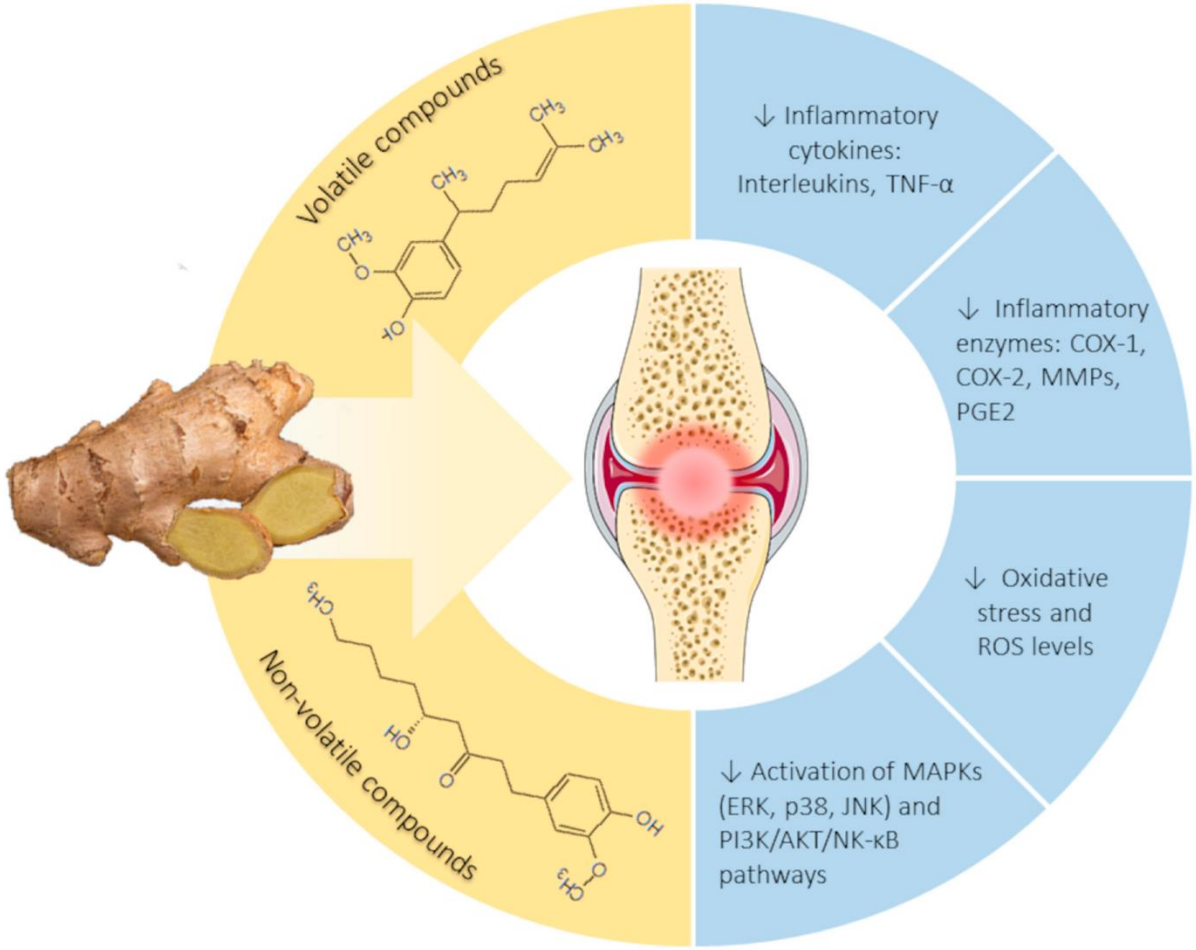

Figure 5: The components and effects of *Zingiber officinale*(ginger) adapted from [19].

4. High fat high carbohydrate animal models

Experimental animal models have been essential tools to deepen human understanding of the occurrence and development of T2DM, providing valuable references for exploring the pathogenesis, aetiology and potential treatment of diabetes[9]. In our laboratory, we use a high fat high carbohydrate induced prediabetic rats which has shown to mimic the human condition in both terms of development and progression[20]. We used rats as they are cost-effective, ease of handling, shares similar numerous pathophysiology and pathology features with humans and a reduced amount of stress by human contact [106]. Accordingly, in this study, we used a diet induced prediabetic male Sprague Dawley rats to investigate the effect of ginger on immune

system and haematological parameters. The rats were induced prediabetics using a high fat high carbohydrate diet.

5. Justification of the study

Prediabetes is a condition characterized by elevated blood glucose levels below the threshold for a diagnosis of T2DM but associated with a higher risk of developing T2DM [11]. This transitional state is associated not only with hyperglycaemia but also with systemic inflammation, immune dysfunction, and haematological abnormalities [107, 108]. Early stage interventions targeting these metabolic and physiological changes are essential for preventing the onset of diabetes and its associated complications [109]. Often focusing on lifestyle modifications and pharmacological intervention that may carry adverse effects and have limitations [110]. Natural compounds have gained attention for their potential among natural remedies, *Zingiber officinale* has been widely recognized for its medicinal properties, including antioxidant, anti-inflammatory, antidiabetic, and immunomodulatory effects [111]. Bioactive compounds in ginger, such as gingerols, shogaols, and paradols, are believed to modulate blood glucose levels, enhance immune function, and improve haematological profiles [112].

However, none of these effects have been investigated during the prediabetic state. In our laboratory, we use a diet-induced animal model of prediabetes which has been shown to mimic the human condition. Accordingly, this study seeks to investigate the effects of a *Zingiber officinale* extract on markers of glucose homeostasis, red blood cell indices and immune function in a diet-induced animal model of prediabetes. the complete medicinal plants still have a hopeful future, as the phytochemical composition and the potential health benefits of many species have not yet been studied or still need to be more deeply investigated [113]. There is notable gap in literature regarding the effects of *Zingiber officinale* [114]. Understanding how *Zingiber officinale* influences the immune system is critical, as abnormalities in blood cell indices and immune response are early indicators of metabolic imbalance and chronic inflammation in prediabetic states [111].

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Chapter 3: Research Manuscript 1

Title: The effects of *Zingiber officinale* (ginger) on inflammation and innate immune system in diet-induced prediabetic rats.

Prologue

The literature review in Chapter 2 summarized key findings, gaps, and inconsistencies in the current understanding of the effects of *Zingiber officinale* (ginger) particularly in the context of diet-induced prediabetes. However, a more structured experimental investigation was required to evaluate how *Zingiber officinale* influences specific inflammatory pathways and innate immune markers in a prediabetic state. Although both prediabetes and chronic inflammation are globally prevalent, the literature review did not adequately address how *Zingiber officinale* interacts with innate immunity under prediabetic conditions in both presence and absence of dietary intervention. This gap prompted the development of a focused animal study to explore the therapeutic potential of *Zingiber officinale* in modulating inflammation and innate immune responses in diet-induced prediabetic rats. To undertake this research, it was necessary to first develop a comprehensive experimental protocol, outlining the study design, inclusion criteria, and methods of analysis, to ensure a transparent and reproducible evaluation of *Zingiber officinale* effects.

This manuscript has been submitted for review in the journal: International Journal of Molecular Sciences as a manuscript titled: “The effects of *Zingiber officinale* (ginger) on inflammation and innate immune system in diet-induced prediabetic rats.” Authored by B Langa, A Khathi and LP Mabuza. This journal is accredited by the Department of Higher Education and Training South Africa and appears in the Scopus accredited list (2024).

Candidate Contribution: B Langa was responsible for brainstorming, designing the study, compiling and analysing all data extracted from the studies, drafting the literature review and editing the final draft of the manuscript.

Abstract

Prediabetes, a state of intermediate hyperglycaemia, and also has also been shown to impair the innate system through low-grade chronic inflammation, moderate increases in pro-inflammatory cytokines and oxidative stress. This study aimed to evaluate the effect of *Zingiber officinale* on inflammation and innate immune system in diet-induced prediabetic rats.

Thirty-six (36) male Sprague Dawley rats were divided into six groups (n=6 per group) and were induced with prediabetes using high carbohydrate for a period of 20 weeks. After induction, the groups were treated as follows for a period of 12 weeks: a prediabetic (PD) control; a dietary intervention (DI) control; *Zingiber officinale* treatment groups in the presence (ZO+DI) and absence [ZO+HFHC] of DI; as well as metformin in the presence (MT+DI) and absence (MT+HFHC) of DI. An additional six animals were kept on a standard diet and served as a non-prediabetic control (NPD). At the end of the treatment period, parameters measured included homeostatic model assessment index (HOMA-IR), body weight, anti-inflammatory cytokine (adiponectin), pro-inflammatory cytokines (TNF α, IL-6 and CRP), neutrophils cell count, MDA levels, nuclear factor erythroid 2-related factor 2 (NFE2L2).

Treatment with *Zingiber officinale* significantly improved glycaemic control and reduced body weights in rats. Additionally, it decreased pro-inflammatory cytokines while enhancing anti-inflammatory cytokine. The expression of NFE2L2 was regulated, leading to reduced levels of neutrophils and lymphocytes. Furthermore, oxidative stress markers were significantly lowered. These findings suggest that Zingiber officinale reduces inflammation and restores the immune balance caused by diet-induced prediabetes.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic condition characterized by peripheral insulin resistance and pancreatic β-cell dysfunction leading to a decrease in glucose transport into muscle cells, increased fat breakdown and increased hepatic glucose production [115]. The chronic hyperglycaemia seen in T2DM has been shown to result in chronic systemic

inflammation [116]. The hyperglycaemia along with the chronic inflammation dysregulate neutrophil and macrophage function, reducing chemotaxis, phagocytosis and reactive oxygen species (ROS) production [50]. The generation of advanced glycation end products (AGEs) and oxidative stress damage cell receptors thus weakening the response to pathogens while persistent inflammation leads to the overproduction of pro-inflammatory cytokines [117].

The onset of T2DM, however, is preceded by prediabetes, which is characterized by impaired fasting glucose IFG) and impaired glucose tolerance (IGT) [118, 119]. Prediabetes is intermediate state where blood glucose levels are higher than normal but below the T2DM threshold [120]. During this state, insulin resistance and β-cell dysfunction begin to develop, leading to subclinical hyperglycaemia [121]. Prediabetes has also been shown to impair the innate system through low-grade chronic inflammation, moderate increases in pro-inflammatory cytokines and oxidative stress [122]. These immune changes driven by AGEs, metabolic stress and altered immune signalling promote infection risk and progression toward T2DM [123].

Studies have shown that a combination of lifestyle and pharmacological intervention can significantly reduce the risk of progression to T2DM in those with impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) [124]. Metformin, a derivation of biguanide is one of the most used drugs to manage T2DMs [125]. Metformin acts directly and indirectly on the liver to lower glucose production, and acts on the gut to increase glucose utilization, increase GLP-1 and alter the microbiome [126]. However, individuals with prediabetes may still progress to develop T2DM due to poor patient compliance whereby patients heavily rely on the pharmacological treatments and neglect the lifestyle interventions thus reducing the efficacy of the drugs. Therefore, there is a necessity to evaluate other alternative treatments such as medicinal plants that may provide therapeutic benefits in both the presence and absence of lifestyle interventions.

Zingiber officinale Roscoe(ginger) is a member of Zingiberaceae family of plants [127]. It contains important proteins compound, including gingerol, gingerdione that have impact microbial activity [128]. It possesses multiple biological activities especially antioxidant and anti-inflammatory and immune modulatory capacities. It exhibits antioxidant properties due its ability to scavenge free radicals and reduce oxidative stress [111]. Gingerol possesses anti-inflammatory properties through multiple mechanisms such as probably inhibiting the activation of NF-kB signaling pathway, which is the target agent in the treatment and control

of diabetes [129]. It triggers the release of anti-inflammatory cytokines while reducing pro-inflammatory cytokines. Ginger extract and gingerols enhanced glucose uptake in L6 myotubes, by enhancing translocation of GLUT4 to the surface membrane and activation of AMPKα1 through a Ca^{2+}/calmodulin-dependent protein kinase pathway [130].

Recent studies have shown that patients with T2DM that were given ginger extracts showed reductive effects of ginger on fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), systolic blood pressure (SBP), diastolic blood pressure (DBP) [131]. However, there have been no studies that have investigated the effects of ginger during the prediabetic state. Investigating the effects of *Zingiber officinale*, known for its anti-inflammatory and immune modulating properties, during prediabetes could elucidate its role in attenuating immune dysfunction and reducing the risk of the onset of T2DM. In our laboratory, we use a high fat, high carbohydrate diet-induced animal model of prediabetes which has been shown to mimic the human condition of prediabetes. Using this animal model, this study sought to investigate the effects of a *Zingiber officinale* extract on markers of inflammation and the innate immune system in diet-induced prediabetes.

2. Materials and methods

2.1. Chemicals and drugs

All chemicals and reagents are of analytical grade and purchased from standard commercial suppliers. Dimethyl sulphoxide (DMSO) 3 ml/kg (Sigma-Aldrich, St Louis, Missouri, United State of America) Metformin 500 mg/kg (Sigma-Aldrich, St Louis, Missouri, United State of America). *Zingiber officinale* was purchased from a local grocery store (Westville, KwaZulu-Natal, South Africa). The concentrations of the drugs and extracts were extrapolated from previous studies and have been shown to have minimal toxicity [132].

2.1.1 *Zingiber officinale* (ginger) root extraction

An aqueous ginger extract was prepared from ginger roots as described by Al-Amin et al. (2006). Briefly, the ginger roots were peeled on crushed ice, and 20 g ginger was cut into small pieces and homogenized (2000 rpm for 10 min) in 75-ml cold, sterile 0.9% NaCl in the presence of some crushed ice. The homogenization was carried out in a blender at high-speed using 2-min bursts for a total of 12 min. The homogenized mixture was filtered three times through a 0.22 μm nylon filter. The clear supernatant fraction was separated and the volume made up to

100 ml with cold normal saline. The concentration of this ginger extract was measured and adjusted to 200 mg/ml. The prepared aqueous extract of ginger root was stored in small samples at -80 °C until use.

2.2. Animals and housing

A sample set of male Sprague-Dawley rats (2 weeks old;150-180 g) bred and housed in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal were used in this study. The animals were housed in a room with a 12-hour light/12 hours dark cycle, room temperature (25°C), relative humidity = 55±5% and noise levels of less than 65 decibels for the duration of the study. The animals in each group had access to food and water *ad libitum*. All animal procedures and housing conditions were approved by the Animal Research Ethics Committee (ethical clearance number: AREC/00006737/2024).

2.3. Induction of prediabetes

An established research protocol was used for the induction of prediabetes (Luvuno et al., 2018). Animals were allowed to acclimatize to their new environment for a week while consuming standard rat chow before commencement of the experimental diet. Procedures involving animals and their care were conducted in conformity with institutional guidelines of the University of KwaZulu-Natal. The animals were housed in a group of six in a type 4 large cages where a well enriched environment was provided. The bedding was changed on every second day. After acclimatization for a week, the animals were then randomly assigned to the following diet groups; Normal diet with drinking water (ND) and high-fat high-carbohydrate (HFHC) diet with both drinking water and 15% fructose dissolved in water for 20 weeks. Prediabetes was induced by allowing the animals to feed on the HFHC diet for 20 weeks. Glucose tolerance was evaluated 5 days after 20 weeks of induction with an oral glucose tolerance test to determine pre-diabetes according to the American Diabetes Association criteria. The animals with fasting blood glucose of more than 5.6 mmol/L were considered pre-diabetic and grouped further for pharmacological studies. The animals that were fed the normal diet were also tested and were found to be normoglycemic and without prediabetes.

2.4. Experimental design and treatment

The sample size of this study was based on previous similar studies, employed to ensuring both scientific validity and ethical compliance by minimizing unnecessary animal use[133]. Thestudy consisted of 7 groups, Group 1: Non-pre-diabetic (NPD, n=6), non-prediabetic rats

fed with normal diet; group 2: Prediabetic (PD, n=6), prediabetic rats fed with high fat high carbohydrate diet; group 3: Dietary intervention (DI, n=6), prediabetic rats fed with normal diet; group 4: Metformin plus dietary intervention (MTF+DI, n=6), prediabetic rats fed with metformin and normal diet; group 5: Metformin plus HFHC diet (MTF+HFHC, n=6), prediabetic rats fed with metformin and HFHC diet; group 6: Ginger plus dietary intervention (ZO+DI, n=6), prediabetic rats fed with ginger normal diet and group 7: Ginger plus HFHC diet (ZO+HFHC, n=6), prediabetic rats fed with ginger and HFHC diet). In each group, monitored parameters including fasting blood glucose (FBG), food intake, fluid intake and urinary output body were measured once after 4 weeks, while body weight was measured once a week at the same time for a duration of 12 weeks. The animals were placed on metabolic cages for 24 hours for monitored parameter measurements and placed back to colony cages once after 4 weeks.

2.5. Blood collection and tissue harvesting

All animals were anaesthetized with Isofor (100 mg/kg)) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) using a gas anesthetic chamber (Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa) for 3 minutes. Blood was collected by cardiac puncture and then injected into individual precooled heparinized containers for immune cell count. The other collected blood was then centrifuged for plasma collection (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes. The spleen, heart, liver and adipose tissue were collected and storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until biochemical assays were done.

2.6. Biochemical analysis

Plasma insulin, nuclear factor erythroid 2-related factor 2 (NF2L2), adiponectin, C-reactive protein (CRP), plasma interleukin 6 (IL-6), and tumor necrosis factor- α (TNF-α) (respectively) were analyzed using separate, specific ELISA kits in accordance with the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China).

2.7. Lipid peroxidation (MDA) measurements

Lipid peroxidation was assessed using an assay technique for thiobarbituric acid reactive substances (TBARS), which is indicated by the presence of malondialdehyde (MDA) (Tsai and Huang, 2015). Tissues (50 mg) were homogenized in 500 µL of 0.2% phosphoric acid. The homogenate was centrifuged at 400 x g for 10 min. Thereafter, 400 µL of the homogenate was

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

Concentration of MDA (Mm) = Average Absorbance / Absorption coefficient (156 mmol-1).

2.8. HOMA-IR Index

HOMA-IR Index Insulin resistance was calculated from fasting blood glucose and insulin levels using the homeostatic model assessment (HOMA) [134]. The HOMA-IR index was calculated using the HOMA2 Calculator v2.2.3 program. Insulin sensitivity was indicated by values less than 1.0, early insulin resistance by values greater than 1.9, and significant insulin resistance by values greater than 2.9.

2.9. Immune cell count analysis

The collected blood (100 µl) was transferred into tubes containing anticoagulants, thereafter monocytes, neutrophils, lymphocytes, eosinophils, basophils and platelet cell count were determined using a hemocytometer (COULTER® Ac.T diff™ Analyzer, Beckman Coulter, United State).

2.10. Statistical analysis

Data were reported as means ± standard error of means (SEM). Statistical analysis was conducted using Graph Pad Prism Instant Software (version 8, graph Pad Software, San Diego,

California, USA). To evaluate differences between the groups, we utilized either a parametric or non-parametric test, depending on the distribution of the data. All tests were parametric. Two-way analysis of variance (ANOVA) was used to analyze differences between the controls and the experimental groups. This was followed by Tukey Kramer post hoc comparison test while. Values of $p < 0.05$ statistical significance between the compared groups.

3.0. Results

3.1. Effect of *Zingiber officinale* on HOMA-IR value after the experimental period.

HOMA-IR was evaluated at the end of the experimental period between all the groups (Table 1). The untreated prediabetic group showed a significant higher HOMA-IR value compared to non-prediabetic group ($p< 0.05$; Table 1). By comparison with the untreated PD group, the rats treated with *Zingiber officinale* combined with dietary intervention (ZO+DI) showed a significantly decreased HOMA-IR value ($p< 0.05$; Table 1) to levels comparable with those of the NPD group. However, the rats that were treated with *Zingiber officinale* in the absence of dietary intervention (ZO+HFHC) showed no difference when compared to the prediabetic control (PD). Similarly, the administration of metformin in the presence of dietary intervention (MT+DI) showed a significantly decreased HOMA-IR value when compared to the PD control while the administration of metformin in the absence of dietary intervention (MT+HFHC) showed no difference when compared to the prediabetic control (PD).

Groups	Mean Fasting serum insulin	Mean Fasting blood glucose	HOMA-IR
NPD	4.81±1.04	4.7±0.43	0.6320 ± 0.2240
PD	8.09±1.88	7.2±0.43	2.7190 ± 0.7040 *
DI	3.64±2.68	5.9±0.62	0.4880 ± 0.1120 α
ZO+DI	3.40±0.78	5.6±0.30	0.7180 ± 0.0780 α
ZO+HFHC	6.87±2.37	7.7±0.45	2.8930 ± 0.4695 *
MT+DI	3.23±1.53	5.8±0.10	0.3345 ± 0.1695 α
MT+HFHC	9.46±2.35	6.7±0.29	3.0730 ± 0.0565 *

* = $p< 0.05$ in comparison to non-prediabetic group, α = $p< 0.05$ in comparison to the prediabetic group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD;

Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and Zing*iber officinale* in the absence of dietary intervention, ZO+HFHC.

3.2. Effect of *Zingiber officinale* on body weight after 12 weeks treatment period.

Figure 1 illustrates the effects of *Zingiber officinale* (ZO) on body weight over a treatment period of 12 weeks. The untreated prediabetic (PD) group and the PD treated groups showed no significant different in body weight at week 0 before treatment (Figure 1).

As the study progressed, the PD control group showed a significant increase in body weight gain when compared with the non-prediabetic (NPD) group over the 12-week period ($p = 0.0136$; Fig 1). The administration of *Zingiber officinale* together with dietary intervention (ZO+ DI) showed a significant reduction in body weight when compared to the untreated PD group over the 12 weeks treatment period ($p = 0.0196$; Fig 1) to levels comparable with those of the NPD group. However, the rats treated with *Zingiber officinale* in the absence of dietary intervention (ZO+HFHC) showed no significant difference when compared to the untreated PD. A trend similar to that of *Zingiber officinale*-treated groups was observed in the groups treated with metformin in the presence and absence of dietary intervention (Fig 1).

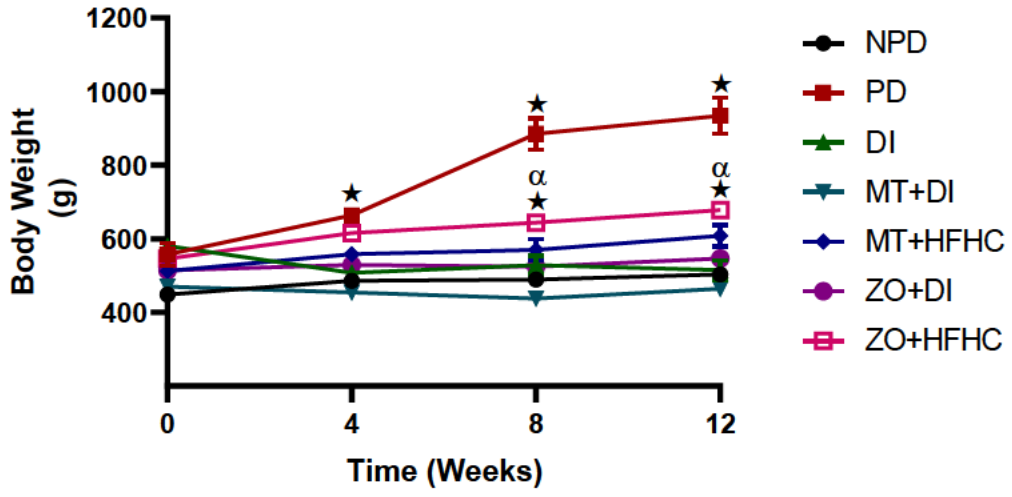

Figure 1: The effects of *Zingiber officinale* on body weight over 12 weeks treatment period. Values are represented as mean ± SEM (n=6) in each group. * = $p< 0.05$ in comparison to non-prediabetic group, α = $p< 0.05$ in comparison to prediabetic group. Non prediabetic, NPD; Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention,

MT+DI; Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC.

3.3. Effect of *Zingiber officinale* on proinflammatory cytokines (TNF alpha, IL-6 and CRP) and anti-inflammatory cytokines (Adiponectin).

Figure 2 illustrates the effect of *Zingiber officinale* (ZO) on adiponectin, TNF α, IL-6 and CRP plasma levels after 12 weeks treatment period (Figure 2). When compared to the NPD group, the untreated PD group showed decreased adiponectin levels ($p = 0.0001$; Fig 2A), increased TNF α levels ($p = 0.0428$; Fig 2B) and increased IL-6 levels ($p< 0.05$; Fig 2C). By comparison of prediabetic control group with the rats treated with *Zingiber officinale* in both absence and presence of dietary intervention (ZO+DI and ZO+HFHC), this group showed a significant increase in adiponectin concentrations ($p< 0.05$; Fig 2A); as well as significant decreases in IL-6 ($p< 0.05$; Fig 2C). By comparison of prediabetic control group with the rats treated with *Zingiber officinale* in both absence and presence of dietary intervention (ZO+DI and ZO+HFHC), this group showed no significant difference in TNF α levels. The administration of metformin only in the presence of dietary intervention showed a similar trend to those seen in *Zingiber officinale*-treated groups however, in the absence of dietary intervention (MT+HFHC), no significant differences were observed.

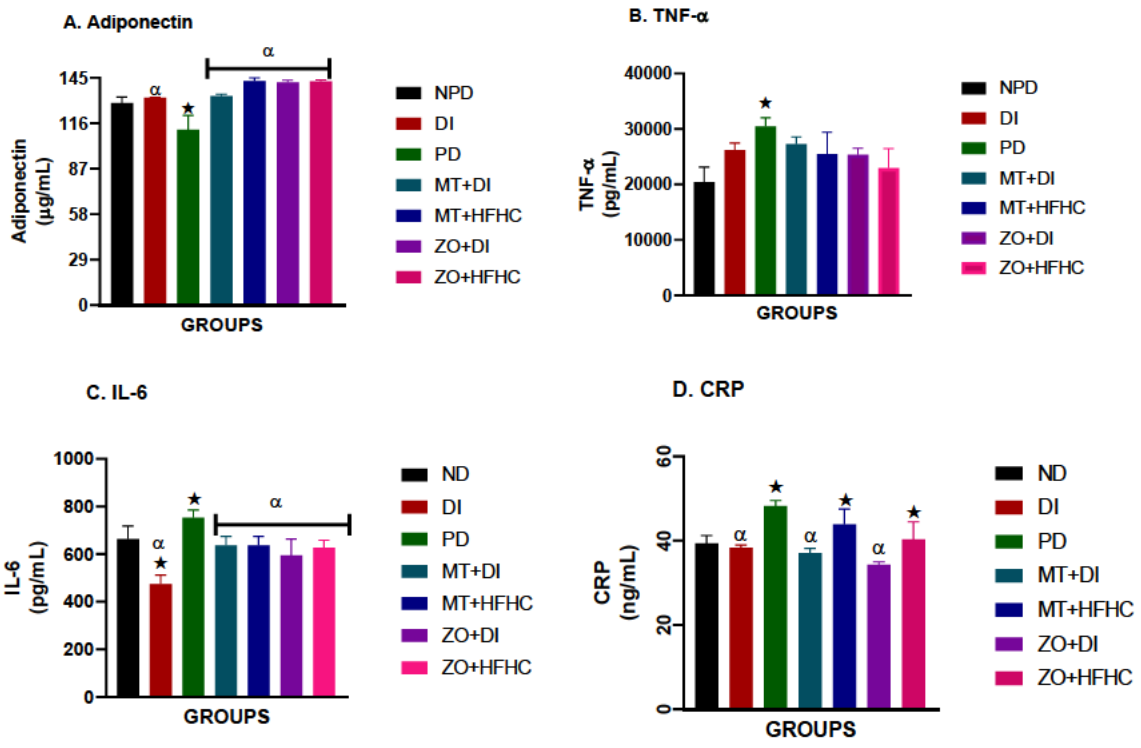

Figure 2: Effect of *Zingiber officinale* on proinflammatory cytokines (TNF alpha, IL-6 and CRP) and anti-inflammatory cytokines (Adiponectin). Figure 2A: Showing the effect of *Zingiber officinale* on adiponectin after 12 weeks treatment period, Figure 2B: Showing the effect of *Zingiber officinale* on TNF alpha after the treatment period, Figure 2C: Showing the effect of *Zingiber officinale* on IL-6 after the treatment period and Figure 2D: Showing the effect of *Zingiber officinale* on CRP after the treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin is the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = $p< 0.05$ in comparison to non-prediabetic group, and α = $p< 0.05$ in comparison to the prediabetic group.

3.4. Effect of *Zingiber officinale* on blood neutrophil count.

Neutrophil count was evaluated in the blood at the end of the experimental period (Figure 3). By comparison with the NPD group, the PD control group showed increased neutrophils cell count level ($p = 0.0002$; Fig 3). In comparison with the untreated prediabetic group, the rats treated with *Zingiber officinale* in both absence and presence of dietary intervention (ZO+DI and ZO+HF) showed a significant decrease in neutrophils ($p= 0.003$, $p= 0.0153$ respectively;

Fig 3) to levels comparable with those of the NPD group. The administration of metformin in both presence and absence of dietary intervention (MT+DI and MT+HFHC) showed similar observation when compared to the PD control group ($p< 0.05$; Fig 3).

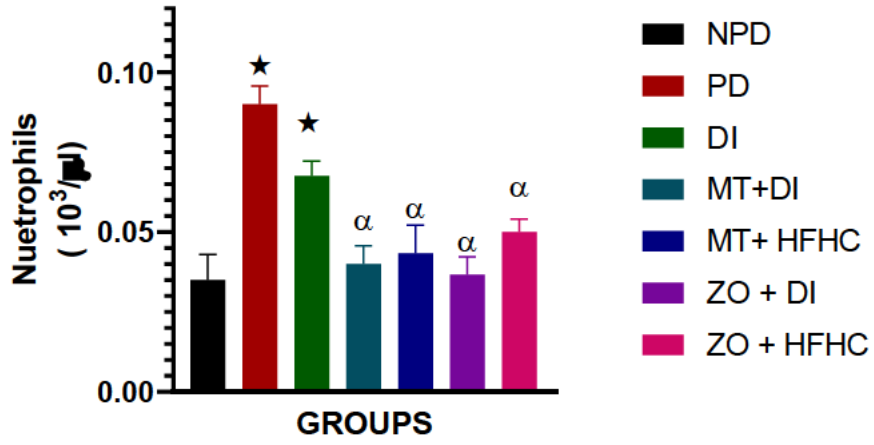

Figure 3: Showing the effect of *Zingiber officinale* on neutrophils after the treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = $p< 0.05$ in comparison to non-prediabetic group, and α = $p< 0.05$ in comparison to the prediabetic group.

3.5. Effect of *Zingiber officinale* on oxidative stress marker, MDA, levels

The oxidative stress marker, MDA, was evaluated at the end the experimental period amongst all the groups (Table 2). In comparison with the control prediabetic group with non-prediabetic group, the prediabetic control group showed a significant increase in levels of MDA ($p< 0.05$; Table 2). By comparison with the untreated PD group, the rats treated with *Zingiber officinale* in the presence of dietary intervention (ZO+DI) showed a significant decreased MDA level ($p< 0.05$; Table 2) to levels comparable with those of the NPD group. However, the rats treated with *Zingiber officinale* in the absence of dietary intervention (ZO+HFHC) showed no significant difference when compared to the PD control group. A trend similar to that of *Zingiber officinale*-treated groups was observed in the groups treated with metformin in the presence and absence of dietary intervention (Table 2).

Table 2: Oxidative stress marker, MDA status between all the groups. Values are represented as mean ± SEM (n=6) in each group.

Groups	MDA (nmol/g tissue)
NPD	0.6320 ± 0.2240
DI	0.4880 ± 0.1120 α
PD	2.7120 ± 0.7040 *
MT+DI	0.3345 ± 0.1695 α
MT+HFHC	3.0730 ± 0.0565 *
ZO+DI	0.7180 ± 0.0780 α
ZO+HFHC	2.8930 ± 0.4695 *

* = $p< 0.05$ in comparison to non-prediabetic group, and α = $p< 0.05$ in comparison to the prediabetic group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC.

3.6. Effect of *Zingiber officinale* on NFE2L2 the antioxidant expression levels.

Figure 4 illustrate the effect of *Zingiber officinale* on NFE2L2 levels after the experimental period. The values are represented as means ± SEM (n=6) for each group. When comparing the prediabetic group with NPD group, PD control group showed a significant decrease in NFE2L2 expression levels ($p< 0.05$; Fig 4). The *Zingiber officinale* treatment group in both absence and presence of dietary intervention showed a significant increase in NFE2L2 expression when compared with the PD control group ($p=0.007$, $p=0.0001$ respectively; Fig 4). Similarly, the *Zingiber officinale* treatment group in both absence and presence of dietary intervention also showed a significant increase in NFE2L2 expression levels when compared to the NPD group ($p= 0.0168$, $p= 0.0013$ respectively; Fig 4). The metformin group in both absence and presence of dietary intervention showed a significant increase in NFE2L2 expression levels when compared to the PD group ($p < 0.05$; Fig 4).

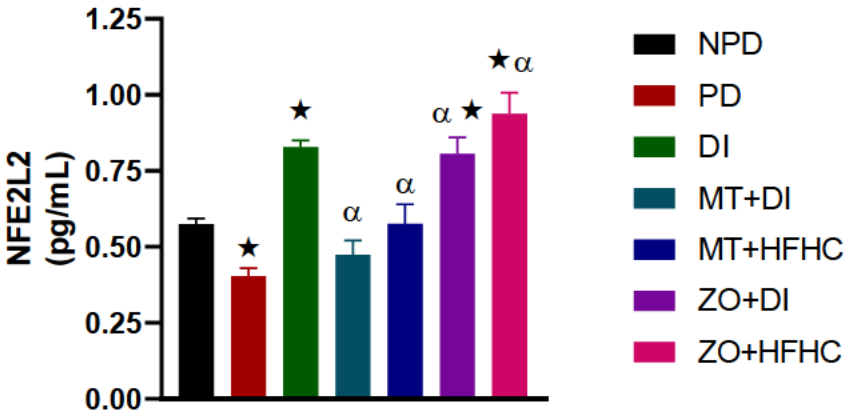

Figure 4: Showing the effect of *Zingiber officinale* on NFE2L2 after the treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = $p < 0.05$ in comparison to non-prediabetic group and α = $p < 0.05$ in comparison to the prediabetic group.

3.7. Effect of *Zingiber officinale* on blood lymphocyte concentration.

Blood lymphocyte concentration was evaluated at the end of the experimental period (Figure 5). When comparing the NPD group with prediabetic group, PD control group showed a significant increase on lymphocyte count ($p < 0.0001$; Fig 5). The rats treated with *Zingiber officinale* in both absence and presence of dietary intervention (ZO+DI and ZO+HFHC) showed a significant decrease in lymphocyte concentration when compared with PD control group ($p < 0.001$, $p = 0.0322$ respectively; Fig 5) to levels comparable with those of the NPD group. Diet-induced prediabetic rats treated with metformin in the presence of dietary intervention (MT+DI) showed a significant decreased when compared with the PD control group ($p = 0.0005$; Fig 5), while the rats that administrated metformin in the absence of dietary intervention (MT+HFHC) showed no significant difference when compared to the untreated PD group.

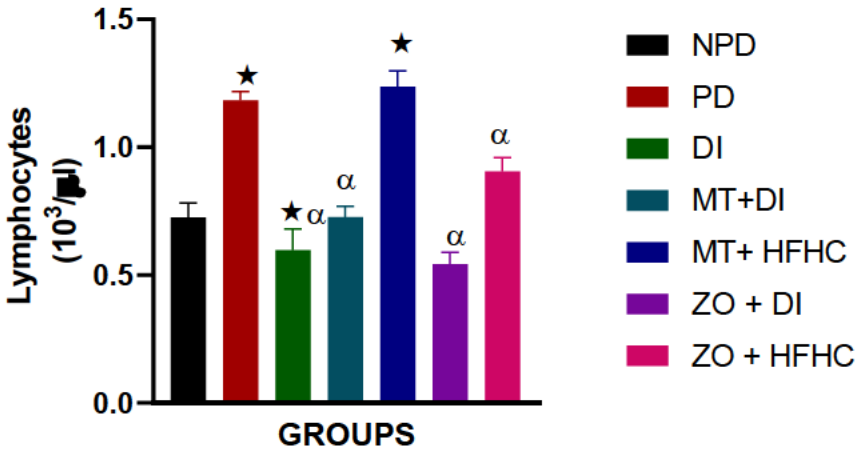

Figure 5: Showing the effect of *Zingiber officinale* on lymphocyte concentration cells after the treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = $p< 0.05$ in comparison to non-prediabetic group, and α = $p< 0.05$ in comparison to the prediabetic group.

4. Discussion

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by persistent hyperglycaemia, resulting from impaired insulin secretion, insulin resistance, or both [22]. Prediabetes is a precursor state of T2DM marked by intermediate hyperglycaemia, where glycaemic levels are elevated but not yet diagnostic of T2DM [12].This condition is associated with low-grade chronic inflammation, oxidative stress and altered immune signalling, all of which impair innate immunity and increase the risk of infections and progression to T2DM [135]. Advanced glycation end-products (AGEs), metabolic stress and dysregulated immune responses further exacerbate immune dysfunction [136]. Prediabetes is often worsened by poor adherence to lifestyle modifications and overreliance on pharmacological interventions [110]. As a result, alternative therapeutic strategies, including the use of medicinal plants, are gaining interest. *Zingiber officinale* (ginger) has demonstrated antioxidant, anti-inflammatory, and immunomodulatory properties [111]. In our laboratory we use a high-fat, high-carbohydrate (HFHC) diet-induced rat model that mimics human prediabetes. This study investigated the effects of a *Zingiber officinale* extract on markers of inflammation and the innate immune response.

The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) is a widely used method to estimate insulin resistance, a key feature of metabolic dysfunction in prediabetes and type 2 diabetes [137, 138]. By calculating the ratio of fasting insulin to fasting glucose levels, the HOMA-IR index provides valuable insight into how effectively the body is utilizing insulin [139]. The increased glucose production in the PD state has been shown to be due to impaired insulin-induced peripheral glucose uptake in insulin-dependent tissue of the prediabetic individuals [140]. This accounts for high fasting insulin, fasting blood glucose and HOMA-IR values in the prediabetic individuals. Indeed, in this study we observed significantly higher HOMA-IR values in the prediabetic control group when compared to the NPD group. The treatment with *Zingiber officinale* in the presence of dietary intervention showed a significant decrease in HOMA-IR value, while in the absence of dietary intervention showed no significant difference when compared to the prediabetic control group. This suggests that *Zingiber officinale* in the presence of dietary intervention was able reduce HOMA-IR due its bioactive compounds such as gingerol and shogaol that may help ameliorate the moderate insulin resistance commonly seen in prediabetes and thus improve glucose homeostasis [141]. In contrast, in the absence of dietary intervention, persistent intake of high fat high carbohydrate diet maintains insulin resistance which likely overwhelms the effects of *Zingiber officinale*. These results were consistent with those observed in the metformin-treated groups [142]. The administration of metformin is often recommended to be combined with DI for maximum efficacy however, patients often neglect this and reduce the efficacy of the drug.

Body weight is a crucial parameter in metabolic diseases like prediabetes and type 2 diabetes [115]. One of the primary factors contributing to weight gain is calorie intake, particularly in the context of a high-fat, high-carbohydrate diet, which is known to promote positive energy balance and fat accumulation [143]. Prediabetes has been shown to cause visceral fat accumulation leading to enlarged adipocytes which accounts by an increased weight gain [144]. Our findings in this study correspond to the previous findings showing the significant increase in body weight gain in the prediabetic control group when compared to the NPD group. The treatment with *Zingiber officinale* in the presence of dietary intervention showed a significantly decrease in body weight, while in the absence of dietary intervention showed no significant difference when compared to the prediabetic control group. Based on this observation, the reduction in body weight in *Zingiber officinale* in the presence of dietary intervention could be attributed to *Zingiber officinale* ability to regulate lipid metabolism by reducing hepatic lipid

accumulation and also ability to enhance insulin sensitivity improving glucose uptake [145]. These findings align with reduced HOMA-IR value as showed with the reduction of body weight gain as it reflects the improved insulin sensitivity.

The balance between pro-inflammatory and anti-inflammatory cytokines plays a critical role in regulating insulin sensitivity, glucose metabolism, and adiposity [146]. Adiponectin is an adipokine that plays a crucial role in regulating insulin sensitivity, inflammation, and metabolic processes [147]. Low plasma levels of adiponectin are associated with increased insulin resistance, obesity, and inflammation, all of which are key features of prediabetes and type 2 diabetes [148]. The results of this study correspond with the previous findings whereby, the untreated PD group showed decreased plasma adiponectin levels when compared to NPD group. The rats treated with *Zingiber officinale* in both absence and presence of dietary intervention showed a significant increase in adiponectin concentration. The enhanced adiponectin levels in the *Zingiber officinale* treated groups may indicate a more direct modulation of adipokine secretion or an effect on adipose tissue function which is adipocyte-endothelial crosstalk [149], as *Zingiber officinale* has been shown to possess anti-inflammatory and metabolic-enhancing properties [150]. This finding is particularly interesting because metformin, a widely used treatment for diabetes, is typically associated with improved insulin sensitivity, yet *Zingiber officinale* seemed to produce a more pronounced effect on adiponectin secretion. These findings further align with the reduction of weight gain and HOMA-IR values as increased adiponectin levels it is known to enhance fatty acid oxidation and glucose uptake by muscle and adipose tissues.

Pro-inflammatory cytokines play a pivotal role in the pathogenesis of insulin resistance and metabolic inflammation [22]. Tumor necrosis alpha (TNF α) is produced by adipocytes and inflammatory cells in response to chronic inflammation [151]. The serum levels of TNF α are strictly related to obesity in T2DM [152]. IL-6 is a cytokine with multidirectional effects THAT has a direct effect on glucose and lipid metabolism and is one of the main cytokines of chronic subclinical inflammation and [62, 153]. CRP is a systemic inflammatory biomarker and has been considered as one of the major causative factors for development of T2DM [66]. TNF-α has been shown increased in obese animals which modulates the insulin action [154]. IL-6 concentration increases in patients with prediabetes which induces hepatic production of CRP [155, 156]. Our findings in this study correspond with previous findings whereby, the untreated

PD group showed increase levels in pro-inflammatory cytokines such as TNFα, IL-6 and CRP when compared to NPD group. The rats treated with *Zingiber officinale* in both absence and presence of dietary intervention showed a significant decrease in IL-6 and CRP, whereas there was no significant difference in TNF α levels. The significant reduction in IL-6 suggests that *Zingiber officinale* has a direct inflammatory effect likely through its bioactive compounds such as gingerol, shogaol and paradol which suppress the activation of NF-kB [111]. Reduction in CRP levels may be due to acute-phase reactant synthesized of the liver in response to IL-6 [157]. The lack of a significant difference in TNF α levels may indicate that *Zingiber officinale* effects on inflammation could be more complex or that the levels of TNF-α measured were not directly responsive to *Zingiber officinale* treatment in this particular animal model. It is also possible that the duration of the experimental period or the specific dietary interventions used may not have been sufficient to elicit a significant change in TNF-α levels, as other studies have reported variable effects of *Zingiber officinale* on TNF-α depending on the context [158]. Furthermore, the observed weight loss and improved insulin sensitivity by reduction of HOMA-IR enhances the adiponectin production and supresses inflammation as reflected by THE reduced pro-inflammatory cytokines.

Neutrophils are the first line of defence against infection, but they also play a key role in the inflammatory process [159]. They are persistently activated by the inflammatory environment [160]. Activated neutrophils produce ROS and release proteases causing oxidative stress [161]. In obesity and insulin resistance, increased levels of neutrophils are often seen as part of the chronic low-grade inflammation associated with these conditions [162]. The results of this study correspond with previous findings as the prediabetic control group showed a significant increase neutrophils cell count when compared to the NPD group. The prediabetic rats treated with *Zingiber officinale* in both the absence and presence of dietary intervention showed a significant decrease in neutrophils cell count when compared to the prediabetic control group. This reduction in neutrophils cell count could suggest that the anti-inflammatory effects of *Zingiber officinale* extend to the immune system, potentially preventing the excessive immune activation observed in metabolic diseases [163]. This reduction of neutrophils cell count further aligns with reduced innate immune activation reflected by improved insulin sensitivity, reduced pro-inflammatory cytokines and increased anti-inflammatory cytokines.

Malondialdehyde (MDA) is a widely-used marker for assessing oxidative stress, which occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants [164]. Elevated MDA levels are indicative of lipid peroxidation, a process in which ROS damage cell membranes, contributing to various metabolic disorders, including insulin resistance, inflammation and cardiovascular complications [165]. MDA levels are typically elevated in conditions of insulin resistance, prediabetes and obesity, as these states are characterized by increased oxidative stress and inflammation [166]. In particular, chronic consumption of a high-fat, high-carbohydrate diet is known to induce oxidative stress, which can worsen insulin resistance and promote inflammatory pathways, further contributing to the metabolic disturbances observed in prediabetes and type 2 diabetes [167]. These findings of the present study align with the previous findings as the prediabetic group showed elevated MDA levels when compared with the NPD group. The rats treated with *Zingiber officinale* in the presence of dietary intervention showed a significant decreased MDA levels when compared with the PD control group. Whereas, in the absence of dietary intervention showed no significant difference. Gingerols, the primary bioactive compounds in *Zingiber officinale*, have been shown to enhance the expression of antioxidant enzymes, which may help protect cells from oxidative damage [168]. However, in the presence of a high fat high carbohydrate diet, the oxidative stress induced by excessive nutrient intake may diminish the antioxidant effects of *Zingiber officinale*, as seen in the findings [169]. As the high fat high carbohydrate diet induces excessive production, it may counteract the antioxidant benefits of *Zingiber officinale.* The observed improved insulin sensitivity and reduced lipid accumulation alleviates oxidative stress reflected by reduced MDA levels which in turn may reduce activation of innate immune cells and lowers systemic inflammation showed by reduction of pro-inflammatory cytokines.

Nuclear factor erythroid 2-related factor 2 (NFE2L2, also known as Nrf2) is a key regulator of antioxidant defence mechanisms in cells. It plays a critical role in protecting cells from oxidative stress by inducing the expression of antioxidant enzymes and other cytoprotective proteins [170]. Activation of NFE2L2 is an important response to oxidative damage, helping to maintain cellular homeostasis and prevent tissue injury associated with metabolic diseases, including diabetes and insulin resistance [171]. However, in prediabetes, Nrf2 activity has been shown to be suppressed, weakening antioxidant defences and allowing oxidative damage to accumulate [172]. The results of this study align with the previous results that the prediabetic control group showed a significant decrease in the NFE2L2 expression levels when compared

to the NPD group. The *Zingiber officinale* treatment group in both absence and presence of dietary intervention also showed a significant increase in NFE2L2 expression levels when compared to the PD control and NPD group. *Zingiber officinale* has been reported to activate NFE2L2 through various signalling pathways, including the PI3K/Akt and MAPK pathways, both of which are involved in regulating cellular responses to oxidative stress (Zhao et al., 2020). The activation of NFE2L2 by *Zingiber officinale* may also involve the upregulation of antioxidant enzymes, thereby reducing oxidative damage caused by high fat high carbohydrates diets and improving insulin sensitivity [163]. While *Zingiber officinale* effects on NFE2L2 expression were evident in the *Zingiber officinale* group, the metformin group did not show the same degree of NFE2L2 upregulation, which might indicate that metformin's effects on oxidative stress and NFE2L2 expression are less pronounced in the absence of a high-fat diet. This could be due to metformin's mechanism of action, which primarily focuses on improving insulin sensitivity and reducing blood glucose levels, rather than directly modulating the oxidative stress response [173]. However, other studies have shown that metformin can also activate NFE2L2 under certain conditions, particularly when oxidative stress is a key factor [170]. The activation of NFE2L2 may enhance the transcription of antioxidant leading to a reduction in oxidative stress resulting in lower IL-6 and CRP. It also supports the adiponectin expression further improving insulin sensitivity.

Lymphocytes, which include T cells, B cells and natural killer (NK) cells, are crucial for immune responses [174]. Their concentration can be influenced by various factors, including inflammation, immune activation, and stress [175]. In the context of metabolic syndrome and prediabetes, changes in lymphocyte populations have been observed, often reflecting alterations in immune function due to chronic low-grade inflammation [175]. Normally, neutrophils and lymphocytes communicate to resolve inflammation but in prediabetes this crosswalk is impaired [176]. Chronic inflammation skews lymphocytes populations which perpetuate inflammation and disrupts immune homeostasis further impairing insulin signaling [116]. The results of this study align with the previous findings the prediabetic control group showed a significant increase in lymphocytes count compared to the NPD group. The rats treated with *Zingiber officinale* in both the absence and presence of dietary intervention showed a significant decrease in lymphocyte concentration when compared with PD control group. These results suggest that *Zingiber officinale* may have an immunosuppressive effect on lymphocytes which may be beneficial in controlling excessive immune activation associated with obesity and insulin resistance [177]. A similar finding was observed in studies showing

that *Zingiber officinale* extract can suppress overactive immune responses, particularly by modulating the function of T cells and macrophages [111]. The increased lymphocyte count in the metformin group in the absence of dietary intervention could indicate that the high fat high carbohydrate diet, despite metformin supplementation, still triggered immune responses, possibly due to the presence of persistent metabolic stress or ongoing inflammation [178]. This finding is consistent with research showing that high fat high carbohydrates diets can alter immune cell distribution and function, which may partially overcome the immune-modulatory effects of metformin [179]. Restored lymphocytes levels indicate improved immune regulation which reduces oxidative stress and inflammation, improving the insulin sensitivity and metabolic balance.

5. Conclusion

Taken together the findings of this study indicate that treatment with *Zingiber officinale* significantly insulin sensitivity as shown by the reduced HOMA-IR values and reduced body weight gain reflecting an enhanced metabolic regulation. A decrease in pro-inflammatory cytokines (IL-6 and CRP) and increased adiponectin levels was observed. Neutrophil's count declined suggesting suppressed innate immune activation, while reduced MDA levels indicated attenuation of oxidative stress. These effects were likely mediated through the activation of NFE2L2, a key regulator of anti-oxidant defence. Additionally, lymphocyte restoration pointed to the improved immune balance and reduced immunosuppression associated with prediabetes. Together, these findings support the protective effect of *Zingiber officinale* in reducing inflammation and restoring the immune balance caused by diet-induced prediabetes.

6. Limitations and future studies

Although the study observed changes in the markers, it did not include detailed molecular assays to confirm activation of specific pathways. Future studies should include the targeted mechanistic studies to how *Zingiber officinale* modulates these pathways at the cellular and molecular levels. Lastly, the study duration was relatively short and may not reflect the long-term efficacy or safety of *Zingiber officinale* administration. Chronic models of prediabetes and longer treatment periods would provide a more comprehensive understanding of its sustained effects on insulin resistance, oxidative stress, and immune modulation.

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Chapter 4: Manuscript 2

Title: The effects of *Zingiber officinale* (ginger) on hematological factors and cardiovascular diseases in diet-induced prediabetic rats.

Bridge

Following the investigation into the effects of *Zingiber officinale* on inflammation and innate immune responses in diet-induced prediabetic rats in Manuscript 1, it became increasingly clear that prediabetes is not only associated with immune dysregulation but also with significant alterations in haematological profiles and increased cardiovascular risk. The initial study demonstrated the immunomodulatory and anti-inflammatory potential of *Zingiber officinale*, highlighting its role in targeting systemic inflammation which is a key contributor to metabolic dysfunction. However, prediabetes is a complex condition that extends beyond immune pathways, often involving changes in blood parameters such as red and white blood cell counts, hemoglobin concentration, and platelet function, all of which may contribute to the onset of cardiovascular complications. Since *Zingiber officinale* is reported to have antioxidant, blood-thinning, and cardioprotective effects, it was important to explore whether these benefits extend to prediabetic conditions. Therefore, this study aimed to assess the effects of *Zingiber officinale* on haematological indices and early cardiovascular complications in diet-induced prediabetic rats. This second phase of research provides a broader understanding of *Zingiber officinale* therapeutic potential in addressing the systemic impact of prediabetes.

This manuscript has been submitted for review in the journal: International Journal of Molecular Sciences as a manuscript titled: "The effects of *Zingiber officinale* (ginger) on hematological factors and cardiovascular complications in diet-induced prediabetic rats." Authored by B Langa, A Khathi and LP Mabuza. This journal is accredited by the Department of Higher Education and Training South Africa and appears in the Scopus accredited list (2024).

Candidate Contribution: B Langa was responsible for brainstorming, designing the study, compiling and analysing all data extracted from the studies, drafting the systematic review and editing the final draft of the manuscript.

Abstract

Prediabetes, a state of intermediate hyperglycaemia, is associated with chronic inflammation, altered haematological parameters and elevated risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disorders. This study aimed to evaluate the effect of a *Zingiber officinale* extract in both the absence and presence of dietary intervention on haematological factors and cardiovascular complications in diet-induced prediabetic rats.

Thirty-six (36) male Sprague Dawley rats were divided into six groups (n=6 per group) and were induced with prediabetes using high carbohydrate for a period of 20 weeks. After induction, the groups were treated as follows for a period of 12 weeks: a prediabetic (PD) control; a dietary intervention (DI) control; *Zingiber officinale* treatment groups in the presence (ZO+DI) and absence [ZO+HFHC] of DI; as well as metformin in the presence (MT+DI) and absence (MT+HFHC) of DI. An additional six animals were kept on a standard diet and served as a non-prediabetic control (NPD). At the end of the treatment period, parameters measured included fasting blood glucose (FBG), oral glucose tolerance test (OGTT), glycated haemoglobin (HbA1c), triglycerides (TG), heart-to-body weight ratio, RBC and platelet indices, as well as mTOR and erythropoietin (EPO) expression.

Treatment with *Zingiber officinale* significantly improved glucose tolerance, reduced HbA1c and triglyceride levels as well as restored red blood cell and platelet indices. Furthermore, modulation of mTOR and EPO expression indicated reduced inflammation and cardiovascular risk. *Zingiber officinale* demonstrated the ability to improve glycaemic control, normalize haematological parameters, and protect against cardiovascular complications in prediabetic rats. These findings suggest its therapeutic potential against PD and in preventing the progression towards developing overt T2DM.

1. Introduction

In patients with T2DM, haematological alterations are associated with production of reactive oxygen species (ROS) as a consequence of a long-term hyperglycaemia [180]. Excessive production of ROS causes oxidative stress, leading to tissue damage, haematological alterations and endothelial and red blood cell (RBC) dysfunction [181]. It has a range of effects on RBC indices including haemoglobin glycation, decreased deformability and decreased longevity [4, 182]. Prediabetes affects the haematological factors and increases the risk of developing cardiovascular disease (CVD) through insulin resistance, chronic low-grade inflammation and oxidative stress [122]. It leads to elevated white blood cell (WBC) count, especially neutrophils, red cell distribution width (RDW) and mean platelet volume (MPV) which is linked to vascular dysfunction and atherogenesis [183, 184]. It alters the haemoglobin (Hb), haematocrit (Hct) and platelet activity contributing to prothrombotic state [185].

The use of a combination of pharmacological and lifestyle interventions has been advised during prediabetes to prevent the progression to overt T2DM. [186]. The pharmacological intervention involves the use of metformin which is an anti-hyperglycaemic drug [187]. The lifestyle intervention includes endurance exercise and healthy diet. [99]. However, individuals with prediabetes may still progress to T2DM due poor patient compliance as they shown to neglect the lifestyle intervention [188]. This reduces the efficacy of drugs like metformin [189]. Therefore, there is the need to find an alternative treatment which can work in present and absence of lifestyle intervention. *Zinger officinale* has been used in traditional medicine for long time [190]. The extracts from this plant have been shown to exhibit a variety of biological activities [191]. It contains important protein compounds including gingerol, gingerdiol, gingerdione which has anti-thrombotic, ant-inflammatory, anti-diabetic, and analgesic properties [192]. Some studies shown that gingerol isolated from Zingiber was shown to exhibit platelet function due to inhabitation of thromboxane formation which also interferes with inflammation processes [179]. *Zinger officinale* extract possesses antioxidation characteristics since it can scavenge superoxide anions and hydroxyl radicals [169]. Some studies have shown that *Zinger officinale* may positively impact lipid metabolism by reducing total cholesterol, LDL cholesterol, triglycerides levels while increasing HDL cholesterol [193]. Some studies had demonstrated that *Zinger officinale* is capable of potentially preventing cardiovascular diseases, associated pathologies that act as cardiovascular diseases risk factors [194].

However, its specific impacts on hematological parameters in the context of prediabetes remain relatively unclear. In our laboratory, we use high fat, high carbohydrate diet-induced animal model of prediabetes which has been shown to mimic the human condition of prediabetes [195]. This research aims to investigate the effects of *Zinger officinale* on hematological factors and cardiovascular diseases in diet-induced prediabetic rats by evaluating the effect on selected red blood cell and platelet indices, lipid profile, and selected markers of cardiovascular disease**.**

2.0. Materials and methods.

2.1.Chemicals and drugs

All chemicals and reagents are of analytical grade and purchased from standard commercial suppliers. Dimethyl sulphoxide (DMSO) 3 ml/kg (Sigma-Aldrich, St Louis, Missouri, United State of America) Metformin 500 mg/kg (Sigma-Aldrich, St Louis, Missouri, United State of America). *Zingiber officinale* was purchased from a local grocery store (Westville, KwaZulu-Natal, South Africa). The concentrations of the drugs and extracts were extrapolated from previous studies and have been shown to have minimal toxicity [132].

2.1.1 *Zingiber officinale* (ginger) root extraction

An aqueous ginger extract was prepared from ginger roots as described by Al-Amin et al. (2006). Briefly, the ginger roots were peeled on crushed ice, and 20 g ginger was cut into small pieces and homogenized (2000 rpm for 10 min) in 75-ml cold, sterile 0.9% NaCl in the presence of some crushed ice. The homogenization was carried out in a blender at high-speed using 2-min bursts for a total of 12 min. The homogenized mixture was filtered three times through a 0.22 μm nylon filter. The clear supernatant fraction was separated and the volume made up to 100 ml with cold normal saline. The concentration of this ginger extract was measured and adjusted to 200 mg/ml. The prepared aqueous extract of ginger root was stored in small samples at -80 °C until use.

2.2. Animals and housing

A sample set of male Sprague-Dawley rats (2 weeks old;150-180 g) bred and housed in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal were used in this study. The animals were housed in a room with a 12-hour light/12 hours dark cycle, room temperature (25°C), relative humidity = 55±5% and noise levels of less than 65 decibels for the duration of the study. The animals in each group had access to food and water *ad libitum*. All animal

procedures and housing conditions were approved by the Animal Research Ethics Committee (ethical clearance number: AREC/00006737/2024).

2.3. Induction of prediabetes

An established research protocol was used for the induction of prediabetes (Luvuno et al., 2018). Animals were allowed to acclimatize to their new environment for a week while consuming standard rat chow before commencement of the experimental diet. Procedures involving animals and their care were conducted in conformity with institutional guidelines of the University of KwaZulu-Natal. The animals were housed in a group of six in a type 4 large cages where a well enriched environment was provided. The bedding was changed on every second day. After acclimatization for a week, the animals were then randomly assigned to the following diet groups; Normal diet with drinking water (ND) and high-fat high-carbohydrate (HFHC) diet with both drinking water and 15% fructose dissolved in water for 20 weeks. Prediabetes was induced by allowing the animals to feed on the HFHC diet for 20 weeks. Glucose tolerance was evaluated 5 days after 20 weeks of induction with an oral glucose tolerance test to determine pre-diabetes according to the American Diabetes Association criteria. The animals with fasting blood glucose of more than 5.6 mmol/L were considered pre-diabetic and grouped further for pharmacological studies. The animals that were fed the normal diet were also tested and were found to be normoglycemic and without prediabetes.

2.4. Experimental design and treatment

The study consisted of 7 groups, Group 1: Non-pre-diabetic (NPD, n=6), non-prediabetic rats fed with normal diet; group 2: Prediabetic (PD, n=6), prediabetic rats fed with high fat high carbohydrate diet; group 3: Dietary intervention (DI, n=6), prediabetic rats fed with normal diet; group 4: Metformin plus dietary intervention (MTF+DI, n=6), prediabetic rats fed with metformin and normal diet; group 5: Metformin plus HFHC diet (MTF+HFHC, n=6), prediabetic rats fed with metformin and HFHC diet; group 6: Ginger plus dietary intervention (ZO+DI, n=6), prediabetic rats fed with ginger normal diet and group 7: Ginger plus HFHC diet (ZO+HFHC, n=6), prediabetic rats fed with ginger and HFHC diet). In each group, monitored parameters including fasting blood glucose (FBG), food intake, fluid intake and urinary output body were measured once after 4 weeks, while body weight and triglycerides was measured once a week at the same time for a duration of 12 weeks. The animals were placed on metabolic cages for 24 hours for monitored parameter measurements and placed back to colony cages once after 4 weeks.

2.5. Blood collection and tissue harvesting

All animals were anaesthetized with Isofor (100 mg/kg)) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) using a gas anesthetic chamber (Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa) for 3 minutes. Blood was collected by cardiac puncture and then injected into individual precooled heparinized containers for immune cell count. The other collected blood was then centrifuged for plasma collection (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes. The spleen, heart, liver and adipose tissue were collected and storage in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until biochemical assays were done.

2.6. Biochemical analysis

Glycated hemoglobin (HbA1C), and mechanistic target of rapamycin (mTOR) (respectively) were analyzed using separate, specific ELISA kits in accordance with the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China) and erythropoietin (EPO) was measured using ELISA kits from ThemoFisher scientific as per manufacturer instructions. The optical density of each well was determined using a Spectrostar nanoplate spectrophotometer (BMG Labtech, Ortenburg, BadenWürtternberg, Germany) at 450 nm.

2.7. Oral Glucose Tolerance (OGTT) Response

An oral glucose tolerance test (OGTT) was conducted following glucose loading to determine the glucose tolerance response of animals. The OGTT responses were monitored in the animals according to a well-established protocol. Briefly, after a 12 h fast, glucose levels were measured (time: 0 min) in all animals. The animals were loaded with glucose (glucose: 0.86 g/kg) through an oral gavage (18-gauge curved 38 mm long gavage needle with a 21/4 mm ball end). Blood was collected using the tail prick method to measure glucose concentration. Glucose concentrations were measured using a Viva check glucometer (Viva check Biotech, Hangzhou, China). The glucose concentrations were measured at 15-, 30-, 60-, and 120-min following glucose loading.

2.8. Hematology analysis

The red blood cell indices and platelet indices were measured together by complete blood count using Yumizen H500 CT hematology analyzer (HORIBA ABX SAS, Montpellier, France).

2.9.Statistical analysis

Data were reported as means ± standard error of means (SEM). Statistical analysis was conducted using Graph Pad Prism Instant Software (version 8, graph Pad Software, San Diego, California, USA). To evaluate whether there were statistically significant differences between the groups, we utilized either a parametric or non-parametric test, depending on the distribution of the data. All the tests were parametric test. Two-way analysis of variance (ANOVA) was used to analyze differences between the controls and the experimental groups. This was followed by Tukey Kramer post hoc comparison test while, Values of $p < 0.05$ statistical significance between the compared groups.

3. Results

3.1. Effects of *Zingiber officinale* on Heart to body weight ratio after 12 weeks treatment period.

Heart to body weight ratio was evaluated after 12 weeks treatment period (Table 1). The prediabetic control group showed a significant increase in heart to body weight ratio when compared with the non-prediabetic group ($p< 0.05$; Table 1). However, administration of *Zingiber officinale* in both the presence and absence of dietary intervention showed a significant decrease in heart to body weight ratio when compared with the prediabetic control group ($p< 0.05$; Table 1). The administration of metformin in the presence of dietary intervention (MT+DI) showed similar observation when compared with the prediabetic control group ($p< 0.05$; Table 1), while the administration of metformin in the absence of dietary intervention (MT+HFHC) showed no significant difference in heart to body weight ratio when compared with the prediabetic control group.

Table 1: Effects of *Zingiber officinale* on Heart to body weight ratio after 12 weeks treatment period. Values are presented as mean ± SEM in each group.

GROUPS	MEAN BODYWEIGHT(g)	MEAN HEART WEIGHT(g)	HEART TO BODY RATIO %
NPD	**472 ± 20.22**	**1.613 ± 0.117**	**0.342 ± 0.031**
DI	**552 ± 26.00 α**	**1.640 ± 0.046 α**	**0.297 ± 0.006 α**
PD	**762 ± 107.2***	**3.024 ± 0.962***	**0.400 ± 0.075***

MT+DI	**464 ± 18.04 α**	**1.503 ± 0.171 α**	**0.324 ± 0.043 α**
MT+HFHC	**608 ± 29.01***	**2.740 ± 0.966***	**0.451 ± 0.069***
ZO+DI	**513 ± 18.34 α**	**1.593 ± 0.015 α**	**0.311 ± 0.015 α**
ZO+HFHC	**671 ± 14.44 ***	**1.820 ± 0.091 α**	**0.271 ± 0.010 α**

* = $p< 0.05$ in comparison to non-prediabetic group, and α = $p< 0.05$ in comparison to the prediabetic group.

Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC.

3.2 Effect of *Zingiber officinale* on Oral glucose tolerance teston pre-treatment and post-treatment.

Figure 1 illustrates the effects of *Zingiber officinale* on OGTT result on pre-treatment (week 0) and post-treatment (week 12) (Figure 1). The results of the OGTT showed varying glucose concentrations over the 2-hour test period (Figure 1). At week 0 pre-treatment, the untreated PD, DI and PD treated groups showed a significant increased fasting blood glucose concentration at time 0 before loading with glucose solution when compared with the NPD group ($p< 0.05$; Fig 1A). After loading the glucose solution, the untreated PD, DI and PD treated groups showed a significant increased blood glucose concentration throughout the 2-hour time period when compared with the NPD group ($p< 0.05$; Fig 1A). At week 12 post-treatment, the untreated PD group showed significant increased fasting blood glucose concentration at time 0 when compared with the NPD group ($p< 0.05$; Fig 1B). The rats treated with *Zingiber officinale* in the presence of dietary intervention (ZO+DI) showed reduced glucose concentration throughout the 2-hour test when compared with the PD control group ($p< 0.05$; Fig 1B), while the rats treated with *Zingiber officinale* in the absence of dietary intervention (ZO+HFHC) showed no significant difference when compared to the PD control group.

A trend similar to that of *Zingiber officinale*-treated groups was observed in the groups treated with metformin in the presence and absence of dietary intervention (Fig 1B).

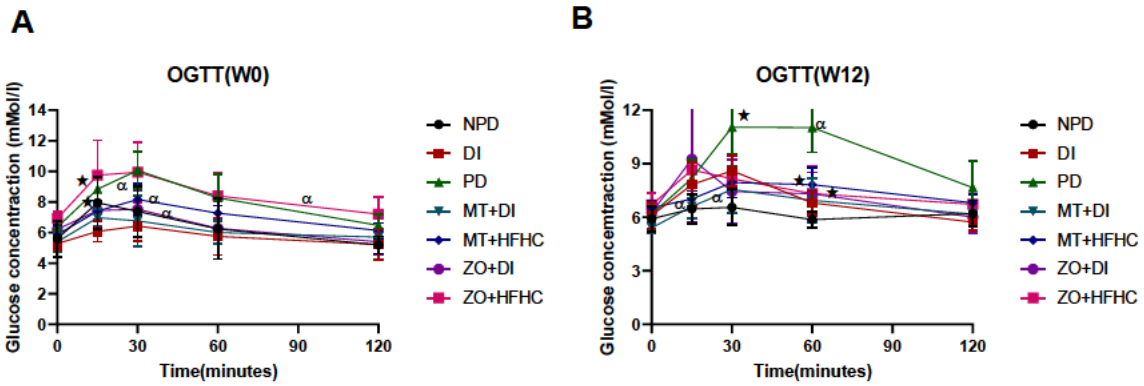

Figure 1: Showing the effect of *Zingiber officinale* on Oral glucose tolerance test on A) pre-treatment week 0 and B) post-treatment week 12. Values are represented as mean ± SEM (n=6) in each group. * = $p < 0.05$ in comparison to non-prediabetic group, α = $p < 0.05$ in comparison to prediabetic group.

Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC.

3.3. Effect of *Zingiber officinale* on glycated hemoglobin (HbA1c) levels.

Figure 2 illustrates the effects of *Zingiber officinale* on glycated haemoglobin after the experimental period. The values are represented as means ± SEM (n=6) for each group. The PD control group showed increase HbA1c levels in comparison with the NPD group ($p = 0.0139$; Fig 2). Treatment with *Zingiber officinale* in both absence and presence of dietary intervention exhibited decrease HbA1c levels when compared with the PD control group ($p = 0.0312$; Fig 2) to levels comparable with those of the NPD group. Similarly, the administration of metformin in both absence and presence of dietary intervention (MT+DI and MT+HFHC) showed similar observation when compared with the PD control group ($p<0.05$; Fig 2).

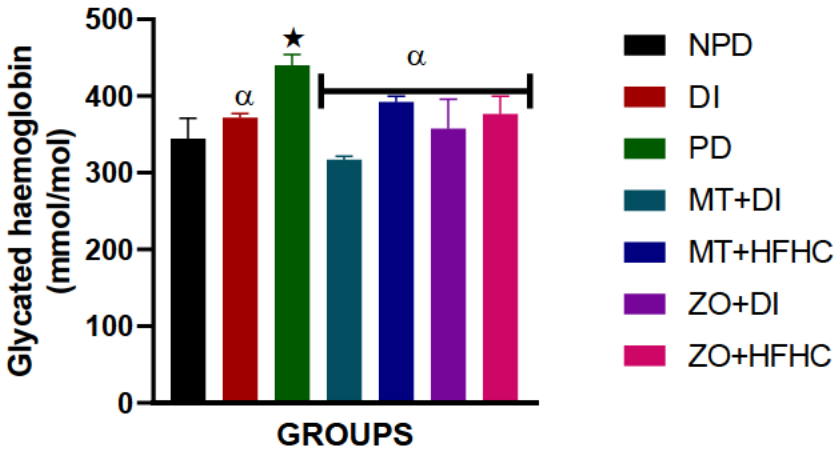

Figure 2: Showing the effect of *Zingiber officinale* on glycated hemoglobin after the treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of the dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of the dietary intervention, ZO+HFHC and ($p < 0.05$) for the significance.

3.4. Effect of *Zingiber officinale* on red blood cells (RBC), red cell distribution width-coefficient of variation (RDW-CV) and red cell distribution width standard deviation (RDW-SD) after 12 weeks treatment period.

Figure 3 illustrates the effect of *Zinger Officinale* on RBCs, RDW-CV and RDW-SD after 12 weeks treatment period. When comparing the NPD group with PD control group, PD control group showed a significant decrease in RBCs concentration after the 12 weeks treatment period ($p<0.05$; Fig 3A). However, when comparing the PD control group with the *Zingiber officinale* treatment group in both absence and presence of dietary intervention it showed a significant increase in RBCs concentrations after 12 weeks treatment period ($p<0.05$; Fig 3A). Similarly, the metformin treatment group in both absence and presence of dietary intervention (MT+DI and MT+HFHC) it showed similar observation when compared to the PD control group. There were no significant differences in red cell distribution width-coefficient of variation (RDW-CV) and red cell distribution width standard deviation (RDW-SD) after 12 weeks treatment period.

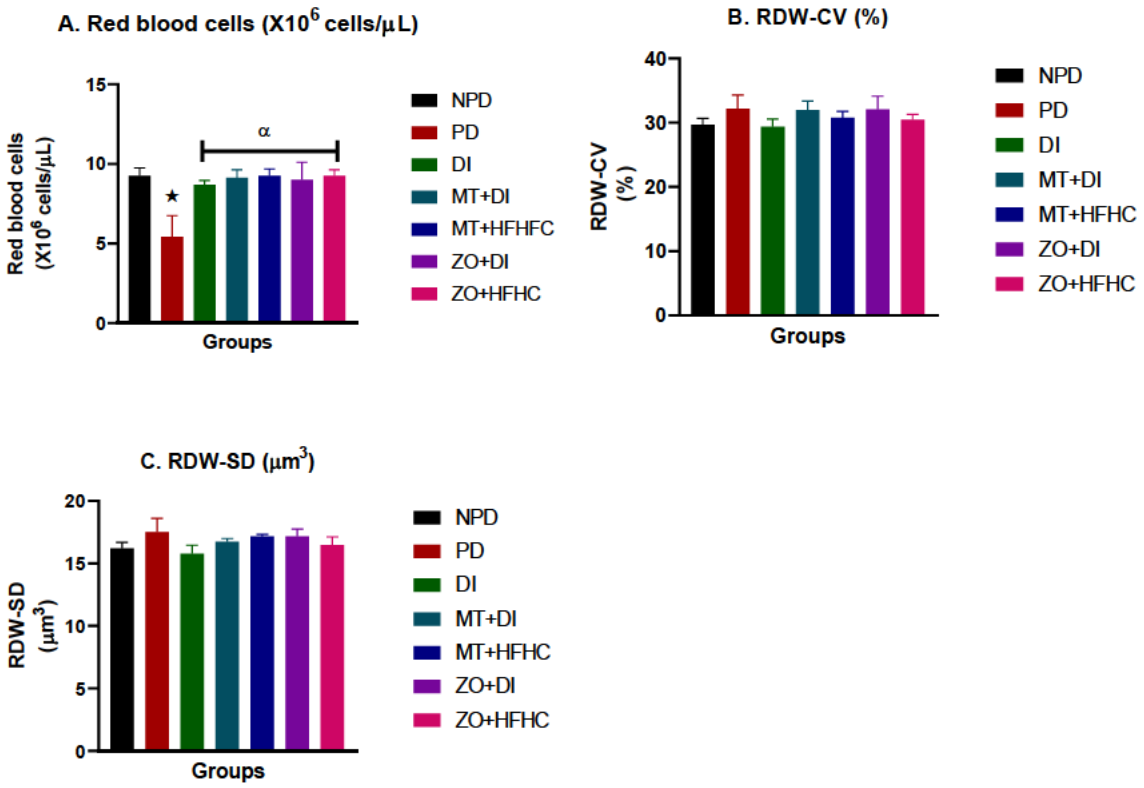

Figure 3: Showing the effects of *Zingiber officinale* on A) red blood cell, B) red cell distribution width-coefficient and C) red cell distribution standard deviation after 12 weeks treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of the dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of the dietary intervention, ZO+HFHC. * = $p< 0.05$ in comparison to non-prediabetic group and α = $p< 0.05$ in comparison prediabetic group.

3.5. Effect of *Zingiber officinale* on hematocrit (HCT) and hemoglobin (HGB) after 12 weeks treatment period.

Figure 4 illustrate the effects of *Zingiber officinale* on hematocrit (HCT) and hemoglobin (HGB) after 12 weeks treatment the experimental period (Figure 4). The prediabetic control group exhibited a significant decrease in hematocrit and hemoglobin concentrations after the 12 weeks treatment period when compared with the non-prediabetic group (p=0.0001, p=0.0001, respectively; Fig 4). Interestingly, treatment with *Zingiber officinale* in both the presence and absence of dietary intervention (ZO+DI and ZO+HFHC) showed a significant increase in hemoglobin and hematocrit concentrations after the 12 weeks treatment period when compared with the prediabetic control group (p=0.001, p=0.0002, respectively; Fig 4) to levels comparable with those of the NPD group. Similarly, the metformin treatment group in both absence and presence of dietary intervention (MT+DI and MT+HFHC) it showed similar observation when compared to the PD control group.

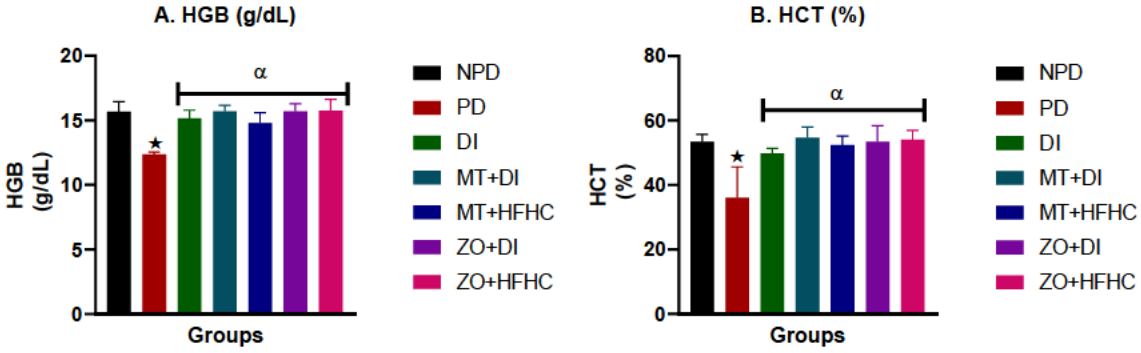

Figure 4: Showing the effects of *Zingiber officinale* on A) HCT and B) HGB after 12 weeks treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = $p< 0.05$ in comparison to non-prediabetic group and α = $p< 0.05$ in comparison prediabetic group.

3.6. Effects of *Zingiber officinale* on mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) after the experimental period.

Figure 5 illustrates the effect of *Zingiber officinale* on MCV, MCH and MCHC after 12 weeks treatment period (Figure 5). In comparison with the NPD group, the PD control group showed a significant increase in MCV, MCH and MCHC after 12 weeks treatment period ($p<0.05$; Fig 5). Interestingly, when compared with the PD group, the *Zingiber officinale* treated groups in both the presence and absence of dietary intervention (ZO+DI and ZO+HFHC) resulted in significant decrease MCV, MCH and MCHC after 12 weeks treatment period ($p=0.0019$, $p<0.0001$, $p<0.001$ respectively; Fig 5) to levels comparable with those of the NPD group. However, the treatment with metformin in both the presence and absence of dietary intervention (MT+DI and MT+HFHC) showed similar observation when compared with the PD control group after 12 weeks treatment period.

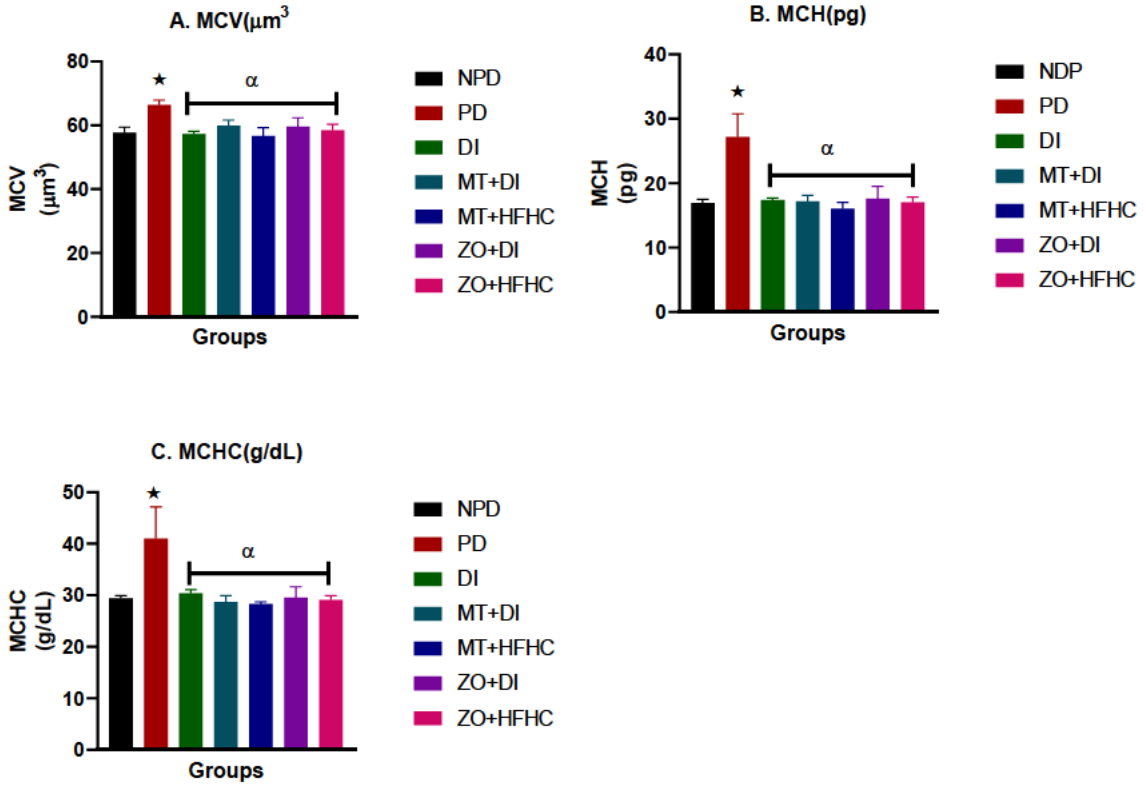

Figure 5: Showing the effect of *Zingiber officinale* on A) MCV, B) MCH and C) MCHC after 12 weeks treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC;

Zingiber officinale in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = p< 0.05 in comparison to non-prediabetic group and α = p< 0.05 in comparison prediabetic group.

3.7. Effects of *Zingiber officinale* on erythropoietin (EPO) levels after 12 weeks treatment period.

Figure 6 illustrates the effect of *Zingiber officinale* on erythropoietin levels after 12 weeks treatment period (Figure 6). The PD control group showed a significant decrease in EPO levels after the 12 weeks treatment period when compared with the NPD group (p= 0.0110; Fig 6). By comparison with the PD control group, the rats treated with *Zingiber officinale* in both absence and presence of dietary intervention (ZO+DI and ZO+HFHC) showed a significant increase in EPO levels after the 12 weeks treatment period (p< 0.05; Fig 6).

Similarly, treatment with metformin in both the presence and absence of dietary intervention (MT+DI and MT+HFHC) showed similar observation when compared with the PD control group after 12 weeks treatment period (p<0.05; Fig 6).

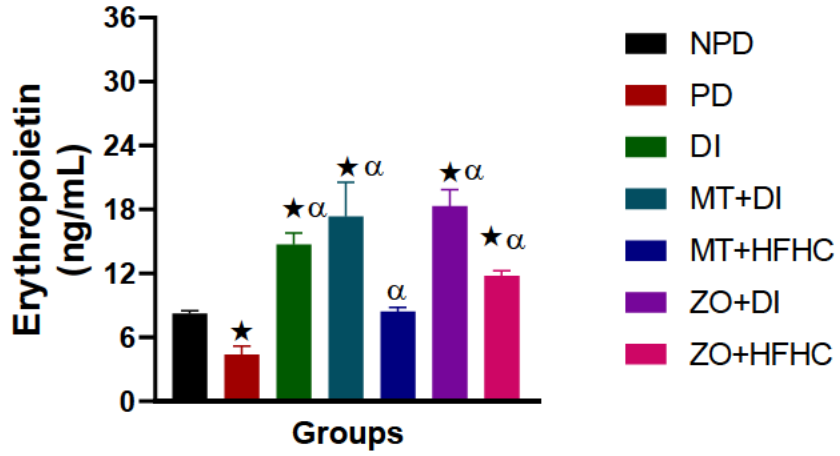

Figure 6: Showing the effect of *Zingiber officinale* on EPO levels after 12 weeks treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of the dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of the dietary intervention, ZO+HFHC. * = p< 0.05 in comparison to prediabetic group, and α = p< 0.05 in comparison to the prediabetic group.

3.8. Effects of *Zingiber officinale* on platelecrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio (P-LCR) after 12 weeks treatment period.

Figure 7 illustrate the effect of *Zingiber officinale* on platelecrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio (P-LCR) after 12 weeks treatment the experimental period (Figure 7). The induction of prediabetes resulted in a significant increase in PCT, MPV, PDW and P-LCR after the 12 weeks treatment period when compared with the NPD group ($p<0.05$; Fig 7). The *Zingiber officinale* treatment group in both presence and absence of dietary intervention (ZO+DI and ZO+HFHC) showed a significant decrease in PCT, MPV, PDW and P-LCR after the 12 weeks treatment period when compared with the PD control group ($p<0.05$; Fig 7) to levels comparable with those of the NPD group.

Similarly, the metformin group in both presence and absence of dietary intervention (MT+DI and MT+HFHC) showed similar observation when compared to the NPD and PD group.

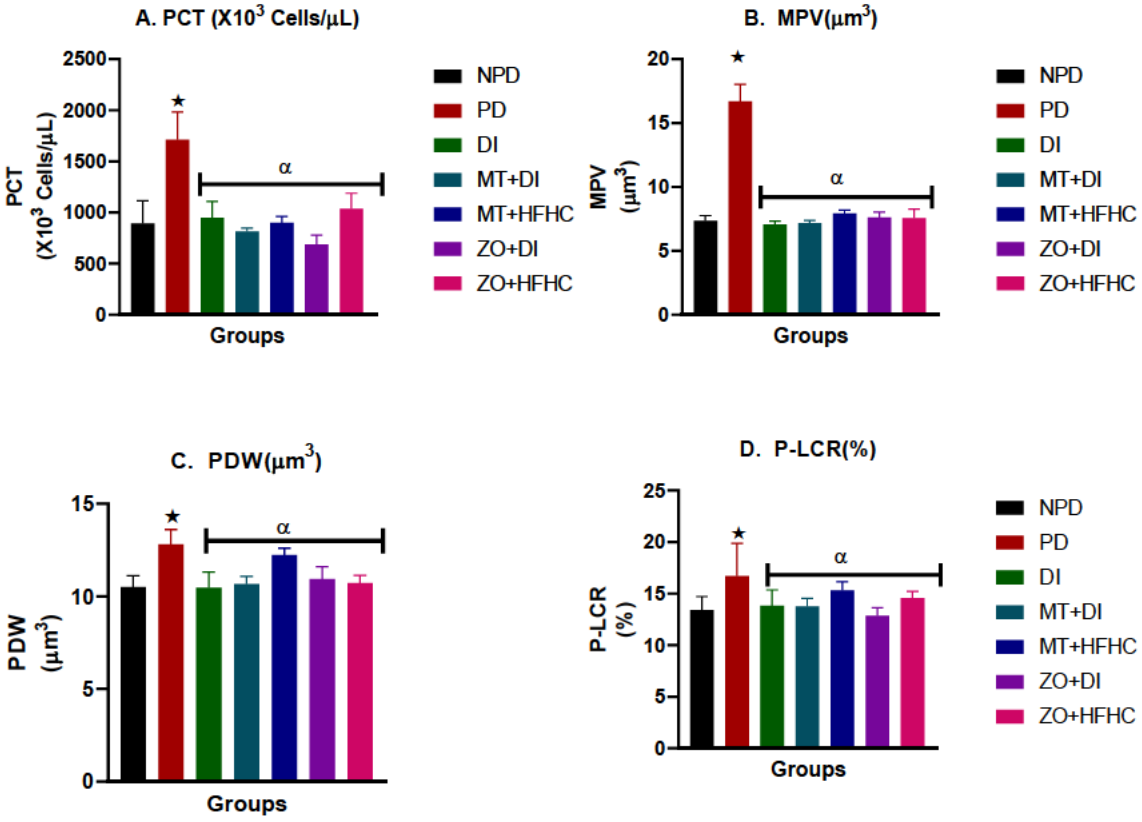

Figure 7: Showing the effect of *Zingiber officinale* on A) PCT, B) MPV, C) PDW and D) P-LCR after 12 weeks treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of the dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of the dietary intervention, ZO+HFHC. * = $p< 0.05$ in comparison to non-prediabetic group and α = $p< 0.05$ in comparison prediabetic group.

3.9. Effect of *Zinger officinale* on mammalian target of rapamycin (MTOR) after 12 weeks treatment period.

Figure 8 illustrates the effects of *Zinger Officinale* on mTOR expression after 12 weeks treatment period. In comparison with the NPD group, the PD control group showed a significant increase is mTOR expression after the 12 weeks treatment period ($p<0.05$; Fig 8).

By comparison with the untreated PD group, the rats that were treated with *Zingiber officinale* in both presence and absence of dietary intervention resulted in significant decrease in mTOR expression after 12 weeks treatment period when compared with the PD control group ($p<0.05$; Fig 8) to levels comparable with those of the NPD group. Similarly, the metformin group in both absence and present of the dietary intervention (MT+DI and MT+HFHC) displayed similar results when compared with the prediabetic control group.

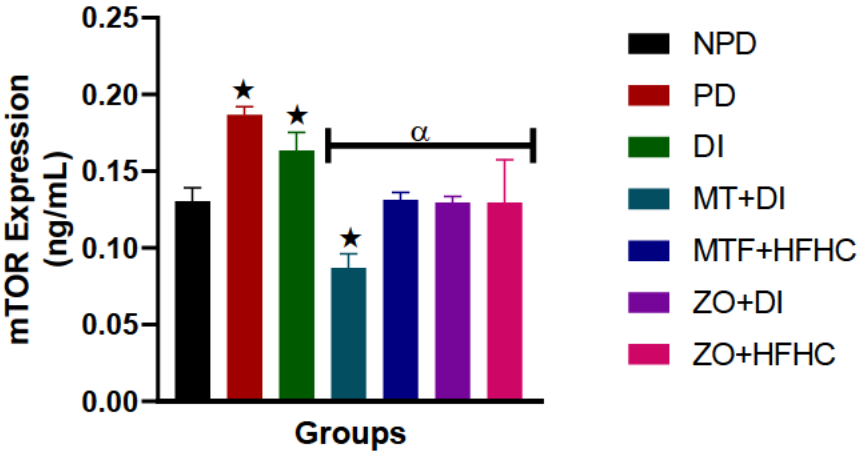

Figure 8: Showing effects of *Zingiber officinale* on MTOR levels after 12 weeks experimental period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in

the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = p< 0.05 in comparison to non-prediabetic group, and α = p< 0.05 in comparison to the prediabetic group.

3.10. Effect of *Zingiber officinale* on plasma triglycerides after 12 weeks treatment period.

Figure 9 illustrates the effect of *Zinger Officinale* on triglycerides after 12 weeks treatment period (Figure 9). When comparing the NPD group with the PD control group, the prediabetic control group showed a significant higher level of triglycerides compared to the non-prediabetic group (p<0.05; Fig 9). When comparing the PD control group with the *Zingiber officinale* treatment group in both presence and absence of dietary intervention (ZO+DI and ZO+HFHC) showed a significant decrease in triglycerides levels (p<0.05; Fig 9) to levels comparable with those in the NPD group. Similarly, the metformin group in both the absence and presence of dietary intervention (MT+DI, MT+HFHC) showed similar results when compared to the PD control group.

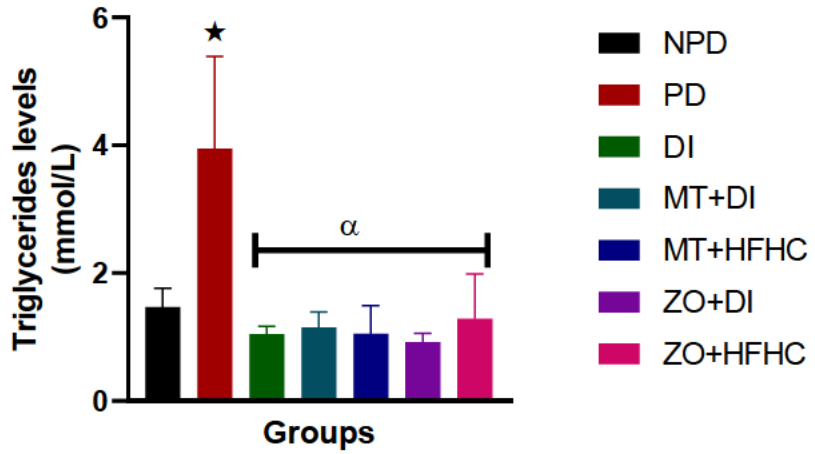

Figure 9: Showing the effect of *Zingiber officinale* on triglycerides after 12 weeks treatment period. Values are represented as mean ± SEM (n=6) in each group. Non prediabetic, NPD, Dietary intervention, DI; Prediabetic, PD; Metformin in the presence of dietary intervention, MT+DI, Metformin in the absence of dietary intervention, MT+HFHC; *Zingiber officinale* in the presence of dietary intervention, ZO+DI; and *Zingiber officinale* in the absence of dietary intervention, ZO+HFHC. * = p< 0.05 in comparison to non-prediabetic group, and α = p< 0.05 in comparison to the prediabetic group.

4. Discussion

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by persistent hyperglycaemia, resulting from impaired insulin secretion, insulin resistance, or both [22]. Prediabetes is a precursor state marked by intermediate hyperglycaemia, where glycaemic levels are elevated but not yet diagnostic of T2DM [12]. Prediabetes affects the haematological factors and increases the risk of developing cardiovascular disease (CVD) through insulin resistance, chronic low-grade inflammation and oxidative stress [196]. It leads to elevated red cell distribution width (RDW) and mean platelet volume (MPV) which is linked to vascular dysfunction and atherogenesis and it also alters the haemoglobin (Hb), haematocrit (Hct) and platelet activity contributing to prothrombotic state [183]. T2DM is often worsened by poor adherence to lifestyle modifications and over-reliance on pharmacological interventions [110]. As a result, alternative therapeutic strategies, including the use of medicinal plants, are gaining interest. *Zingiber officinale* (ginger) has demonstrated to exhibit a variety of biological activities as it contains important protein compounds including gingerol, gingerdiol, gingerdione which has anti-thrombotic, anti-inflammatory, anti-diabetic and analgesic properties [190, 197]. However, its specific impacts on hematological parameters in the context of prediabetes remain relatively unclear. In our laboratory, we use a high fat high carbohydrate diet-induced animal model of prediabetes which has been shown to mimic the human condition of prediabetes [198]. This research aims to investigate the effects of *Zinger officinale* on hematological factors and cardiovascular diseases in diet-induced prediabetic rats by evaluating the effect on selected red blood cell and platelet indices, lipid profile, and selected markers of cardiovascular disease.

The heart-to-body weight ratio is a key indicator used to assess cardiovascular health and overall cardiac function [199]. This ratio helps to evaluate whether there is any hypertrophy of the heart, which is often seen in conditions like obesity, type 2 diabetes and prediabetes [200]. In the prediabetic state, there is an imbalance in this ratio which suggest cardiovascular issues, particularly if the heart is disproportional larger compared to the body weight, which could be the sign of strain or adaptation due to metabolic disturbances [201]. Indeed, in this study we observed a significant increase in heart to body weight ratio when compared to the NPD group. However, administration of *Zingiber officinale* in both the presence and absence of dietary intervention showed a significant decrease in heart to body weight ratio when compared with the prediabetic control group. These results were consistent with those observed in the metformin in the presence of dietary intervention group. Based on this observation, *Zingiber*

officinale may have a protective effect on cardiovascular health that it exhibits using its ability to improve the lipid profile and regulate body mass [149]. Metformin in the absence of dietary intervention had no significant difference when compared to the PD control group. This could be attributed to the reported reduced efficacy of metformin when it is not combined with dietary intervention [202]. This suggests that *Zingiber officinale* may have subtle effect on cardiovascular health, potentially reducing the strain on the heart despite the high fat high carbohydrate diet.

The oral glucose tolerance test (OGTT) is commonly used to assess the body's ability to metabolize glucose and to detect abnormalities, such as those seen in prediabetes [203]. Previous studies have shown that diet-induced prediabetic animals exhibit impaired glucose tolerance and fasting glucose levels as a results of insulin resistance, key characteristics of prediabetes [204]. The findings observed in this study correspond to the previous findings showing a significant increased blood glucose concentration in PD control group when compared to the NPD group. The treatment with *Zingiber officinale* in the presence of dietary intervention showed a reduced blood glucose concentration while in the absence of dietary intervention showed no significant difference. Based on this observation, the reduction in blood glucose concentration in *Zingiber officinale* in the presence of dietary intervention could be attributed to *Zingiber officinale* insulin-sensitizing properties which enhance the glucose uptake in tissues, improving overall glucose metabolism [205]. Both metformin and *Zingiber officinale* have beneficial effects on glucose metabolism but their mechanisms of actions differ. While metformin primarily acts through the AMPK pathway to improve insulin sensitivity [206], *Zingiber officinale* exerts its effects through a combination of reducing inflammation, enhancing insulin receptor function and regulating lipid metabolism [207]. These findings align with the reduced heart to body weight ratio as showed with reduced blood glucose concentration reflects improve overall glucose metabolism.

Glycated haemoglobin (HbA1c) is a key marker used to assess long-term blood glucose control [208]. It reflects the average blood glucose levels over the past two to three months and is widely used to diagnose and monitor diabetes and prediabetes [12]. Elevated HbA1c levels are indicative of poor blood glucose control and are associated with an increased risk of complications related to diabetes, including cardiovascular disease, kidney damage, and neuropathy. These results in this study aligns with the previous findings as PD control group

showed elevated HbA1c levels when compared to the NPD group. Treatment with *Zingiber officinale* in both absence and presence of dietary intervention exhibited decrease HbA1c levels when compared with the PD control group. These results were consistent with those observed in the metformin-treated groups. The obtained results may be attributed to the *Zingiber officinale* anti-oxidant and anti-inflammatory effects and also inhibiting the hepatic gluconeogenesis (Mohamed, El-samie et al.2022). These findings align with improved insulin sensitivity and glycaemic control observed in the OGTT results.

Pathological alterations in RBC morphology and functions related to chronic hyperglycaemia not only mechanistically underpin T2DM complications but can also be triggered by multisystemic changes such as the accumulation of toxins and altered cellular signalling [78]. Prediabetes leads to decreased red blood count and also leads to RBD dysfunction that may lead to deranged tissue oxygenation through a variety of mechanisms [181]. These results in this study correspond to previous finding as the PD control group showed a significant decrease in RBC concentration when compared to the NPD group. However, when comparing the PD control group with the *Zingiber officinale* treatment group, in both the absence and presence of dietary intervention, it showed a significant increase in RBCs concentrations. These results were consistent with those observed in the metformin-treated groups. This suggests that *Zingiber officinale* administration as well as metformin might enhance the red blood cells production or reduce plasma volume, which can improve the overall blood health which is often impaired in prediabetic states due to oxidative stress and inflammation [209]. By counteracting these factors, *Zingiber officinale* may help support healthier blood profiles in prediabetic and CVD conditions [210]. Improved glycaemic control reduces glucotoxicity and cardiac strain which reduces oxidative stress which supports erythropoiesis contributing to increased RBC count together indicating enhance cardiovascular function and metabolic balance.

Hematocrit is a critical measurement that indicates the proportion of red blood cells in the total blood volume and [211, 212]. In prediabetes, hematocrit and hemoglobin levels may be reduced due to inflammation, oxidative stress and altered erythropoiesis [213]. The results in this study align with the previous studies as the prediabetic control group exhibited a significant decrease in both hematocrit and hemoglobin concentration when compared to the NPD group. Interestingly, treatment with *Zingiber officinale* in both the presence and absence of dietary intervention showed a significant increase in hemoglobin and hematocrit concentrations after the 12 weeks treatment period when compared with the prediabetic control group. These results

were consistent with those observed in the metformin-treated groups. This observation may be attribute to the previously observed anti-inflammatory and anti-oxidant properties of *Zingiber officinale* which potentially improve erythropoiesis and anti-oxidant status, which in turn, protect erythroid cells from oxidative damage and support the red blood cell maturation [214, 215]. This results in elevated hemoglobin synthesis and increased hematocrit, reflecting improved oxygen-carrying capacity in prediabetic conditions. Reduced oxidative stress supports erythropoiesis leading to increased RBC count, hematocrit and hemoglobin levels [216]. Together these changes indicate enhance cardiovascular function and improved oxygen delivery, contributing to overall metabolic balance.

Red blood cell indices such as Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), and Mean Corpuscular Haemoglobin Concentration (MCHC) provide important insights into the blood's oxygen-carrying capacity and overall health [217]. These biomarkers are important for assessing the health of the blood, particularly in context of cardiovascular diseases (CVD) and metabolic disorders such as prediabetes [218]. In the prediabetic state, the red blood indices are elevated [95]. In this study the results correspond to the previous findings, as the prediabetic control group showed a significant increase in MCV, MCHC and MCV levels when compared to the NPD group. The *Zingiber officinale* treated groups in both the presence and absence of dietary intervention resulted in significant decrease in MCV, MCH and MCHC levels when compared to PD control group and also to comparable with those of the NPD group. These results were consistent with those observed in the metformin-treated groups. This observation is attributed to *Zingiber officinale* bioactive compounds such as 6-gingerol, shogaol and zingerone which exhibit a strong anti-oxidant and anti-inflammatory properties [219]. This limits lipid peroxidation and inflammation, as well as also prevents abnormal enlargement of RBCs and excessive hemoglobin loading contributing to the reduction of MCV, MCHC, and MCH regardless of dietary intervention [220]. Improved red blood cell function together with glycaemic control reduces tissue hypoxia and cardiac strain. Together, this change suggests the restoration of haematological function and cardiovascular efficiency.

Erythropoietin (EPO) is a glycoprotein hormone is the principal regulator of erythropoiesis through stimulating the differentiation and proliferation of hematopoietic stem cells, as well as preventing apoptosis [221]. The results in this study align with the previous findings as the

prediabetic control group showed a significant low EPO level when compared to the NPD group. Treatment with *Zingiber officinale* in both absence and presence of dietary intervention showed a significant increase in EPO levels when compared to the PD group. These results were consistent with those observed in the metformin-treated group. This observation may be attributed to *Zingiber officinale* ability to alleviate the renal oxidative stress, improve tissue oxygenation and kidney function therefore enhancing the EPO production [222]. EPO reduces the production of the pro-inflammatory mediators and increases the production of anti-inflammatory cytokines [223]. These findings align with the observed improved red cell function and glycaemic control as it further supports sustains the EPO production and erythroid recovery.

Increased MPV has been observed in T2DM patient leading to CVD [79]. Prediabetes causes an increase in platelet reactivity through direct effects and by promoting glycation of platelet proteins on the platelet surface, this glycation decreases membrane fluidity and tends to activate the platelets [224]. The findings in this study align with the previous studies, as the prediabetic control group showed a significant increase in PCT, MPV, PDW and P-LCR. The *Zingiber officinale* treatment group in both presence and absence of dietary intervention showed a significant decrease in PCT, MPV, PDW and P-LCR levels when compared with the PD control group to levels comparable with those of the NPD group. These results were consistent with those observed in the metformin-treated groups. These observations are attribute to *Zingiber officinale* anti-thrombotic effect [225]. This reduction implies that *Zingiber officinale* and metformin may help reduce platelet activation and inflammation, potentially lowering the risk of thrombotic complications. By improving platelet function and potentially reducing excessive platelet aggregation, this may reduce some of the cardiovascular risks associate with prediabetes. Collectively, these findings point to restoration of haematological balance, reduce cardiovascular risk, and improved metabolic and vascular function.

The mammalian target of rapamycin (mTOR) is a protein kinase that controls cellular metabolism, catabolism, immune responses, autophagy, survival, proliferation, and migration, to maintain cellular homeostasis [226]. Elevated mTOR activity has been linked to increased inflammation, oxidative stress, and impaired cellular function, which can contribute to the development of cardiovascular diseases (CVD) and other metabolic complications [227]. The results in this study align with the previous findings showed by the significant increase in mTOR levels when compared to the NPD group.

Treatment with *Zingiber officinale* in both presence and absence of dietary intervention resulted in significant decrease in mTOR expression when compared with the PD control group to levels comparable with those of the NPD group. These results were consistent with those observed in the metformin-treated group. The *Zingiber officinale* administration may have a protective effect against the dysregulation of mTOR signalling in prediabetes [228]. The reduction of mTOR activity could help reduce the metabolic dysfunction and inflammation which contribute to CVDs. Some studies have shown that *Zingiber officinale* is a potent inhibitor of mTOR pathway [229]. These haematological improvements, along with observed glycaemic control suggest that *Zingiber officinale* enhances cardiovascular function and reduces oxidative, suppressing mTOR and restoring metabolic homeostasis.

Triglycerides are nonpolar lipid molecules composed of a glycerol molecule associated with three fatty acids molecules and they represent main form of lipid storage and energy in the human organism [230].. The findings in this study align with the previous findings as showed the prediabetic control group had a significant increased triglycerides levels when compared to the NPD group. Treatment with the *Zingiber officinale* treatment group in both presence and absence of dietary intervention showed a significant decrease in triglycerides levels to levels comparable with those in the NPD group. These results were consistent with those observed in the metformin-treated groups. This suggest that both *Zingiber officinale* and metformin may have a role in reducing triglycerides, potentially through mechanisms such as improving insulin sensitivity, reducing inflammation, or altering lipid metabolism [22, 145]. These observations support the conception that *Zingiber officinale* have a beneficial effect on lipid metabolism, helping to prevent the accumulation of triglycerides that is often seen in prediabetes and T2DM.

The reduction in systemic inflammation and oxidative stress may contribute to the haematological improvements, which could in turn reduce the cardiovascular risks.

5. Conclusion.

Taken together the findings indicate that *Zingiber officinale* treatment significantly improved cardiovascular and haematological parameters in diet-induced prediabetic rats. The reduction in blood glucose, triglycerides and heart to body weight ratio indicates improved metabolic and cardiac function. Increased RBC count, hemoglobin, hematocrit and EPO levels alongside with reduced MCV, MCH and MCHC suggest the enhanced erythropoiesis and red cell quality. Furthermore, the decrease in PCT, MPV, PDW, and P-LCR reflects the reduced platelet activation while mTOR suppression points to decreased metabolic stress and inflammation.

These findings suggest that *Zingiber officinale* has the ability restore haematological factors and prevents the cardiovascular risks in prediabetes.

6. Limitation and future studies

Although the heart-to-body weight ratio was measured in this study, no direct functional cardiac assessment was included. While heart-to-body weight ratio provides a useful gross anatomical indicator of cardiac hypertrophy or enlargement, it does not reflect cardiac performance, contractility, or electrical activity. Future studies should incorporate functional cardiac assessments such as echocardiography or electrocardiography to evaluate the physiological impact of prediabetes and *Zingiber officinale* treatment on cardiac function. Additionally, histological evaluation of cardiac tissue could provide insight into structural changes, fibrosis, or early signs of cardiovascular pathology that may not be apparent from gross measurements alone.

Furthermore, although selected haematological and immune markers were assessed, this study did not include molecular assays. Measuring circulating markers gives valuable information on systemic changes; however, it does not directly elucidate the underlying molecular mechanisms responsible for these alterations. Future studies should employ molecular techniques such as gene expression analysis, protein phosphorylation studies, or pathway-specific assays to confirm the involvement of specific signaling pathways. Such approaches would provide a deeper understanding of how *Zingiber officinale* exerts its effects on immune function, haematological parameters, and cardiovascular risk in prediabetes.

Lastly, this study focused on a single dose and duration of *Zingiber officinale* treatment. Dose-response relationships and long-term effects remain unclear, and future investigations should explore these aspects to optimize therapeutic strategies. Addressing these limitations would strengthen the translational relevance of the findings and provide a more comprehensive understanding of the interplay between prediabetes, haematology, immune function, and potential interventions.

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Chapter 5: Synthesis and conclusion

Type 2 diabetes mellitus (T2DM) is strongly associated with cardiovascular disease (CVD) and the dysregulation of immune function [231]. The prevalence rate of CVD and immune function dysregulation is higher in adults with T2DM than in those without T2DM [232](Filh, 2019). These immune changes which are driven by AGEs, metabolic stress and altered immune signalling, promote infection risk and progression toward T2DM [123]. This risk increases progressively with increasing fasting plasma glucose levels, even before it reaches sufficient levels for the diagnosis of T2DM [233]. Prior to the onset of type 2 diabetes mellitus , individuals may exhibit a condition known as prediabetes[9]. Prediabetes is a condition of intermediate hyperglycaemia where the glycaemic parameters are above the homeostatic range but below the threshold for a diagnosis of T2DM [10]. As a result of progressive β-cell dysfunction leading to deterioration of glucose homeostasis and eventually the development of insulin resistance which is a key underlying aetiology [12]. In prediabetes, insulin resistance and elevated glucose levels stimulate pro-inflammatory pathways, leading to increased cytokine production and altered immune cell function [121]. These metabolic changes also affect the heart and blood vessels by promoting cellular stress, inflammation leading to cardiovascular risks [122].

Medicinal plants have been identified as a promising future treatment for the management of health care in the twenty-first century [234]. *Zingiber officinale* has been shown to possessed multiple biological activities especially antioxidant, anti-thrombotic, anti-inflammatory and immune modulatory capacities [235]. However, no studies have investigated the effects of *Zingiber officinale* on these parameters during prediabetic state. Therefore, this study sought to the effect of *Zingiber officinale* (ginger) on inflammation, haematological factors, immune system and cardiovascular disease in diet-induced prediabetic rats. To begin, A literature review was conducted. This chapter exhibited that in prediabetes, either innate immunity, adaptive immunity or both are involved or interacted with each other [236]. It also affects immune cell lineage development, function, and migration [50]. Haematological changes have been reported in T2DM and prediabetes as these play a major role in CVD complications [237]. Haematological changes can be caused by several factors including increased production of reactive oxygen species (ROS) [238, 239]. Increased production of ROS resulting in oxidative stress, is implicated in tissue damage and haematological changes such as red blood cell (RBC) dysfunction, platelets (PLT) hyperactivity and endothelial dysfunction [238].

Once diagnosed with prediabetes, ameliorative strategies that include a combination of dietary modification and pharmacological treatment are recommended, nevertheless, patients tend to rely on the pharmacotherapy and neglect the dietary modification thus reducing the efficacy of the drugs [240]. The literature findings summarised key findings and highlighted gaps which prompted the need to find alternative or novel drugs that can mitigate the inflammation, immune dysfunction, irregulated haematological factors and CVDs in both the presence and absence of dietary intervention.

In the first manuscript of the study, we investigated effect of *Zingiber officinale* in inflammation and innate immune system in diet-induced prediabetic rats. The induction of prediabetes by high fat, high carbohydrate diet resulted in the increased body weight gain through increased caloric intake and mTOR expression in prediabetic rats. This, in turn, resulted in increased glucose levels, insulin levels and HOMA-IR values in the prediabetic rats. Treatment with *Zingiber Officinale* restored body weight gain, caloric intake and mTOR expression. These findings were associated with decreased glucose, insulin and HOMA-IR values in the prediabetic treated rats. *Zingiber Officinale* has been reported to possessed 6-gingerol which plays a key role in glucose homeostasis via amelioration of glucose stimulated insulin secretion and dramatically improving glucose tolerance through increasing the expression of Glut-4 glucose transporters [241].

Impaired glucose metabolism leads to excessive production of reactive oxygen species (ROS), which overwhelms the antioxidant system thus resulting into oxidative stress which damages immune cells and impairing their function [242]. In the prediabetic group, there was a significant increase in malondialdehyde (MDA) levels, indicating elevated oxidative stress due to excessive ROS production. This was accompanied by a decrease in nuclear factor erythroid 2-related factor 2 (NFE2L2) expression, suggesting impaired activation of the antioxidant defence system. Treatment with *Zingiber officinale* and dietary intervention significantly reduced MDA levels, indicating a decrease in lipid peroxidation and oxidative stress. Furthermore, *Zingiber officinale* upregulated NFE2L2 expression, reflecting enhanced activation of the antioxidant response pathway. Additionally, neutrophils and lymphocytes cell counts were elevated in the prediabetic rats, reflecting immune system activation likely driven by oxidative stress and inflammation. Treatment with *Zingiber officinale* resulted in reduction of neutrophils and lymphocytes cell counts, suggesting the restoration of immune balance and reduced inflammation in the prediabetic treated rats.

Hyperglycaemia together with oxidative stress are contributing risk factors for imbalance of pro and anti-inflammatory cytokines [242]. This dysregulation led to elevated proinflammatory cytokine that promote chronic inflammation response [243]. Hyperglycaemia resulted in decreased adiponectin levels with increased IL-6, TNF-α and CRP levels in the prediabetic rats. Treatment with *Zingiber Officinale* resulted in decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines suggesting that *Zingiber officinale* exerts anti-inflammatory effects by suppressing pro-inflammatory cytokines and enhancing anti-inflammatory mediators, thereby contributing to the mitigation of chronic low-grade inflammation in prediabetic conditions [244].

In the second manuscript of the study, we investigated the effect of *Zingiber officinale* on haematological factors and selected markers of cardiovascular function in diet-induced prediabetic rats. The high fat high carbohydrate diet results in the increased glucose concentration and HbA1c levels in the prediabetic rats. Treatment with *Zingiber officinale* restored glycaemic control as observed through decreased blood glucose and HbA1c concentrations in the treated groups.

Hyperglycaemia causes red blood cell glycation reducing their oxygen carrying capacity while increasing oxidative damage which contribute to CVD [245]. In the prediabetic control group, this was reflected by a significant reduction in hemoglobin (Hb), hematocrit (HCT), and red blood cell count (RBC), indicating impaired erythropoiesis and reduced oxygen transport capacity [246]. Additionally, mean corpuscular volume (MCV) and red cell distribution width (RDW) were elevated, suggesting altered red cell morphology and size variability, consistent with oxidative stress and inflammation-induced erythrocyte damage [247]. Treatment with *Zingiber officinale* led to an improvement in RBC indices, as evidenced by increased Hb, HCT, RBC count and EPO production, along with normalization of MCV and a reduction in RDW. These findings indicated a protective role of *Zingiber officinale* in maintaining red blood cell integrity and function, likely through its antioxidant and anti-inflammatory properties [248].

Hyperglycaemia also activates platelet hyperactivity creating a prothrombotic state [249]. In the prediabetic control group, this was reflected by an increased in platelet count, mean platelet volume (MPV), and platelet distribution width (PDW), indicating heightened platelet activation and aggregation potential [250]. Treatment with *Zingiber officinale* reduced platelet count, MPV, and PDW, suggesting an anti-thrombotic effect and improved platelet regulation [251]. Elevated glucose increases insulin secretion which leads to high triglycerides (TGs) and

heart to body weight ratio in prediabetic group indicating lipid build-up and hypertrophy of the heart. *Zingiber officinale* treatment reduced TGs and heart to body weight ratio, suggesting protection against lipid-induced cardiac dysfunction and hypertrophy of the heart [252]. These results, for the first time, showed that *Zingiber officinale* is able to reduce inflammation, restore immunity and haematological parameters and lower the risk of cardiovascular disease in diet-induced prediabetic rats.

Conclusion

Zingiber officinale demonstrated significant protective effects against prediabetes-related complications by improving glucose uptake and insulin signaling, thereby enhancing metabolic health. Treatment with *Zingiber officinale* modulated the immune system by reducing pro-inflammatory cytokines and increasing anti-inflammatory responses, while also improving haematological parameters such as red blood cell indices and platelet function. These effects collectively contributed to the prevention of cardiovascular dysfunction in prediabetic rats. Importantly, the beneficial effects of *Zingiber officinale* were observed both in the presence and absence of dietary intervention, highlighting its potential as a therapeutic agent for managing prediabetes and its associated immune, haematological, and cardiovascular complications.

Shortfall and future studies

Although the study observed changes in the markers, it did not include detailed molecular assays to confirm activation of specific pathways. Future studies should include the targeted mechanistic studies to how *Zingiber officinale* modulates these pathways at the cellular and molecular levels. Lifestyle modification is not only limited to the dietary intervention, but it also includes other factors such as moderate exercise. However, the current study only evaluated dietary intervention as the one of the prediabetic strategies. Therefore, in future, the effect of exercise should be investigated as one of the prevention and management of prediabetics progression and link it with the dietary intervention. Further studies are needed to fully understand the long-term effects and the optimal dosage, these findings will contribute to the growing body of evidence supporting the use of ginger as an adjunct treatment in managing prediabetes related CVS complications, immune and haematological function. The studies in human population may fully evaluate *Zingiber officinale* therapeutic efficacy and its role in cardiovascular disease preventions.

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Appendices

AREC Research Application (R) (The protectiveeffects of zingiber officcinale(ginger) extract with endurance exerise on haemotological factors and immue system in diet-induced prediabetic rats, (AREC/00006737/2024)) Langa, Bonakele (219013848)

NB:
Please click on **Edit** on the top right of the screen to view the full information and make changes to the application. If there is no edit button visible to you, you may be unable to edit the application as it is may be with someone else at the moment.
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Type of ethics review: AREC Research Application (R)

Title: The protectiveeffects of zingiber officcinale(ginger) extract with endurance exerise on haemotological factors and immue system in diet-induced prediabetic rats

Date of approval: 11.06.2024

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