Title

Effect of *Moringa oliefera* on palmitate-induced insulin resistance on C2C12 skeletal muscle cells

FT Mthiyane

orcid.org/0000-0001-8109-8940

Dissertation submitted in partial fulfilment of the requirements for the degree Master of Science in Biochemistry at the North-West University

Supervisor: Professor SE Mazibuko-Mbeje

Co- supervisor: Dr NH Hlengwa

Examination: August 2025

DECLARATION

I, Fikile Thubelihle Mthiyane, certify that this thesis submitted for the MSc degree in Biochemistry at the North-West University, titled "***Effect of Moringa oliefera on palmitate-induced insulin resistance of C2C12 mouse skeletal muscle cells***," is my original work. I certify that no other institution has used this work for a different degree and that all content has been appropriately cited in the text and references.

Candidate: Fikile Thubelihle Mthiyane

Signature..... **Date:** 08th of April 2025

Supervisor: Prof Sithandiwe E Mazibuko Mbeje

Signature:..... **Date:** 08th of April 2025

Co-supervisor: Dr NH Hlengwa

Signature:..... **Date:** 08th of April 2025

ACKNOWLEDGMENTS

To God be the glory.

Psalm 127: 1

“Unless the LORD builds the house, its builders labor in vain. Unless the LORD watches over the city, the watchmen stand guard in vain. In vain, you rise early and stay up late, toiling for food to eat for he grants sleep to those He loves.”

My gratitude to my supervisor Professor S.E Mazibuko-Mbeje for your valuable patience and feedback. I want to thank you for taking your time to teach me about the pros and cons of science, you had a lot on your plate, but you made sure that you made time whenever I needed assistance with my project. You went the extra mile for me, and I am genuinely grateful to you.

Thank you to Dr Hlengwa and Dr Ndlovu for the scientific input for this project.

I would like to acknowledge and thank Khanyisani, Cebile, Snenhlanhla and Ndivhuwo for helping me out during the course of my degree, you were patient with me you answered my questions no matter how silly I felt they were, you would go as far as helping me in the lab and that I highly appreciate.

I highly appreciate you Sibongiseni Mpanza for your constant and non-wavering support, you supported me from the very first day you heard about this MSc offer which would take me 825 km away from home but still you supported me and you believed in me and you have rooted for me since day one. I have felt your support and it has warmed my little heart.

To my family, thank you for constantly supporting me, and thank you for being there for me throughout this journey.

Most importantly, I give thanks to God Almighty for this lovely opportunity to do this MSc in Biochemistry. I am in awe of the great work that He has done for me during the course of my MSc.

I believe that it would have not been possible if You had not sustained me on this journey so I give all the praise to God.

ABSTRACT

Type 2 diabetes has become a global epidemic with an impact on the global economy and healthcare systems. Diet rich in saturated fatty acids, such as palmitate, is attributes to the onset of insulin resistance, which is the main contributing factor of type 2 diabetes. Skeletal muscle, that accounts for 75% of glucose homeostasis, has been reported to be affected by high levels of saturated fatty acid leading to the development of insulin resistance. Natural compounds have gained interest for their health benefits. These benefits are attributed to their polyphenols found in plants and have been demonstrated to have ameliorative effects against insulin resistance and the advancement of diabetes. Therefore, in this study, we aimed to investigate the natural plant *Moringa oliefera* rich in polyphenols on palmitate-induced insulin resistance in C2C12 mouse skeletal cells. The bioactivity of *Moringa oliefera* in healthy C2C12 skeletal cells was examined in the study's first objective. In growth media containing 10% FBS, C2C12 myoblasts were cultivated until they got to an 80–90% confluence. The cells were then treated with 2% FBS for 3 days to induce myotubule differentiation. The quercetin (1 µM) and insulin (1 µM) were utilized as positive controls, and the C2C12 skeletal cells were exposed during the course of 2, 4, 8, 16, and 24 hours at various concentrations (0.001, 0.01, 0.1, 1, 10, and 100 µg/mL). The MTT assay was then used to examine how *Moringa oliefera* affected cellular activity and cytotoxicity. Due to the significant impact *Moringa oliefera* had on cellular activity at this time, an exposure length of 8 hours was established to be used for all relevant experiments with all the dosages. Thereafter, the effect of *Moringa oliefera* on glucose utilization and glycogen concentration was investigated using a glucometer and the glycogen assay respectively in treated cells for 8 hours. The second aim of this study focused on exploring the effect *Moringa oliefera* on cellular activity and cytotoxicity of palmitate-induced insulin resistance. Briefly, insulin resistance was produced by treating the cells with 0.75 mM palmitate conjugated with 2 %BSA for 16 hours. Thereafter treated with various doses of *Moringa oliefera* (0.001, 0.01, 0.1,1,10, and 100 µg/mL), for 8 hours, and insulin (1 µM), in the last 15 minutes. In the insulin-resistant cells the effect of *Moringa oliefera* on glucose utilization was also assessed. Using the quantitative real-time polymerase chain reaction (qPCR), the expression of mRNA related to the insulin signaling pathway (*Insr, Pkcθ, Pi3k*) enzymatic antioxidant activity (*Sod1* and *Gss*), and glucose metabolism (*As160*) were measured. The first objective, results showed that *Moringa oliefera* had no impact on cell viability when exposed for a brief period of time (2 and 4 hours), but it had a substantial impact when exposed for eight hours. It's interesting to note that treating the cells for 16 hours had a significant effect only at 0.01 and 0.1 µg/mL, and that after 24 hours of exposure to *Moringa oliefera*, there were marginal dose-dependent reductions in cell viability of the C2C12 skeletal cells however which did not translate to any toxicity. The observations showed that *Moringa oliefera* had no effect on glucose utilization in healthy cells however it significantly enhanced glycogen concentrations at these doses 0.01, 0.1, 1 and 10 µg/mL. For the second objective, results showed that palmitate , palmitate induced insulin resistance in C2C12 skeletal cells and this was evident by the decrease in cell viability but *Moringa oliefera* significantly reversed this damage by increasing cell viability in all doses tested. *Moringa oliefera* significantly enhanced glucose uptake at 10 µg/mL. The mRNA expression of *Insr* was increased by the addition of insulin and interestingly, *Moringa oliefera* was able to improve the activity of the *Insr* mRNA, had a significant increase at 1, 10 and at 100 µg/mL. It's noteworthy that palmitate enhanced *Pkcθ* expression confirming insulin resistance, *Moringa oliefera* was able to reverse this by lowering *Pkcθ* expression at all doses tested. In terms of insulin dependent pathway insulin significantly increased the expression of *Pi3k*, and *Moringa oliefera* significantly upregulated *Pi3k* expression at all the doses. The *As160* mRNA expression was reduced by palmitate treatment but *Moringa oliefera* was able to increase the mRNA expression at all the doses, surprisingly, dose 100 µg/mL showed more effect. The *Sod1* enzymatic antioxidant activity was also increased, with a notable improvement seen at 0.1 µg/mL.

At 0.01 and 0.1 µg/mL, furthermore, mRNA activity was also enhanced after treatment with our plant of interest. In conclusion, our results show that *Moringa oliefera* had no toxic effect in all concentration tested. The effects of palmitate-induced insulin resistance could be mitigated by *Moringa oliefera* by lowering *Pkcθ* and activating *Insr*, Pi*3k* and *As160,* suggesting increased in glucose metabolism. Furthermore, the modulation of enzymatic antioxidants *Sod1* and *Gss* by *Moringa oliefera,* suggests that *Moringa oliefera* has some antioxidant activity.

Key terms

Moringa oliefera, skeletal muscle, insulin resistance, oxidative stress, type 2 diabetes

ABBREVIATIONS

Acetyl-CoA	: Acetyl Coenzyme A
ABTS	:2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
ATP	: Adenosine triphosphate
AKT	: Protein kinase B
AMPK	:5' adenosine monophosphate-activated protein kinase
BMI	: Body mass index
BCL-2	: B-cell lymphoma 2
B2M	: Beta-2 microglobulin
CAT	: Catalase
CCAAT	: Cytosine-cytosine-adenosine-adenosine-thymidine
COX-2	: Cyclooxygenase-2
C/EBPα	: CCAAT/Enhancer Binding Protein α
DAG	: Diacylglycerol
DPPH	:2,2-diphenylpicrylhydrazyl
DPBS	: Phosphate buffer saline
DMEM	: Dulbecco's modified eagle medium
ER	: Endoplasmic reticulum
FFA	: Free fatty acids
FAOD	: Fatty acid oxidation disorders
FBS	: Foetal bovine serum
FDA	: Food and Drug Association
FPG	: Fasting plasma glucose
GLUT4	: Glucose transporter 4
GSH	: Glutathione
GST	: Glutathione S-transferase
GPx	: Glutathione peroxidase
G-6-P	: Glucose-6-phosphate
HbA1c	: Hemoglobin A1C
HFD	: High-fat diet

HO-1 : Heme oxygenase 1

IRS1 *:* Insulin receptor substrate 1

INSR *:* Insulin receptor

IKKB : Inhibitor of KappaB Kinase

IR : Insulin resistance

iNOS : Inducible nitric oxide synthase

JNK :c-Jun N terminal kinases

LC-FA :Long-chain fatty acyl-CoA

MCP-1 :Monocyte chemoattractant protein-1

MDA :Malondialdehyde

MAPK :Mitogen-activated protein kinase

MTT :3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide

NADH :Nicotinamide adenine dinucleotide hydrogen

NAD+ :Nicotinamide adenine dinucleotide

NF-Kb :Nuclear factor kappa B

NRF2 :Nuclear factor erythroid 2–related factor 2

$ONOO^-$ **:**Peroxynitrite

•OH :Hydroxyl radical

$O_2^{\cdot -}$;Superoxide anion

PDE-5 :Phosphodiesterase type 5

p53 :Tumor protein p53 gene

PPARα *:*Peroxisome proliferator–activated receptor α

PPARy *:*Peroxisome Proliferator Activated Receptor Gamma

PGCC1α *:*Peroxisome proliferator-activated gamma coactivator 1-alpha

PI3K *:*Phosphoinositide 3-kinase

PKCθ :Protein kinase C theta

qPCR :Quantitative polymerase chain reaction

ROS :Reactive oxygen species

SOD1 :Superoxide dismutase

STZ :Streptozotocin

SREBP1c :Sterol regulatory element-binding transcription factor 1c

SIRT1	:Sirtuin 1
SLCA2	:Solute carrier 2A
T2DM	:Type 2 diabetes mellitus
T1DM	:Type 1 diabetes mellitus
TNF-α	:Tumor necrosis factor-alpha
VEGF	:Vascular endothelial growth factor

Declaration i

Acknowledgments ii

ABSTRACT iii

Abbreviations v

LIST OF FIGURES xii

OUTPUTS xiii

CHAPTER 1 1

INTRODUCTION 2

Aim 4

Objectives 4

LITERATURE REVIEW 6

A review on the antidiabetic properties of *Moringa oleifera* extracts: Focusing on oxidative stress and inflammation as main therapeutic targets 6

Abstract 6

1. Introduction 8

2. An overview of *Moringa oleifera* and its diverse biological properties 9

3. Oxidative stress and inflammation as prominent mechanisms involved in diabetes-induced complications 10

4. The antioxidant properties of *Moringa oleifera* 12

5. *Moringa oleifera* extracts improve markers of oxidative stress and inflammation in pre-clinical models of T1D 12

6. Moringa oleifera extracts improve markers of oxidative stress and inflammation in pre-clinical models of T2D 18

7. Clinical translation 21

8. Summary and future perspective 21

Funding statement 23

Abbreviations 23

Acknowledgements 23

Declaration of competing interest 23

Author's contribution 24

Data availability statement 24

References 25

CHAPTER 3 34

3 Materials and Methods 35

3.1. Materials, reagents and methods 35

3.2. General overview of in vitro models 35

3.3.1. Thawing of C2C12 mouse skeletal muscle cells 36

3.3.2. Sub-culturing and cell counting and seeding of cells 36

3.3.3. Differentiation of myoblast into myotubes 36

3.3.4. Preparation of *Moringa oliefera* treatment 37

3.4. Dose and time study 37

3.4.1. Bioactivity of *Moringa oliefera* in C1C12 myotubules ... 37
3.4.2. The 3- [4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay (MTT assay) in normal cells ... 38
3.4.3. Glucose uptake ... 39
3.4.4. Glycogen assay ... 39
3.5. Insulin resistance model ... **40**
3.5.1. Preparation of palmitate and induction of insulin resistance ... 40
3.5.2. Extract and compounds preparations and treatment of cells ... 41
3.6. The 3- [4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay (MTT assay) ... **42**
3.7. Glucose uptake ... **42**
3.8. RNA extraction ... **42**
3.9. RNA quantification using a Nanodrop spectrophotometer ... **43**
3.10. Quantification of the quality of RNA using gel electrophoresis ... **44**
3.11. Reverse transcription of RNA into cDNA ... **44**
3.12. Quantitative real-time PCR analysis ... **45**
3.13. Statistical analysis ... **46**
CHAPTER 4 ... **47**
4. RESULTS ... **47**
4.1. Objective 1: To investigate the bioactivity of *Moringa oliefera* in normal C2C12 skeletal muscle cells. ... **47**
4.1.1. The effect of the dose and time of *Moringa oliefera* on cell viability in C2C12 skeletal muscle cells ... 47
4.1.2 The effect of *Moringa oliefera* on glucose utilization and glycogen concentration in C2C12 skeletal cells ... 50
4.2. Objective 2: To investigate the effect of *Moringa oliefera* leaf extract on palmitate-induced insulin resistant C2C12 skeletal muscle cells at a cellular level. ... **51**
4.2.1. Effect of *Moringa oliefera* on cell viability and cytotoxicity in palmitate induced-insulin resistant C2C12 skeletal cells ... 51
4.2.2. Effect of *Moringa oliefera* on glucose metabolism ... 51
4.3. Objective 3: To investigate the effect of the leaf extract on the expression of the insulin signalling and anti-inflammatory genes (*Insr Pkcθ, Pi3k, As160, Sod1,* and *Gss,*) ... **53**
4.3.1. Effect of *Moringa oliefera* on insulin signaling pathway in palmitate induced insulin resistance in C2C12 skeletal muscle cells ... 53
4.2.4. Effect of *Moringa oliefera* on insulin signaling pathway in palmitate induced insulin resistance in C2C12 skeletal muscle cells ... 54
4.2.5. The effect of *Moringa oliefera* on enzymatic antioxidant activity of superoxide dismutase (*Sod1*) and glutathione (*Gss*) ... 55
5. Discussion ... **57**
5.1. Summary ... **57**
5.2. Effect of *Moringa oliefera* on cell viability in normal C2C12 skeletal cells ... 57
5.3. Effect of *Moringa oliefera* on glucose uptake and glycogen concentration in normal C2C12 skeletal muscle cells ... **58**
5.4. Protective potential of *Moringa oliefera* in palmitate induced insulin resistant C2C12 skeletal cells ... **59**
5.5. Activity of glucose uptake in palmitate induced insulin-resistant C2C12 skeletal cells after treating with *Moringa oliefera* ... **59**
5.6. Impact of *Moringa oliefera* on insulin signaling pathway ... **60**
5.7. Effect of *Moringa oliefera* on enzymatic antioxidant activity ... **62**
6. Conclusion ... **63**

7. RECOMMENDATIONS FOR FUTURE STUDIES 64

8. Study limitation 64

CHAPTER 6 65

9. References 66

APPENDIX 2 83

Buffer and media preparations 83

APPENDIX 3 84

LIST OF TABLES

Table 1: Studies on the effect of *Moringa oleifera* extracts targeting markers of oxidative stress and inflammation in preclinical models of type 1 diabetes. 15

Table 2: An overview of studies on the effects of *Moringa oleifera* extracts targeting markers of oxidative stress and inflammation in preclinical models of type 2 diabetes. 19

Table 3: The different cell densities used for seeding in different multi-well plates. 36

Table 4: Components used for the glycogen assay 40

Table 5: Reaction components of cDNA synthesis 45

Table 6: Probes used for PCR 45

Table 7: Components used for the PCR reaction 46

Table 8: List of reagents used in the study 78

Table 9: List of equipment and software 80

Table 10: List of consumables 81

Table 11: Components used for the Kreb's ringer bicarbonate buffer in 500 mL 83

Table 12: Preparation of Sorenson's buffer 84

Table 13: Averages of the NanoDrop readings used for synthesis of cDNA for experiment 1 85

Table 14: Averages after the NanoDrop readings of RNA used for the synthesis of cDNA for experiment 2 85

Table 15: NanoDrop readings that were averaged for the calculations of cDNA synthesis for experiment 3 86

LIST OF FIGURES

Figure 1. *Moringa oleifera* and chemical structures of its major flavonoids myricetin, quercetin and kaempferol. 10

Figure 2. An overview of pathological mechanisms implicated in the development of diabetes mellitus. 11

Figure 3. An overview of therapeutic mechanisms associated with the ameliorative effects of *Moringa oleifera* extracts in preclinical models of diabetes. 22

Figure 4: An overview of the preparation of *Moringa oliefera* and relevant controls for the dose-time study to fulfil the first objective of this study in normal C2C12 skeletal cells. 38

Figure 5: A schematic diagram of the insulin resistance model. 41

Figure 6: Insulin resistant C2C12 skeletal cells treated with *Moringa oliefera* for 8 hours in 96 well plates for MTT, glucose assay and in 6-well plates for RNA extraction for qPCR. 42

Figure 7: The effect of *Moringa oliefera* extract on cell viability in C2C12 skeletal muscle cells treated for 2 hrs (A), 4 hrs (B), 8 hrs (C), 16 hrs (D) and 24 hrs (E).. 49

Figure 9: Effect of *Moringa oliefera* on cell viability in insulin resistant C2C12 skeletal cells. 51

Figure 10: Effect of *Moringa oliefera* on glucose uptake.. 52

Figure 11: The effect of *Moringa oliefera* on the mRNA expression of Insr (A) and *Pkcθ* (B). 53

Figure 12: To assess the effect that *Moringa oilefera* has on the insulin signaling pathway *Pi3k*(A) and *As160* (B) 54

Figure 13: Effect of *Moringa oliefera* on enzymatic antioxidant activity *Sod1*(A) and *Gss*(B)

55

Figure 14: An overview of the proposed mechanism of action used by *Moringa oliefera* 64

Figure 15: RNA quantification of the treated C2C12 skeletal cells using an agarose gel electrophoresis. 84

OUTPUTS

Publications Related to Masters

Fikile T. Mthiyane, Phiwayinkosi V. Dludla, Khanyisani Ziqubu, Sinenhlanhla X. H. Mthembu, Ndivhuwo Muvhulawa, Nokulunga Hlengwa, Bongani B. Nkambule and Sithandiwe E. Mazibuko-Mbeje. ,2022. A review on the antidiabetic properties of *Moringa oleifera* extracts: Focusing on oxidative stress and inflammation as main therapeutic targets. Fronteirs in Pharmacology,13, p.940572. https://doi.org/10.3389/fphar.2022.940572

Collaborations

Mazibuko-Mbeje, S.E., Mthembu, S.X., Tshiitamune, A., Muvhulawa, N., **Mthiyane, F.T.**, Ziqubu, K., Muller, C.J. and Dludla, P.V., 2021. Orientin Improves Substrate Utilization and the Expression of Major Genes Involved in Insulin Signaling and Energy Regulation in Cultured Insulin-Resistant Liver Cells. Molecules, 26(20), p.6154. https://doi.org/10.3390/molecules26206154

Conference presentation

Tsholofelo P Moloi at the South African Society of Biochemistry and Molecular Biology. Congress 2024, 7-10 July 2024. Effect of *Moringa oleifera* on palmitate-induced mitochondrial dysfunction in C2C12 skeletal muscle cells.

CHAPTER 1

This chapter introduces the study and covers aims and objectives.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is amongst the top 10 predominant reasons of death around the world, affecting roughly 537 million people in the past year (2021), and these numbers are expected to rise to 643 and 783 million by 2030 and 2045 respectively (International Diabetes Federation Diabetes Atlas/Tenth Edition, 2021; The Top 10 Causes of Death, 2020). Currently there are approximately 589 million adults aged between 20-79 that are living with diabetes globally and this is according to the 11th edition (2025) of the International Diabetes Federation (IDF) Diabetes Atlas. IDF further claims that about 43%(which is approximately 252 million people) of these adults are undiagnosed meaning that there are some gaps in the detection and maybe 4 out of 5 of them live in low and middle class countries. While the number of individuals living with diabetes increases so does the death rate, in 2024 alone, there were approximately 3.54 million deaths among the age groups mentioned above. Several factors contribute to the increasing prevalence of T2DM such as physical inactivity, genetics, unhealthy diet, environmental factors, inflammation, oxidative stress, mitochondrial dysfunction, obesity, hypertension, heart disease, liver disease, kidney disease and insulin resistance (Alam Miah & Yousuf, 2018; Fletcher et al., 2002; Galicia-Garcia et al., 2020). When oxidative stress is increased it tends to cause deleterious damage that leads to the onset of insulin resistance, cardiovascular diseases and T2DM (Burgos-Morón et al., 2019; Hurrle & Hsu, 2017; Li et al., 2023; Tangvarasittichai, 2015). Insulin resistance was of interest, as it plays a major role in developing T2DM (Kahn et al., 2006). Insulin resistance is defined as an inability of cells to respond to the actions of insulin leading to high levels of circulating glucose and reduction glucose uptake in several organs such as skeletal muscle (Lee et al., 2022). Skeletal muscle accounts for 75% whole body insulin-stimulated glucose uptake, it is known to play a central role in metabolic health therefore, it can be a target organ for therapeutics research focusing on insulin resistance and T2DM (Bayol et al., 2014; Merz & Thurmond, 2020).

Insulin resistance can target several peripheral tissues such as adipose, liver and muscle leading to an ectopic fat accumulation (Lopez-Yus et al., 2024; Snel et al., 2012). The accumulation of free fatty acids within a cell, particularly linoleic and palmitic acid, causes their enzymatic activation into long-chain fatty acyl-CoA (LC-FA-CoA) derivatives. These are then broken down into intermediates like diacylglycerol (DAG) and ceramides that inhibit insulin signalling pathways and reduce glucose transport in cells, thereby perpetuating insulin resistance in skeletal muscle. These FFA intermediates upregulate the expression of serine/threonine kinases like protein kinase theta (PKC θ), Jun N-terminal kinase and IKB-kinase-β which then phosphorylates the insulin-receptor substrate (IRS ser307) on the serine residues and the activation of the serine kinases lead to the inactivation of the phosphatidyl-inositol-3-kinase (PI3K) leading to the reduction of translocation of glucose by glucose transporter 4 (GLUT4) (Boden, 1999; Corcoran et al., 2007; Czech et al., 2013; Mazibuko-Mbeje et al., 2013; Snel et al., 2012; Trouwborst et al., 2018; Zhang et al., 2010).

Moreover, inappropriate modern lifestyle such as inactivity, over-eating and stress intensify the level of insulin resistance (Chen et al., 2023; Marušić et al., 2021; Sato, 2020; Snel et al., 2012). A nutritional and lifestyle intervention has been suggested to be the primary factor for ameliorating insulin resistance (Kelly, 2000; Khalafi et al., 2023; Menichini et al., 2020; Mirabelli et al., 2020; Trouwborst et al., 2018). It has been reported that a low-fat diet, low glycaemic index diet, low-carbohydrate diet and high-fibre diet contribute to weight reduction in overweight individuals and also improve insulin resistance (Aas et

al., 2023; Mao et al., 2021; Weickert, 2012; Weickert & Pfeiffer, 2018). A low glycaemic index diet helps improve lipid profiles whilst a high-fibre diet helps increase post-meal satiety and reduces postprandial glucose (Weickert, 2012; Weickert & Pfeiffer, 2018). In addition to diet, physical activity has been reported to improve insulin sensitivity (Consitt et al., 2019; Lin et al., 2022). In older adults, physical activity in particular endurance training was reported to enhance skeletal muscle protein kinase B (PKB/AKT) activity (Consitt et al., 2019).

In support of diet studies, over the years plant polyphenols have become popular with researchers because of their health benefits and their rich antioxidant status (Cory et al., 2018; Tsao, 2010). Polyphenols are most present in foods such as fruits, vegetables, cereals and beverages. It has also been reported that consuming a diet rich in polyphenols for a longer period can give protection against the onset and the damage of diseases like cancers, cardiovascular diseases, osteoporosis, neurodegenerative diseases and diabetes (Chung S Yang et al., 2001; Niwano et al., 2022; Pandey & Rizvi, 2009; Scalbert et al., 2005). Apart from having antioxidant benefits, polyphenols were also reported to have anti-inflammatory benefits by interacting with gene expression and signalling genes, and other evidence proves that they play a role in improving glucose tolerance and insulin sensitivity (Cory et al., 2018; Kang et al., 2019; Mthiyane et al., 2022; Silveira et al., 2019).

Polyphenols play a huge role in carbohydrate metabolism and glucose uptake in the muscle and adipocytes by translocating GLUT4 to the plasma membrane through the activation of AMPK (Chiș et al., 2024; Giacometti et al., 2020; Naz et al., 2023; Shahwan et al., 2022). These plants possess chemical compounds such as aspalathin which are found in abundance in rooibos tea and have been reported to reverse palmitate-induced insulin resistance in C2C12 skeletal muscle and 3T3-L1 fat cells by regulating the energy metabolism and insulin signalling genes including the PI3K/AKT pathway and AMPK, this polyphenol also reduces hepatic insulin resistance by regulating the PI3K/AKT and AMPK pathways (Mazibuko-Mbeje et al., 2013; Mazibuko-Mbeje et al., 2019; Mazibuko et al., 2015). Accumulative research shows that epigallocatechin-3-gallate (EGCG) the main catechin present in green tea, scientifically known as *Camellia sinensis* has powerful antioxidant properties and it is an antitumor and an antiangiogenic agent. It induces cell apoptosis whilst promoting cell growth arrest which then leads to the altering of the expression of the cell cycle regulatory proteins which activate the killer caspases, and suppress the NFκB activation (Farhan, 2022; Gupta et al., 2008; Gupta et al., 2004; Singh et al., 2011). NFkB plays a critical role in disrupting the insulin signaling pathway which further leads to the interruption of glucose uptake and insulin resistance. This pathway exacerbates insulin resistance in the target tissues by intensifying oxidative stress, this then leads to the decrease of *SOD1* activity.

Moringa oliefera is the plant of interest, which has gained a lot of popularity in many parts of the world due to its health benefits (Islam et al., 2021; Milla et al., 2021; Patil et al., 2022). This plant has been reported to be rich in polyphenols such as kaempferol, myricetin, quercetin, rutin, chlorogenic acid, glucosinolates and isothiocyanates that exhibit anti-inflammatory, antihypertensive, antioxidant, anticancer and anti-diabetic properties and it has also been shown to improve insulin sensitivity (Abd Rani et al., 2018; Adel-Mehraban et al., 2024; Kim et al., 2014; Kou et al., 2018; Nova et al., 2020; Oguntibeju et al., 2020; Pareek et al., 2023; Parveen et al., 2024). Polyphenols found in *Moringa oliefera*

such as quercetin and kaempferol have been reported to have many health benefits such as enhancing and stimulating glucose uptake and decreasing hyperglycaemia, it also regulates signalling pathways involved in glucose metabolism, reduce oxidative stress while improving insulin resistance (Ahmad et al., 2023; Dhanya, 2022; Mahaveerchand & Abdul Salam, 2024; Shi et al., 2019; Sok Yen et al., 2021). In another study by (Duranti et al., 2021) they have reported that *Moringa oliefera* influenced oxidative metabolism in C2C12 skeletal muscle cells through the SIRT-1-PPARα pathway. Another study by (Mohamed et al., 2019) reported that it ameliorates high-fat diet-induced insulin resistance in the liver and improves testicular function. However, little is known about the mechanism of action of *Moringa oliefera* on palmitate induced insulin resistance in C2C12 skeletal muscle cells which mimic daily fat that we consume as humans. Hence this study aims to investigate the effect of *Moringa oliefera* on palmitate-induced insulin resistance on C2C12 skeletal muscle cells.

Aim

- To investigate the effect of *Moringa oliefera* extract on palmitate-induced insulin resistance in C2C12 skeletal muscle cells.

Objectives

- To investigate the bioactivity of *Moringa oliefera* in normal C2C12 skeletal muscle cells
- To investigate the effect of *Moringa oliefera* leaf extract on palmitate-induced insulin resistant C2C12 skeletal muscle cells at a cellular level
- To investigate the effect of the leaf extract on the expression of the insulin signalling and antioxidant genes (*Insr Pi3k*, *As160* and *Pkcθ, Sod1, Gss,*)**.**

CHAPTER 2

This chapter gives details on the background of the study as a literature review, this review was already published (Mthiyane et al., 2022, hence it is represented it is on the paper).

LITERATURE REVIEW

A review on the antidiabetic properties of *Moringa oleifera* extracts: Focusing on oxidative stress and inflammation as main therapeutic targets

Fikile T. Mthiyane[1], Phiwayinkosi V. Dludla[2], Khanyisani Ziqubu[1], Sinenhlanhla X.H. Mthembu[1,2], Ndivhuwo Muvhulawa[1], Nokulunga Hlengwa[3], Bongani B. Nkambule[4], Sithandiwe E. Mazibuko-Mbeje[1]

[1] Department of Biochemistry, North-West University, Mafikeng Campus, Mmabatho 2735, South Africa

[2] Biomedical Research and Innovation Platform, South African Medical Research Council, Tygerberg 7505, South Africa

[3] Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa,3886, South Africa.

[4] School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban 4000, South Africa.

Correspondence:

Sithandiwe E. Mazibuko-Mbeje, PhD

Department of Biochemistry, Faculty of Natural and Agricultural Sciences, North-West University Mafikeng Campus, Private X 2046, Mmabatho 2375, South Africa, Email: 36588296@nwu.ac.za. Tel.: +27 18 399 2854

Abstract

Natural plants have been used for centuries as medicine or for food consumption, in part due to their high nutritional content. *Moringa oleifera* is one of the popular plants that has shown significant health benefits. Certainly, preclinical evidence summarized in the current review supports the beneficial effects of *Moringa oleifera* leaf extracts in combating the prominent characteristic features of diabetes mellitus. This includes effective control of blood glucose or insulin levels, enhancement of insulin tissue sensitivity, improvement of blood lipid profiles, and protecting against organ damage under sustained conditions of hyperglycemia. Interestingly, as major complications implicated in the progression of diabetes, including organ damage, *Moringa oleifera* could efficiently block the detrimental effects of oxidative stress and inflammation in these preclinical models. Notably, these extracts showed enhanced effects in strengthening intracellular antioxidant defences like catalase, superoxide dismutase, and glutathione to lower lipid peroxidation products and reduce prominent pro-inflammatory markers such as tumor necrosis factor-α, interleukin (1L)-β, IL-6, monocyte chemoattractant protein-1 and nitric oxide synthase. Although limited clinical studies have been done on the antidiabetic properties of *Moringa*

oleifera, current findings provide an important platform for future research directed at developing this plant as a functional food to manage diabetic complications.

Keywords:

Diabetes; oxidative stress; inflammation*; Moringa oleifera*; antioxidants; therapeutic targets.

1. Introduction

According to the World Health Organization, diabetes mellitus is amongst the top 10 leading causes of mortality and morbidity around the world [1]. Diabetes is a metabolic disorder that is characterized by a state of hyperglycemia that occurs alongside dysregulations in insulin levels and in some cases, it arises concurrently to overweight and obesity [2]. Indeed, excessive body fat or obesity remains the major culprit in the development of type 2 diabetes (T2D), which is the predominant form of diabetes [2]. The rapid increase in cases of diabetes, especially T2D, raises concerns, also highlighting an urgent need to investigate effective therapies to curb this disease [3]. Accumulative research has focused on understanding the pathophysiological mechanisms implicated in diabetes-associated complications, which is essential to discover effective therapies that can improve metabolic function and prevent multiple organ failure in those affected by this condition [4-6].

As a prime example, inflammation and oxidative stress, which normally emerge as a result of an abnormal pro-inflammatory response, or an exacerbated production of free radical species are increasingly recognized as the key abnormalities implicated in the development and acceleration of diabetes-linked complications [4]. Notably, oxidative stress is linked with the activation of protein kinase C (PKC), which is normally consistent with impaired insulin signaling and tissue damage in experimental models of diabetes [4]. Importantly, this content supports the hypothesis by Randle and colleagues [7] which stated that alteration in the uptake and metabolism of glucose and free fatty acids may be an instrumental process in the pathogenesis of insulin resistance, the major characteristic feature of T2D. Indeed, many diverse biochemical mechanisms, extending beyond inflammation and oxidative stress or activation of PKC, are implicated in the development of T2D [4].

Literature suggests that effective modulation of energy metabolism and insulin signaling through the regulation of AMP-activated protein kinase (AMPK) or phosphoinositide 3-kinase/ protein kinase B (PI3K/AKT) pathways appears to reverse some devastating outcomes of diabetes [8-10]. In fact, natural plants and related bioactive compounds are increasingly screened for their antidiabetic properties. Some natural plants have shown anti diabetic properties through lowering blood glucose and modulation of AMPK/PI3K/AKT pathways [11-13]. Consistently, our group continues to review literature on the impact of plants like *Camellia sinensis*, including prominent bioactive compounds such as gallic acid and isoorientin for their ameliorative effect extending beyond regulation essential metabolic pathways [14-16].

Moringa oleifera is a medicinal plant that has gained a lot of interest for its diverse biological properties. Reviewed evidence indicates its biological capabilities expand to protecting against complications linked with heart disease, cancer, fatty liver, and diabetes [17-19]. Furthermore, a previously published review supports the beneficial effects of *Moringa oleifera* in improving blood glucose control in experimental models of diabetes [3]. While such information already affirms the hypoglycaemic potential of this medicinal plant, data regarding the prominent biochemical mechanisms implicated in the antidiabetic effects of *Moringa oleifera* have not been critically reviewed. There is less information documented on the mechanisms of action of *Moringa oleifera* on its protective effect against the diabetes and related complications. The current study provides a brief overview on *Moringa oleifera* as medicinal plant, followed by its therapeutic mechanisms in controlling diverse diabetic complications. Mostly focusing on

understanding the modulatory effects of this medicinal plant in mechanisms of inflammation and oxidative stress in a diabetic state.

This current review includes evidence that was obtained from a search done (from inception until end of December 2021) on major electronic databases/search engines such as PubMed, Google Scholar and ScienceDirect. The systematic search was conducted using the following Medical Subject Heading (MeSH) terms "*Moringa oleifera*", "diabetes mellitus", "glucose metabolism", "insulin resistance", "oxidative stress", and "inflammation" as well as relevant synonyms. EndNote20 desktop software (Elsevier, Amsterdam, The Netherlands) was used for references and identification of duplicated studies. Preclinical studies reporting on the mechanisms of *Moringa oleifera* in diabetes models and related metabolic syndrome was included in this review. However, review papers, and encyclopaedias were excluded but screened for primary findings.

2. An overview of *Moringa oleifera* and its diverse biological properties

Moringa oleifera (shown in Figure 1) is a fast-growing tree that is classified as a vegetable that also serves as a medicinal plant [20, 21]. This miracle tree originates from the sub-Himalayan parts of India and it can be grown in both tropical and subtropical regions and is able to withstand droughts and mild frosty weather, hence it can be cultivated anywhere in the world [22]. This plant has gained medical and socioeconomic popularity because it has shown great health benefit and it is easy to cultivate [23, 24]. Traditionally, it is used in diets to maintain healthy skin and it has also been used as a decoction to relieve stress and also provided energy [25, 26]. All the parts of the plant can be used in a diet or as medicine since they are rich in minerals, proteins, vitamins, polyphenols, flavonoids, glucosinolates, isothiocyanates, alkaloids [20, 21]. The leaves can be eaten raw, dried or taken as an infusion of an aqueous extract, while the bark is boiled in water or soaked in alcohol to make drinks and infusions that help with toothaches, stomach aches, the same is done to the roots [27]. Furthermore, the leaves are utilized the most for medicinal purposes and they are a great source of prominent anti-inflammatory and antioxidant flavonoids, namely myrecytin, quercetin and kaempferol [17]. Interestingly, these bioactive compounds are known to contain potential anticancer, hypolipidemic, hypotensive and antidiabetic properties, antioxidant and anti-inflammatory [17]. Other documented uses for this medicinal plant include its application as a diuretic, a testosterone stimulant, an antifungal and as an antibacterial [25, 26]. It can also be used to relieve a sore throat and relieve the symptoms of influenza and as an anti-inflammatory [25].

Certainly, evidence has grown that *Moringa oleifera* contains hypoglycemic effects in diabetic animal models, including its associated complications such as oxidative stress and inflammation [28-30].

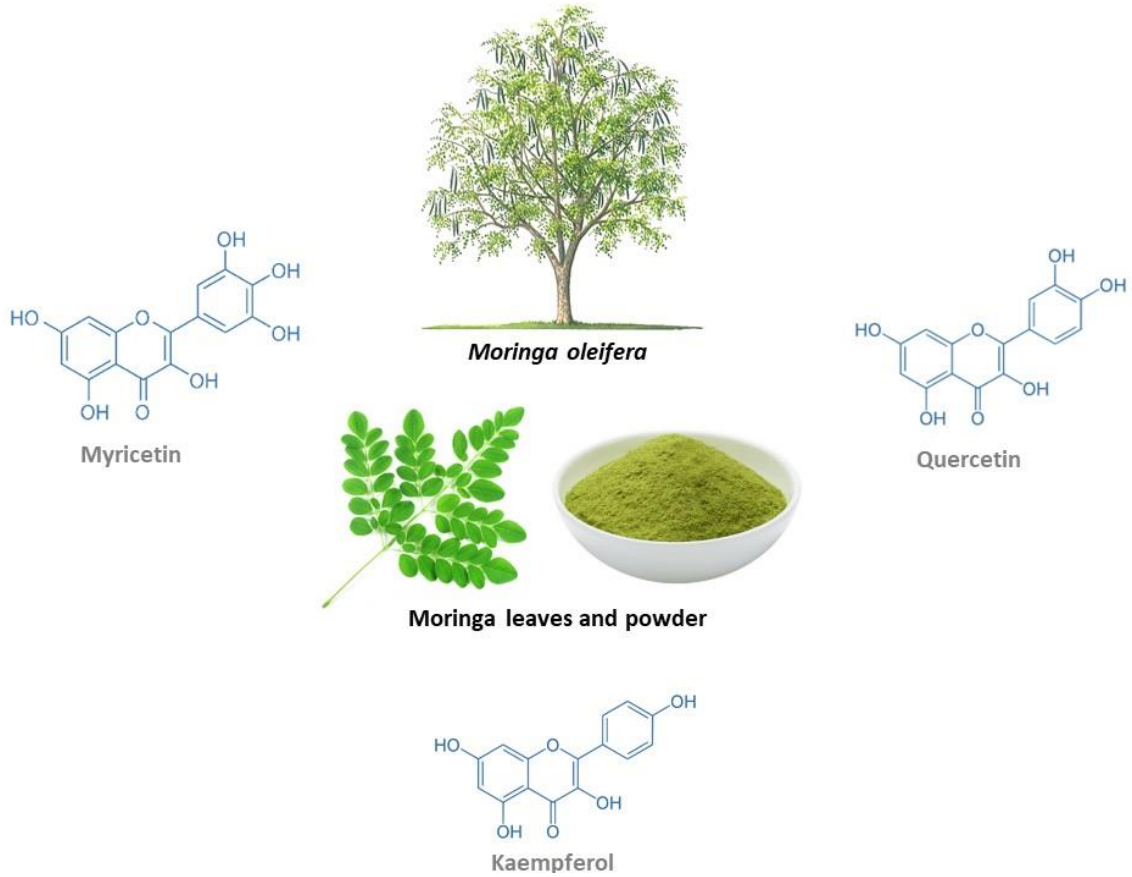


Figure 1. *Moringa oleifera* and chemical structures of its major flavonoids myricetin, quercetin and kaempferol.

3. Oxidative stress and inflammation as prominent mechanisms involved in diabetes-induced complications

Several pathophysiological mechanisms have been implicated in the aggravation of diabetes-related complications. For instance, individuals with T2D already present dyslipidemic features which is normally characterised by the increased accumulation of lipids and these can easily be attached by free radicals to generate oxidative products [31, 32]. This consequence is referred to as lipid peroxidation, and it remains as one of the vital parameters used to track the devastating outcomes of oxidative stress in conditions of diabetes or any related metabolic disease [33-35]. Within the pathological state, free radicals can be generated through impaired mitochondrial function, or enhanced activities of some enzyme complexes such as NADPH oxidases, in a process like oxidative stress that is known to deplete intracellular antioxidant systems [36, 37]. Generally, oxidative stress is generated as a disparity in the production of reactive oxygen species (ROS) or reactive nitrogen species, in comparison to counteractive activity of antioxidants in diabetes [38]. Some of the prominent free radical molecules include hydroxyl radical (•OH), superoxide anion ($O_2^{\bullet-}$), peroxynitrite ($ONOO^-$), and all these molecules are crucial for efficient metabolic process in a physiological state [39, 40]. Also, individuals with T2D display classic signatures of oxidative stress by presenting significantly decreased levels of antioxidant mechanisms such

as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and heme oxygenawe-1 (HO-1) [41-43]. In diabetes or related metabolic complications, uncontrolled ROS can induce damage to the lipids, proteins, and nucleic acids which lead to impaired signalling mechanisms and activation of pro-inflammatory response [39, 44]. Figure 2 gives an overview of some of the pathophysiological mechanisms implicating the detrimental effects of oxidative stress and inflammation during the pathogenesis of diabetes.

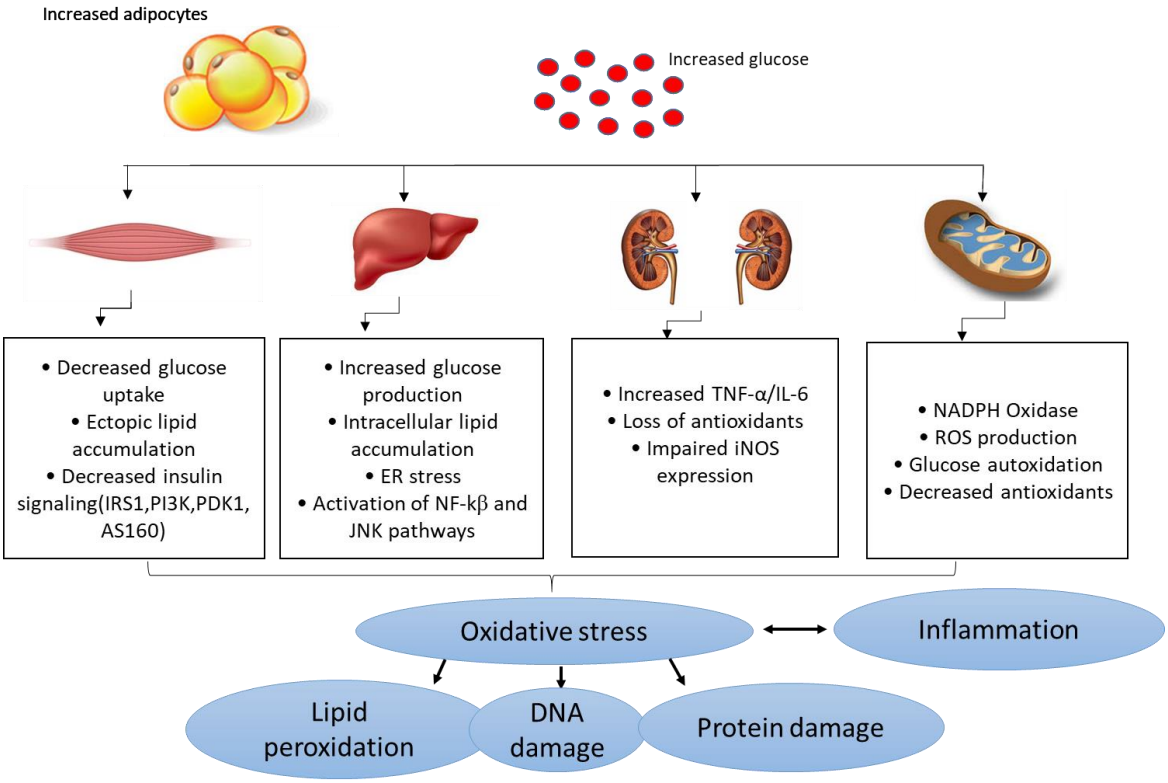


Figure 2. An overview of pathological mechanisms implicated in the development of diabetes mellitus. Briefly, overnutrition (characterized by increased adipocyte size) and consistent increased levels of glucose (a state of hyperglycemia) may induce detrimental effects in major organs of the body including the skeletal muscle, liver, and kidneys, through enhanced oxidative stress and exacerbated inflammation. This consequence is characterized by impaired glucose homeostasis/insulin signaling, ectopic lipid accumulation, mitochondrial dysfunction, endoplasmic reticulum (ER) stress insufficient or decreased antioxidant responses/increased ROS (reactive oxygen species) production and altered actions of inducible nitric oxide synthase (iNOS) and lipid peroxidation/DNA damage. Along with raised pro-inflammatory markers like tumor necrosis factor-alpha (TNF-α), like nuclear factor kappa β (NF-κβ), c-Jun N-terminal kinases (JNK) and interleukin-6 (IL-6).

Currently, both oxidative stress and inflammation have been subject to ongoing research to improve metabolic function in conditions of metabolic syndrome [45-47]. Also, accumulative research has evaluated the use of dietary compounds with antioxidant and anti-inflammatory effects such as salvianolic acid, aspalathin and resveratrol in combination with common drugs like metformin to lower glucose as well as attenuate the detrimental effects of oxidative stress and inflammation [48-50]. This has been an especially important aspect to uncover to improve the long-term protective effects of metformin. The

latter is the first line drug for diabetes which works by lowering blood glucose, body weight and lipid levels in the body it also mediates the activation of the AMPK pathway. On the other hand, other antidiabetic drugs like the thiozonidediones have been used to manage T2D, and function by activating peroxisome proliferator activated receptor gamma (PPARγ) and mediate adipogenesis and the uptake of fatty acids in the adipocytes [51]. This class of drugs improve insulin sensitivity by reducing the circulating fatty acids in the peripheral tissues and can control the production of hormones such as adiponectin to improve metabolic function [51]. However, like metformin, thiozonidediones are known to present various side effects [52], and their long-term protection effect against deteriorated metabolic function is not proven. This fact, has created opportunities to evaluate alternative regimes, including medicinal plants like *Moringa oleifera* with antioxidant and anti-inflammatory properties for their capacity to improve metabolic function in conditions of T2D or metabolic syndrome [53, 54]. This is especially important since most medicinal plants and bioactive compounds are known to play a major role in cellular detoxifying mechanisms, especially in part activating nuclear factor-erythroid factor 2-related factor 2 (Nrf2), the major antioxidant response element involved in the attenuation of oxidative stress and an undesired pro-inflammatory response in a disease state [55-57].

4. The antioxidant properties of *Moringa oleifera*

Antioxidants are important substances that aid in eliminating oxidizing agents. Any imbalance of antioxidants caused by oxidative stress may lead to tissue damage [58]. This may further prompt the disruption of lipids, membranes, nucleic acids and proteins which may further cause detrimental effect and metabolic complications [59, 60]. For years, the first line of drugs for metabolic complications such as diabetes and related metabolic disorders have been metformin, thiazolidinediones and rosiglitazone but literature has proven that plant polyphenols and their bioactive compounds play a role in alleviating diabetes, especially targeting oxidative stress and inflammation to promote human health [61-64]. For example, a study showed that *Moringa oleifera* has great scavenging activity, as measured through the DPPH (1,1-diphenyl-2-picrylhydrazyl (DPPH)-2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) [65]. As discussed below, preclinical evidence strongly suggests that this plant targets oxidative stress and inflammation biomarkers to attenuate diabetes and related metabolic complications without causing any adverse complications.

5. *Moringa oleifera* extracts improve markers of oxidative stress and inflammation in pre-clinical models of T1D

Type 1 diabetes mellitus represents approximately 10% of all diagnosed cases of diabetes mellitus, with abnormally increased glucose levels “a state of hyperglycemia” being the major culprit implicated in the detrimental effects associated with this condition [2]. Generally accepted as an autoimmune disease that is categorized by immune-mediated damage to pancreatic β-cells, a persistent state of hyperglycemia is accredited for the damaging effects on major organs of the body in those with T1D [66]. Accordingly, successful establishment of experimental models of T1D, characterized by chronic hyperglycemia, have been predominantly accomplished by employing chemicals that destroy the activity of pancreatic β-cells,

triggering impaired insulin secretion [67]. Persistent/sustained hyperglycemia is perhaps the main consequence responsible for major organ damage, especially through destructive mechanisms involving activation of oxidative stress and pro-inflammatory response [38]. Thus far, different animals, mostly rodents have been administering different chemicals such as streptozotocin and alloxan to generate preclinical models of T1D [67]. Despite their usefulness in understanding the pathogenesis of T1D, these experimental models have become relevant for screening novel drugs for their potential antidiabetic properties [68]. In fact, besides their potential capacity to reduce the abnormally elevated levels of glucose, increasing research has actively screened novel therapies for their ameliorative effects against oxidative stress and inflammation to alleviate organ damage within a state of T1D [69-71]. This has especially been relevant for plant sources like *Moringa oleifera*, with known antioxidant and anti-inflammatory properties [72].

Evidence summarized in Table 1 reports on the impact of *Moringa oleifera* extracts on modulating markers of oxidative stress and inflammation in pre-clinical models of T1D. Importantly, most of these findings indicate that chemical exposure to STZ and alloxan, followed by a state of hyperglycemia, remains a principal method used to induce T1D in these animals. Consequently, most studies showed that *Moringa oleifera* extracts (at varied doses between 100-300 mg/kg) could effectively ameliorate hyperglycemia, when used for a period starting from 2 weeks [73, 74], to an average time of 6-weeks [75, 76], or even in treatments lasting 8-weeks [77, 78]. Interestingly, treatment with *Moringa oleifera* leaf extracts proved effective in wound healing and tissue regeneration in animals exposed to sustained levels of hyperglycemia, when used for an estimated time of 3 weeks [79, 80]. In addition to wound healing properties, the extracts *Moringa oleifera* showed enhanced protective effects against damage to various organs, including the liver and kidneys, in these preclinical models of T1D [76, 81-83]. The antidiabetic properties of these extracts extend to preventing cognitive or erectile dysfunction in rats, by mainly reducing the activities of enzymes like acetylcholinesterase, angiotensin-I converting enzyme and butyrylcholinesterase [74, 84]. Apparently, the ameliorative effects against oxidative stress or undesired pro-inflammatory response remain the predominant mechanisms by which *Moringa oleifera* extracts protect against complications of T1D in preclinical models.

For instance, through blockade of lipid peroxidation as well as by reinforcing intracellular antioxidant capacity, as demonstrated by reduced levels of peroxidation products like MDA/ thiobarbituric acid reactive species (TBARS) and elevated antioxidant defences such as SOD, GSH, GST and CAT, *Moringa oleifera* extracts showed enhanced effects in protecting against the detrimental effects of oxidative stress in preclinical models of T1D [77, 85-87]. In support of this mechanistic insight, it has long been established that induction of diabetes in rats with STZ or alloxan favors uncontrolled availability of lipid peroxidation products, while consequently suppressing the intracellular antioxidant defences [88, 89]. This process prompts excess free radical production, as also observed in patients with T1D [90], while the accompanied hyperglycemic state may directly contribute to oxidative stress-induced organ damage [88]. Besides the harmful effects associated with lipid peroxidation, evidence summarized in Table 1 indicates that treatment with *Moringa oleifera* extracts for 3 weeks remains effective in targeting other sources of oxidative stress like the mitochondria to ease complications linked with T1D. In actual fact, Alejandra Sánchez-Muñoz and colleagues showed that a leaf extract of this plant improved mitochondrial respiration, while increasing levels of intracellular antioxidants like GSH, glutathione reductase and HO-1 activity, to reduce excess production of ROS liver cells of STZ-induced diabetic rats [91]. Generally,

these results are of interest as many studies indicate that mitochondria remain one of the major therapeutic targets to ameliorate hyperglycemia-induced oxidative damage [38, 92, 93].

Consistent with attenuating the destructive effects of oxidative stress, presented evidence showed that *Moringa oleifera* extracts could effectively reduce the elevated levels of pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, cyclooxygenase-2 (COX-2), nitric oxide synthase (iNOS), and chemokine (MCP-1) to protect against exacerbated inflammation, under sustained conditions of hyperglycemia [76, 79, 80]. Significantly, such positive effects were associated with reduced nephrotoxic and hepatotoxic damage [83], including upregulation of an angiogenic marker vascular endothelial growth factor (VEGF) to protect against hyperglycemia-induced wound injury in a preclinical model of T1D [79]. This is an essential result since tenacious hyperglycemia, seen in T1D, is already known to mediate iNOS induction, leading to the activation of protein kinase enzymes such as PKC/ c-Jun N-terminal kinases (JNKs)/ mitogen-activated protein kinase (MAPK) to propagate the detrimental effects of inflammation [38]. Notably, activation of such pro-inflammatory mechanisms can directly cause excess generation of oxidation products that precede the onset of atherosclerosis and endothelial dysfunction, which are major risk factors for the development of cardiovascular diseases [94, 95]. Although there was limited information on its cardioprotective effects, much evidence suggests *Moringa oleifera* extracts can significantly decrease pro-inflammatory and apoptotic markers such as TNF-α, IL-1β, IL-6, NF-κβ, caspase 3, caspase 9, and tumor protein (p53) to alleviate the detrimental effects of hyperglycemia in preclinical models of T1D [76, 79, 80, 83]. Overall, summarized evidence supports the beneficial effects of *Moringa oleifera* in lowering hyperglycemia in addition to ameliorating the detrimental effects of oxidative stress and inflammation in preclinical models of T1D. Some other takeaways from the current results indicate that most studies observed therapeutic effects when using doses between 100-300 mg/kg [73, 80, 86, 91], with an average dose of 250 mg/kg commonly exploited [75, 76, 82, 87]. Also, most studies reported on the use of leaf extracts over seed extracts of this plant (Table 1). This could be supported by available evidence already supporting the strong antioxidant properties of leaf extracts of over seed extracts [96, 97].

Table 1: Studies on the effect of *Moringa oleifera* extracts targeting markers of oxidative stress and inflammation in preclinical models of type 1 diabetes.

Author, year	Experimental model, effective dose and intervention period	Experimental outcome
Jaiswal et al., 2013 [85]	Streptozotocin (STZ)-induced diabetes in Wistar rats treated with 200 mg/kg *Moringa oleifera* leaf extract for 3 weeks	Ameliorated oxidative stress by significantly increasing the antioxidant activity of superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT) while decreasing the lipid peroxide levels
Yassa and Tohamy, 2014 [77]	STZ-induced diabetes in Sprague Dawley rats treated with 200 mg/kg *Moringa oleifera* extract for 8 weeks	Lowered fasting plasma glucose (FPG) levels, reversed pancreatic damage, while also enhancing glutathione (GSH) and reducing malondialdehyde (MDA) pancreatic concentrations
Al-Malki et al., 2015 [86]	STZ-induced diabetes in albino rats were treated with 50 and 100 mg/kg with *Moringa oleifera* seed extract for 4 weeks	Decreased FPG, and increased the concentration of antioxidants like SOD, CAT and GSH in serum and kidney. Moreover, treatment decreased the concentration of interleukin (IL)-6 and lipid peroxides (MDA) in the serum and kidney tissue homogenate
Muhammad et al., 2016 [79]	STZ-nicotinamide induced diabetes in Wistar rats treated with 0.5%,1% and 2% w/w *Moringa oleifera* leaf aqueous fractions for 3 weeks	Decreased wound size under sustained hyperglycemic condition and improved wound contraction, and tissue regeneration. This was associated with reduced inflammatory mediator such as tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, cyclooxygenase-2 (COX-2), nitric oxide synthase (iNOS) and upregulation of an angiogenic marker vascular endothelial growth factor in wound tissue
Tuorkey, 2016 [73]	Alloxan-induced diabetes albino mice treated with 100 mg/kg of *Moringa oleifera* aqueous extract 2 weeks	Significantly decreased FPG and plasma insulin levels, concomitant to reversing insulin resistance. Total antioxidant capacity increased while the levels of creatinine and urea significantly declined. While cluster of differentiation (CD)44 was not changed, CD69 and interferon gamma I (NF-γ) were increased by treatment
Abd Eldaim et al., 2017 [82]	Alloxan-induced diabetes in Wistar rats treated with 250 mg/kg *Moringa oleifera* leaf extract for 2.5 weeks	Prevented hepatic damage and normalized the reduced hepatic levels of glutathione (GSH), as well as the activities of SOD and CAT, while also reducing blood glucose levels, hepatic lipid peroxidation

Omodanisi et al., 2017 [75]	STZ-induced diabetes in Wistar rats treated with 250 mg/kg *Moringa oleifera* leaf extract for 6 weeks	Reduced FPG and biomarkers of oxidative stress and inflammation. Specifically, reduced the level of lipid peroxidation (MDA), and improved antioxidant such as CAT, SOD, GSH, glutathione peroxidase (GPx), whilst decreasing pro-inflammatory makers such as tumor necrosis factor-alpha (TNF-α) and IL-6
Omodanisi et al., 2017 [76]	STZ-induced diabetes in Wistar rats treated with 250 mg/kg *Moringa oleifera* aqueous extract for 6 weeks	Reduced hepatic enzyme markers and normalized lipid profile parameters, while enhancing antioxidant capacity and alleviating inflammatory biomarkers of the liver. Specifically, reduced levels of MDA and increased endogenous antioxidants (SOD, CAT, GSH, GPx), while decreased inflammatory cytokines (IL-1α, IL-6, IL-12, IL-18, TNF-α) and (chemokine: MCP-1) in the serum, liver; kidney and erythrocytes
Raafat and Hdaib, 2017 [87]	Alloxan-induced diabetes albino rats treated with 250 mg/kg *Moringa oleifera* leaf extract for 2.5 weeks Alloxan-induced diabetes Swiss-Webster mice treated with 40,60 and 80 μg/mL *Moringa oleifera* seed extract for 8 weeks	Reduced FPG and hindered lipid peroxidation, whilst increasing hepatic GSH levels, as well as the activities of SOD and CAT, and the gene expression of glycogen synthase while reducing pyruvate carboxylase caspase 3 gene expression Decreased FPG and inhibited alpha glucosidase activity. Serum insulin levels and serum CAT levels were significantly increased whilst lipid peroxidation and glycated hemoglobin (HbA1C) were decreased
Alejandra Sánchez-Muñoz et al., 2018 [91]	STZ-induced diabetes in Wistar rats treated with 200 mg/kg *Moringa oleifera* leaf extract for 3 weeks	Ameliorated oxidative stress-induced modification in liver mitochondria, in part by improving mitochondrial respiration, as well as enhancing the antioxidant levels of GSH, glutathione reductase and heme oygenase-1 (HO-1) activity, which decreasing lipid peroxidation (MDA) and production of reactive oxygen species
Azevedo et al., 2018 [80]	STZ-induced diabetes in Wistar adult rats were treated with 100 mg/kg of *Moringa oleifera* leaf extract for 3 weeks	Displayed wound healing properties and significantly reduced glycemia accompanied by a decreased in pro-inflammatory markers such as TNF-α, IL-β and IL-6 in the serum

Oboh et al., 2018 [74]	STZ-induced diabetes in Wistar rats treated with 2% and 4% *Moringa oleifera* leaf and seed extracts for 2 weeks	Both extracts reduced FPG, prevented cognitive dysfunction-induced by chronic hyperglycemia by reducing the activities of acetylcholinesterase, angiotensin-I converting enzyme and butyrylcholinesterase. This was concomitant to the increase in antioxidant molecules such as CAT, GST and GPx, as well as a decrease in lipid peroxidation (MDA) level in the brain
Aju et al., 2019 [78, 98]	STZ-induced diabetes in Sprague-Dawley rats treated with 300 mg/kg body weight leaf (methanol) extract for 8.5 weeks	Significantly decreased FPG and glycated hemoglobin but increased plasma insulin levels. The antioxidant enzymes like SOD, CAT, GPx and glutathione-reductase and non-enzymatic antioxidant GSH were increased causing a decrease in hydroperoxides, conjugated dienes and lipid peroxidation
Oguntibeju et al., 2020 [83]	STZ-induced diabetes Wistar rats treated with 250 mg/kg leaf extract of *Moringa oleifera* for 6 weeks	Reduced nephrotoxic and hepatotoxic damage evident by a decrease in serum creatinine, albumin and bilirubin. Likewise, the inflammatory cytokines interleukin (IL)-1α, IL-12 and IL-18, and apoptotic markers caspase 3, caspase 9, B-cell lymphoma 2(BCL-2), NF-κβ, and p53 were decreased
Sierra-Campos et al., 2020 [99]	Alloxan-induced diabetes in Wistar rats treated with 200 mg/kg leaf extract of *Moringa oleifera* for 3 weeks	Displayed antidiabetic effects by increasing the levels of serum paraoxonase 1 and liver cytosolic CAT
Oldoni et al., 2021 [81]	STZ-induced diabetes in Wistar rats treated with 500 mg/kg crude leaf extract of *Moringa oleifera* (hydroalcoholic extract was produced by using ethanol: water (80:20 v/v)) for 6.5 weeks	Reduced FPG and protected against oxidative damage in liver and kidney by enhancing endogenous antioxidant defenses such as CAT, GST and non-protein thiol groups, while reducing lipid peroxidation in these tissues
Oyeleye et al., 2021 [84]	STZ-induced diabetes in Wistar rats treated with 2 and 4% of *Moringa oleifera* leaf and seed extracts for 2 weeks	Both extracts reversed diabetes-induced erectile dysfunction by reducing FPG, as well as blocking lipid peroxidation by decreasing thiobarbituric acid reactive species (TBARS) levels. Treatments also attenuated the activity of phosphodiesterase type 5 (PDE-5) and arginase but improved the levels of nitric oxide

6. Moringa oleifera extracts improve markers of oxidative stress and inflammation in pre-clinical models of T2D

Type 2 diabetes, remains the major form of diabetes, contributing to approximately 90% to all global cases of this condition, as regularly reported by the International Diabetes Federation [2]. Modifiable risk factors, mostly involving sedentary lifestyle, taking place together with overnutrition are to blame for increased cases of T2D, as these factors cause overweight and obesity [100]. In such conditions, increased nutrient availability may drive excessive fat accumulation in key body areas such as the liver, skeletal muscle, blood circulation and heart muscle, leading to the development of pathological complications like non-alcoholic fatty liver disease, muscle wasting or sarcopenia and cardiovascular diseases [100, 101]. Just like in T1D, hyperglycemia remains the major pathological feature of T2D. Besides hyperglycemia, patients with T2D are known to present with insulin resistance and a cluster of other irregularities such as abnormal blood lipid profiles, as observed through aberrant levels of triglycerides, total cholesterol, and low-density lipoprotein cholesterol [102, 103]. However, defects in insulin signaling or a state of insulin resistance has been seen as an early sign of T2D manifestation, which is likely to occur simultaneous to other metabolic dysregulations, including enhanced inflammatory signaling, generation of oxidative products and initiation of endoplasmic reticulum (ER) stress pathways [104]. These are among the most explored pathological mechanisms in preclinical models of T2D. For example, rodents exposed to a high fat diet (HFD) or its combination with low dose STZ [30, 105-108], as well as gene-defiant mice such as those considered leptin resistance (*db/db*) [109] are known to progressively develop T2D, including its complications involving oxidative stress and inflammation. This explains, the surge in use of these preclinical models to test novel treatments against T2D.

Table 2 gives an overview of information on the antidiabetic properties of *Moringa oleifera* extracts, including its modulatory effects on markers of oxidative stress and inflammation in pre-clinical models of T2D. Most importantly, summarized evidence showed that these extracts were effective in reducing body weight, body fat mass and fasting plasma glucose levels, which are the major characteristic features of T2D [106, 108, 109]. Consistent with evidence summarized in Table 1, blocking hepatic lipid accumulation, in part through effectively modulating the makers of oxidative stress and inflammation such as antioxidants like CAT, SOD, MDA content, uncoupling protein 2/3, TNF-α, 1L-β, IL-6, IL-2 and MCP-1 remains the major mechanism of action of these extracts [107, 110]. Some evidence showed these extracts could improve lipid profiles and reduce the expression of genes involved in energy metabolism or fat synthesis such as fatty-acid synthase, lipoprotein lipase, CCAAT-enhancer-binding protein homologous-α (C/EBPα), sterol regulatory element-binding protein1c (SREBP1c), within the skeletal muscle [109, 110]. Partially indicating that *Moringa oleifera* extracts may be a potent remedy to decrease excess body fat or ameliorate complications linked with obesity, as reviewed elsewhere [111]. Of further interest, some evidence indicated that *Moringa oleifera* extracts could target early pathological signs of T2D, such as improving glucose tolerance and insulin levels, while enhancing insulin sensitivity and glucose homeostasis in tissues of these preclinical models [106, 107]. This hypothesis remains to be confirmed in other experimental models of T2D, however provides necessary information to guide future research.

Table 2: An overview of studies on the effects of *Moringa oleifera* extracts targeting markers of oxidative stress and inflammation in preclinical models of type 2 diabetes.

Author, year	Experimental model, effective dose and intervention period	Experimental outcome
Waterman et al., 2015 [105]	High fat diet (HFD)-fed C57BL/6L mice treated with 5% *Moringa oleifera* concentrate (delivering 66 mg/kg/d of moringa isothiocyanates)	Improved glucose tolerance and insulin signaling and did not develop fatty liver disease. Treatment also reduced plasma insulin, leptin, resistin, cholesterol, interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), and lowered hepatic glucose-6-phosphatase expression
Joung et al., 2017 [110]	HFD-induced glucose intolerance C57BL/6 mice treated with 250 mg/kg *Moringa oleifera* leaf extract for 10 weeks	Did not affect body weights but reduced hepatic lipid accumulation. Also, reduced HFD-induced endoplasmic reticulum stress, oxidative stress, and lipotoxicity in quadriceps muscles. Reduced the expression of genes involved in energy metabolism such as fatty-acid synthase, lipoprotein lipase, CCAAT-enhancer-binding protein homologous-α (C/EBPα), sterol regulatory element-binding protein 1c (SREBP1c), within the skeletal muscle. Oxidative and inflammatory markers such as uncoupling protein 2/3, TNF-α, 1L-β, IL-6, IL-2 and monocyte chemoattractant protein-1 (MCP-1) were improved
Tang et al., 2017 [109]	Type 2 diabetic (*db/db*) mice treated with 150 mg/kg *Moringa oleifera* leaf ethanolic extract for 5 weeks	Reduced fasting plasma glucose (FPG) and increased insulin levels, while improving lipid profiles by decreasing concentrations of triglycerides and low-density lipoprotein. Also, protected against renal damage by decreasing pro-inflammatory markers such as TNF-α, IL-1β, IL-6, cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) in renal tissue
Jaja-Chimedza et al., 2018 [106]	HFD-induced obese C57Bl/6J mice treated with *Moringa oleifera* seed, containing 0.54 and 0.73% of extract supplemented in diet for 12 weeks	Reduced body weight, decreased adiposity, improved glucose tolerance, decreased inflammatory gene expression, and increased antioxidant gene expression. Specific, inflammatory genes that were decreased included IL-1β, IL-6 and TNF-α, while oxidative genes improved included iNOS and NADPH dehydrogenase [quinone] 1 (NQO1), in some of the tissues (liver, jejunum, ileum and colon)
Chin et al., 2019 [30]	HFD and streptozotocin-induced diabetes in Sprague Dawley rats treated with 0.5% standardized aqueous *Moringa oleifera* leaf	Significantly improved wound healing, and this was in part by effective modulation of pro-inflammatory markers and growth factors including TNF-α, IL-6, MCP-1, vascular endothelial growth factor, epidermal growth factor in the wound site

	extract-loaded hydrocolloid film for 3 weeks	
Mohamed et al., 2019 [107]	HFD-induced insulin resistant in Sprague Dawley rats treated with 300 mg/kg *Moringa oleifera* aqueous extract for 4 weeks	Reversed hepatic insulin insensitivity and this was linked to up-regulation of genes involved in insulin receptors and glucose uptake such as insulin receptor, insulin receptor substrate-1 and glucose transporter (GLUT) 4. Also improved hepatic antioxidants like catalase (CAT) and superoxide dismutase (SOD), while decreasing level of lipid peroxidation, the malonaldehyde (MDA) content
El-Shehawi et al., 2021 [108]	HFD-induced obese Wistar rats treated with 300 mg/kg *Moringa oleifera* leaf extract for 14 weeks	Reduced body weight and body fat mass, while also decreasing FPG, insulin, and leptin levels, while increased adiponectin. Consistently, treatment improved lipid profiles like serum total cholesterol, triacylglycerol, and low-density lipoprotein, while enhancing hepatic antioxidant enzymes such as SOD, CAT. Lipid peroxidation (MDA) and some pro-inflammatory markers like nuclear factor kappa β (NF-κβ)- P65 were decreased

7. Clinical translation

Preclinical studies are important to understand the pathophysiological mechanisms implicated in disease development, and this aspect remains essential to explore the therapeutic potential of plant extracts and their derivative compounds [112, 113]. Whereas screening plant extracts and their biological compounds using preclinical models has become a routine procedure to determine effective doses, pharmacokinetic profile and evaluate relevant toxicological aspects before commencement of clinical trials [112, 113]. Although accumulative literature supports the beneficial effects of plant extracts and naturally derived compounds against diabetes [114, 115], persistent setbacks have been the limited number of studies entering clinical trial phase, which is a vital component in drug development. Likewise, there has been a limited number of studies on the antidiabetic properties of *Moringa oleifera* extracts. At present, only three randomized controlled trials have been published on the antidiabetic potential of *Moringa oleifera*. In 2016, Anthanont and co-workers showed that capsules of *Moringa oleifera* leaf powder (at a dose of 4 g), taken after an overnight fast and every two weeks, could significantly increase insulin secretion in healthy subjects [116]. Leone and colleagues demonstrated that randomly giving Saharawi people with diabetes a traditional meal supplemented with 20 g leaf powder of *Moringa oleifera* on 2 different days could improve postprandial blood glucose response when compared to nondiabetic controls [117]. While Gómez-Martínez and co-workers reported that giving subjects with prediabetes six daily capsules of *Moringa oleifera* leaf powder (2400 mg/day) improved fasting blood glucose and glycated hemoglobin (HbA1c) when compared to the controls. These results are consistent with some preclinical findings [106, 107], indicating that *Moringa oleifera* leaf extracts might be useful to treat complications identified during the early development of T2D. Nonetheless, despite accumulative literature, as reviewed elsewhere [118, 119], indicating that this plant might present with important antidiabetic properties, more needs to be done to confirm these in clinical settings. It remains crucial, to evaluate whether *Moringa oleifera* leaf extracts can alleviate oxidative stress and inflammation, to verify preclinical findings.

8. Summary and future perspective

The swift prevalence of diabetes warrants urgent investigation into novel therapies to protect and better manage this chronic medical condition [2]. Metformin and insulin, which are commonly used antidiabetic therapies, have certainly prolonged the lives of patients with diabetes [120-122]. Correspondingly, other effective interventions like physical exercise and caloric restriction can be used to manage diabetes [123, 124], however only a few individuals can constantly adhere to such strenuous interventions. Besides lowering glucose or improving insulin sensitivity, it has become imperative to uncover therapies that can target the amelioration of both oxidative stress and inflammation, as the prime dysregulations implicated in the pathogenesis of diabetes [125-127]. This also explains the surge in research investigating the biological properties of nutritional plant sources like rooibos (*Aspalathus linearis*) and broccoli (*Brassica oleracea var. italica*) with abundant antioxidants properties in combating metabolic complications like oxidative stress and inflammation [93, 128].

Plants have been studied for their therapeutic properties and they are also cheap, easily accessible and safer than synthetic conventional drugs [3]. There is growing evidence that plants not only serve as a food source but as medicine, nutraceuticals and so forth [24]. Also they contain a body of polyphenols,

vitamins, flavonoids, alkaloids and other important phytochemicals. *Moringa oleifera* has been proven in a number of studies to alleviate insulin resistance by activating the insulin-independent pathway PI3K/AKT and also through AMPK pathway in the skeletal muscle and it can also improve skeletal muscle oxidative metabolism through the NAD-dependent deacetylase (SIRT1)-PPARα pathway and also through improving fatty acid peroxidation [28, 129].

In fact, overwhelming evidence summarized in this review supports the beneficial effects of *Moringa oleifera* in improving blood glucose levels, lipid profiles and insulin sensitivity, in addition to protecting against hepatic or nephrotic damage in preclinical models of T1D/T2D (Table 1 and 2). Interestingly, these extracts show enhanced effects in strengthening intracellular antioxidants like CAT, SOD, GSH, and GST to lower lipid peroxidation products MDA/TBARS, and reduce prominent pro-inflammatory markers like TNF-α, 1L-β, IL-6, MCP-1, COX-2, and nitric oxide synthase in these models. Figure 3 summarizes some therapeutic effects in protecting against oxidative stress and inflammation associated with *Moringa oleifera* extracts in preclinical models of diabetes. Furthermore, the current literature review indicates the common use of leaf extracts of *Moringa oleifera*, within a range 100-300 mg/kg, from initial treatment duration of 2 weeks up until 8 weeks (Table 1 and 2). This further sets a platform for future research (which is currently limited) directed at developing *Moringa oleifera* as a functional food to manage diabetes mellitus.

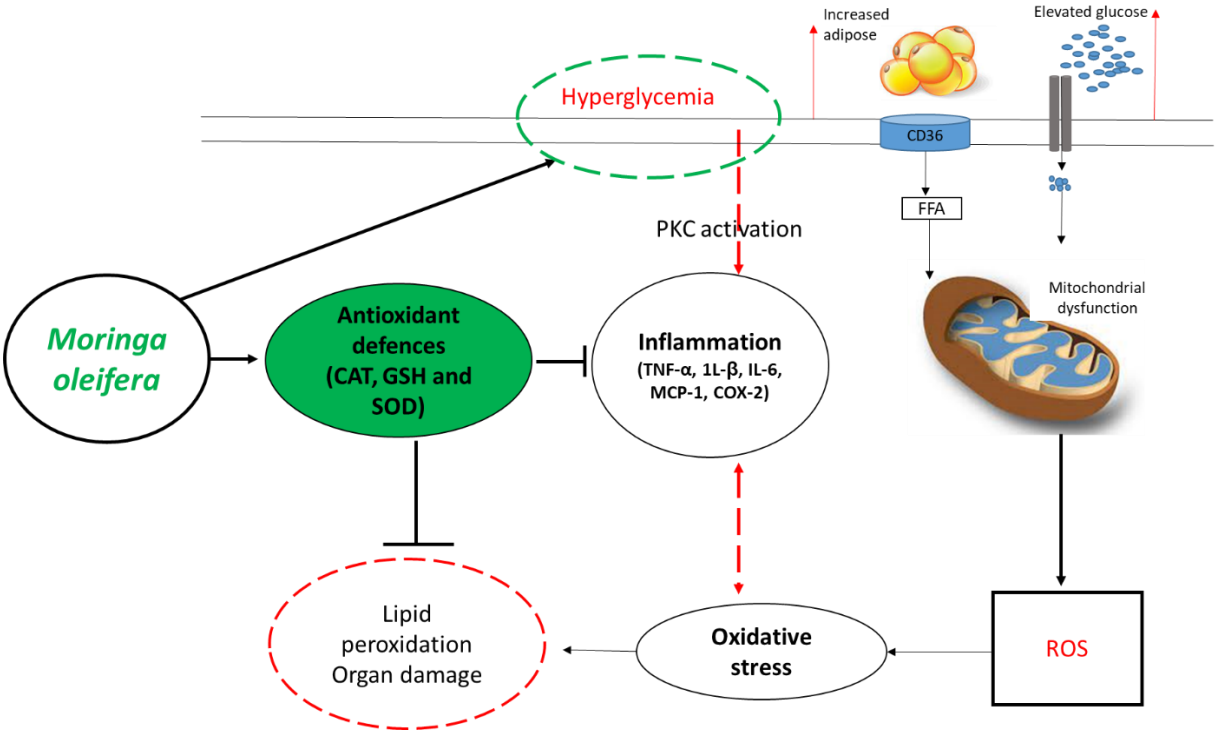


Figure 3. An overview of therapeutic mechanisms associated with the ameliorative effects of *Moringa oleifera* extracts in preclinical models of diabetes. Briefly, overwhelming evidence supports the beneficial effects of this plant in enhancing intracellular antioxidants such as catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD) to block the detrimental effects reactive oxygen species (ROS), lipid peroxidation and organ damage. This is in part by also improving glucose control (hyperglycemia) and reducing prominent pro-inflammatory markers like tumor necrosis factor-alpha (TNF-α), interleukin (1L)-β, IL-6, monocyte chemoattractant protein-1 (MCP-1) and COX-2-cyclooxygenase-2 (COX-2).

Abbreviations: CD36-cluster of differentiation 36; FFA-free fatty acid; PKC-protein kinase C. Indicators: red lines-detrimental effects, bold lines/green lines-protective effects of *Moringa oleifera* extracts.

Funding statement

This work was funded by the National Research Foundation (NRF) of South Africa Thuthuka Programme grant 128296 and NRF support for rated scientist 113674 and SAMRC EIP grant to SE Mazibuko-Mbeje. Baseline funding from the Biomedical Research and Innovation Platform of the South African Medical Research Council (SAMRC) and Northwest University is also acknowledged. Grant holders acknowledge that opinions, findings and conclusions, or recommendations expressed in any publication generated by the NRF or SAMRC-supported research are those of the authors and that the NRF or SAMRC accepts no liability whatsoever in this regard.

Abbreviations

AMPK-AMP-activated protein kinase, **AKT**-protein kinase B, **ACOX1**-peroxisomal acyl-CoA oxidase 1, **BCL-2**-B-cell lymphoma 2, **CAT**-catalase, **C/EBPα**-enhancer-binding protein, **COX-2**-cyclooxygenase-2, **GPx**-glutathione reductase, **GST**-glutathione-S-transferase, **GLUT4**-glucose transporter 4, **GSH**-glutathione, **HbA1C**-glycated hemoglobin, **HO-1**-heme oygenase-1, **NJK**-c-Jun N-terminal kinases, **MDA**-malonaldehyde, **iNOS**- inducible nitric oxide synthase, **INF-γ**-interferon gamma,IL-interleukin, **MAPK**- mitogen-activated protein kinase, **MCP-1**-monocyte chemoattractant protein-1, **NF-κβ**-nuclear factor kappa β, **T1D/T1D**-type 1/2 diabetes mellitus, **TNF-α**-tumor necrosis factor alpha, **TBARS**- thiobarbituric acid reactive species, **PDE-5**-phosphodiesterase type 5, **PKC**-protein kinase C, **PPARγ**-peroxisome proliferator-activated receptor gamma, **SIRT1**-sirtuin 1, **NAD**-dependent deacetylase, **SOD**-superoxide dismutase, **SREBP1c**-sterol regulatory element-binding protein 1c, **VEGF**-vascular endothelial growth factor.

Acknowledgements

The work reported herein was made possible through funding by the South African Medical Research Council through its division of Research Capacity Development under the Early Investigators Programme from funding received from the South African National Treasury. The content hereof is the sole responsibility of the authors and do not necessarily represent the official views of the SAMRC. The North-West University, and the University of Zululand is also acknowledged. All the content expressed in this review is the official views of the authors and do not represent that of the North-West University.

Declaration of competing interest

The authors declare no conflict of interest.

Author’s contribution

F.T. Mthiyane and S.E. Mazibuko-Mbeje–concept and original draft. All authors, including F.T. Mthiyane P.V. Dludla, K. Ziqubu, S.X.H. Mthembu, N. Muvhulawa, N. Hlengwa, B.B. Nkambule and S.E. Mazibuko-Mbeje wrote and approved the final manuscript.

Data availability statement

All the data used to support the findings of this study are included within the article. The raw data can be available on request after the publication.

References

1. World Health Organization (WHO), The top ten leading causes of death. Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death. Accessed 02 January 2022.
2. International Diabetes Federation (IDF), IDF Diabetes Atlas 2021. Available at: https://diabetesatlas.org/atlas/tenth-edition/. Accessed 03 January 2022.
3. Ahmad, J., I. Khan, I., and R. Blundell, R. Moringa oleifera and glycemic control: A review of current evidence and possible mechanisms. "Moringa oleifera and glycemic control: A review of current evidence and possible mechanisms." Phytotherapy research, 2019. 33(11): p. 2841-2848.
4. King, G.L., and Brownlee, M. The cellular and molecular mechanisms of diabetic complications. Endocrinology and Metabolism Clinics. 1996. 25(2): p. 255-70.
5. Fowler, M.J. Diabetes: Magnitude and Mechanisms. 2007. 25(1): p. 25-28.
6. Wei Q, Qi, L., Lin, H., Liu, D., Zhu, X., Dai, Y., Waldron, R.T., Lugea, A., Goodarzi, M.O., Pandol, S.J., Li, L. Pathological Mechanisms in Diabetes of the Exocrine Pancreas: What's Known and What's to Know. Frontiers in Physiology. 2020. 11: p. 1394-1394.
7. Randle, P.J., Garland, P.B., Hales, C.N., Newsholme, E.A. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus The Lancet. 1963. 281(7285): p. 785-789.
8. Huang, X., Liu, G., Guo, J., & Su, Z. The PI3K/AKT pathway in obesity and type 2 diabetes. International journal of biological sciences, 2018. 14(11): p. 1483-1496.
9. Long, Y.C., and Zierath, J.R. AMP-activated protein kinase signaling in metabolic regulation. Journal Clinical Investigation, 2006. 116(7): p. 1776-83.
10. Mazibuko-Mbeje, S. E., Dludla, P. V., Nkambule, B. B., Obonye, N., & Louw, J. The role of glucose and fatty acid metabolism in the development of insulin resistance in skeletal muscle. Muscle cell and tissue-current status of research field. 2018
11. Francini, F., Schinella, G.R., and Ríos, J.L. Activation of AMPK by Medicinal Plants and Natural Products: Its Role in Type 2 Diabetes Mellitus. Mini Reviews in Medicinal Chemistry 2019. 19(11): p. 880-901.
12. Mazibuko-Mbeje SE, Dludla PV, Roux C, Johnson R, Ghoor S, Joubert E, Louw J, Opoku AR, Muller CJ. Aspalathin-Enriched Green Rooibos Extract Reduces Hepatic Insulin Resistance by Modulating PI3K/AKT and AMPK Pathways. International Journal of Molecular Sciences. 2019. 20(3).
13. Costa, I. S., Medeiros, A. F., Piuvezam, G., Medeiros, G. C., Maciel, B. L., & Morais, A. H. A. Insulin-Like Proteins in Plant Sources: A Systematic Review. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. 2020. 13: p. 3421-3431.
14. Dludla, P.V., Nkambule, B.B., Jack, B., Mkandla, Z., Mutize, T., Silvestri, S., Orlando, P., Tiano, L., Louw, J., and Mazibuko-Mbeje, S.E. Inflammation and Oxidative Stress in an Obese State and the Protective Effects of Gallic Acid. Nutrients. 2019. 11(1): p. 23.
15. Ziqubu, K., Dludla, P.V., Joubert, E., Muller, C.J., Louw, J., Tiano, L., Nkambule, B.B., Kappo, A.P., and Mazibuko-Mbeje, S.E. Isoorientin: A dietary flavone with the potential to ameliorate diverse metabolic complications. Pharmacological Research, 2020. 158: p. 104867.
16. Dludla, P.V., Nkambule, B.B., Mazibuko-Mbeje, S.E., Nyambuya, T.M., Mxinwa, V., Mokgalaboni, K., Ziqubu, K., Cirilli, I., Marcheggiani, F., Louw, J., and Tiano, L. Adipokines as a

therapeutic target by metformin to improve metabolic function: A systematic review of randomized controlled trials. Pharmacological research. 2021. 163: p. 105219.
17. Vergara-Jimenez, M., Almatrafi, M.M., and Fernandez, M.L. Bioactive Components in Moringa Oleifera Leaves Protect against Chronic Disease. Antioxidants (Basel), 2017. 6(4).
18. Paikra, B.K., Dhongade, H.K.J., and Gidwani, B. Phytochemistry and Pharmacology of Moringa oleifera Lam. J Pharmacopuncture. 2017. 20(3): p. 194-200.
19. Abd Rani, N.Z., Husain, K., and Kumolosasi, E. Moringa Genus: A Review of Phytochemistry and Pharmacology. Frontiers in Pharmacology. 2018. 9: p. 108.
20. Gopalakrishnan L, D.K., Kumar D.S. Moringa oleifera: A review on nutritive importance and its medicinal application. Food Science and Human Wellness. 2016. 5(2): p. 49-56.
21. Trigo, C., Castello, M.L. Ortola, M.D., Garcia-Mares, F.J. and Desamparados Soriano, M. Moringa oleifera: An Unknown Crop in Developed Countries with Great Potential for Industry and Adapted to Climate Change. Foods. 2020. 10(1).
22. Gopalakrishnan, L., Doriya, K., and Kumar, D.S. Moringa oleifera: A review on nutritive importance and its medicinal application. Food Science and Human Wellness. 2016. 5(2): p. 49-56.
23. Zhu, Y., Yin, Q., and Yang, Y. Comprehensive Investigation of Moringa oleifera from Different Regions by Simultaneous Determination of 11 Polyphenols Using UPLC-ESI-MS/MS. Molecules (Basel, Switzerland), 2020. 25(3): p. 676.
24. Alegbeleye, O.O. How Functional Is Moringa oleifera: A Review of Its Nutritive, Medicinal, and Socioeconomic Potential. Food and Nutrition Bulletin. 2018. 39(1): p. 149-170.
25. Mishra, G., Singh, P., Verma, R., Kumar, S., Srivastav, S., Jha, K.K., and Khosa, R.L. Traditional uses, phytochemistry and pharmacological properties of Moringa oleifera plant: An overview. Der Pharmacia Lettre. 2011. 3: p. 141-164.
26. Senthilkumar, R.P., Bhuvaneshwari, V., Malayaman, V., Ranjithkumar, R. and Sathiyavimal, S. Phytochemical Screening of Aqueous Leaf Extract of Sida acuta Burm. F. and its Antibacterial Activity. 2018.
27. Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., and Bertoli, S. Cultivation, Genetic, Ethnopharmacology, Phytochemistry and Pharmacology of Moringa oleifera Leaves: An Overview. International Journal of Molecular Sciences. 2015. 16(6): p. 12791-835.
28. Bao, Y., Xiao, J., Weng, Z., Lu, X., Shen, X. and Wang, F. A phenolic glycoside from Moringa oleifera Lam. improves the carbohydrate and lipid metabolisms through AMPK in db/db mice. Food Chemistry. 2020. 311: p. 125948.
29. Balakrishnan, B.B., Krishnasamy, K., Mayakrishnan, V., and Selvaraj, A. Moringa concanensis Nimmo extracts ameliorates hyperglycemia-mediated oxidative stress and upregulates PPARγ and GLUT4 gene expression in liver and pancreas of streptozotocin-nicotinamide induced diabetic rats. Biomedicine Pharmacotherapy. 2019. 112: p. 108688.
30. Chin, C.Y., P.Y. Ng, and S.F. Ng, Moringa oleifera standardised aqueous leaf extract-loaded hydrocolloid film dressing: in vivo dermal safety and wound healing evaluation in STZ/HFD diabetic rat model. Drug Delivery and Translational Research. 2019. 9(2): p. 453-468.
31. Biswas, S., Das, R., and Banerjee, E.R. Role of free radicals in human inflammatory diseases. Aims Biophysics. 2017. 4(August): p. 596-614.

32. Ito, F., Sono, Y., and Ito, T. Measurement and Clinical Significance of Lipid Peroxidation as a Biomarker of Oxidative Stress: Oxidative Stress in Diabetes, Atherosclerosis, and Chronic Inflammation. Antioxidants. 2019. 8(3).
33. Rahimi, R., Nikfar, S., Larijani, B., and Abdollahi, M. A review on the role of antioxidants in the management of diabetes and its complications. Biomedicine & Pharmacotherapy, 2005. 59(7): p. 365-373.
34. Grotto, D., Maria, L.S., Valentini, J., Paniz, C., Schmitt, G., Garcia, S.C., Pomblum, V.J., Rocha, J.B.T., and Farina, M. Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. Quimica Nova. 2009. 32(1): p. 169-174.
35. Augustine, R., Hasan, A., Dalvi, Y.B., Rehman, S.R.U., Varghese, R., Unni, R.N., Yalcin, H.C., Alfkey, R., Thomas, S., and Al Moustafa, A.E. Growth factor loaded in situ photocrosslinkable poly (3-hydroxybutyrate-co-3-hydroxyvalerate)/gelatin methacryloyl hybrid patch for diabetic wound healing. Materials Science and Engineering: C. 2021. 118: p. 111519.
36. He, L., He, T., Farrar, S., Ji, L., Liu, T. and Ma, X. Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. Cellular Physiology and Biochemistry. 44(2): p. 532-553.
37. Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., and Malik, A.B. Reactive oxygen species in inflammation and tissue injury. Antioxidants & redox signaling, 2014. 20(7): p. 1126-1167.
38. Giacco, F. and Brownlee, M. Oxidative stress and diabetic complications. Antioxidants & Redox Signaling. 2010. 107(9): p. 1058-70.
39. Burgos-Morón, E., Abad-Jiménez, Z., Martínez de Marañón, A., Iannantuoni, F., Escribano-López, I., López-Domènech, S., Salom, C., Jover, A., Mora, V., Roldan, I., and Solá, E. Relationship between Oxidative Stress, ER Stress, and Inflammation in Type 2 Diabetes: The Battle Continues. Journal of clinical medicine. 2019. 4;8(9):1385.
40. Chandra, K., Singh, P., Dwivedi, S., and Jain, S.K. 2019. Diabetes Mellitus and Oxidative Stress: A Co-relative and Therapeutic Approach. Journal of Clinical & Diagnostic Research, 13(5).
41. Sharma, P., Jha, A.B., Dubey, R.S. and Pessarakli, M. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. Journal of Botany. 2012. 2012.
42. Matough, F.A., Budin, S.B., Hamid, Z.A., Alwahaibi, N. and Mohamed, J. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos University Medical Journal, 2012. 12(1): p. 5-18.
43. Dumanović, J., Nepovimova, E., Natić, M., Kuča, K. and Jaćević, V. The Significance of Reactive Oxygen Species and Antioxidant Defense System in Plants: A Concise Overview. Frontiers in Plant Science, 2021. 11: p. 2106-2106.
44. Kim, J.H., Cho, J.H., Kim, S.R., and Hur, Y.B. Toxic effects of waterborne ammonia exposure on hematological parameters, oxidative stress and stress indicators of juvenile hybrid grouper, Epinephelus lanceolatus Epinephelus fuscoguttatus. Environmental Toxicology and Pharmacology. 2020. 80: p. 103453.
45. Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., Ferrucci, L., Gilroy, DW., Fasano, A., Miller, G.W., Miller, A.H. Chronic inflammation in the etiology of disease across the life span. Nature Medicine. 2019. 25(12):1822-32.

46. Monserrat-Mesquida, M., Quetglas-Llabrés, M., Capó, X., Bouzas, C., Mateos, D., Pons, A., Tur, J.A., and Sureda, A. Metabolic Syndrome Is Associated with Oxidative Stress and Proinflammatory State. Antioxidants. 2020. 9(3).
47. D'Oria, R., Schipani, R., Leonardini, A., Natalicchio, A., Perrini, S., Cignarelli, A., Laviola, L. and Giorgino, F. Review Article the role of oxidative stress in cardiac disease: From physiological response to injury factor. Oxidative Medicine and Cellular Longevity. 2020. 2020.
48. Frendo-Cumbo, S., MacPherson, R.E, and Wright, D.C. Beneficial effects of combined resveratrol and metformin therapy in treating diet-induced insulin resistance. Physiology Reports. 2016. 4(15).
49. Dludla, P.V., Gabuza, K.B., Muller, C.J.F., Joubert, E., Louw, J., and Johnson, R. Aspalathin, a C-glucosyl dihydrochalcone from rooibos improves the hypoglycemic potential of metformin in type 2 diabetic (db/db) mice. Physiological Resesearch. 2018. 67(5): p. 813-818.
50. Wu, P., Yan, Y., Ma, L.L., Hou, B.Y., He, Y.Y., Zhang, L., Niu, Z.R., Song, J.K., Pang, X.C., Yang, X.Y. and Du, G.H. Effects of the Nrf2 Protein Modulator Salvianolic acid A alone or combined with metformin on diabetes-associated macrovascular and renal injury. The Journal of Biological Chemistry. 2016. 291(42): p. 22288-22301.
51. Greenfield, J.R., and Chisholm, D.J. Experimental and clinical pharmacology Thiazolidinediones – mechanisms of action. 2004(3): p. 67-70.
52. DeFronzo, R., Fleming, G.A., Chen, K. and Bicsak, T.A. Metformin-associated lactic acidosis: Current perspectives on causes and risk. Metabolism. 2016. 65(2): p. 20-9.
53. Rena, G., Pearson, E.R., and Sakamoto, K. Molecular mechanism of action of metformin: old or new insights? Diabetologia. 2013: p. 1898-1906.
54. Yendapally, R., Sikazwe, D., Kim, S.S., Ramsinghani, S., Fraser-Spears, R., Witte, A.P. and La-Viola, B. A review of phenformin, metformin, and imeglimin. Drug Development Research. 2020. 81(4): p. 390-401.
55. Minhaj S., Ahmed U., Luo L., Namani A., Wang X. J., Tang X. Nrf2 signaling pathway: Pivotal roles in inflammation. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2016. 1863(2):585-97.
56. Ma, Q., Role of nrf2 in oxidative stress and toxicity. Annual Review of Pharmacology and Toxicology. 2013; 53:401
57. Dludla, P.V., Muller, C.J., Joubert, E., Louw, J., Essop, M.F., Gabuza, K.B., Ghoor, S., Huisamen, B. and Johnson, R. Aspalathin Protects the Heart against Hyperglycemia-Induced Oxidative Damage by Up-Regulating Nrf2 Expression. Molecules. 2017. 22(1).
58. Kurutas, E.B., The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutrition Journal. 2016. 15(1): p. 71.
59. Phaniendra, A., and Babu, D. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. Indian journal of clinical biochemistry. 2015. 30(1): p. 11-26.
60. Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D. and Bitto, A. Oxidative Stress: Harms and Benefits for Human Health. Oxidative Medicine and Cellular Longevity. 2017. 2017: p. 8416763.
61. Marimoutou, M., Le Sage, F., Smadja, J., Lefebvre d'Hellencourt, C., Gonthier, M.P., and Silva, R.D. Antioxidant polyphenol-rich extracts from the medicinal plants Antirhea borbonica, Doratoxylon apetalum and Gouania mauritiana protect 3T3-L1 preadipocytes against H2O2,

TNFα and LPS inflammatory mediators by regulating the expression of superoxide dismutase and NF-κB genes. Journal of Inflammation. 2015. 12(1): p. 10.
62. Singh, G., Passsari, A.K., Leo, V.V., Mishra, V.K., Subbarayan, S., Singh B.P., Kumar B, Kumar S., Gupta V.K., Lalhlenmawia, H., Nachimuthu, SK. Evaluation of Phenolic Content Variability along with Antioxidant, Antimicrobial, and Cytotoxic Potential of Selected Traditional Medicinal Plants from India. Frontiers in Plant Science. 2016. 7: p. 407-407.
63. Do, B.H., Hoang, N.S., Nguyen, T.P.T., Ho, N.Q.C., Le, T.L., and Doan, C.C. Phenolic Extraction of Moringa Oleifera Leaves Induces Caspase-Dependent and Caspase-Independent Apoptosis through the Generation of Reactive Oxygen Species and the Activation of Intrinsic Mitochondrial Pathway in Human Melanoma Cells. Nutrition and Cancer. 2021. 73(5): p. 869-888.
64. Taïlé, J., Arcambal, A., Clerc P., Gauvin-Bialecki, A., Gonthier, M.P. Medicinal Plant Polyphenols Attenuate Oxidative Stress and Improve Inflammatory and Vasoactive Markers in Cerebral Endothelial Cells during Hyperglycemic Condition. Antioxidants. 2020 2;9(7): 573.
65. Pakade, V., C.E., Chimuka, L. Comparison of antioxidant activity of Moringa oleifera and selected vegetables in South Africa. South African Journal of Science. 1996. 109(3-4).
66. Roep, B.O., Thomaidou, S., van Tienhoven, R., and Zaldumbide, A. Type 1 diabetes mellitus as a disease of the β-cell (do not blame the immune system?). Nature Reviews Endocrinology. 2021. 17(3): p. 150-161.
67. Kottaisamy, C.P.D., Raj, D.S., Prasanth Kumar, V., and Sankaran, U. Experimental animal models for diabetes and its related complications-a review. Laboratory Animal Research. 2021. 37(1): p. 23.
68. King, A.J., The use of animal models in diabetes research. British Journal Pharmacology. 2012. 166(3): p. 877-94.
69. Perreault, L., Skyler,J.S., and Rosenstock,J. Novel therapies with precision mechanisms for type 2 diabetes mellitus. Nature Reviews Endocrinology. 2021. 17(6): p. 364-377.
70. Mokgalaboni, K., Nkambule, B.B., Ntamo, Y., Ziqubu, K., Nyambuya, T.M., Mazibuko-Mbeje, S.E., Gabuza, K.B., Chellan, N., Cirilli, I., Tiano, L., and Dludla, P.V. Vitamin K: A vital micronutrient with the cardioprotective potential against diabetes-associated complications. Life Sciences. 2021. 286: p. 120068.
71. Mima, A. Inflammation and oxidative stress in diabetic nephropathy: new insights on its inhibition as new therapeutic targets. Journal Diabetes Research. 2013. 2013: p. 248563.
72. Xu, Y.B., Chen, G.L., and Guo,M.Q. Antioxidant and Anti-Inflammatory Activities of the Crude Extracts of Moringa oleifera from Kenya and Their Correlations with Flavonoids. Antioxidants (Basel), 2019. 8(8).
73. Tuorkey, M.J. Effects of Moringa oleifera aqueous leaf extract in alloxan induced diabetic mice. Interventional Medicine Applied Science, 2016. 8(3): p. 109-117.
74. Oboh, G., Oyeleye, S.I., Akintemi, O.A. and Olasehinde, T.A. Moringa oleifera supplemented diet modulates nootropic-related biomolecules in the brain of STZ-induced diabetic rats treated with acarbose. Metabolic Brain Disease. 2018. 33(2): p. 457-466.
75. Omodanisi, E.I., Y.G. Aboua, and O.O. Oguntibeju, Assessment of the Anti-Hyperglycaemic, Anti-Inflammatory and Antioxidant Activities of the Methanol Extract of Moringa Oleifera in Diabetes-Induced Nephrotoxic Male Wistar Rats. Molecules. 2017. 22(4).

76. Omodanisi, E.I., Aboua, Y.G., Chegou, N.N. and Oguntibeju, O.O. Hepatoprotective, Antihyperlipidemic, and Anti-inflammatory Activity of Moringa oleifera in Diabetic-induced Damage in Male Wistar Rats. Pharmacognosy Research. 2017. 9(2): p. 182-187.
77. Yassa, H.D., and Tohamy, A.F. Extract of Moringa oleifera leaves ameliorates streptozotocin-induced Diabetes mellitus in adult rats. Acta Histochem. 2014. 116(5): p. 844-54.
78. Aju, B.Y., Rajalakshmi, R., and Mini, S. Protective role of Moringa oleifera leaf extract on cardiac antioxidant status and lipid peroxidation in streptozotocin induced diabetic rats. Heliyon, 2020. 6(1): p. e03146.
79. Muhammad, A.A., Arulselvan, P., Cheah, P.S., Abas, F. and Fakurazi, S. Evaluation of wound healing properties of bioactive aqueous fraction from Moringa oleifera Lam on experimentally induced diabetic animal model. Drug Design, Development and Therapy. 2016. 10: p. 1715-30.
80. Azevedo, Í.M., Araújo-Filho, I., Teixeira, M.M.A., Moreira, M.D.F.D.C. and Medeiros, A.C. Wound healing of diabetic rats treated with Moringa oleifera extract. Acta Cirúrgica Brasileira. 2018. 33(9): p. 799-805.
81. Oldoni, T.L.C., Merlin, N., Bicas, T.C., Prasniewski, A., Carpes, S.T., Ascari, J., de Alencar, S.M., Massarioli, A.P., Bagatini, M.D., Morales, R., and Thomé, G. Antihyperglycemic activity of crude extract and isolation of phenolic compounds with antioxidant activity from Moringa oleifera Lam. leaves grown in Southern Brazil. Food Research International. 2021. 141: p. 110082.
82. Abd Eldaim, M.A., Shaban Abd Elrasoul, A., and Abd Elaziz, S.A. An aqueous extract from Moringa oleifera leaves ameliorates hepatotoxicity in alloxan-induced diabetic rats. Biochemistry and Cell Biology. 2017. 95(4): p. 524-530.
83. Oguntibeju, O.O., Aboua, G.Y., and Omodanisi, E.I. Effects of Moringa oleifera on oxidative stress, apoptotic and inflammatory biomarkers in streptozotocin-induced diabetic animal model. South African Journal of Botany. 2020. 129: p. 354-365.
84. Oyeleye, S.I., Ojo, O.R., and Oboh, G. Moringa oleifera leaf and seed inclusive diets influenced the restoration of biochemicals associated with erectile dysfunction in the penile tissue of STZ-induced diabetic male rats treated with/without Acarbose drug. Journal of Food Biochemistry. 2021. 45(3): p. e13323.
85. Jaiswal, D., Rai, P.K., Mehta, S., Chatterji, S., Shukla, S., Rai, D.K., Sharma, G., Sharma, B. and Watal, G. Role of Moringa oleifera in regulation of diabetes-induced oxidative stress. Asian Pacific Journal of Tropical Medicine. 2013. 6(6): p. 426-32.
86. Al-Malki, A.L., and El Rabey, H.A. The antidiabetic effect of low doses of Moringa oleifera Lam. seeds on streptozotocin induced diabetes and diabetic nephropathy in male rats. BioMed research international. 2015. 2015: p. 381040.
87. Raafat, K., and Hdaib, F. Neuroprotective effects of Moringa oleifera: Bio-guided GC-MS identification of active compounds in diabetic neuropathic pain model. Chinese Journal of Integrative Medicine. 2017. 12:1-0.
88. Maritim, A.C., Sanders, A., and Watkins Iii, J.B. 3rd, Diabetes, oxidative stress, and antioxidants: a review. Journal of Biochemical and Molecular Toxicology. 2003;17(1):24-38.
89. Davì, G., Falco, A., and Patrono, C. Lipid peroxidation in diabetes mellitus. Antioxidants & Redox Signaling. 2005. 7(1-2): p. 256-68.
90. Domínguez, C., Ruiz, E., Gussinye, M., and Carrascosa, A. Oxidative stress at onset and in early stages of type 1 diabetes in children and adolescents. Diabetes Care. 1998. 21(10): p. 1736-42.

91. Alejandra Sánchez-Muñoz, M., Valdez-Solana, M.A., Campos-Almazán, M.I., Flores-Herrera, O., Esparza-Perusquía, M., Olvera-Sánchez, S., García-Arenas, G., Avitia-Domínguez, C., Téllez-Valencia, A., and Sierra-Campos, E. Streptozotocin-Induced Adaptive Modification of Mitochondrial Supercomplexes in Liver of Wistar Rats and the Protective Effect of Moringa oleifera Lam. Biochemistry Research International.2018. 2018: p. 5681081.
92. Teodoro, J.S., Nunes, S., Rolo, A.P., Reis, F., and Palmeira, C.M. Therapeutic Options Targeting Oxidative Stress, Mitochondrial Dysfunction and Inflammation to Hinder the Progression of Vascular Complications of Diabetes. Frontiers in Physiology. 2018. 9: p. 1857.
93. Dludla, P.V., Joubert, E., Muller, C.J., Louw, J. and Johnson, R. Hyperglycemia-induced oxidative stress and heart disease-cardioprotective effects of rooibos flavonoids and phenylpyruvic acid-2-O-β-D-glucoside. Nutrition & Metabolism. 2017. 14: p. 45.
94. Rose, B.A., Force, T. and Wang, Y. Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. Physiological Reviews. 2010. 90(4): p. 1507-46.
95. Mokgalaboni, K., Dludla, P.V., Nyambuya, T.M., Yakobi, S.H., Mxinwa, V., and Nkambule, B.B. Monocyte-mediated inflammation and cardiovascular risk factors in type 2 diabetes mellitus: A systematic review and meta-analysis of pre-clinical and clinical studies. JRSM Cardiovascular Disease. 2020. 9: p. 2048004019900748.
96. Ilyas, M., Arshad, M.U., Saeed, F. and Iqbal, M. Antioxidant potential and nutritional comparison of moringa leaf and seed powders and their tea infusions. The Journal of Animal & Plant Sciences. 2015. 25(1): p. 226-233.
97. Saini, R.K., Sivanesan, I. and Keum, Y.S. Phytochemicals of Moringa oleifera: a review of their nutritional, therapeutic and industrial significance. 3 Biotech. 2016. 6(2): p. 203
98. Aju, B.Y., Rajalakshmi, R., and Mini, S. Protective role of Moringa oleifera leaf extract on cardiac antioxidant status and lipid peroxidation in streptozotocin induced diabetic rats. Heliyon, 2019. 5(12): p. e02935.
99. Sierra-Campos, E., Valdez-Solana, M., Avitia-Domínguez, C., Campos-Almazán, M., Flores-Molina, I., García-Arenas, G. and Téllez-Valencia. Effects of Moringa oleifera Leaf Extract on Diabetes-Induced Alterations in Paraoxonase 1 and Catalase in Rats Analyzed through Progress Kinetic and Blind Docking. Antioxidants (Basel). 2020. 9(9).
100. Grundy, S.M. Metabolic syndrome update. Trends Cardiovasc Med, 2016. 26(4): p. 364-73.
101. Chait, A., and Den Hartigh, L.J. Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease. Frontiers in cardiovascular medicine.2020. 7: p. 22.
102. Oguntibeju, O.O., Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. International journal of physiology, pathophysiology and pharmacology. 2019. 11(3): p. 45-63.
103. Stanford, K.I., and Goodyear, L.J. Exercise and type 2 diabetes: molecular mechanisms regulating glucose uptake in skeletal muscle. Advances in physiology education. 2014. 38(4): p. 308-14.
104. Muoio, D.M., and Newgard, C.B. Mechanisms of disease: Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nature reviews Molecular cell biology.2008. 9(3): p. 193-205.
105. Waterman, C., Rojas-Silva, P., Tumer, T.B., Kuhn, P., Richard, A.J., Wicks, S., Stephens, J.M., Wang, Z., Mynatt, R., Cefalu, W., and Raskin, I. Isothiocyanate-rich Moringa oleifera extract

reduces weight gain, insulin resistance, and hepatic gluconeogenesis in mice. Molecular Nutrition & Food Research. 2015. 59(6): p. 1013-24.
106. Jaja-Chimedza, A., Zhang, L., Wolff, K., Graf, B.L., Kuhn, P., Moskal, K., Carmouche, R., Newman, S., Salbaum, J.M., and Raskin, I. A dietary isothiocyanate-enriched moringa (Moringa oleifera) seed extract improves glucose tolerance in a high-fat-diet mouse model and modulates the gut microbiome. Journal of Functional Foods. 2018. 47: p. 376-385.
107. Mohamed, M.A., Ahmed, M.A., and El Sayed, R.A. Molecular effects of Moringa leaf extract on insulin resistance and reproductive function in hyperinsulinemic male rats. Journal of Diabetes & Metabolic Disorders. 2019. 18(2): p. 487-494.
108. El-Shehawi, A.M., Alkafafy, M., El-Shazly, S., Sayed, S., Farouk, S., Alotaibi, S., Madkour, D.A., Khalifa, H.K., and Ahmed, M.M. Moringa oleifera leaves ethanolic extract ameliorates high fat diet-induced obesity in rats. Journal of King Saud University – Science. 2021. 33(6): p. 101552.
109. Tang, Y., Choi, E.J., Han, W.C., Oh, M., Kim, J., Hwang, J.Y., Park, P.J., Moon, S.H., Kim, Y.S., and Kim, E.K. Moringa oleifera from Cambodia Ameliorates Oxidative Stress, Hyperglycemia, and Kidney Dysfunction in Type 2 Diabetic Mice. Journal of Medicinal Food. 2017. 20(5): p. 502-510.
110. Joung, H., Kim, B., Park, H., Lee, K., Kim, H.H., Sim, H.C., Do, H.J., Hyun, C.K. and Do, M.S. Fermented Moringa oleifera Decreases Hepatic Adiposity and Ameliorates Glucose Intolerance in High-Fat Diet-Induced Obese Mice. Journal of Medicinal Food. 2017. 20(5): p. 439-447.
111. Redha AA, P.S., Riva A., Petrangolini G., Peroni G., Nichetti M., Lannello G., Naso M, Faliva MA, Rondanelli, M. Novel insights on anti-obesity potential of the miracle tree, Moringa oleifera: A systematic review. Journal of Functional Foods. 2021. 84(2021): p. 104600.
112. Bonjour, J.P., Ammann, P., and Rizzoli, R. Importance of preclinical studies in the development of drugs for treatment of osteoporosis: a review related to the 1998 WHO guidelines. Osteoporosis International. 1999. 9(5): p. 379-93.
113. Steinmetz, K.L., and Spack, E.G. The basics of preclinical drug development for neurodegenerative disease indications. BMC Neurology. 2009. 9 Suppl 1(Suppl 1): p. S2.
114. Hung, H.Y., Qian, K., Morris-Natschke, S.L., Hsu, C.S., and Lee, K.H. Recent discovery of plant-derived anti-diabetic natural products. Natural Product Reports.2012. 29(5): p. 580-606.
115. Jugran, A.K., Rawat, S., Devkota, H.P., Bhatt, I.D., and Rawal, R.S. Diabetes and plant-derived natural products: From ethnopharmacological approaches to their potential for modern drug discovery and development. Phytotherapy Research. 2021. 35(1): p. 223-245.
116. Anthanont, P., Lumlerdkij, N., Akarasereenont, P., Vannasaeng, S., and Sriwijitkamol, A. Moringa Oleifera Leaf Increases Insulin Secretion after Single Dose Administration: A Preliminary Study in Healthy Subjects. Journal of Medicine Association Thailand, 2016. 99(3): p. 308-13.
117. Leone, A., Bertoli, S., Di Lello, S., Bassoli, A., Ravasenghi, S., Borgonovo, G., Forlani, F., and Battezzati, A. Effect of Moringa oleifera Leaf Powder on Postprandial Blood Glucose Response: In Vivo Study on Saharawi People Living in Refugee Camps. Nutrients. 2018. 10(10).
118. Watanabe, S., Okoshi, H., Yamabe, S., and Shimada, M. Moringa oleifera Lam. in Diabetes Mellitus: A Systematic Review and Meta-Analysis. Molecules. 2021. 26(12).
119. Fatoumata, B.A., MamadouSadou, B.A.H., Mohamet, S., Joseph, K.S., Modou, M.G., and El, H.B. Antidiabetic properties of Moringa oleifera: A review of the literature. Journal of Diabetes and Endocrinology. 2020. 11(1): p. 18-29.
120. Bailey, C.J. Metformin: historical overview. Diabetologia, 2017. 60(9): p. 1566-1576.

121. Foretz, M., Guigas, B., Bertrand, L., Pollak, M. and Viollet, B. Metformin: from mechanisms of action to therapies. Cell Metabolism. 2014. 20(6): p. 953-66.
122. Joya-Galeana, J., Fernandez, M., Cervera, A., Reyna, S., Ghosh, S., Triplitt, C., Musi, N., DeFronzo, R.A., and Cersosimo, E. Effects of insulin and oral anti-diabetic agents on glucose metabolism, vascular dysfunction and skeletal muscle inflammation in type 2 diabetic subjects. Diabetes Metabolism Research and Reviews. 2011. 27(4): p. 373-82.
123. Nyawo, T.A., Pheiffer, C., Mazibuko-Mbeje, S.E., Mthembu, S.X., Nyambuya, T.M., Nkambule, B.B., Sadie-Van Gijsen, H., Strijdom, H., Tiano, L., and Dludla, P.V. Physical Exercise Potentially Targets Epicardial Adipose Tissue to Reduce Cardiovascular Disease Risk in Patients with Metabolic Diseases: Oxidative Stress and Inflammation Emerge as Major Therapeutic Targets. Antioxidants (Basel). 2021. 10(11).
124. Shakoor, H., Apostolopoulos, V., Feehan, J., Ali, H.I., Ismail, L.C., Al Dhaheri, A.S.O.S., and Stojanovska, L. Effect of Calorie Restriction and Exercise on Type 2 Diabetes. Prilozi (Makedon Akad Nauk Umet Odd Med Nauki). 2021. 42(1): p. 109-126.
125. Vikram, A., Tripathi, D.N., Kumar, A., and Singh, S. Oxidative stress and inflammation in diabetic complications. International Journal of Endocrinology.2014. 2014: p. 679754.
126. Mahlangu, T., Dludla, P.V., Nyambuya, T.M., Mxinwa, V., Mazibuko-Mbeje, S.E., Cirilli, I., Marcheggiani, F., Tiano, L., Louw, J., and Nkambule, B.B. A systematic review on the functional role of Th1/Th2 cytokines in type 2 diabetes and related metabolic complications. Cytokine. 2020. 126: p. 154892.
127. Mokgalaboni, K., Ntamo, Y., Ziqubu, K., Nyambuya, T.M., Nkambule, B.B., Mazibuko-Mbeje, S.E., Gabuza, K.B., Chellan, N., Tiano, L., and Dludla, P.V. Curcumin supplementation improves biomarkers of oxidative stress and inflammation in conditions of obesity, type 2 diabetes and NAFLD: updating the status of clinical evidence. Food and Function. 2021. 12(24): p. 12235-12249.
128. Hwang, J.H., and Lim, S.B. Antioxidant and Anti-inflammatory Activities of Broccoli Florets in LPS-stimulated RAW 264.7 Cells. Preventive Nutrition and Food Science.2014. 19(2): p. 89-97.
129. Duranti, G., Maldini, M., Crognale, D., Sabatini, S., Corana, F., Horner, K., and Ceci, R. Moringa oleifera leaf extract influences oxidative metabolism in C2C12 myotubes through SIRT1-PPARα pathway. Phytomedicine Plus. 2021. 1(1): p. 100014-100014.

CHAPTER 3

This chapter reports on the methodology used in this project.

3 Materials and Methods

3.1. Materials, reagents and methods

All materials, reagents, equipment, and consumables used in this study are listed in appendix 1

3.2. General overview of in vitro models

The end goal of the research is to reach a point of conducting large random controlled trials that will confirm the efficacy of therapies, therefore, in vitro models are an important part of research that assists with gathering basic evidence on emerging and promising therapies to help us reach these large random controlled trials (Hammal et al., 2022). This type of methodology is done in controlled set ups such as pH, temperature, and nutrients. It allows the evaluation of physiological or molecular phenomena with no distractions that are present in whole organisms, and it is ethically correct and has greater accuracy and not to mention that it saves time. In this research project, we assessed the effect of *Moringa oliefera* on cell viability and its cytotoxicity using the immortalized cell line C2C12 that was derived from mouse skeletal muscle cells. This cell line was selected on the basis that it mimics the human skeletal muscle cell which is accountable for ~75% of whole-body glucose removal and responds to insulin effectively. (Dimopoulos et al., 2006; Yang et al., 2013) hence it can be a useful model to study the effect of *Moringa oliefera* on glucose metabolism.

3.3. Cell culture

3.3.1. Thawing of C2C12 mouse skeletal muscle cells

Murine C2C12 cells (ATCC, Cat No: CRL 172) (P13) were a gift from the North-West University Potchefstroom campus. The 1 mL vial of cells was thawed as rapidly as possible at 37°C in a NUVE NB20 water bath (Nüve, Turkey), leaving a little bit of ice inside the vial. Thereafter the vial was sprayed with 70% ethanol and placed inside a laminar cabinet, whereby the cells were transferred from the vial by a pipette (1000 µL) into a 15 mL centrifuge tube containing 10 mL of pre-warmed Dulbecco's modified eagle's medium (DMEM with 10% foetal bovine serum (FBS) and 1% pen/strep) and the vial was rinsed with 1 mL and added to the 15 mL tube. Cells were transferred from the tube into the 75 cm^2 flask. The flask was gently swirled to evenly distribute the cells and was then placed inside the incubator set at 37 °C in 5% CO_2 at humidified air. After 24 hours the media was replaced with fresh 10% FBS. Thereafter, cells were kept at 37°C 5% CO_2, 95% humidified air until they reached an 80-90% confluency.

3.3.2. Sub-culturing and cell counting and seeding of cells

Upon 80-90% confluency, cells were rinsed with 80 mL Dulbecco's phosphate buffer saline (DPBS), and trypsinised with 2 mL of trypsin for 3 -5 min. After they had dislodged the trypsin reaction was stopped by adding 8mL of 10% FBS, thoroughly mixed and added into a 15 mL tube, centrifuged at 800 rpm for 5 minutes with an NF 200 centrifuge (Nüve, Turkey). Thereafter, the supernatant was discarded, whilst the pellet was gently broken and resuspended in 5 mL of 10% FBS and cells were mixed up and down with pipetting. Thereafter a portion (100 µL) of the cell suspension was mixed with 0.4% (w/v) trypan blue in DPBS and 10 µL of the cell suspension was then added into the counting slide and they were counted using the automated Invitrogen Countess 3 (ThermoFischer Scientific, Waltham, Massachusetts,) chamber according to the manufacturer's recommendations. After counting, cells were either in DMEM containing 10%FBS and 7% dimethyl sulfoxide (DMSO) at a density of $1X10^6$ cell/mL and kept at -80 until further use. Alternatively, after counting cells were seeded for various experiments (MTT assay, glucose uptake, glycogen assay and PCR analysis) at a density of $2.5X\ 10^4$ cells/mL as described on table 3. Thereafter cells were cultured at 37° C in 5% CO_2 in humidified air until they were 90% confluent.

Table 3: The different cell densities used for seeding in different multi-well plates.

Cell type	Plate type	Cell concentration	Volume(µL)	Cell density
C2C12	96-well plate	$2.5x10^4$ cells/mL	200 µL	$5x10^3$ cells/well
	6-well plate	$2.5x10^4$ cells/mL	3000 µL	$7.5x10^4$ cells/well

3.3.3. Differentiation of myoblast into myotubes

After myoblast cells had reached 90% confluency, they were differentiated into myotubules, this was achieved by replacing the normal growth medium containing 10% FBS growth media with a differentiation medium containing 2% FBS and the cells were incubated at 37° C in 5% CO_2 and humidified air for 3 days until they formed myotubules. Thereafter relevant treatment was initiated.

3.3.4. Preparation of *Moringa oliefera* treatment

A 1000 µg/mL stock, of MO leaf powder (from Thabaneng Agricultural Projects) was prepared by carefully weighing (10 mg) and dissolving it in 100% DMSO. After vortex-mixing the solution for ten to thirty seconds, it was aseptically filter-sterilized using a sterile 0.22 µm membrane filter. To maintain consistency and sterility, stocks were made fresh every day right before use.

3.4. Dose and time study

3.4.1. Bioactivity of *Moringa oliefera* in C1C12 myotubules

We achieved our first objective, by investigating the bioactivity of *Moringa oliefera* through conducting a dose and time study in normal differentiated C2C12 cells (Fig 4). To achieve this a freshly prepared stock solution of 1000 µg/mL *Moringa oliefera* stock was further diluted in Kreb's buffer that contained 8mM glucose using a dilution factor of 1:10 to yield a final concentration of 0.001, 0.01, 0.1, 1, 10 and 100 µg/mL. Cells were then treated at various time points (2, 4, 8, 16 and 24 hours) with various concentrations of *Moringa oliefera* (0.001, 0.01, 0.1, 1, 10 and 100 µg/mL) (Fig 4). Insulin was used as a positive control and it was added during the last 15 minutes. Thereafter MTT, glucose uptake and glycogen assay were performed. At the end of this dosing study, one-time point (8 hrs) was chosen for the second objective of this study insulin resistance model.

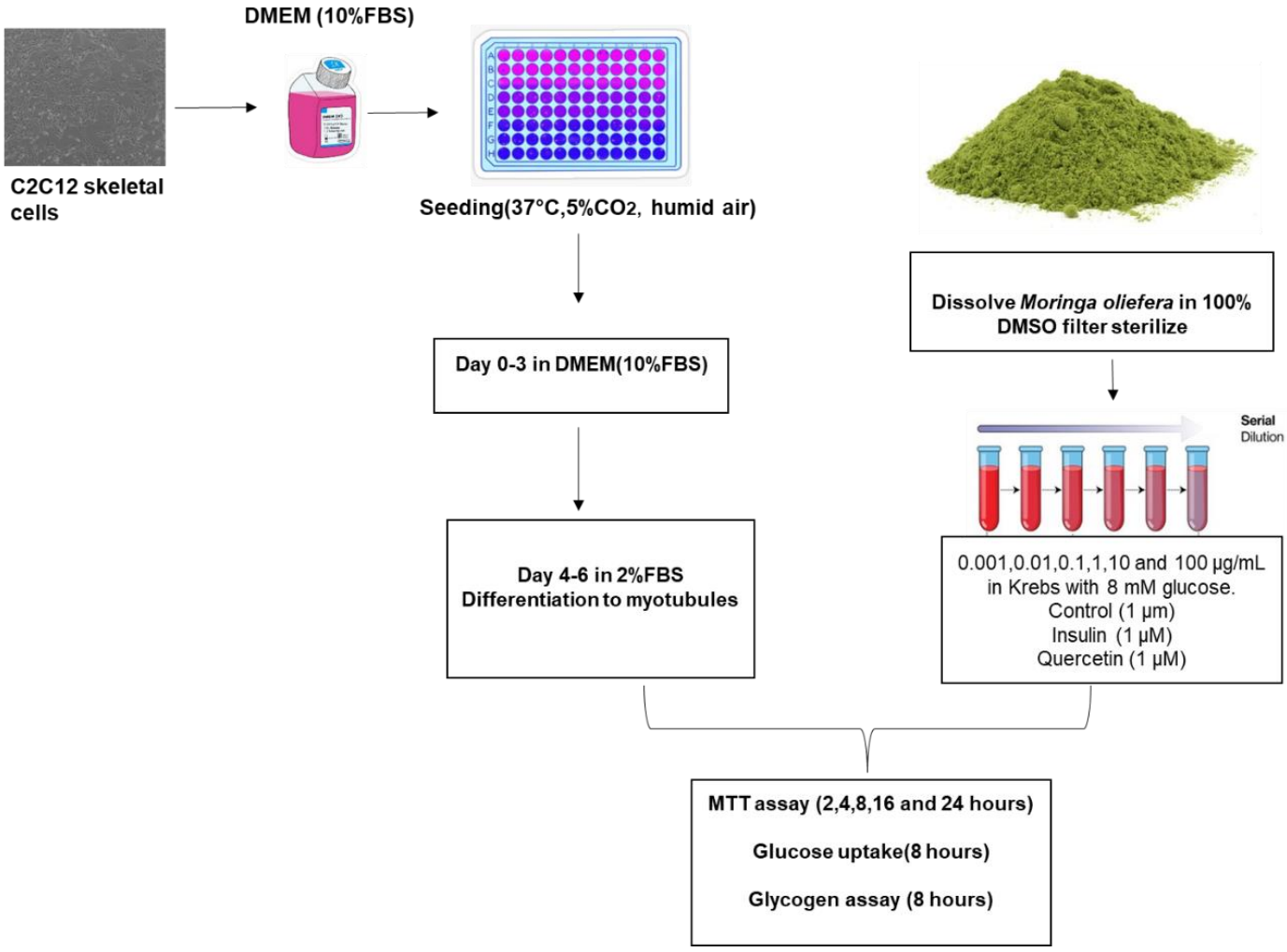


Figure 4: An overview of the preparation of *Moringa oliefera* and relevant controls for the dose-time study to fulfil the first objective of this study in normal C2C12 skeletal cells.

3.4.2. The 3- [4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay (MTT assay) in normal cells

After treating cells with the relevant treatments, MTT assay was performed to test the cytotoxicity of *Moringa oliefera* in C2C12 myotubules. MTT is a colorimetric assay that is based on the redox based colorimetric readout and cellular reducing power. In this assay the viable cells reduce the yellow tetrazolium salt into a purple dye by the transfer of electrons from NADP or NADPH to MTT, leading to the disruption of the tetrozole ring that later forms an insoluble purple dye.This dye is than sulubilized and quantified using a spectrophotometer at 570 nm. The MTT assay was performed as previously described by Mosman in (1983) with slight modifications. Briefly, a stock solution of MTT (5 mg/mL) was prepared by dissolving the MTT powder in DPBS. The media with or without *Moringa oleifera* extract was transferred into new plates for other assays, thereafter 50 µL of Kreb's buffer with 8mM glucose and 50 µL of MTT solution were added into each well. The plate was incubated for 30 minutes at

37°C, followed by the addition of 100 µL DMSO into each well to solubilize MTT. The Sorenson's Glycine Buffer (pH 7.4) was then added to each well to stop the MTT reaction and the plate was wrapped in foil and shaken on an orbital shaker for 15 minutes, after shaking, the absorbance was read at an optical density (OD) of 570 nm using the MultiSkan Go plate reader (Thermo Fisher Scientific, Waltham, Massachusetts).

3.4.3. Glucose uptake

A glucometer is an enzymatic electrochemical biosensor, which uses glucose oxidase (GOx) or a glucose dehydrogenase (GDH) to oxidise glucose. There is a mediator molecule (e.g. ferricyanide or ferrocene) which carriers electrons to the electrodes and the resulting charge within the fixed sample volume is linearly proportional to the glucose concentration. The glucometer works the same in culture media, and the anticipated outcomes is that the cells treated with palmitate would have a larger residual glucose because GLUT4 translocation is impeded, whereas the cells treated with MO would have a lower residual glucose because GLUT4 has recently been repaired. (GlucCell meter, Accu-chek Active Kit, Dischem, South Africa) following manufactures recommendations. Briefly, the samples were thawed on ice, and 1.5 µL of the sample was used to measure the glucose remaining in the cell culture supernatant. The initial 8 mM glucose media that was used was first measured, the samples were taken and placed onto the strip and within 15 seconds of adding the sample the reading of the glucose (in mmol/L) level in the media was recorded. The results were subtracted from the 8 mM glucose blank and calculated into a percentage.

3.4.4. Glycogen assay

The glycogen assay measures the amount of glycogen, which is a branched polysaccharide of glucose that is found stored in cells or tissues. Glycogen can either be broken down enzymatically or chemically into glucose units and this is achieved by using the enzyme amyloglucosidase. This enzyme hydrolyzes the α-1,4 and α-1,6 glycosidic bonds found in glycogen to give free glucose. The free glucose is quantified using a glucose assay since most are enzyme based and the common detection systems include hexokinase enzyme which converts glucose to glucose-6-phosphate, glucose-6-phosphate dehydrogenase this converts G-6-phosphate to 6-phosphogluconolactone and this will produce NADP or NADPH from NAD+ OR NADP+. It is then quantified by using a standard curve of known concentrations of glucose or glycogen in the sample based on the released amount of glucose.

The glycogen assay was performed only in the first objective of this study (normal cells treated with *Moringa oliefera* only) using a glycogen assay kit following the manufactures recommendations. Briefly, media collected after treating the C2C12 cells with various concentrations of *Moringa oliefera* were used to make a standard curve (0, 0.125, 0.25, 0.5, 0.75, 1 and 2 mg/µL) in assay buffer, and added into the 96-well plate then 5 µL of the sample was added into the plate followed by 45 µL of the hydrolysis buffer to make a total volume of 50 µL, thereafter 2 µL of the hydrolysis enzyme mix (Table 4) was added into each well except into the wells with the samples that were to be used as a blank. The plate was then incubated at room temperature and protected from light for 30 minutes. During the incubation, a master mix consisting of the development buffer, development enzyme mix, and fluorescent peroxidase substrate

was prepared and placed on ice for further use. After 30 minutes, 50 µL of the master mix was then added to all the wells of the plate including the ones without the hydrolysis buffer. The plate was then incubated again at room temperature away from the light for 30 minutes. When the 30 minutes was up the plate was read at 570 nm with a plate reader. The results were subtracted from the blank. **Table 4:** Components used for the glycogen assay

Table 4: Components used for the glycogen assay

Components	**Quantity**
Hydrolysis buffer	45 µL
Hydrolysis enzyme mix	2 µL
Master mix (development buffer, development enzyme mix, and fluorescent peroxidase)	50 µL

3.5. Insulin resistance model

3.5.1. Preparation of palmitate and induction of insulin resistance

For our second objective, palmitate, a known inducer of insulin resistance was used as a model in the study as described by Mazibuko et al., 2013. Briefly, to prepare palmitate stock, 96,15 mg was weighed and dissolved in 5 mL of 100 % ethanol, heated at 95˚C for 5 minutes and then filter sterilized to yield a concentration of 75 mM palmitate, stock was kept at 4ºC for a maximum of 2 months. For a working solution, a palmitate cocktail of 0.75 mM was prepared from the stock in DMEM with 4.5 g/L glucose and 2 % BSA. To ensure that palmitate is conjugated, the palmitate cocktail was placed in a water bath for 1 hour at 37 ºC. Following conjugation, C2C12 myotubes were pre-treated with 0.75 mM palmitate for 16 hours at 37°C in 5% CO2-humidified air (Fig 5). In a study by Mazibuko et al (2011) they reported that ethanol (>0.01%) does not affect the cells negatively and that the palmitate doesn't change the pH of the media it remains at 7.4-7.5.

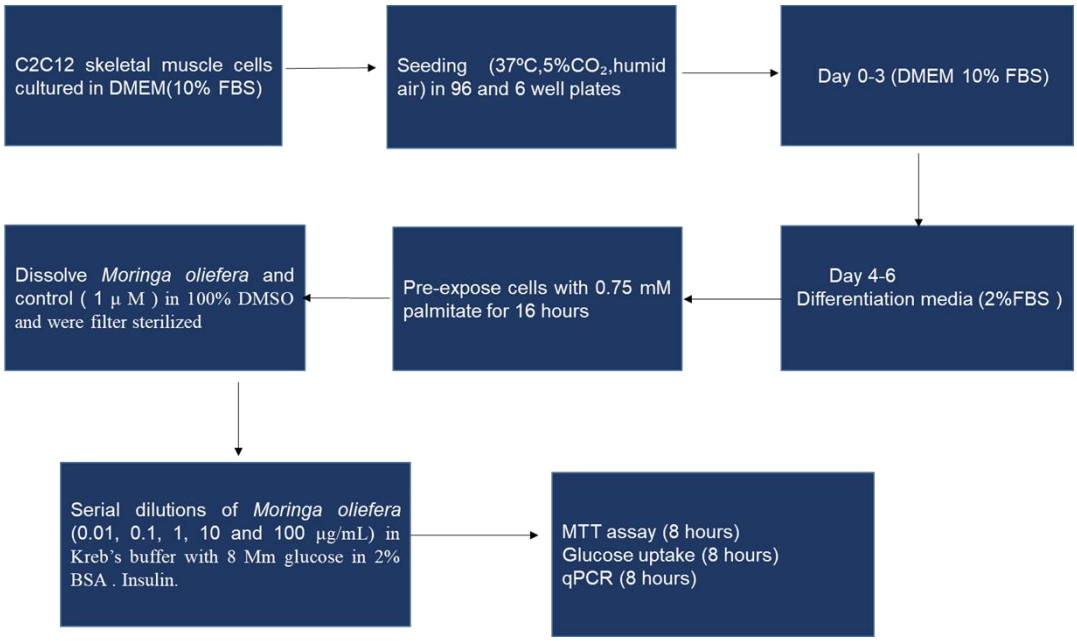


Figure 5: A schematic diagram of the insulin resistance model. The C2C12 skeletal cells were maintained in 10%FBS and then differentiated with 2%FBS into myotubules. Thereafter they were pre-exposed with 0.75 mM palmitate for 16 hours to induce insulin resistance. *Moringa oliefera* was prepared to be investigated against insulin resistance.

3.5.2. Extract and compounds preparations and treatment of cells

After induction of insulin resistance for 16 hrs, the cells were serum and glucose starved with Kreb's buffer without glucose and serum for 30 minutes to help bring the cells to the same cell cycle, so they start at the same rate or time. Thereafter, treated with various doses of *Moringa oliefera* (0.001; 0.01; 0.1; 1; 10, and 100 µg/mL) in Kreb's buffer containing 8 mM glucose, and 2% BSA for 8 hours. The final concentration of DMSO was <0.01 % which is deemed safe to use in culture (Jack et al., 2018). Insulin (1 µM) was used as a positive control, and it was added in the last 15 minutes. To counteract DMSO (0.01%) prepared in Kreb's buffer containing 8 mM glucose and 0.01% DMSO was used as normal vehicle control (Fig 5). Thereafter media was collected for glucose assay, MTT was conducted and 6 well plates with the cells were stored at -80°C to be used later for the extraction of RNA (ribonucleic acid) and the media was transferred into Eppendorf tubes and placed in -20°C until further use.

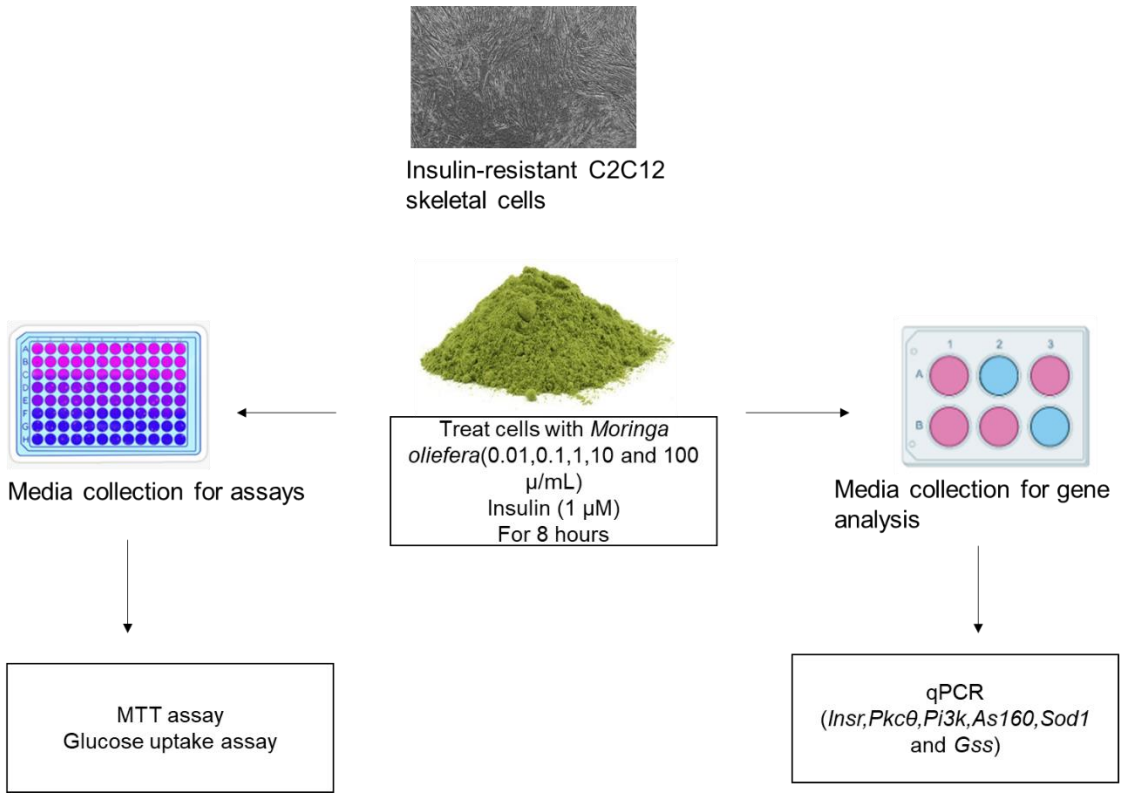


Figure 6: Insulin resistant C2C12 skeletal cells treated with *Moringa oliefera* for 8 hours in 96 well plates for MTT, glucose assay and in 6-well plates for RNA extraction for qPCR.

3.6. The 3- [4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay (MTT assay)

After inducing insulin resistance and treating the cells with the relevant treatments as per figure 5 and 6 above, MTT assay was performed as previously explained in section 3.6.2.

3.7. Glucose uptake

To monitor glucose uptake, media that was collected after treatment (Fig 6) was used to measure the rate of glucose uptake using a glucometer (GlucCell meter, Accu-Chek Active Kit, Dischem, South Africa) following manufactures recommendations as stated in 3.6.3.

3.8. RNA extraction

The extraction of RNA is crucial for inactivating RNase, which degrades RNA, and employing chaotropic chemicals like guanidinium thiocyanate accelerates protein denaturation, thereby preserving RNA integrity. RNA is highly sensitive; so, rapidity and cleanliness (RNAse-free environment) are essential, and a controlled pH is critical, as a low pH facilitates RNA separation during phenol-chloroform extraction. The total ribonucleic acid (RNA) was extracted from the treated C2C12 myotubes to perform a

polymerase chain reaction (PCR) for gene analysis. Briefly, TriZol was added to each well of the plates and they were incubated at room temperature for 10 minutes, then the cells were scrapped off using a cell scrapper. The cells were then collected into 1.5 mL Eppendorf tubes and 200 µL of chloroform was added into the tubes. The tubes were then placed at room temperature for 10 minutes. During the 10 minutes, the cells were vortexed 4 times for 15 seconds followed by centrifugation at 18 400 x g for 20 minutes at 4°C. After centrifugation, the supernatant was transferred into a new tube, without disturbing the middle and bottom layer which was discarded. Isopropanol (0.5 mL) was then added into the tubes and mixed thoroughly, and the tubes were placed on ice for 10 minutes. Then the tubes were removed from the ice and centrifuged again at the above-mentioned speed, time and temperature, after this spin a pellet was formed, and the supernatant was discarded. The pellet was washed with 75% ethanol and centrifuged at 12 000 x g for 10 minutes at 4°C. The supernatant was then discarded, and this was repeated twice, thereafter, the supernatant was discarded and the tubes were allowed to air dry for 15 minutes before adding 30 µL of RNASe-free water which depended on the size of the RNA pellet and it was stored at -80°C freezers until needed.

3.9. RNA quantification using a Nanodrop spectrophotometer

To determine the concentration of RNA, a NanoDrop One spectrophotometer from Thermo Fisher Scientific (Waltham, Massachusetts) was used as per the manufacturers' recommendations. Briefly, the machine was first blanked with 1 µL RNase-free water, followed by the reading of RNA samples. This was measured two times by adding a 1 µL sample of it on the Nanodrop and the readings were extracted and calculated for 2 µg cDNA synthesis.

3.10. Quantification of the quality of RNA using gel electrophoresis

The agarose gel electrophoresis is used to separate RNA molecules based on size and the uniform negative charge that is given by their phosphate backbone, and when an electric field is applied the RNA migrates to the positive electrode, and smaller fragments move faster and travel further through the gel matrix than larger ones and this results in a size- based separation. In order to visualize the integrity of the RNA fragments the gel is stained with a fluorescent dye (ethidium bromide) and high quality RNA will have 2 sharp bands corresponding to the ribosomal RNA 28S and 18S band whereas bad quality RNA will appear smeared below the rRNA bands. The quality of RNA was quantified using agarose gel electrophoresis. The agarose powder was weighed (1 g) and dissolved in 100 ml of 1X TAE buffer and then heated in the microwave until the agarose was dissolved. Ethidium bromide (1.5 µL) was added and the gel was swirled a little. The gel was then poured into a casting tray with the comb inserted and left until the gel become solid. The comb was removed after the gel had solidified. The samples were prepared by mixing 1 µL of the 6 X loading dye with 5 µL of the RNA and it was then loaded in the wells. The gel was then covered with the 1X TAE buffer and the voltage was set at 100 V for 50 minutes. After this, the gel was visualized using the Gel Doc™ XR+ machine with image Lab™ software (Bio-Rad, Hercules, California, USA).

3.11. Reverse transcription of RNA into cDNA

The enzyme reverse transcriptase is an RNA-dependent DNA polymerase originated from retroviruses. It reads the RNA template and synthesizes the complementary DNA strand using deoxyribonucleotide triphosphates (dNTPs), which requires a primer to begin the synthesis. The synthesis begins at the 3'-OH end with the DNA primer and works backwards to achieve a DNA-RNA-protein flow. This conversion step is critical because it stabilizes RNA, which is known to be unstable due to its single stranded structure, and it enables enzymatic amplification. Converting RNA to cDNA facilitates crucial, reliable, and sensitive gene expression research.

The process to conduct the quantitative polymerase chain reaction (qPCR), requires that the RNA must first be reverse transcribed into complementary DNA (cDNA), this was done using a Quantitect Reverse Transcription kit. Briefly, the RNA was placed on ice to thaw and then a master mix was prepared by adding Quantiscript RT, Quantiscript RT-Buffer, and RT Primer Mix in a tube and placed on ice until further use. Thereafter gDNA Wipe-out buffer (2 µL) was added into a small PCR tube containing RNA template (12 µL) to make a final concentration of 2 µg, the tubes were then incubated in the Eppendorf cycler at 42°C for 2 minutes and were immediately placed on ice thereafter. After the incubation, the master mix (6 µL) was then added to all the tubes (Table 5), and the tubes were further incubated at 42°C for 30 minutes and at 95°C for 3 minutes and then placed on ice. After this incubation, the cDNA was then diluted with 180 µL of RNASe-free water to bring up to a total volume of 200 µL. This was then mixed thoroughly, and the cDNA was ready to be used for qPCR.

Table 5: Reaction components of cDNA synthesis

Components	**Quantity**
gDNA Wipe-out buffer	2 µL
RNA template	12 µL
Quantiscript Reverse Transcriptase	1 µL
Quantiscript RT-Buffer	4 µL
RT Primer Mix	1 µL

3.12. Quantitative real-time PCR analysis

The molecular mechanism of *Moringa oliefera* in C2C12 skeletal muscles, was investigated by performing qPCR using the previously prepared cDNA. This was used to analyze the expression of the target genes (Table 6 expression of a *Gss* (glutathione synthetase), *Sod1* (superoxide dismutase), *As160* (Akt substrate of 160 kDa), *Insr* (insulin receptor), *Pi3k* (Phosphatidylinositol 3-kinase), *Pkc θ* (Protein kinase C θ). *B2m* (Beta-2- microglobulin) a well-known housekeeping gene was used as an internal control for the normalization. (Beta-2-microglobulin) Each qPCR reaction were prepared in separate Eppendorf tubes and kept on ice until further use. . Complementary DNA (4 µL) was added to the 96-well plate thereafter the master mix (6 µL) was added into the plate (Table 7). Thereafter, the plate was placed into the StepOne™ Real-Time PCR System machine (Thermo Fischer Scientific, Waltham, Massachusetts) and ran according to manufactures recommendation (50°C for 2 minutes, 95°C for 2 minutes, 95°C for 15 seconds and 60°C for 1 minute). The gene expression was normalized to the housekeeping gene *B2m*. The B2m gene was selected to standardize our data since it has shown steady gene expression in similar cell types and reduces error due to sample variance and processing. The relative expression was estimated with the ΔΔCt technique. After subtracting B2m's Ct from the target gene's Ct (ΔCt), we compared the ΔCt values to a calibrator sample to obtain ΔΔCt. This guarantees that the reported differences reflect actual changes in our gene expression and not experimental variances. Results were reported as the mean ±SD of 3 independent experiments at 100%.

Table 6: Probes used for PCR

Name	**Number**
Beta-2-microglobulin	Mm00437762_m1
Insulin receptor gene	Mm01211875_m1
Akt substrate of 160 kDa	Mm00557659_m1
Phosphatidylinositol 3-kinase	Mm01282781_m1
Protein kinase C θ	Mm01340228_m1
Superoxide Dismutase 1	Mm01344233_g1
Glutathione synthetase	Mm01246544_m1

Table 7: Components used for the PCR reaction

Components	**Quantity**
TaqMan master mix	5 µL
Probe	0.5 µL
Water	0.5 µL
cDNA	4 µL

3.13. Statistical analysis

All the values were expressed as the mean of ± standard error of the mean (SEM) and Graph Pad Prism™ 8.0.1.244 was used for statistical analysis. Comparisons of groups were performed by one-way ANOVA, post-hoc or student t-test where applicable. The Turkey post-hoc test was used to compare the significance of the rest of the groups to the control group. Significance was established at a p-value of < 0.05.

CHAPTER 4

This chapter provides a summary of the research we conducted for this project, and it will give a report of the findings of section 1 which is our objective 1 and section 2 and that is objective 2 and 3 of the study.

4. RESULTS

4.1. Objective 1: To investigate the bioactivity of *Moringa oliefera* in normal C2C12 skeletal muscle cells.

4.1.1. The effect of the dose and time of *Moringa oliefera* on cell viability in C2C12 skeletal muscle cells

The effect of *Moringa oliefera* extract on cell viability in C2C12 skeletal muscle cells was investigated using MTT assay a well-known assay for its cytotoxicity studies (Mosmann, 1983). The respective dosages (0.001,0.01,0.1,1,10 and 100 µg/mL) of *Moringa oliefera* were tested at different exposure periods (2,4,8,16 and 24 hours). Positive control insulin (1µM) had no effect in all time points tested. Similarly, quercetin (1µM) a known flavonoid present in *Moringa oliefera* had no significant effect on cell viability in all-time points tested (Fig 7 A- E). Treatment for 2 hours with *Moringa oliefera* did not affect cell viability in all concentrations tested (Fig 7 A), similar results were obtained at 4 hours (Fig 7 B). Exposing the cells for 8 hours yielded significant increases in cell viability at doses 0.1 ($p< 0.001$),1 and 10 µg/mL ($p<0.01$) and 100 µg/mL ($p<0.001$) (Fig 7 C). At 16 hours cell viability was increased at a of dose 0.01 µg/mL (<0.05) and 10 µg/mL ($p<0.05$) respectively (Fig 7 D). Incubating with *Moringa*

oliefera for 24 hours the results show a significant increase at 0.001 ($p<0.01$) and 0.01 µg/mL ($p<0.05$) and slight decreases in cell viability from 0.1-100 µg/mL in a dose-dependent manner (Fig 7 E). Based on our result, for further studies on bioavailability and glucose uptake, only one time point was selected (8 hrs) for further assays.

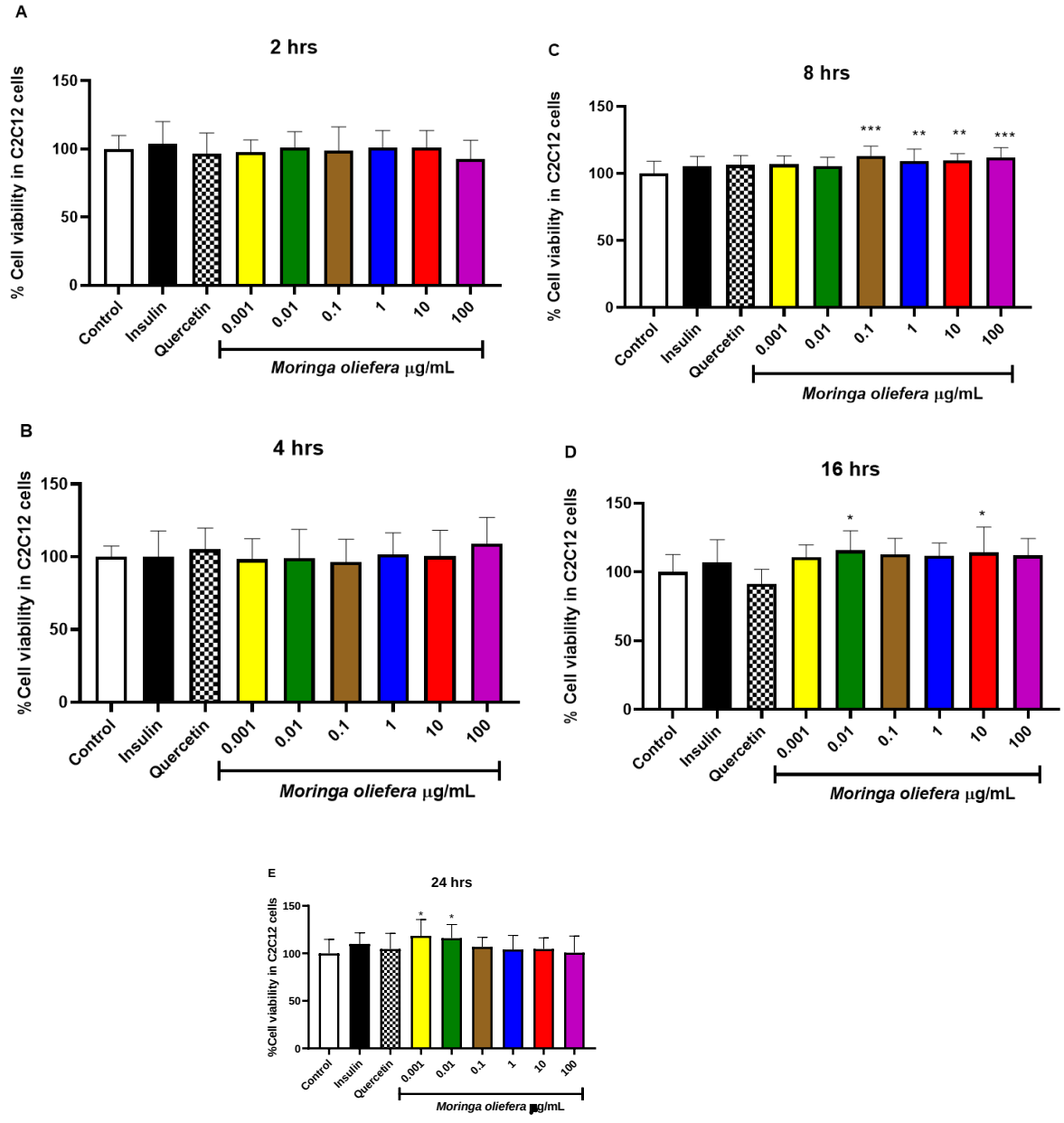


Figure 7: The effect of *Moringa oliefera* extract on cell viability in C2C12 skeletal muscle cells treated for 2 hrs (A), 4 hrs (B), 8 hrs (C), 16 hrs (D) and 24 hrs (E). Differentiated C2C12 skeletal cells were treated with 8 mM glucose containing various doses of *Moringa oliefera* (0.001,0.01,0.1,1,10 and 100 µg/mL) at various time points (2,4,8,16 and 24) insulin and quercetin (1 µM) were used as a positive control with insulin added the last for 15 minutes. Thereafter MTT assay was performed. Results were reported as the ±SD mean of 3 independent experiments. Significance was reported as *$p<0.05$, **$p<0.001$, ***$p<0.0001$, ****$p<0.0001$ relevant to control at 100%.

4.1.2 The effect of *Moringa oliefera* on glucose utilization and glycogen concentration in C2C12 skeletal cells

The subsequent experiments were based on the 8 hrs exposure period due to the significant effects *Moringa oliefera* displayed on cell viability of C2C12 skeletal cells in all the dosages. Glucose uptake was measured after 8 hrs of treatment with *Moringa oliefera*. The positive control and *Moringa oliefera* had no significant effect on glucose uptake (Fig 8 A). Thereafter the glycogen content was assessed using the glycogen assay and the results showed that *Moringa oliefera* significantly increased glycogen content in doses 0.001-10 µg/mL ($p>0.05$), ($p>0.001$), ($p<0.001$), ($p<0.05$) compared to the control respectively (Fig 8 B).

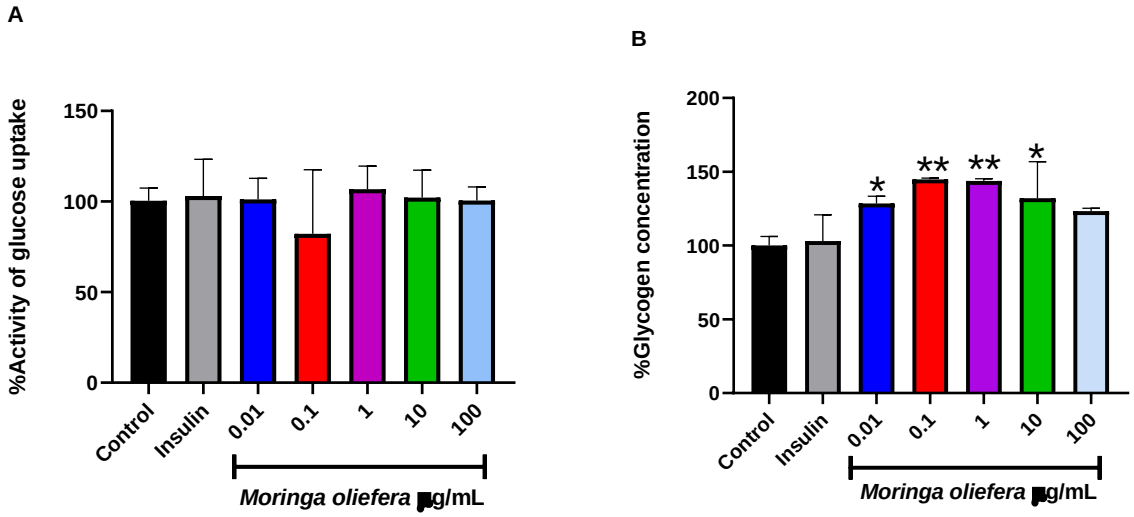


Figure 8: Effect of *Moringa oliefera* of glucose uptake (A) and glycogen content (B) in normal C2C12 skeletal cells. Differentiated C2C12 skeletal muscles were treated with *Moringa oliefera* (M), 8 mM glucose for 8 hrs, and insulin (1 µM) for 15 minutes to assess the effect it will have on the glucose uptake and glycogen content using the glycogen assay. The results were reported as the mean of 3 independent experiments ±SD relative to the control. Significance was reported as *** $p<0.0001$, **$p<0.001$ and *$p<0.05$ versus the control.

4.2. Objective 2: To investigate the effect of *Moringa oliefera* leaf extract on palmitate-induced insulin resistant C2C12 skeletal muscle cells at a cellular level.

4.2.1. Effect of *Moringa oliefera* on cell viability and cytotoxicity in palmitate induced-insulin resistant C2C12 skeletal cells

In order to achieve the second objective, this study investigated the effect of *Moringa oliefera* on cell viability in palmitate-induced insulin-resistance C2C12 skeletal muscle cells. Insulin increased cell viability compared to normal control. Treating the cells with 0.75 mM palmitate for 16 hrs significantly reduced cell viability ($p<0.0001$). In the palmitate-treated cells, we observed an increase in cell viability after the addition of insulin ($p<0.001$) and ($p<0.0001$) compared to palmitate control and normal control. The cellular activity was significantly increased at all the doses 0.001 µg/mL ($p<0.0001$),0.01 µg/mL($p<0.0001$) 1 µg/mL and 100 µg/mL($p<0.0001$) respectively compared to palmitate (Fig 9).

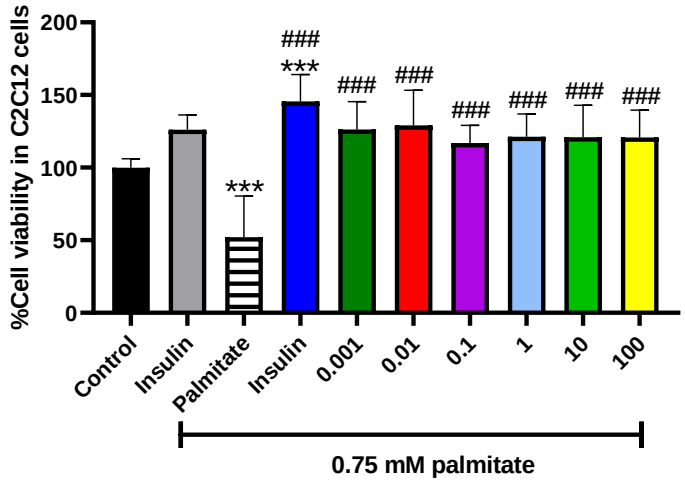


Figure 9: Effect of *Moringa oliefera* on cell viability in insulin resistant C2C12 skeletal cells. The cells were cultured in 8 Mm glucose with or without palmitate (P) (0.75 Mm) for 16 hrs and different doses of *Moringa oliefera* for 8 hrs, insulin (I) (1 µM) was added in the last 15 minutes, cell viability was assessed with the aid of the MTT assay. Results were expressed as the mean of ±SD of 3 independent experiments. Significance denoted with * $p<0.01$, **$p<0.001$, ***$p<0.0001$ compared to the control and ###$p<0.0001$ compared to palmitate.

4.2.2. Effect of *Moringa oliefera* on glucose metabolism

The outcome of *Moringa oliefera* regarding glucose uptake in insulin resistant C2C12 skeletal muscle cells was investigated. The positive control insulin had no effect, while palmitate showed to reduce glucose uptake within the C2C12 skeletal cells, however this was not significant. In insulin resistant cells, insulin was able to significantly increase the glucose uptake ($p<0.001$). Treatment with *Moringa oliefera* significantly increased glucose uptake at dose 10 µg/mL ($p<0.05$) compared to the palmitate (Fig 10).

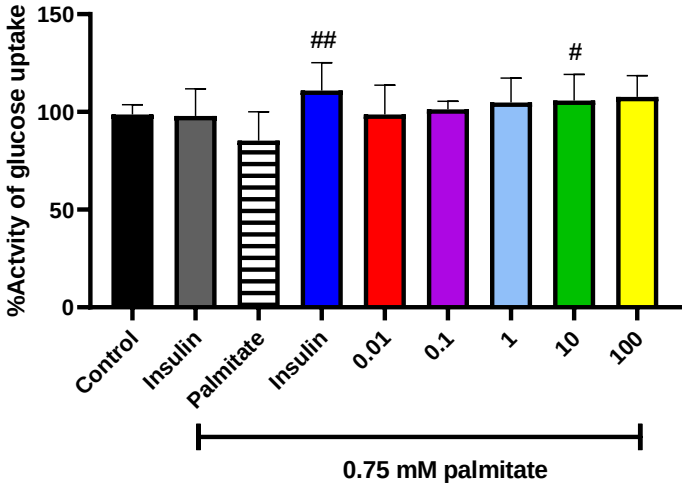


Figure 10: Effect of *Moringa oliefera* on glucose uptake.C2C12 cells cultured in 8 mM glucose were with or without 0.75 mM palmitate treated with *Moringa oliefera* for 8 hrs and insulin (1 µM) for 15 minutes. Substrate consumption was measured in culture media by a monitoring blood glucose monitoring system and the results were subtracted from 8 mM glucose. The results were reported as the mean of 3 independent experiments ±SD. Significance was denoted by #p<0.05 and ##p<0.001 compared to palmitate control.

4.3. Objective 3: To investigate the effect of the leaf extract on the expression of the insulin signalling and anti-inflammatory genes (*Insr Pkcθ, Pi3k, As160, Sod1,* and *Gss,*)

4.3.1. Effect of *Moringa oliefera* on insulin signaling pathway in palmitate induced insulin resistance in C2C12 skeletal muscle cells

Treating the C2C12 skeletal muscle cells with 0.75 mM palmitate for 16 hrs did induce insulin resistance, as the results indicated that palmitate reduced mRNA expression of *Insr* and the addition of insulin ($p<0.001$) in the insulin resistant cells there was a noticeable rise in the *Insr* mRNA expression. *Moringa oliefera* had no effect at 0.01 µg/mL, however there was a meaningful elevation at 1 ($p<0.05$),10 ($p<0.05$) and 100 µg/mL ($p<0.05$) compared to palmitate treated cells (Fig 11 A). In terms of *Pkcθ* gene expression, a known marker for insulin resistance, our results indicate that palmitate increased the mRNA expression of *Pkcθ* ($p<0.0001$) compare to normal control (Fig 11 B). The addition of *Moringa oliefera* for 8 hrs and insulin (1 µM) for 15 minutes reduced this effect significantly with insulin reducing mRNA expression of *Pkcθ* ($p<0.0001$), while *Moringa oliefera* treated cells reduced *Pkcθ* at 0.01-100 µg/mL ($p<0.0001$) compared to palmitate control respectively (Fig 11 B).

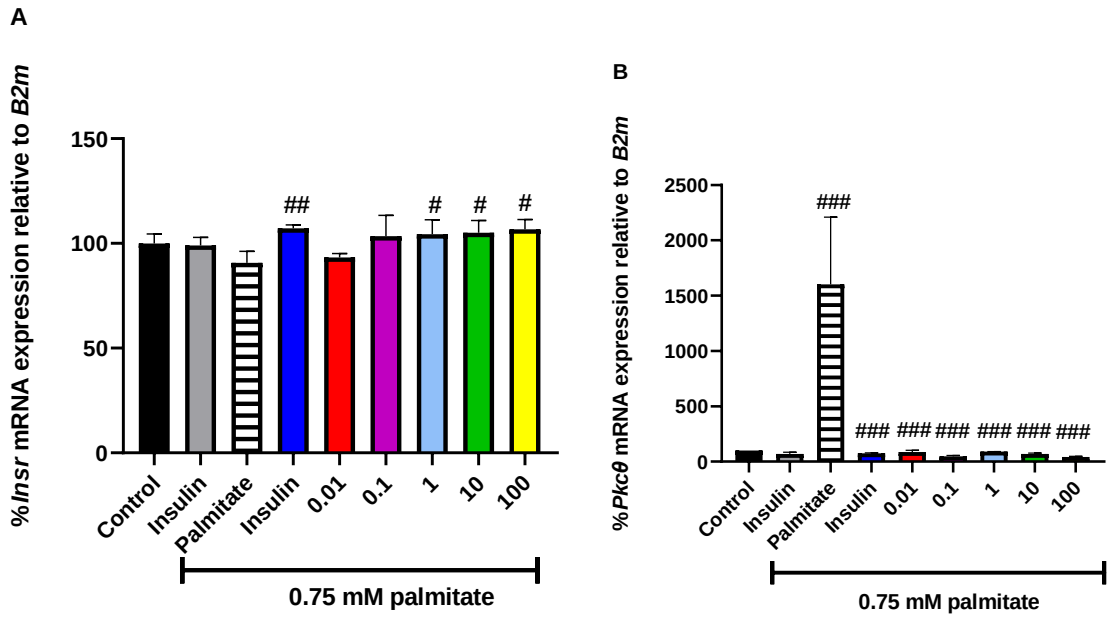


Figure 11: The effect of *Moringa oliefera* on the mRNA expression of Insr (A) and *Pkcθ* (B). C2C12 skeletal muscle cells were cultured in 8 mM glucose with or without 0.75 mM palmitate with *Moringa oliefera* for 8 hrs and for 15 minutes with insulin (1 µM). Thereafter mRNA that was quantified for qPCR analysis for *Insr* and *Pkcθ*, the results were normalized to the housekeeping gene *B2m*. The results were expressed as the ±SD mean of 3 independent experiments relative to the control. Significance was reported as *$p<0.05$, **$p<0.001$, ***$p<0.0001$ to the control and #$p<0.05$, ###$p<0.0001$ compared to palmitate.

4.2.4. Effect of *Moringa oliefera* on insulin signaling pathway in palmitate induced insulin resistance in C2C12 skeletal muscle cells

Insulin had no effect on the mRNA expression of *Pi3k* compared to the control, whilst palmitate decreased the expression of *Pi3k* ($p<0.0001$). The addition of insulin in insulin resistant cells had a significant effect, as it increased the mRNA expression ($p<0.0001$). *Moringa oliefera* significantly increase *Pi3k* in all concentration tested (0.01 ($p<0.0001$) ,0.1 ($p<0.0001$),1 ($p<0.0001$),10 ($p<0.0001$) compared to palmitate control while 100 µg/mL increased by $p<0.05$ compared to normal cells and ($p<0.0001$) compared to palmitate control (Fig 12A). Furthermore, to evaluate the ability of *Moringa oliefera* to increase glucose metabolism, expression of *As160,* an AKT substrate that is responsible for the insulin-stimulated translocation of GLUT4 to the plasma membrane was evaluated. Our results showed that palmitate slightly decreased *As160* mRNA expression showing a reduction in glucose metabolism, but with the addition of insulin ($p<0.0001$) there was a significant increase of *As160* expression confirming a reduction in insulin resistance in the C2C12 skeletal cells. Addition of *Moringa oliefera* did not elicit any statistically significant increases on mRNA expression at concentrations 0.01.0.1,1 and 10 µg/mL however at 100 µg/mL ($p<0.05$) the increase was significant (Fig 12 B).

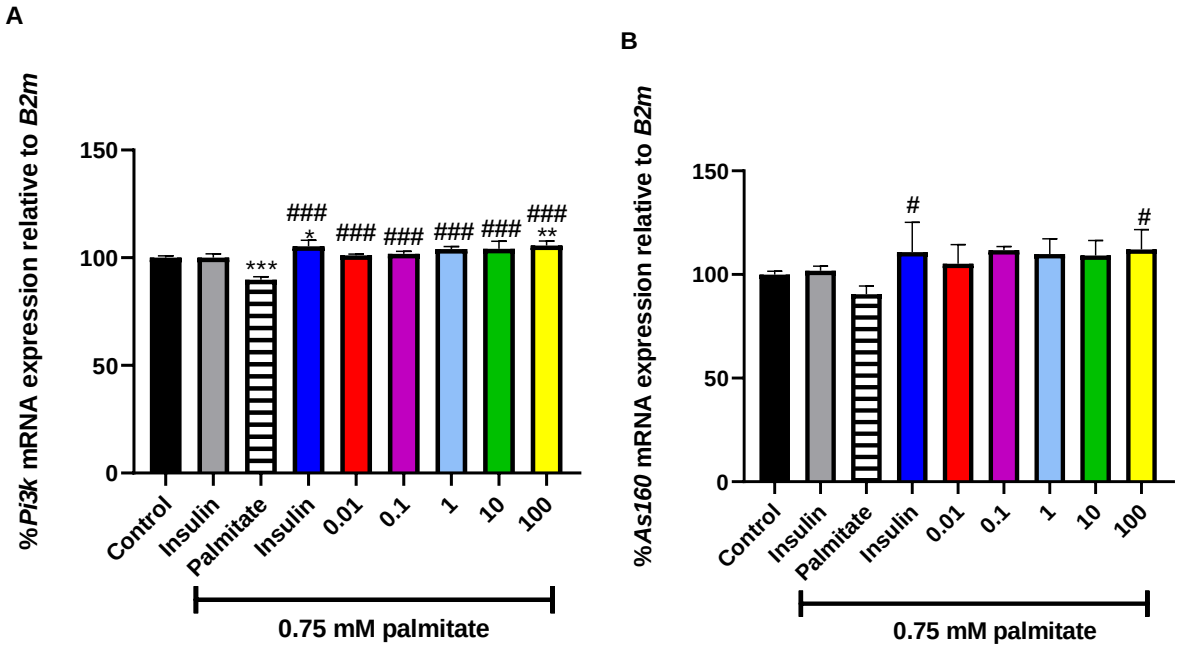


Figure 12: To assess the effect that *Moringa oilefera* has on the insulin signaling pathway *Pi3k*(A) and *As160* (B) the cells were cultured in 8 mM glucose with or without 0.75 mM palmitate (P) for 16 hrs to induce insulin resistance, thereafter they were treated with insulin (I) (1 µM) for 15 minutes and *Moringa oliefera* for 8 hrs. The mRNA was extracted from the cells and subjected to qPCR and the results were normalized to the housekeeping gene *B2m*. The results were expressed as the ±SD mean of 3 independent experiments relative to the control. One-way analysis of variance (ANOVA) was and Tukey post hoc test used for statistical analysis. Significance was reported as *$p<0.05$, , **$p<0.001$, ***$p<0.0001$ to the control and #$p<0.01$ and ###$p<0.0001$ versus palmitate.

4.2.5. The effect of *Moringa oliefera* on enzymatic antioxidant activity of superoxide dismutase (*Sod1*) and glutathione (*Gss*)

Oxidative stress could be the main therapeutic target of which *Moringa oliefera* could target to combat or reverse diabetes. Insulin had no effect on *Sod1* expression, but palmitate significantly reduced the mRNA expression of *Sod1* ($p<0.001$) compared to normal control (Fig 13A). In the insulin resistant cells insulin showed a slight increase on mRNA expression. Our results showed that there was a slight increase in *Sod1* activity at doses 0.01,1 and 10 µg/mL ($p<0.01$) compared to the palmitate control. Unexpectedly at 0.01 µg/mL a reduction of *Sod1* activity was observed compared to the normal control. The mRNA expression of *Gss* was measured (Fig 13B), our results showed that insulin significantly increased the mRNA expression of *Gss* activity ($p<0.0001$) interestingly, palmitate decreased the mRNA expression of *Gss*, however this was not significant. *Moringa oliefera* also increased antioxidant enzyme activity significantly at 0.01 and 0.1 µg/mL ($p<0.01$) respectively.

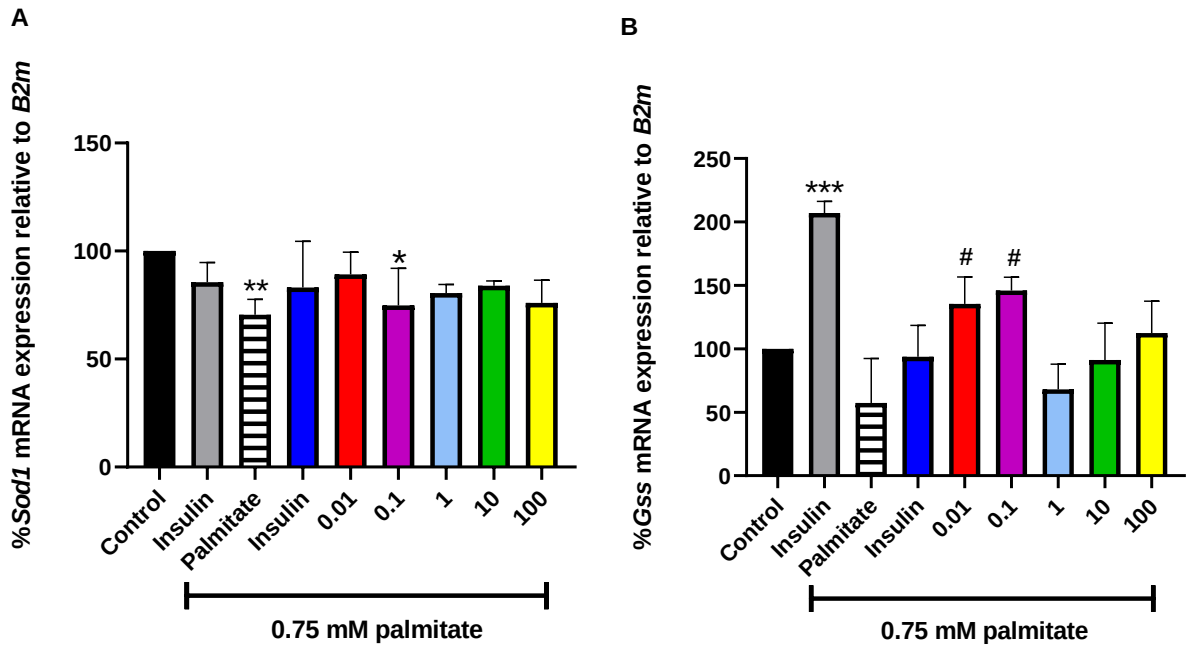


Figure 13: Effect of *Moringa oliefera* on enzymatic antioxidant activity *Sod1*(A) and *Gss*(B). The C2C12 skeletal cells cultured in 8 mm glucose with or without 0.75 palmitate (P) were treated with *Moringa oliefera* for 8 hrs and insulin (I) (1 µM) for 15 minutes. The cells were lysed and mRNA was extracted then it was quantified by qPCR. Results were normalized to the housekeeping gene *B2m.* Significance was denoted by *$p<0.01$, **$p<0.001$, ***$p<0.0001$ and #$p<0.01$ compared to palmitate compared to the control.

CHAPTER 5

This is the chapter that gives a discussion on the findings of this project.

5. Discussion

5.1. Summary

Skeletal muscle plays a pivotal role in metabolic health and accounts for 70–80% of peripheral glucose clearance; as a result, muscle mass must be kept in good condition at all times through exercise, dietary or hormonal therapy, or even plant polyphenols to prevent against disease development (Bayol et al., 2014; Duranti et al., 2021; Frontera & Ochala, 2015; Nikawa et al., 2021). A poor diet rich in saturated fatty acids, such as palmitate has been reported to inhibit glucose metabolism and raises the amount of free fatty acids circulating in the skeletal muscle, which leads to muscular insulin resistance. Increased levels of free fatty acids in in vitro model have been shown to activate *Pkcθ, Irs1(ser^{307}),* which in turn deactivates *Irs1* and other pathways like the PI3K/AKT pathway, leading to a reduction in insulin sensitivity (Mazibuko-Mbeje et al., 2013; Szendroedi et al., 2014). T2DM is characterized by insulin resistance, which is brought on by a decrease in insulin sensitivity. About 90% of diabetic cases are type 2 diabetes (T2DM), which is distinguished by a chronic hyperglycemia (Hong et al., 2023).Due to their sedentary lifestyles and heavy fat and carbohydrate diets, adults are the ones who experience it most frequently(Hong et al., 2023). We know that changing one's diet and lifestyle can help with type 2 diabetes (T2DM); nevertheless, in today's hectic environment, it can be challenging for people to maintain such rigorous lifestyle changes. For this reason, medications or other therapies are essential to managing T2DM. Clinical medications like biguanide and sulfonylureas are used, but they carry a risk of resistance and side effects. As a result, new and natural therapies must be created to manage resistance and potential side effects (Abdelmoneim et al., 2015; Bell, 2006; Blahova et al., 2021; Monami et al., 2013)

In light of its many therapeutic benefits, including those related to cardiovascular health, antihypertensive, anticancer, antifatigue, antioxidant, and antidiabetic effects, *Moringa oliefera* was of interest in this particular study (Dong et al., 2024; Mthiyane et al., 2022; Sultan et al., 2023). The ability of the plant *Moringa oliefera* to reduce blood glucose levels brought on by diabetogenic medications or a high-fat diet makes it special (Bassogog et al., 2024). Specifically focusing on Akt1, GADPH, and the PI3K-Akt signaling pathway, the plant can improve insulin resistance(Hong et al., 2023) . In order to repair the dysfunction caused by oxidative stress, it targets enzymes such as SOD, CAT, and GSH. Additionally, it reduces MDA, a biomarker of lipid peroxidation (Mohlala et al., 2023; Reda et al., 2023; Wu et al., 2023). According to Siahaan (Siahaan et al., 2022) and Xiao (Xiao et al., 2020), the plant in question targets and lowers TNF-α, which also reduces inflammation.

5.2. Effect of *Moringa oliefera* on cell viability in normal C2C12 skeletal cells

Natural plants have gained popularity due to that they are rich in polyphenols that can play a role in the management of diseases like T2DM. Therefore, the intention of this research project was to examine the potential effect of *Moringa oliefera* in normal and insulin resistance C2C12 mouse skeletal muscle cells. In terms of cell viability, our findings demonstrated that shorter exposures to *Moringa oliefera* (2 and 4 hours) had no impact on the viability of the cells, whereas 8 hours treatment resulted in significantly higher metabolic activity in the C2C12 skeletal cells. These results are similar to those reported by Yun et al in (2020), where they conducted cell viability in C2C12 cells treated with (0, 0.05, 0.10, 0.25, and 0.5 mg/mL) *Moringa oliefera* for 24 hours (Yun et al., 2020). Furthermore, our results showed that *Moringa*

oliefera continued to be stable and increased cell viability even at 16 hours, however, quercetin showed to lose its activity after 16hrs (Sidha Bhagawan et al., 2017). Interestingly, after 24 hours of exposure, cell viability was reduced in a dose-dependent manner, suggesting instability of the extract at 24 hrs and these results are in contradiction, *Moringa oliefera* has been investigated for its impact on cell viability and toxicity using different cell types, such as HepG2 cells, and it was found to be more effective at higher concentrations (500 µg/mL) and to have no adverse effects on these cells even after 24 hours exposure (Sosa-Gutiérrez et al., 2018). In a study utilizing *Caco-2 cells*, *Moringa oliefera* 0.025,0.05,0.075,0.1 and 0.125 mg mL^{-1} , also enhanced the cellular activity of the cells after 24 and 48 hours treatment (Castro-López et al., 2020). All subsequent tests were predicated on the time point and doses used in the initial 8 hours of treatment of healthy cells with *Moringa oliefera*, which increased metabolic activity at all doses.

5.3. Effect of *Moringa oliefera* on glucose uptake and glycogen concentration in normal C2C12 skeletal muscle cells

The findings show that exposure of the C2C12 skeletal cells with *Moringa oliefera* had partial effects on glucose uptake in all the doses. This data suggests that *Moringa oliefera* could maintain basal glucose levels normal and allowing for everyday bodily functions. (Khan et al., 2017) showed that the oral administration of *Moringa oliefera* to rats in normoglycemia had no significant effect on blood glucose levels and this is a bit similar to the results observed on the C2C12 skeletal cells. Evidence shows that a substantial amount of glycogen is stored in skeletal muscle (~500 g) and it is used as the main energy substrate (Jensen et al., 2011). This data showed that *Moringa oliefera* significantly enhanced insulin stimulated glycogen content, suggesting that it has ability to increase glucose uptake or store it for late use. This suggests that *Moringa oliefera* extract may increase the activity of glycogen synthase and other enzymes involved in the metabolism of glycogen, including hexokinase (HK), glucokinase (GK), and phosphofructokinase (PFK).

According to several researchers, aberrant insulin action to store glucose as glycogen is the fundamental cause of insulin resistance (Crosson et al., 2003; Levin et al., 2001; Velho et al., 1996; Warram et al., 1990) . Since our plant is known to improve insulin-stimulated glycogen synthesis, we anticipated a rise in glycogen content after treating with *Moringa oliefera*. Several studies, showed decreased glucose metabolism, higher plasma free fatty acids, and impaired insulin signaling pathway result in lower levels of insulin-stimulated muscle glycogen synthesis and glycogen synthase activity in T2D patients (Cline et al., 1999; He & Kelley, 2004; Henry et al., 1996; Nikoulina et al., 2000; Vats et al., 2004). We reported that *Moringa oliefera* significantly increased glycogen content in the current study, and these results are consistent with a study by (Olayaki et al., 2015) that showed *Moringa oliefera* has hypoglycemic effects as a result of its capacity to increase glycogen content in rats with alloxan-induced diabetes. Similarly our results showed that increase in glycogen content in *Moringa oliefera* treated cells possible reduce hyperinsulinemia and hyperglycemic condition by storing the majority of the glucose as glycogen (Sylow et al., 2021), in accordance with research. These findings suggest that *Moringa oliefera* could have adaptogenic effects, but this could be further explored in in vivo studies.

5.4. Protective potential of *Moringa oliefera* in palmitate induced insulin resistant C2C12 skeletal cells

A well-established model on insulin resistance was used in this study. The findings indeed show that 0.75 mM palmitate caused insulin resistance in C2C12 cells as cell viability was reduced by palmitate and improved by *Moringa oliefera* after 8 hrs of treatment. These observations show that *Moringa oliefera* improved insulin resistance caused by palmitate by increasing cell viability. Mazibuko et al (2013) reported similar results but using *Aspalathus linearis* commonly known as rooibos, they reported that fermented rooibos extract and aspalathin-enriched green rooibos extract does improve mitochondrial activity in C2C12 skeletal cells in insulin-resistant cells. Another study reported that epigallocatechin gallate (EGCG) was able to reverse the effects of palmitate induced insulin resistance in C2C12 skeletal cells after 48 hrs, and 60 hrs with curcumin and after 72 hrs of exposure with epicatechin gallate (ECG) at doses 1,5,10 and 20 µM (Deng et al., 2012).

5.5. Activity of glucose uptake in palmitate induced insulin-resistant C2C12 skeletal cells after treating with *Moringa oliefera*

Through measuring glucose uptake in culture media, the investigation into substrate consumption revealed that *Moringa oliefera* modestly enhanced glucose utilization. *Moringa oliefera* only elicited a significant effect on glucose uptake at 10 µg/mL but could not at other doses, maybe the cells could not completely take up the raw plant due to its solubility and there could have been a problem with the binding affinity of *Moringa oliefera* and the C2C12 skeletal cells. A similar tendency was noticed in a study using yeast cells that indicated that the *Moringa oliefera* leaf powder increased glucose translocation in a dose-dependent manner. The results demonstrated a dose-dependent increase in glucose absorption by the C2C12 cells with a considerable increase at 100 µg/mL. The effect of aqueous extract of *Moringa oliefera* on glucose uptake in isolated rat skeletal muscle cells was further examined in the same study. The results showed that glucose uptake increased dose-dependently, with maximum activity being recorded at the highest dose of 100 µg/mL. (Khan et al., 2017). These findings suggest that *Moringa oliefera* may act as an antihyperglycemic drug and have an impact on glucose uptake in insulin-resistant cells. Interestingly, Khan et al (2017) used a water extract of *Moringa oliefera* leaves and yeast cells and a longer exposure period, but our findings had a similar effect to his on glucose uptake. Given that Aini and colleagues (Aini et al., 2023) reported that giving 450 mg/kg of *Moringa oliefera* to diabetic rats induced with alloxan significantly reduced blood glucose levels, increased liver glycogen content, and even repaired liver cell damage, it is true that *Moringa oliefera* improves persistent hyperglycemia. These findings highlight the critical role that *Moringa oliefera* plays in improving glucose metabolism overall and mitigating the majority of the risk factors associated with type 2 diabetes. In one study, alloxan-induced diabetic mice were used to assess the diabetic effect of a mixed leaf and seed extract of *Moringa oliefera*. After giving the mice, a dose of 500 mg/kg, the researchers in this study discovered that the combined extracts considerably increased glucose tolerance by lowering blood glucose levels (Aljazzaf et al., 2023).

Numerous researchers have suggested that the antihyperglycemic and hypoglycemic properties of *Moringa oliefera* can improve glucose metabolism by promoting the release of insulin, which will increase glucose uptake and glycogen synthesis (Nova et al., 2020). The results of the current study, show that the glucose biomarkers were significantly and moderately improved by *Moringa oliefera* and by

these observations in agreement with other studies, it is safe to suggest that *Moringa oliefera* is an antidiabetic therapy that targets insulin-dependent glucose biomarkers (As160, *Pi3k*, glucose blood levels).

5.6. Impact of *Moringa oliefera* on insulin signaling pathway

Additionally, palmitate triggered and upregulated *Pkcθ* expression in C2C12 skeletal muscle cells. *Pkcθ* phosphorylates *Irs1 (ser^{307})* is known to impair the insulin signaling pathway (PI3K/AKT pathway). This study demonstrated *Moringa oliefera's* ability to suppress *Pkcθ* activation. Other researchers that used C2C12 myotubes cultured in 0.75 mM palmitate for 18 hours showed that palmitate does indeed induce insulin resistance through the phosphorylation of *Pkcθ* and these results are consistent with those of the current study (Wang et al., 2010). One study showed that the activation of *Pkcθ* induced insulin stimulated vasoconstriction through inhibiting *Akt* and the stimulation of extracellular signal–related kinase (*ERK1/2)* in muscle resistance arteries, linking *Pkcθ* activation to insulin resistance (Bakker et al., 2008). A study that used rodents fed a high fat diet (palmitic acid) reported that indeed it does cause hypothalamic insulin resistance by increasing DAG levels which then facilitated the translocation of *Pkcθ* in the rodents (Benoit et al., 2009). Interestingly literature shows that plants do attenuate insulin resistance through the reduction of *Pkcθ* translocation (Dube et al., 2007; Lee & Lin, 1997; Li et al., 2011).

An unexpected finding in this study was that palmitate did not significantly increase insulin resistance. This finding could be attributed to the cells' affinity for palmitate or to a slight contraction of the cells that led to acute insulin resistance (Nieuwoudt et al., 2017). It will be difficult to determine whether the acute insulin resistance is caused by palmitate-induced insulin resistance because we were not examining how contraction shields muscle from that. Nevertheless, research can be done to find the answers to these unexpected findings. The contraction may have happened as the cells were being moved during the experiment, acting as a little electrical pulse stimulant(Lambernd et al., 2012). The results are likely to have a negative impact on the study, |since it could be possible that our insulin resistance model did not induce insulin resistance, but it may be beneficial in preventing palmitate-induced insulin resistance using muscle contractions and this needs to be looked into.

The insulin receptor plays a crucial part in the insulin signaling pathway and when the effect of *Moringa oliefera* was investigated on the pathway with the aid of C2C12 skeletal muscle cells and the results were satisfactory. When insulin binds to the insulin receptor it causes a cascade of pathways to be phosphorylated such as the *Irs1* thereafter the PI3K/AKT pathway, and this then allows glucose to be translocated with the GLUT4 transporters into the cell (Stöckli et al., 2011). The results showed that with the addition of insulin to the insulin resistant cells there was a significant increase in the mRNA expression of *Insr.* Furthermore, our research revealed that *Moringa oliefera* enhanced *Insr's* mRNA expression, with the highest level of expression occurring at 1,10 and 100 µg/mL. An in vivo study reported that *Moringa oliefera* reversed hepatic insulin insensitivity in rats fed a high fructose diet through upregulating the insulin receptor gene and these results are in agreement with our results (Mohamed et al., 2019) .One particular study used the extract of a white sweet potato on insulin resistant C2C12 skeletal muscles and displayed similar results as that of *Moringa oliefera*. Different concentrations

of the sweet potato were used and the highest concentration (200 µg/mL) yielded results consistent with our study (Shyur et al., 2021). The sweet potato extract demonstrated that it has the similar effects on insulin resistance as *Moringa oliefera* does. Epicatechin (20 mg/kg body weight) prevented the downregulation of *Insr* in the insulin resistant adipose tissue of mice fed a high fat diet even though these results were observed in an animal study but they were consistent to our results (Cremonini et al., 2016).Interestingly, 0.5 and 1% of Korean red ginseng (Panax ginseng) improved insulin sensitivity by enhancing the mRNA expression of *Insr* in insulin resistant skeletal muscle cells of *ob/ob* mice and these results are consistent to ours (Cheon et al., 2015). These findings on *Moringa oliefera* suggest that it can modulate insulin signaling pathway by upregulating the insulin receptor.

The PI3K/AKT pathway is also an important part of the insulin signaling pathway therefore we investigated the effect that *Moringa oliefera* would have on the *Pi3k* gene and the results showed that *Moringa oliefera* has good effects on this pathway. Insulin had similar effects on *Pi3k* expression as that of *Insr* and interestingly, *Moringa oliefera* was able to significantly upregulate the activity of *Pi3k* from the lowest dose to the highest dose. The findings on the impact that *Moringa oliefera* on *Pi3k* expression were similar with those of (Khan et al., 2017), who employed lower quantities of the plant using rat skeletal muscle and they found that even at lower doses, it demonstrated considerable antidiabetic potential. Another study found that an isothiocyanate-rich *Moringa oleifera* extract improved insulin sensitivity by upregulating *Pi3k* levels in the liver of rats fed a very high fat diet and these results corroborate our findings on *Moringa oliefera* in skeletal muscle cells (Waterman et al., 2015). Apart from *Moringa oliefera* other plants such as *Atractylode japonia koidzumi* have been studied and they have shown similar results as the ones that were observed in this current study. The roots of this plant were researched and employed on 3T3-L1 adipocytes and they activated the insulin signaling pathway by upregulating *Pi3k* levels suggesting that it has antidiabetic effects (Han et al., 2012). Literature also reports that guava leaf extract alleviated hepatic insulin insensitivity in KK-Ay type 2 diabetic rats by increasing the mRNA expression of *Pi3k* gene (Yang et al., 2020) .

We further observed that the addition of insulin to the insulin resistant cells significantly upregulated the *As160* levels, however, *Moringa oliefera* was only able to elicit a significant increase in *As160* levels at 100 µg/mL. A tangeretin from citrus fruit was used on insulin resistant C2C12 myotubes from mice fed a high fat diet to assess if they could phosphorylate *As160* and their results are in agreement with the ones obtained in this study. Berberine was assessed on insulin resistant myotubes on *As160* level and their findings showed that it increased the phosphorylation of this gene as our plant did (Kim et al., 2012). Thujone which is a monoterpene, present in antidiabetic herbs was reported that it upregulated *As160* levels in palmitate induced insulin resistant C2C12 skeletal muscle cells corroborating with our results. The insulin resistant skeletal muscle cells were treated for 6-12 hrs with 0.01 mg/mL Thujone to get complete restoration of *As160* phosphorylation (Alkhateeb & Bonen, 2010). The polyphenols that are present in *Moringa oliefera* and other plants contribute to the plants having medicinal effects against diseases such as cancer, cardiovascular diseases and neurodegenerative diseases (Ayoka et al., 2022; Burgos-Morón et al., 2019; Kashyap et al., 2022; Kou et al., 2018; Singh et al., 2020).

5.7. Effect of *Moringa oliefera* on enzymatic antioxidant activity

We found that *Moringa oliefera* was able to activate the antioxidant enzymes *Sod1* and *Gss*, indicating that our plant may be utilized as an alternative therapy to synthetic medication.

A study by Ceci et al (2022) reported that *Moringa oliefera* enhances deficient antioxidant enzyme activity in C2C12 cells treated with 1/1000 and 1/100 working solution; these results are consistent with the findings of this investigation and provide further evidence of the antioxidant ability of *Moringa oliefera*. At the lowest concentrations of 0.01 and 0.1 µg/mL, *Moringa oliefera* considerably boosted *Gss* activity. Analogous findings were found with *Sod1* activity. *Moringa oliefera* modulates redox state in C2C12 cells by altering total antioxidant capacity and enhancing the activity of antioxidant enzymes (SOD, CAT, GSH, GST, and GPx), according to research by (Duranti et al., 2021), and these findings corroborate our findings from this study. The disruption of the insulin signaling system is caused by oxidative stress, which is a fundamental underlying cause of insulin resistance (Rains & Jain, 2011). By scavenging free radicals, antioxidants are crucial in the prevention of oxidative stress, which therefore enables them to defend against both communicable and non-communicable diseases (Sreelatha & Padma, 2009). Interestingly, other plants also exhibit antioxidant effects similar to that of *Moringa oliefera*. *Mimosa pudica* is one plant that was evaluated for its antioxidant activity and the results showed that it increased *Sod* and *Gss* (*Gsh*) activity in the brains of rats and the highest concentrations (200 and 400 mg/kg) gave significant results (Patro et al., 2016). Furthermore, *Ipomoea batatas* (orange-fleshed sweet potato) is one plant that attenuated oxidative stress by increasing the *Gsh* levels in insulin resistant C2C12 skeletal muscle cells (Ayeleso et al., 2018). In addition to these plants saffron extract increased the antioxidant activity of *Sod* in the lens tissue of streptozotocin-induced diabetic rats (Talebanzadeh et al., 2018). Fermented rooibos extract is also among the plants that have antioxidant activity, and it was reported that it attenuated oxidative stress in H9c2 cardiomyocytes by enhancing the antioxidant enzymatic activity of *Sod* and *Gsh* (Dludla et al., 2020). Sage (*Salvia officinalis* L.) is also one plant that that has antioxidant activity like the plant of interest *Moringa oliefera.* Furthermore, sage was used on *Caco-2* and it attenuated oxidative stress through increasing cellular *Gsh* after 24 hours of treatment (Aherne et al., 2007). Zou and colleagues demonstrated that oregano oil exhibits protective effects against oxidative stress by increasing the mRNA expression of *Sod1* in IPEC-J2 at 12 hrs of treatment (Zou et al., 2016). In a study done by Ertik and colleagues, there is evidence that *Moringa oliefera* reversed oxidative damage induced by valproate in muscles by increasing glutathione, superoxide dismutase, glutathione peroxidase, total antioxidant status, aryl esterase and sodium/potassium ATPase (Ertik et al., 2023). This evidence on these plants shows that plants exhibit antioxidant properties and this is consistent with *Moringa oliefera* that was investigated in this current study.

6. CONCLUSION

Firstly, *Moringa oliefera* had no toxic effects on the C2C12 skeletal cells and it was able to improve cellular activity suggesting *Moringa oliefera* to having protective effects. In our study the results show that the effects of palmitate-induced insulin resistance could be mitigated by *Moringa oliefera* by lowering *Pkcθ* and activating *Insr* and *Pi3k*, the plant was able to sensitize the insulin signaling pathway. The activation of *As160*, boosted glucose uptake and improved glycogen content in C2C12 skeletal muscle cells, it also improved overall glucose metabolism. The enzymatic antioxidants *Sod1* and *Gss* were activated by *Moringa oliefera* suggesting that oxidative stress was mitigated by the plant of interest. These findings imply that this mechanism of action is how *Moringa oliefera* reduces insulin resistance at a molecular level in skeletal muscle, however it should be studied further until it reaches to translational research or clinical trials. The findings of our research demonstrate the potential therapeutic value of *Moringa oliefera* for the treatment of diabetes by reducing oxidative stress, insulin resistance and improving glucose metabolism in skeletal muscle cells (Fig 14).

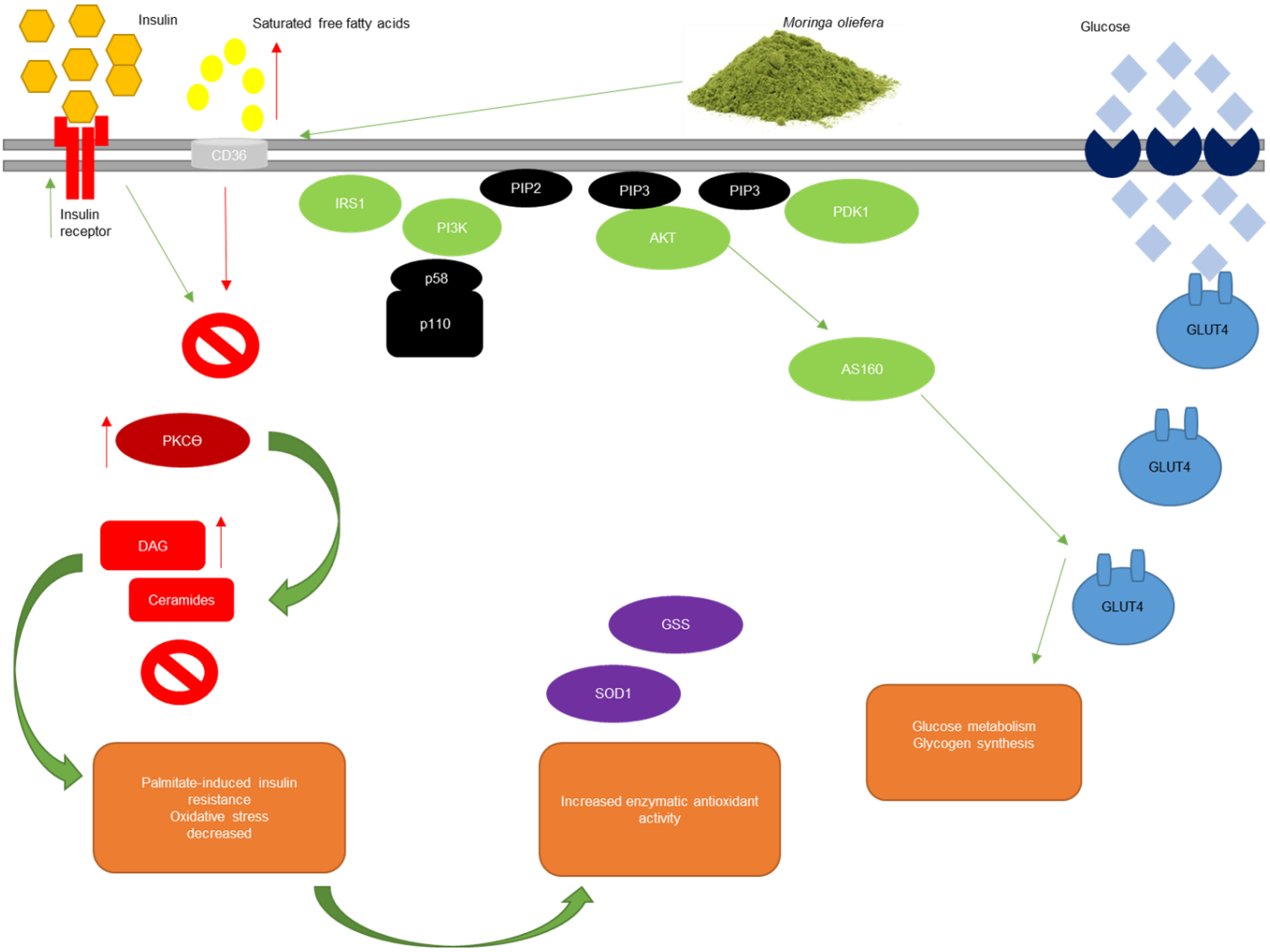


Figure 14: An overview of the proposed mechanism of action used by *Moringa oliefera* to attenuates insulin resistance in C2C12 skeletal muscle cells that is caused by palmitate. Palmitate a saturated fatty acid induced insulin resistance in the in vitro model by increasing diacylglycerol (DAG) and ceramides. It reduced cell viability and increased the expression of *Pkcθ* and then *Insr* expression decreased followed by the decrease of *Pi3k*, *Sod1* and *Gss* and this affected glucose metabolism and glycogen synthesis in the skeletal cells. *Moringa oliefera* counteracted this effect and enhanced the function of *Insr* in turn decreasing *PkcΘ* expression which decreased the ceramides and DAG resulting in the phosphorylation of *Irs1* and PI3K/AKT pathway and this activated the *As160* resulting in the translocation of GLUT4 vesicles to the membrane to transport glucose into the cell. *Sod1* and *Gss* activity were also increased therefore, *Moringa oliefera* has the potential to improve insulin sensitivity in skeletal muscle cells.

7. RECOMMENDATIONS FOR FUTURE STUDIES

In the future, the Western blot method will have to be used to examine how *Moringa oliefera* affects the expression of the AMPK and AKT pathways at the protein level and also to confirm these results at a molecular level. It would also be advisable to look at how the different extracts of *Moringa oliefera* and the extraction techniques affect the plant's ability to ameliorate skeletal muscle insulin resistance. A drug-herb interaction study can be done to investigate the pharmacokinetic or pharmacodynamics activities of the plant. Lastly, to learn more about the impact of *Moringa oliefera* on skeletal muscle insulin resistance and its mode of action, an in vivo investigation is necessary.

8. STUDY LIMITATION

Like most or many other studies we also encountered problems, one of them being the possibility that insulin resistance was not really confirmed in our model, due to the fact that when the insulin resistant cells were treated with insulin control they responded well to insulin. The study was limited in that we could not confirm the mechanism of action of *Moringa oliefera* at a protein level with the Western blot technique meaning we did not get to explore the effect it has on AMPK and AKT pathway. Due to time, we also couldn’t assess how *Moringa oliefera* affects glycogen content in the insulin resistant C2C12 skeletal muscle cells. We also could not do the liquid chromatography-mass spectrometry due to time and constraints such as our budget.

CHAPTER 6

This chapter shows the references used for this study.

9. References

Aas, A.-M., Axelsen, M., Churuangsuk, C., Hermansen, K., Kendall, C. W. C., Kahleova, H., Khan, T., Lean, M. E. J., Mann, J. I., Pedersen, E., Pfeiffer, A., Rahelić, D., Reynolds, A. N., Risérus, U., Rivellese, A. A., Salas-Salvadó, J., Schwab, U., Sievenpiper, J. L., Thanopoulou, A., . . . Nutrition Study Group of the European Association for the Study of, D. (2023). Evidence-based European recommendations for the dietary management of diabetes. *Diabetologia*, *66*(6), 965-985. https://doi.org/10.1007/s00125-023-05894-8

Abd Rani, N. Z., Husain, K., & Kumolosasi, E. (2018). Moringa Genus: A Review of Phytochemistry and Pharmacology [Review]. *Frontiers in Pharmacology*, *9*. https://doi.org/10.3389/fphar.2018.00108

Abdelmoneim, A. S., Eurich, D. T., Light, P. E., Senior, P. A., Seubert, J. M., Makowsky, M. J., & Simpson, S. H. (2015). Cardiovascular safety of sulphonylureas: over 40 years of continuous controversy without an answer. *Diabetes Obes Metab*, *17*(6), 523-532. https://doi.org/10.1111/dom.12456

Adel-Mehraban, M. S., Tabatabaei-Malazy, O., Manayi, A., Alatab, S., Mohseni, S., Fana, S. E., Asili, P., Bahramsoltani, R., Esmaeili, F., & Azizi, B. (2024). Antioxidative and Anti-inflammatory Effects of Plant-derived Hypoglycemic Medicines: An In vivo/In vitro Systematic Review. *Curr Top Med Chem*. https://doi.org/10.2174/0115680266295032240415064750

Aherne, S. A., Kerry, J. P., & O'Brien, N. M. (2007). Effects of plant extracts on antioxidant status and oxidant-induced stress in Caco-2 cells. *British Journal of Nutrition*, *97*(2), 321-328. https://doi.org/10.1017/S0007114507250469

Ahmad, S., Pandey, A. R., Rai, A. K., Singh, S. P., Kumar, P., Singh, S., Gulzar, F., Ahmad, I., Sashidhara, K. V., & Tamrakar, A. K. (2023). Moringa oleifera impedes protein glycation and exerts reno-protective effects in streptozotocin-induced diabetic rats. *J Ethnopharmacol*, *305*, 116117. https://doi.org/10.1016/j.jep.2022.116117

Aini, Q., Samingan, S., & Sabri, M. (2023). Ethanolic Extract of Moringa oleifera Leaves Improves Glucose Tolerance, Glycogen Levels, and Liver Health in Alloxan-Induced Diabetic Rats. *Elkawnie*, *9*(2). https://doi.org/10.22373/ekw.v9i2.17585

Alam Miah, M. B., & Yousuf, M. A. (2018). Analysis the significant risk factors on type 2 diabetes perspective of Bangladesh. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, *12*(6), 897-902. https://doi.org/https://doi.org/10.1016/j.dsx.2018.05.012

Aljazzaf, B., Regeai, S., Elghmasi, S., Alghazir, N., Balgasim, A., Hdud Ismail, I. M., Eskandrani, A. A., Shamlan, G., Alansari, W. S., Al-Farga, A., & Alghazeer, R. (2023). Evaluation of Antidiabetic Effect of Combined Leaf and Seed Extracts of Moringa oleifera (Moringaceae) on Alloxan-Induced Diabetes in Mice: A Biochemical and Histological Study. *Oxid Med Cell Longev*, *2023*, 9136217. https://doi.org/10.1155/2023/9136217

Alkhateeb, H., & Bonen, A. (2010). Thujone, a component of medicinal herbs, rescues palmitate-induced insulin resistance in skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *299*(3), R804-R812.

Ayeleso, T. B., Ramachela, K., & Mukwevho, E. (2018). Aqueous-methanol extracts of orange-fleshed sweet potato (Ipomoea batatas) ameliorate oxidative stress and modulate type 2

diabetes associated genes in insulin resistant C2C12 cells. *Molecules (Basel, Switzerland)*, *23*(8), 2058.

Ayoka, T. O., Ezema, B. O., Eze, C. N., & Nnadi, C. O. (2022). Antioxidants for the Prevention and Treatment of Non-communicable Diseases. *Journal of Exploratory Research in Pharmacology*, *7*(3), 178-188.

Bakker, W., Sipkema, P., Stehouwer, C. D., Serne, E. H., Smulders, Y. M., van Hinsbergh, V. W., & Eringa, E. C. (2008). Protein kinase C theta activation induces insulin-mediated constriction of muscle resistance arteries. *Diabetes*, *57*(3), 706-713. https://doi.org/10.2337/db07-0792

Bassogog, C. B. B., Nyobe, C. E., Sabine, F. Y., Dupon, A. A. B., Ngui, S. P., Minka, S. R., Laure, N. J., & Mune, M. A. M. (2024). Protein hydrolysates of Moringa oleifera seed: Antioxidant and antihyperglycaemic potential as ingredient for the management of type-2 diabetes. *Heliyon*, *10*(7).

Bayol, S., Bruce, C., & Wadley, G. (2014). Growing healthy muscles to optimise metabolic health into adult life. *Journal of developmental origins of health and disease*, *5*, 1-15. https://doi.org/10.1017/S2040174414000452

Bell, D. S. (2006). Do sulfonylurea drugs increase the risk of cardiac events? *Cmaj*, *174*(2), 185-186. https://doi.org/10.1503/cmaj.051237

Benoit, S. C., Kemp, C. J., Elias, C. F., Abplanalp, W., Herman, J. P., Migrenne, S., Lefevre, A. L., Cruciani-Guglielmacci, C., Magnan, C., Yu, F., Niswender, K., Irani, B. G., Holland, W. L., & Clegg, D. J. (2009). Palmitic acid mediates hypothalamic insulin resistance by altering PKC-theta subcellular localization in rodents. *J Clin Invest*, *119*(9), 2577-2589. https://doi.org/10.1172/jci36714

Blahova, J., Martiniakova, M., Babikova, M., Kovacova, V., Mondockova, V., & Omelka, R. (2021). Pharmaceutical Drugs and Natural Therapeutic Products for the Treatment of Type 2 Diabetes Mellitus. *Pharmaceuticals*, *14*(8), 806. https://www.mdpi.com/1424-8247/14/8/806

Boden, G. (1999). Free fatty acids, insulin resistance, and type 2 diabetes mellitus. *Proc Assoc Am Physicians*, *111*(3), 241-248. https://doi.org/10.1046/j.1525-1381.1999.99220.x

Burgos-Morón, E., Abad-Jiménez, Z., Martinez de Maranon, A., Iannantuoni, F., Escribano-López, I., López-Domènech, S., Salom, C., Jover, A., Mora, V., & Roldan, I. (2019). Relationship between oxidative stress, ER stress, and inflammation in type 2 diabetes: the battle continues. *Journal of clinical medicine*, *8*(9), 1385.

Castro-López, C., Gonçalves, C., Ventura-Sobrevilla, J. M., Pastrana, L. M., Aguilar-González, C. N., & Martínez-Ávila, G. C. G. (2020). Moringa oleifera—Storage Stability, In Vitro-Simulated Digestion and Cytotoxicity Assessment of Microencapsulated Extract. *Processes*, *8*(7), 770. https://www.mdpi.com/2227-9717/8/7/770

Chen, Y. K., Liu, T. T., Teia, F. K. F., & Xie, M. Z. (2023). Exploring the underlying mechanisms of obesity and diabetes and the potential of Traditional Chinese Medicine: an overview of the literature. *Front Endocrinol (Lausanne)*, *14*, 1218880. https://doi.org/10.3389/fendo.2023.1218880

Cheon, J.-M., Kim, D.-I., & Kim, K.-S. (2015). Insulin sensitivity improvement of fermented Korean Red Ginseng (Panax ginseng) mediated by insulin resistance hallmarks in old-

aged ob/ob mice. *Journal of Ginseng Research*, *39*(4), 331-337. https://doi.org/https://doi.org/10.1016/j.jgr.2015.03.005

Chiș, A., Noubissi, P. A., Pop, O.-L., Mureșan, C. I., Fokam Tagne, M. A., Kamgang, R., Fodor, A., Sitar-Tăut, A.-V., Cozma, A., Orășan, O. H., Hegheș, S. C., Vulturar, R., & Suharoschi, R. (2024). Bioactive Compounds in Moringa oleifera: Mechanisms of Action, Focus on Their Anti-Inflammatory Properties. *Plants*, *13*(1), 20. https://www.mdpi.com/2223-7747/13/1/20

Chung S Yang, Janelle M Landau, Mou-Tuan Huang, a., & Newmark, H. L. (2001). INHIBITION OF CARCINOGENESIS BY DIETARY POLYPHENOLIC COMPOUNDS. *Annual Review of Nutrition*, *21*(1), 381-406. https://doi.org/10.1146/annurev.nutr.21.1.381

Cline, G. W., Petersen, K. F., Krssak, M., Shen, J., Hundal, R. S., Trajanoski, Z., Inzucchi, S., Dresner, A., Rothman, D. L., & Shulman, G. I. (1999). Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. *N Engl J Med*, *341*(4), 240-246. https://doi.org/10.1056/nejm199907223410404

Consitt, L. A., Dudley, C., & Saxena, G. (2019). Impact of Endurance and Resistance Training on Skeletal Muscle Glucose Metabolism in Older Adults. *Nutrients*, *11*(11), 2636. https://doi.org/10.3390/nu11112636

Corcoran, M. P., Lamon-Fava, S., & Fielding, R. A. (2007). Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise. *Am J Clin Nutr*, *85*(3), 662-677. https://doi.org/10.1093/ajcn/85.3.662

Cory, H., Passarelli, S., Szeto, J., Tamez, M., & Mattei, J. (2018). The Role of Polyphenols in Human Health and Food Systems: A Mini-Review [Mini Review]. *Frontiers in nutrition*, *5*. https://doi.org/10.3389/fnut.2018.00087

Cremonini, E., Bettaieb, A., Haj, F. G., Fraga, C. G., & Oteiza, P. I. (2016). (-)-Epicatechin improves insulin sensitivity in high fat diet-fed mice. *Arch Biochem Biophys*, *599*, 13-21. https://doi.org/10.1016/j.abb.2016.03.006

Crosson, S. M., Khan, A., Printen, J., Pessin, J. E., & Saltiel, A. R. (2003). PTG gene deletion causes impaired glycogen synthesis and developmental insulin resistance. *The Journal of clinical investigation*, *111*(9), 1423-1432. https://doi.org/10.1172/JCI17975

Czech, M. P., Tencerova, M., Pedersen, D. J., & Aouadi, M. (2013). Insulin signalling mechanisms for triacylglycerol storage. *Diabetologia*, *56*(5), 949-964. https://doi.org/10.1007/s00125-013-2869-1

Deng, Y.-T., Chang, T.-W., Lee, M.-S., & Lin, J.-K. (2012). Suppression of Free Fatty Acid-Induced Insulin Resistance by Phytopolyphenols in C2C12 Mouse Skeletal Muscle Cells. *Journal of Agricultural and Food Chemistry*, *60*(4), 1059-1066. https://doi.org/10.1021/jf204496f

Dhanya, R. (2022). Quercetin for managing type 2 diabetes and its complications, an insight into multitarget therapy. *Biomedicine & pharmacotherapy*, *146*, 112560. https://doi.org/https://doi.org/10.1016/j.biopha.2021.112560

Dimopoulos, N., Watson, M., Sakamoto, K., & Hundal, H. S. (2006). Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. *Biochem J*, *399*(3), 473-481. https://doi.org/10.1042/bj20060244

Dludla, P. V., Johnson, R., Mazibuko-Mbeje, S. E., Muller, C. J. F., Louw, J., Joubert, E., Orlando, P., Silvestri, S., Chellan, N., Nkambule, B. B., Essop, M. F., & Tiano, L. (2020).

Fermented rooibos extract attenuates hyperglycemia-induced myocardial oxidative damage by improving mitochondrial energetics and intracellular antioxidant capacity. *South African Journal of Botany*, *131*, 143-150. https://doi.org/https://doi.org/10.1016/j.sajb.2020.02.003

Dong, W., Bian, X., Wan, M., Jin, L., Wang, Y., Jing, C., Yao, Z., Gao, W., Xi, Z., & Guo, C. (2024). Moringa oleifera leaf extracts improve exercise performance in young male adults: A pilot study. *Phytomedicine*, *131*, 155751. https://doi.org/https://doi.org/10.1016/j.phymed.2024.155751

Dube, J. J., Bhatt, B. A., Dedousis, N., Bonen, A., & O'Doherty, R. M. (2007). Leptin, skeletal muscle lipids, and lipid-induced insulin resistance. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *293*(2), R642-R650.

Duranti, G., Maldini, M., Crognale, D., Horner, K., Dimauro, I., Sabatini, S., & Ceci, R. (2021). Moringa oleifera Leaf Extract Upregulates Nrf2/HO-1 Expression and Ameliorates Redox Status in C2C12 Skeletal Muscle Cells. *Molecules (Basel, Switzerland)*, *26*(16), 5041. https://doi.org/10.3390/molecules26165041

Ertik, O., Magaji, U. F., Sacan, O., & Yanardag, R. (2023). Effect of Moringa oleifera leaf extract on valproate-induced oxidative damage in muscle. *Drug Chem Toxicol*, *46*(6), 1212-1222. https://doi.org/10.1080/01480545.2022.2144876

Farhan, M. (2022). Green Tea Catechins: Nature’s Way of Preventing and Treating Cancer. *International journal of molecular sciences*, *23*(18), 10713. https://www.mdpi.com/1422-0067/23/18/10713

Fletcher, B., Gulanick, M., & Lamendola, C. (2002). Risk Factors for Type 2 Diabetes Mellitus. *Journal of Cardiovascular Nursing*, *16*(2). https://journals.lww.com/jcnjournal/Fulltext/2002/01000/Risk_Factors_for_Type_2_Diabetes_Mellitus.3.aspx

Frontera, W. R., & Ochala, J. (2015). Skeletal Muscle: A Brief Review of Structure and Function. *Calcified Tissue International*, *96*(3), 183-195. https://doi.org/10.1007/s00223-014-9915-y

Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K. B., Ostolaza, H., & Martín, C. (2020). Pathophysiology of Type 2 Diabetes Mellitus. *International journal of molecular sciences*, *21*(17), 6275. https://doi.org/10.3390/ijms21176275

Giacometti, J., Muhvić, D., Grubić-Kezele, T., Nikolić, M., Šoić-Vranić, T., & Bajek, S. (2020). Olive Leaf Polyphenols (OLPs) Stimulate GLUT4 Expression and Translocation in the Skeletal Muscle of Diabetic Rats. *International journal of molecular sciences*, *21*(23). https://doi.org/10.3390/ijms21238981

Gupta, J., Siddique, Y., Beg, T., Ara, G., & Afzal, M. (2008). A review on the beneficial effects of tea polyphenols on human health. *International Journal of Pharmacology*, *4*(5), 314-338.

Gupta, S., Hastak, K., Afaq, F., Ahmad, N., & Mukhtar, H. (2004). Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor kappaB and induction of apoptosis. *Oncogene*, *23*(14), 2507-2522.

Han, Y., Jung, H. W., & Park, Y.-K. (2012). The roots of Atractylodes japonica Koidzumi promote adipogenic differentiation via activation of the insulin signaling pathway in 3T3-L1 cells.

BMC complementary and alternative medicine, *12*(1), 154. https://doi.org/10.1186/1472-6882-12-154

He, J., & Kelley, D. E. (2004). Muscle glycogen content in type 2 diabetes mellitus. *Am J Physiol Endocrinol Metab*, *287*(5), E1002-1007. https://doi.org/10.1152/ajpendo.00015.2004

Henry, R. R., Ciaraldi, T. P., Abrams-Carter, L., Mudaliar, S., Park, K. S., & Nikoulina, S. E. (1996). Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-dependent diabetes mellitus subjects. Biochemical and molecular mechanisms. *J Clin Invest*, *98*(5), 1231-1236. https://doi.org/10.1172/jci118906

Hong, Z., Xie, J., Hu, H., Bai, Y., Hu, X., Li, T., Chen, J., Sheng, J., & Tian, Y. (2023). Hypoglycemic effect of Moringa oleifera leaf extract and its mechanism prediction based on network pharmacology. *Journal of Future Foods*, *3*(4), 383-391. https://doi.org/https://doi.org/10.1016/j.jfutfo.2023.03.009

Hurrle, S., & Hsu, W. H. (2017). The etiology of oxidative stress in insulin resistance. *Biomed J*, *40*(5), 257-262. https://doi.org/10.1016/j.bj.2017.06.007

Islam, Z., Islam, S. M. R., Hossen, F., Mahtab-ul-Islam, K., Hasan, M. R., & Karim, R. (2021). Moringa oleifera is a Prominent Source of Nutrients with Potential Health Benefits. *International Journal of Food Science*, *2021*, 6627265. https://doi.org/10.1155/2021/6627265

Jensen, J., Rustad, P., Kolnes, A., & Lai, Y.-C. (2011). The Role of Skeletal Muscle Glycogen Breakdown for Regulation of Insulin Sensitivity by Exercise [Review]. *Frontiers in Physiology*, *2*. https://doi.org/10.3389/fphys.2011.00112

Kahn, S. E., Hull, R. L., & Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*, *444*(7121), 840-846. https://doi.org/10.1038/nature05482

Kang, G. G., Francis, N., Hill, R., Waters, D., Blanchard, C., & Santhakumar, A. B. (2019). Dietary Polyphenols and Gene Expression in Molecular Pathways Associated with Type 2 Diabetes Mellitus: A Review. *International journal of molecular sciences*, *21*(1). https://doi.org/10.3390/ijms21010140

Kashyap, P., Kumar, S., Riar, C. S., Jindal, N., Baniwal, P., Guiné, R. P. F., Correia, P. M. R., Mehra, R., & Kumar, H. (2022). Recent Advances in Drumstick (Moringa oleifera) Leaves Bioactive Compounds: Composition, Health Benefits, Bioaccessibility, and Dietary Applications. *Antioxidants*, *11*(2), 402. https://www.mdpi.com/2076-3921/11/2/402

Kelly, G. (2000). Insulin resistance: Lifestyle and nutritional interventions. *Alternative medicine review : a journal of clinical therapeutic*, *5*, 109-132.

Khalafi, M., Azali Alamdari, K., Symonds, M. E., Rohani, H., & Sakhaei, M. H. (2023). A comparison of the impact of exercise training with dietary intervention versus dietary intervention alone on insulin resistance and glucose regulation in individual with overweight or obesity: a systemic review and meta-analysis. *Critical Reviews in Food Science and Nutrition*, *63*(28), 9349-9363. https://doi.org/10.1080/10408398.2022.2064424

Khan, W., Parveen, R., Chester, K., Parveen, S., & Ahmad, S. (2017). Hypoglycemic Potential of Aqueous Extract of Moringa oleifera Leaf and In Vivo GC-MS Metabolomics [Original Research]. *Frontiers in Pharmacology*, *8*. https://doi.org/10.3389/fphar.2017.00577

Kim, H.-S., Quon, M. J., & Kim, J.-a. (2014). New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. *Redox Biology*, *2*, 187-195. https://doi.org/https://doi.org/10.1016/j.redox.2013.12.022

Kim, M. S., Hur, H. J., Kwon, D. Y., & Hwang, J.-T. (2012). Tangeretin stimulates glucose uptake via regulation of AMPK signaling pathways in C2C12 myotubes and improves glucose tolerance in high-fat diet-induced obese mice. *Molecular and Cellular Endocrinology*, *358*(1), 127-134. https://doi.org/https://doi.org/10.1016/j.mce.2012.03.013

Kou, X., Li, B., Olayanju, J. B., Drake, J. M., & Chen, N. (2018). Nutraceutical or Pharmacological Potential of Moringa oleifera Lam. *Nutrients*, *10*(3), 343. https://doi.org/10.3390/nu10030343

Lambernd, S., Taube, A., Schober, A., Platzbecker, B., Görgens, S. W., Schlich, R., Jeruschke, K., Weiss, J., Eckardt, K., & Eckel, J. (2012). Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. *Diabetologia*, *55*(4), 1128-1139. https://doi.org/10.1007/s00125-012-2454-z

Lee, S. F., & Lin, J. K. (1997). Inhibitory effects of phytopolyphenols on TPA-induced transformation, PKC activation, and c-jun expression in mouse fibroblast cells. *Nutrition and Cancer*, *28*(2), 177-183. https://doi.org/10.1080/01635589709514572

Lee, S. H., Park, S. Y., & Choi, C. S. (2022). Insulin Resistance: From Mechanisms to Therapeutic Strategies. *Diabetes Metab J*, *46*(1), 15-37. https://doi.org/10.4093/dmj.2021.0280

Levin, K., Daa Schroeder, H., Alford, F. P., & Beck-Nielsen, H. (2001). Morphometric documentation of abnormal intramyocellular fat storage and reduced glycogen in obese patients with Type II diabetes. *Diabetologia*, *44*(7), 824-833. https://doi.org/10.1007/s001250100545

Li, H., Ren, J., Li, Y., Wu, Q., & Wei, J. (2023). Oxidative stress: The nexus of obesity and cognitive dysfunction in diabetes. *Front Endocrinol (Lausanne)*, *14*, 1134025. https://doi.org/10.3389/fendo.2023.1134025

Li, Y., Zhao, S., Zhang, W., Zhao, P., He, B., Wu, N., & Han, P. (2011). Epigallocatechin-3-O-gallate (EGCG) attenuates FFAs-induced peripheral insulin resistance through AMPK pathway and insulin signaling pathway in vivo. *Diabetes Research and Clinical Practice*, *93*(2), 205-214. https://doi.org/https://doi.org/10.1016/j.diabres.2011.03.036

Lin, Y., Fan, R., Hao, Z., Li, J., Yang, X., Zhang, Y., & Xia, Y. (2022). The Association Between Physical Activity and Insulin Level Under Different Levels of Lipid Indices and Serum Uric Acid [Original Research]. *Frontiers in Physiology*, *13*. https://doi.org/10.3389/fphys.2022.809669

Lopez-Yus, M., Hörndler, C., Borlan, S., Bernal-Monterde, V., & Arbones-Mainar, J. M. (2024). Unraveling Adipose Tissue Dysfunction: Molecular Mechanisms, Novel Biomarkers, and Therapeutic Targets for Liver Fat Deposition. *Cells*, *13*(5), 380. https://www.mdpi.com/2073-4409/13/5/380

Mahaveerchand, H., & Abdul Salam, A. A. (2024). Environmental, industrial, and health benefits of Moringa oleifera. *Phytochemistry Reviews*. https://doi.org/10.1007/s11101-024-09927-x

Mao, T., Huang, F., Zhu, X., Wei, D., & Chen, L. (2021). Effects of dietary fiber on glycemic control and insulin sensitivity in patients with type 2 diabetes: A systematic review and meta-analysis. *Journal of Functional Foods*, *82*, 104500. https://doi.org/https://doi.org/10.1016/j.jff.2021.104500

Marušić, M., Paić, M., Knobloch, M., & Liberati Pršo, A.-M. (2021). NAFLD, Insulin Resistance, and Diabetes Mellitus Type 2. *Canadian journal of gastroenterology & hepatology*, *2021*, 6613827-6613827. https://doi.org/10.1155/2021/6613827

Mazibuko-Mbeje, S., Muller, C., Joubert, E., De Beer, D., Johnson, R., Opoku, A. R., & Louw, J. (2013). Amelioration of palmitate-induced insulin resistance in C2C12 muscle cells by rooibos (Aspalathus linearis). *Phytomedicine : international journal of phytotherapy and phytopharmacology*, *20*. https://doi.org/10.1016/j.phymed.2013.03.018

Mazibuko-Mbeje, S. E., Dludla, P. V., Roux, C., Johnson, R., Ghoor, S., Joubert, E., Louw, J., Opoku, A. R., & Muller, C. J. (2019). Aspalathin-enriched green rooibos extract reduces hepatic insulin resistance by modulating PI3K/AKT and AMPK pathways. *International journal of molecular sciences*, *20*(3), 633.

Mazibuko, S. E., Joubert, E., Johnson, R., Louw, J., Opoku, A. R., & Muller, C. J. (2015). Aspalathin improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. *Mol Nutr Food Res*, *59*(11), 2199-2208. https://doi.org/10.1002/mnfr.201500258

Menichini, D., Petrella, E., Dipace, V., Di Monte, A., Neri, I., & Facchinetti, F. (2020). The Impact of an Early Lifestyle Intervention on Pregnancy Outcomes in a Cohort of Insulin-Resistant Overweight and Obese Women. *Nutrients*, *12*(5). https://doi.org/10.3390/nu12051496

Merz, K., & Thurmond, D. (2020). Role of Skeletal Muscle in Insulin Resistance and Glucose Uptake. *Comprehensive Physiology*, *10*, 785-809. https://doi.org/10.1002/cphy.c190029

Milla, P. G., Peñalver, R., & Nieto, G. (2021). Health Benefits of Uses and Applications of Moringa oleifera in Bakery Products. *Plants (Basel)*, *10*(2). https://doi.org/10.3390/plants10020318

Mirabelli, M., Russo, D., & Brunetti, A. (2020). The Role of Diet on Insulin Sensitivity. *Nutrients*, *12*(10). https://doi.org/10.3390/nu12103042

Mohamed, M. A., Ahmed, M. A., & El Sayed, R. A. (2019). Molecular effects of Moringa leaf extract on insulin resistance and reproductive function in hyperinsulinemic male rats. *Journal of Diabetes & Metabolic Disorders*, *18*(2), 487-494.

Mohlala, K., Offor, U., Monageng, E., Takalani, N. B., & Opuwari, C. S. (2023). Overview of the Effects of Moringa oleifera Leaf Extract on Oxidative Stress and Male Infertility: A Review. *Applied Sciences*, *13*(7), 4387. https://www.mdpi.com/2076-3417/13/7/4387

Monami, M., Genovese, S., & Mannucci, E. (2013). Cardiovascular safety of sulfonylureas: a meta-analysis of randomized clinical trials. *Diabetes Obes Metab*, *15*(10), 938-953. https://doi.org/10.1111/dom.12116

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, *65*(1-2), 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

Mthiyane, F. T., Dludla, P. V., Ziqubu, K., Mthembu, S. X. H., Muvhulawa, N., Hlengwa, N., Nkambule, B. B., & Mazibuko-Mbeje, S. E. (2022). A Review on the Antidiabetic

Properties of Moringa oleifera Extracts: Focusing on Oxidative Stress and Inflammation as Main Therapeutic Targets. *Front Pharmacol*, *13*, 940572. https://doi.org/10.3389/fphar.2022.940572

Naz, R., Saqib, F., Awadallah, S., Wahid, M., Latif, M. F., Iqbal, I., & Mubarak, M. S. (2023). Food Polyphenols and Type II Diabetes Mellitus: Pharmacology and Mechanisms. *Molecules*, *28*(10), 3996. https://www.mdpi.com/1420-3049/28/10/3996

Nieuwoudt, S., Mulya, A., Fealy, C. E., Martelli, E., Dasarathy, S., Naga Prasad, S. V., & Kirwan, J. P. (2017). In vitro contraction protects against palmitate-induced insulin resistance in C2C12 myotubes. *Am J Physiol Cell Physiol*, *313*(5), C575-c583. https://doi.org/10.1152/ajpcell.00123.2017

Nikawa, T., Ulla, A., & Sakakibara, I. (2021). Polyphenols and Their Effects on Muscle Atrophy and Muscle Health. *Molecules (Basel, Switzerland)*, *26*(16), 4887. https://doi.org/10.3390/molecules26164887

Nikoulina, S. E., Ciaraldi, T. P., Mudaliar, S., Mohideen, P., Carter, L., & Henry, R. R. (2000). Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. *Diabetes*, *49*(2), 263-271. https://doi.org/10.2337/diabetes.49.2.263

Niwano, Y., Kohzaki, H., Shirato, M., Shishido, S., & Nakamura, K. (2022). Anti-Osteoporotic Mechanisms of Polyphenols Elucidated Based on In Vivo Studies Using Ovariectomized Animals. *Antioxidants*, *11*(2), 217. https://www.mdpi.com/2076-3921/11/2/217

Nova, E., Redondo-Useros, N., Martínez-García, R. M., Gómez-Martínez, S., Díaz-Prieto, L. E., & Marcos, A. (2020). Potential of Moringa oleifera to Improve Glucose Control for the Prevention of Diabetes and Related Metabolic Alterations: A Systematic Review of Animal and Human Studies. *Nutrients*, *12*(7). https://doi.org/10.3390/nu12072050

Oguntibeju, O. O., Aboua, G. Y., & Omodanisi, E. I. (2020). Effects of Moringa oleifera on oxidative stress, apoptotic and inflammatory biomarkers in streptozotocin-induced diabetic animal model. *South African Journal of Botany*, *129*, 354-365. https://doi.org/https://doi.org/10.1016/j.sajb.2019.08.039

Olayaki, L. A., Irekpita, J. E., Yakubu, M. T., & Ojo, O. O. (2015). Methanolic extract of Moringa oleifera leaves improves glucose tolerance, glycogen synthesis and lipid metabolism in alloxan-induced diabetic rats. *J Basic Clin Physiol Pharmacol*, *26*(6), 585-593. https://doi.org/10.1515/jbcpp-2014-0129

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity*, *2*(5), 270-278. https://doi.org/10.4161/oxim.2.5.9498

Pareek, A., Pant, M., Gupta, M. M., Kashania, P., Ratan, Y., Jain, V., Pareek, A., & Chuturgoon, A. A. (2023). Moringa oleifera: An Updated Comprehensive Review of Its Pharmacological Activities, Ethnomedicinal, Phytopharmaceutical Formulation, Clinical, Phytochemical, and Toxicological Aspects. *Int J Mol Sci*, *24*(3). https://doi.org/10.3390/ijms24032098

Parveen, S., Shehzadi, S., Shafiq, N., Rashid, M., Naz, S., Mehmood, T., Riaz, R., K, S. A., Nafidi, H. A., & Bourhia, M. (2024). A discovery of potent kaempferol derivatives as multi-target medicines against diabetes as well as bacterial infections: an in silico approach. *J Biomol Struct Dyn*, 1-23. https://doi.org/10.1080/07391102.2024.2308773

Patil, S. V., Mohite, B. V., Marathe, K. R., Salunkhe, N. S., Marathe, V., & Patil, V. S. (2022). Moringa Tree, Gift of Nature: a Review on Nutritional and Industrial Potential. *Current Pharmacology Reports*, *8*(4), 262-280. https://doi.org/10.1007/s40495-022-00288-7

Patro, G., Bhattamisra, S. K., Mohanty, B. K., & Sahoo, H. B. (2016). In vitro and In vivo Antioxidant Evaluation and Estimation of Total Phenolic, Flavonoidal Content of Mimosa pudica L. *Pharmacognosy Res*, *8*(1), 22-28. https://doi.org/10.4103/0974-8490.171099

Rains, J. L., & Jain, S. K. (2011). Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med*, *50*(5), 567-575. https://doi.org/10.1016/j.freeradbiomed.2010.12.006

Reda, R. M., Helmy, R. M. A., Osman, A., Ahmed, F. A. G., Kotb, G. A. M., & El-Fattah, A. H. A. (2023). The potential effect of Moringa oleifera ethanolic leaf extract against oxidative stress, immune response disruption induced by abamectin exposure in Oreochromis niloticus. *Environmental Science and Pollution Research*, *30*(20), 58569-58587. https://doi.org/10.1007/s11356-023-26517-0

Sato, Y. (2020). Overview of Exercise Prescription for Diabetes Patients and Its Application in Japan. *Journal of Science in Sport and Exercise*, *2*(3), 226-235. https://doi.org/10.1007/s42978-020-00061-6

Scalbert, A., Manach, C., Morand, C., Rémésy, C., & Jiménez, L. (2005). Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr*, *45*(4), 287-306. https://doi.org/10.1080/10408690590960

Shahwan, M., Alhumaydhi, F., Ashraf, G. M., Hasan, P. M. Z., & Shamsi, A. (2022). Role of polyphenols in combating Type 2 Diabetes and insulin resistance. *International Journal of Biological Macromolecules*, *206*, 567-579. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2022.03.004

Shi, G.-J., Li, Y., Cao, Q.-H., Wu, H.-X., Tang, X.-Y., Gao, X.-H., Yu, J.-Q., Chen, Z., & Yang, Y. (2019). In vitro and in vivo evidence that quercetin protects against diabetes and its complications: A systematic review of the literature. *Biomedicine & pharmacotherapy*, *109*, 1085-1099. https://doi.org/https://doi.org/10.1016/j.biopha.2018.10.130

Shyur, L. F., Varga, V., Chen, C. M., Mu, S. C., Chang, Y. C., & Li, S. C. (2021). Extract of white sweet potato tuber against TNF-α-induced insulin resistance by activating the PI3K/Akt pathway in C2C12 myotubes. *Bot Stud*, *62*(1), 7. https://doi.org/10.1186/s40529-021-00315-8

Siahaan, S. C. P. T., Santoso, B., & Widjiati. (2022). Effectiveness of Moringa oleifera Leaves on TNF-α Expression, Insulin Levels, Glucose Levels and Follicle Count in Rattus norvegicus PCOS Model. *Diabetes, Metabolic Syndrome and Obesity*, *15*(null), 3255-3270. https://doi.org/10.2147/DMSO.S385492

Sidha Bhagawan, W., Atmaja, R., & Atiqah, S. (2017). OPTIMIZATION AND QUERCETIN RELEASE TEST OF MORINGA LEAF EXTRACT (MORINGA OLEIFERA) IN GEL-MICROEMULSION PREPARATION. *Journal of Islamic Pharmacy*, *2*, 34. https://doi.org/10.18860/jip.v2i2.4508

Silveira, A. C., Dias, J. P., Santos, V. M., Oliveira, P. F., Alves, M. G., Rato, L., & Silva, B. M. (2019). The Action of Polyphenols in Diabetes Mellitus and Alzheimer's Disease: A Common Agent for Overlapping Pathologies. *Curr Neuropharmacol*, *17*(7), 590-613. https://doi.org/10.2174/1570159x16666180803162059

Singh, A. K., Rana, H. K., Tshabalala, T., Kumar, R., Gupta, A., Ndhlala, A. R., & Pandey, A. K. (2020). Phytochemical, nutraceutical and pharmacological attributes of a functional crop Moringa oleifera Lam: An overview. *South African Journal of Botany*, *129*, 209-220. https://doi.org/https://doi.org/10.1016/j.sajb.2019.06.017

Singh, B. N., Shankar, S., & Srivastava, R. K. (2011). Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochem Pharmacol*, *82*(12), 1807-1821. https://doi.org/10.1016/j.bcp.2011.07.093

Snel, M., Jonker, J. T., Schoones, J., Lamb, H., de Roos, A., Pijl, H., Smit, J. W. A., Meinders, A. E., & Jazet, I. M. (2012). Ectopic Fat and Insulin Resistance: Pathophysiology and Effect of Diet and Lifestyle Interventions. *International Journal of Endocrinology*, *2012*, 983814. https://doi.org/10.1155/2012/983814

Sok Yen, F., Shu Qin, C., Tan Shi Xuan, S., Jia Ying, P., Yi Le, H., Darmarajan, T., Gunasekaran, B., & Salvamani, S. (2021). Hypoglycemic Effects of Plant Flavonoids: A Review. *Evidence-Based Complementary and Alternative Medicine*, *2021*, 2057333. https://doi.org/10.1155/2021/2057333

Sosa-Gutiérrez, J. A., Valdéz-Solana, M. A., Forbes-Hernández, T. Y., Avitia-Domínguez, C. I., Garcia-Vargas, G. G., Salas-Pacheco, J. M., Flores-Herrera, O., Téllez-Valencia, A., Battino, M., & Sierra-Campos, E. (2018). Effects of Moringa oleifera Leaves Extract on High Glucose-Induced Metabolic Changes in HepG2 Cells. *Biology*, *7*(3), E37. Retrieved 2018/06//, from http://europepmc.org/abstract/MED/29949946

https://doi.org/10.3390/biology7030037

https://europepmc.org/articles/PMC6164683

https://europepmc.org/articles/PMC6164683?pdf=render

Sreelatha, S., & Padma, P. (2009). Actividad antioxidante y contenido fenólico total de las hojas de Moringa oleifera en dos etapas de madurez. *Alimentos de plantas para la nutrición humana*, *64*(4), 303-311.

Stöckli, J., Fazakerley, D. J., & James, D. E. (2011). GLUT4 exocytosis. *Journal of cell science*, *124*(24), 4147-4159.

Sultan, R., Ahmed, A., Wei, L., Saeed, H., Islam, M., & Ishaq, M. (2023). The anticancer potential of chemical constituents of Moringa oleifera targeting CDK-2 inhibition in estrogen receptor positive breast cancer using in-silico and in vitro approches. *BMC Complementary Medicine and Therapies*, *23*(1), 396. https://doi.org/10.1186/s12906-023-04198-z

Sylow, L., Tokarz, V. L., Richter, E. A., & Klip, A. (2021). The many actions of insulin in skeletal muscle, the paramount tissue determining glycemia. *Cell metabolism*, *33*(4), 758-780. https://doi.org/https://doi.org/10.1016/j.cmet.2021.03.020

Szendroedi, J., Yoshimura, T., Phielix, E., Koliaki, C., Marcucci, M., Zhang, D., Jelenik, T., Müller, J., Herder, C., Nowotny, P., Shulman, G. I., & Roden, M. (2014). Role of diacylglycerol activation of PKCθ in lipid-induced muscle insulin resistance in humans. *Proc Natl Acad Sci U S A*, *111*(26), 9597-9602. https://doi.org/10.1073/pnas.1409229111

Talebanzadeh, S., Ashrafi, M., Kazemipour, N., Erjaee, H., & Nazifi, S. (2018). Evaluation of the effects of saffron aqueous extract on oxidative stress in the lens of streptozotocin-induced diabetic rats. *Biomedical Research and Therapy*, *5*(4), 2133-2141.

Tangvarasittichai, S. (2015). Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. *World J Diabetes*, *6*(3), 456-480. https://doi.org/10.4239/wjd.v6.i3.456

Trouwborst, I., Bowser, S. M., Goossens, G. H., & Blaak, E. E. (2018). Ectopic Fat Accumulation in Distinct Insulin Resistant Phenotypes; Targets for Personalized Nutritional Interventions. *Frontiers in nutrition*, *5*, 77-77. https://doi.org/10.3389/fnut.2018.00077

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), 1231-1246. https://doi.org/10.3390/nu2121231

Vats, V., Yadav, S. P., & Grover, J. K. (2004). Ethanolic extract of Ocimum sanctum leaves partially attenuates streptozotocin-induced alterations in glycogen content and carbohydrate metabolism in rats. *Journal of Ethnopharmacology*, *90*(1), 155-160. https://doi.org/https://doi.org/10.1016/j.jep.2003.09.034

Velho, G., Petersen, K. F., Perseghin, G., Hwang, J. H., Rothman, D. L., Pueyo, M. E., Cline, G. W., Froguel, P., & Shulman, G. I. (1996). Impaired hepatic glycogen synthesis in glucokinase-deficient (MODY-2) subjects. *J Clin Invest*, *98*(8), 1755-1761. https://doi.org/10.1172/jci118974

Wang, X., Yu, W., Nawaz, A., Guan, F., Sun, S., & Wang, C. (2010). Palmitate Induced Insulin Resistance by PKCtheta-Dependent Activation of mTOR/S6K Pathway in C2C12 Myotubes. *Exp Clin Endocrinol Diabetes*, *118*(09), 657-661. https://doi.org/10.1055/s-0030-1252069

Warram, J. H., Martin, B. C., Krolewski, A. S., Soeldner, J. S., & Kahn, C. R. (1990). Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Annals of internal medicine*, *113*(12), 909-915.

Waterman, C., Rojas-Silva, P., Tumer, T. B., Kuhn, P., Richard, A. J., Wicks, S., Stephens, J. M., Wang, Z., Mynatt, R., & Cefalu, W. (2015). Isothiocyanate-rich Moringa oleifera extract reduces weight gain, insulin resistance, and hepatic gluconeogenesis in mice. *Molecular nutrition & food research*, *59*(6), 1013-1024.

Weickert, M. O. (2012). Nutritional modulation of insulin resistance. *Scientifica*, *2012*, 424780-424780. https://doi.org/10.6064/2012/424780

Weickert, M. O., & Pfeiffer, A. F. (2018). Impact of Dietary Fiber Consumption on Insulin Resistance and the Prevention of Type 2 Diabetes. *The Journal of Nutrition*, *148*(1), 7-12. https://doi.org/10.1093/jn/nxx008

Wu, Y., Yang, X., Hu, Y., Hu, X., Zhang, Y., An, T., Lv, B., Tao, S., Liu, Q., & Jiang, G. (2023). Moringa oleifera leaf supplementation relieves oxidative stress and regulates intestinal flora to ameliorate polycystic ovary syndrome in letrozole-induced rats. *Food Sci Nutr*, *11*(9), 5137-5156. https://doi.org/10.1002/fsn3.3473

Xiao, X., Wang, J., Meng, C., Liang, W., Wang, T., Zhou, B., Wang, Y., Luo, X., Gao, L., & Zhang, L. (2020). Moringa oleifera Lam and its Therapeutic Effects in Immune Disorders [Mini Review]. *Frontiers in Pharmacology*, *11*. https://doi.org/10.3389/fphar.2020.566783

Yang, M., Wei, D., Mo, C., Zhang, J., Wang, X., Han, X., Wang, Z., & Xiao, H. (2013). Saturated fatty acid palmitate-induced insulin resistance is accompanied with myotube loss and the impaired expression of health benefit myokine genes in C2C12 myotubes. *Lipids Health Dis*, *12*, 104. https://doi.org/10.1186/1476-511X-12-104

Yang, Q., Wen, Y.-M., Shen, J., Chen, M.-M., Wen, J.-H., Li, Z.-M., Liang, Y.-Z., & Xia, N. (2020). Guava leaf extract attenuates insulin resistance via the PI3K/Akt signaling

pathway in a type 2 diabetic mouse model. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *13*, 713.

Yun, Y. R., Oh, S. J., Lee, M. J., Choi, Y. J., Park, S. J., Lee, M. A., Min, S. G., Seo, H. Y., & Park, S. H. (2020). Antioxidant activity and calcium bioaccessibility of Moringa oleifera leaf hydrolysate, as a potential calcium supplement in food. *Food Sci Biotechnol*, *29*(11), 1563-1571. https://doi.org/10.1007/s10068-020-00820-9

Zhang, L., Keung, W., Samokhvalov, V., Wang, W., & Lopaschuk, G. D. (2010). Role of fatty acid uptake and fatty acid β-oxidation in mediating insulin resistance in heart and skeletal muscle. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1801*(1), 1-22. https://doi.org/https://doi.org/10.1016/j.bbalip.2009.09.014

Zou, Y., Wang, J., Peng, J., & Wei, H. (2016). Oregano Essential Oil Induces SOD1 and GSH Expression through Nrf2 Activation and Alleviates Hydrogen Peroxide-Induced Oxidative Damage in IPEC-J2 Cells. *Oxidative medicine and cellular longevity*, *2016*, 5987183. https://doi.org/10.1155/2016/5987183

APPENDIX 1

Table 8: List of reagents used in the study

Product	**Catalogue number**	**Company**
Agarose powder	A2929-100G	Sigma-Aldrich, St Louis, MO, USA
Ambion nuclease free water not DEPC treated	210038	Ambion, Austin, TX, USA Invitrogen
Cell culture water	17-724F	Lonza, Walkersville, MD, USA
Calcium chloride	C4901-100G	Sigma-Aldrich, St Louis, MO, USA
Chloroform	0757	AMRESCO, Solon, OH, USA
Dimethyl sulfoxide (DMSO)	D5879-1L	Sigma-Aldrich, St Louis, MO, USA
D (+) Glucose anhydrous	SAAR2676020EM	Merck, Kilsyth, Australia
Dulbecco's modified Eagle medium (DMEM)	12-604F	Lonza, Walkersville, MD, USA
Dulbecco's phosphate buffer saline (DPBS)	BE17-513F	Lonza, Walkersville, MD, USA
6X DNA loading dye EA	01203062	Thermo Fisher Scientific, Waltham, MA, USA
Ethidium bromide	15585-011	Invitrogen, Carlsbad, CA, USA
Fetal bovine serum	10499-044	Gibco Thermo Fisher Scientific, Waltham, MA, USA
Fatty free acid bovine serum albumin	SH30574.02	HylClone laboratories GE Healthcare Lifesciences, Chicago, Illinios
Glycine	G7126	Sigma-Aldrich, St Louis, MO, USA
HEPES	15630080	Thermo Fisher Scientific, Waltham, MA, USA
Isopropanol	563935	Sigma-Aldrich, St Louis, MO, USA
Insulin solution human	19278-5ML	Sigma-Aldrich, St Louis, MO, USA
Moringa oliefera powder		Thabaneng Agricultural Projects
Murine C2C12 skeletal muscle cells	CRL 1722	American Type Culture Collection (Manassas, VA, USA)

Magnesium chloride	M8266-1KG	Sigma-Aldrich, St Louis, MO, USA
RNase AWAY™ Surface Decontaminant	7002PK	Thermo Fisher Scientific, Waltham, MA, USA
QuantiTect reverse transcription kit	205313	Qiagen, Hilden, Germany
Quercetin	Q4951-10G	Sigma-Aldrich, St Louis, MO, USA
Sodium chloride	SAAR5822320EM	Sigma-Aldrich, St Louis, MO, USA
Sodium bicarbonate	SO510	Melford Biolaboratories Ltd, Chelsworth, UK
Thiazolyl blue tetrazolium bromide	M5655-1G	Thermo Fisher Scientific, Waltham, MA, USA
Trizol	15596026	Ambion Life Technologies, Carlsbad, CA
Trypsin	CC-5012	Lonza, Walkersville, MD, USA
TaqMan master mix	4444963	Thermo Fisher Scientific, Waltham, MA, USA
Potassium chloride	P9541-500G	Sigma-Aldrich, St Louis, MO, USA
Palmitic acid	P5585-10G	Sigma-Aldrich, St Louis, MO, USA
Penicillin/streptomycin	15140122	Gibco Thermo Fisher Scientific, Waltham, MA, USA

Table 9: List of equipment and software

Equipment name	**Catalogue number**	**Company**
Aspirator	04012019060039	Monitoring and Control Laboratories, Frankenwald, Sandton, South Africa
Analogue Vortex Mixer	E-VM-A	United Scientific PTY LTD, Western Cape, South Africa,
Centrifuge	15227589	Thermo Fisher Scientific, Waltham, MA, USA
Cell countess II	2185A20066697	Invitrogen Thermo Fisher Scientific, Waltham, MA, USA
Cell scrappers	541070	Greiner bio-one, Frickenhausen, Germany
Corning disposal Pasteur glass pipettes	28721525	Sigma-Aldrich, St Louis, MO, USA
Gel Doc Image lab software	Version 6.1	Bio-Rad, Hercules, CA, USA
Gel Doc XR+ System	1708182	Bio-Rad, Hercules, CA, USA
GraphPad Prism ®	GraphPad Software Inc, CA, USA	8.0.1.244
Dri-Block heating block	002895-A	Techne, Cambridge, UK
EVOS Microscope	AMF4300	Invitrogen Thermo Fisher Scientific, Waltham, MA, USA
Filter systems	8-0000-230514	Thermo Fisher Scientific, Waltham, MA, USA
Facemasks	1530722WJ	
Hood	A12-109-01	Lasec Bio Rad
Incubator	162004-21576	Thermo Fisher Scientific, Waltham, MA, USA
Microsoft office	Word/Excel/PowerPoint	Microsoft Corporation, WA, USA
InsMark pH Meter IS128	85001128	United Scientific PTY LTD, CA, South Africa
Kern ADB weighing scale	WE21001062	Kern, Ayala Avenue, Makati, Philippines

Multi-channel pipettes		EINS SCI, JHB South Africa
Multispin 12 microcentrifuge	BST-201700 2013	ESAMED, Via Spagna, Italy
Microwave	14264879	Safeway, South Africa,
MultiSkan GO plate reader	1510-04302	Thermo Fisher Scientific, Waltham, MA, USA
Nanodrop OneC Microvolume	AZY2123199	Thermo Fisher Scientific, Waltham, MA, USA
NUVE NF200 benchtop centrifuge	03.15110	NÜVE, Saracalar, Akyurt, Turkey
NUVE NB20 Water bath	03.3444	NÜVE, Saracalar, Akyurt, Turkey
PCR MicroAMP Fast 96-well reaction plates	4346907	Thermo Fischer Scientific Applied Biosystems, Waltham, MA, USA
PowerPac basic system	041BR314041	Bio-Rad, Hercules, CA, USA
SL8R Centrifuge	75007224	Thermo Fischer Scientific Waltham, MA, USA
StepOne Plus Real-Time PCR system machine	4376600	Thermo Fischer Scientific, Applied Biosystems Waltham, MA, USA
T100 thermal cycler	186-1096	Bio-Rad, Hercules, CA, USA
3D Plate Shaker, Analogue and digital	TC33418	World of Science, CA, South Africa
Vortex	CLS6776	Corning, MA, USA

Table 10: List of consumables

Product name	**Catalogue**	**Company**
Bio-pure pipetting reservoir	Z679968	Sigma-Aldrich, St Louis, MO, USA
Microplates, 96-well	655101	Greiner bio-one, Frickenhausen, Germany

Microcentrifuge tubes	655101	Labcon plastique, Chamdor South Africa
75cm2 Flasks	C7231	Greiner bio-one, Frickenhausen, Germany
25cm2 Flask	708001	Whitehead Scientific, CA, South Africa,
96-well plates	20160030507B	Whitehead Scientific, CA, South Africa
6-well plates	701001	Whitehead Scientific, CA, South Africa
Optical adhesive covers slips	703001	Thermo Fischer Scientific Applied Biosystems, Waltham, MA, USA
Pasteur pipettes (3ml)	4311971	Whitehead Scientific, CA, South Africa
Sleeve covers	01252021	Labfriend, New South Wales, Australia
Shoe covers		Labfriend, New South Wales, Australia
Syringe Filters		Sigma-Aldrich, St Louis, MO, USA
Serological pipettes	CLS431212	Sigma-Aldrich, St Louis, MO, USA
Syringe Filters	CLS4500	Sigma-Aldrich, St Louis, MO, USA

Serological pipettes	CLS431212	Sigma-Aldrich, St Louis, MO, USA

APPENDIX 2

Buffer and media preparations

Freezing media for the cryopreservation of the cells

The media used to freeze the C2C12 cells had DMEM that was supplemented with 90% fetal bovine serum (FBS) and 7% (v/v) dimethyl sulfoxide (DMSO).

The preparation of 75 mM palmitate stock

The amount of 96.15 mg of palmitic acid was dissolved in 5 mL of 100% absolute ethanol to make a stock of 75 mM and it was heated at 90°C for 5 minutes. The working solution was diluted by 1:100 in Kreb's to make final concentration of 0.75mM.

Preparation of *Moringa oliefera*

The amount of 10 mg of *Moringa oliefera* was weighed and dissolved in 1 mL of 100% DMSO.

Table 11: Components used for the Kreb's ringer bicarbonate buffer in 500 mL

Reagent	**Molecular weight**	**Final concentration**	**Weight**
NaCl	58.44	115 mM	3.36 g
NaHCO3	84.01	24 mM	1.008 g
KCL	74.55	5 mM	0.186 g
MgCL2	95.21	1 mM	0.048 g
CaCl2	110.98	2.5 mM	0.139 g
BSA		0.1% (w/v)	0.5 g
HEPES	238.3	10 mM	5 mL

Sorenson's glycine buffer

Sorenson's buffer was prepared by dissolving 0.751 g glycine and 0.584 g NaCl in 100 mL distilled water. The pH of the buffer was adjusted to pH10.5 with 0.1 mM NaOH.

Table 12: Preparation of Sorenson's buffer

Chemical	Final concentration	Amount for 100 mL
Glycine	0.1 M	0.751 g
NaCl	0.1 M	0.584 g

APPENDIX 3

The quality of RNA of the treated C2C12 skeletal muscle cells using the agarose gel electrophoresis is shown in the figure below.

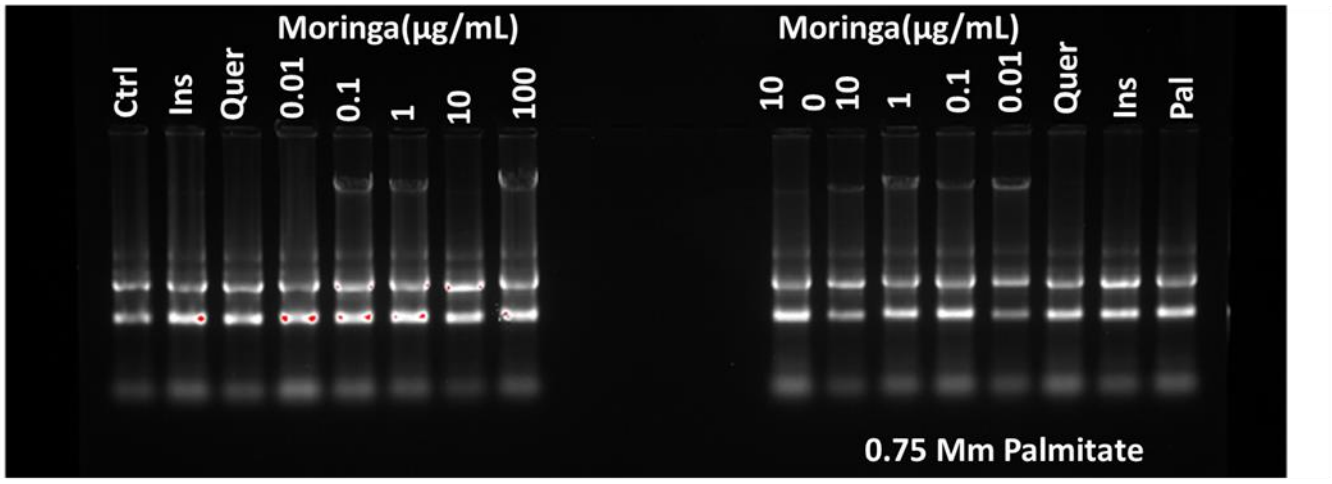


Figure 15: RNA quantification of the treated C2C12 skeletal cells using an agarose gel electrophoresis. Total mRNA was extracted from the treated C2C12 skeletal cells and after extraction, the quality was assessed using the agarose gel electrophoresis in order to assess whether the quality would be good enough to use for quantitative polymerase chain reaction (qPCR). The purpose of quantifying the gel like this is to see if the RNA fragments are intact or not and here we observed that our RNA was mostly intact. The concentrations *Moringa oliefera* showed clear bands which are indicative of intact RNA. The bands also showed that they have a lower molecular weight because they seem to have migrated further down the gel. In some cases, RNA (lower than 18 rRNA) that has smaller molecular weight could tell us that it is degraded. The bands on the left represent the normal cells treated with only *Moringa oliefera* and on the right are the insulin resistant cells treated with *Moringa oliefera*. Ctrl (control), Ins (insulin), Quer (quercetin), Pal (palmitate) ,0.01,0.1,1,10 and 100 µg/mL represents the different doses of *Moringa oliefera* used for treating the cells.

Table 13: Averages of the NanoDrop readings used for synthesis of cDNA for experiment 1

Sample	**information**	**RNA (ng/µL)**	**Average**	**RNA (µg/µL)**	**µL RNA needed for 2 µg**	**µL H2O needed for 12 µL**
Sample 1	Control	1496.88				
	Control	1581.33	1539.10	1.54	1.30	10.70
Sample 2	Insulin	1128.81				
	Insulin	1108.49	1118.65	1.12	1.79	10.21
Sample 3	Palmitate	599.564				
	Palmitate	633.173	616.3685	0.62	3.24	8.76
Sample 4	Pal+Ins	708.913				
	Pal+Ins	796.337	752.625	0.75	2.66	9.34
Sample 5	Pal+Mor 0.01	756				
	Pal+Mor 0.01	729	742.5	0.74	2.69	9.31
Sample 6	Moringa 10	1914				
	Moringa 10	1591	1752.5	1.75	1.14	10.86
Sample 7	Pal+Mor 0.1	724				
	Pal+Mor 0.1	691	707.5	0.71	2.83	9.17
Sample 8	Pal+Mor 1	706				
	Pal+Mor 1	874	790	0.79	2.53	9.47
Sample 9	Pal+Mor 10	896				
	Pal+Mor 10	914	905	0.91	2.21	9.79
Sample 10	Pal+Mor 100	950				
	Pal+Mor 100	959	954.5	0.95	2.10	9.90

Table 14: Averages after the NanoDrop readings of RNA used for the synthesis of cDNA for experiment 2

Sample	Information	RNA (ng/µL)	Average	RNA (µg/µL)	µL RNA needed for 2 µg	µL H2O needed for 12 µL
Sample 11	Control	815.459				
	Control	841.864	770.5	0.77	2.60	9.40
Sample 12	Insulin	813.854				
	Insulin	985.307	1195.5	1.20	1.67	10.33
Sample 13	Palmitate	880.002				
	Palmitate	432.925	1088	1.09	1.84	10.16
Sample 14	Pal+Ins	458.608				
	Pal+Ins	941.191	1000.5	1.00	2.00	10.00
Sample 15	Pal+Mor 0.01	1066.26				
	Pal+Mor 0.01	1065.426	553	0.55	3.62	8.38
Sample 16	Pal+Mor 0.1	1118.021				
	Pal+Mor 0.1	863.491	575.5	0.58	3.48	8.52
Sample 17	Pal+Mor 1	883.923				
	Pal+Mor 1	724.102	1217.5	1.22	1.64	10.36
Sample 18	Pal+Mor 10	739.176				
	Pal+Mor 10	916.802	1636	1.64	1.22	10.78
Sample 19	Pal+Mor 100	998.902				
	Pal+Mor 100	1058.467	1316	1.32	1.52	10.48

Table 15: NanoDrop readings that were averaged for the calculations of cDNA synthesis for experiment 3

Sample	information	RNA (ng/µL)	Average	RNA (µg/µL)	µL RNA needed for 2 µg	µL H2O needed for 12 µL
Sample 20	Control	1755.867				

	Control	1499.723	803.415	0.803415	2.489373487	9.510626513
Sample 21	Insulin	879.307				
	Insulin	891.693	1275.6905	1.2756905	1.567778391	10.43222161
Sample 22	Palmitate	918.622				
	Palmitate	812.689	1123.043	1.123043	1.78087571	10.21912429
Sample 23	Pal+Ins	1029.326				
	Pal+Ins	1161.047	1095.1865	1.0951865	1.826172985	10.17382701
Sample 24	Pal+Mor 0.01	544.248				
	Pal+Mor 0.01	1091.114	597.253	0.597253	3.348664636	8.651335364
Sample 25	Pal+Mor 0.1	1112.055				
	Pal+Mor 0.1	1079.641	1141.5755	1.1415755	1.751964719	10.24803528
Sample 26	Pal+Mor 1	1100.297				
	Pal+Mor 1	859.61	988.117	0.988117	2.024051808	9.975948192
Sample 27	Pal+Mor 10	893.786				
	Pal+Mor 10	1279.458	920.4605	0.9204605	2.17282545	9.82717455
Sample 28	Pal+Mor 100	1363.645				
	Pal+Mor 100	1398.52	1448.089	1.448089	1.38113058	10.61886942

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

17 November 2021

RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 17/11/2021, the NWU-AnimCareREC hereby clears your study as a no risk study. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Effect of *Moringa oleifera* in palmitate-induced insulin resistant C2C12 skeletal muscle
Principal Investigator/Study Supervisor/Researcher: Prof SE Mazibuko-Mbeje
Student: F Mthiyane - 37345109

Ethics number:

N	W	U	-	0	1	5	0	1	-	2	1	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: No Risk
Commencement date: 17/11/2021

Risk: **No Risk**

General conditions:

The following general terms and conditions will apply:

- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research;*
  - *withdraw or postpone clearance if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

Fikile Mthiyane

ORIGINALITY REPORT

25%	21%	16%	10%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	repository.nwu.ac.za Internet Source	2%
2	Submitted to North West University Student Paper	2%
3	uzspace.unizulu.ac.za:8080 Internet Source	1%
4	scholar.sun.ac.za Internet Source	1%
5	www.frontiersin.org Internet Source	1%
6	Submitted to University of Stellenbosch, South Africa Student Paper	1%
7	www.mdpi.com Internet Source	1%
8	ulspace.ul.ac.za Internet Source	1%
9	(5-15-14) http://196.21.83.35/bitstream/handle/10530/618/AN%2	1%