Effects of *Momordica balsamina* on glucose handling in high fat high carbohydrate induced prediabetic rat model and glucose handling in C2C12 induced insulin resistant cell lines: Effects on selected metabolic markers

by

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Preface

Ingestion of high fat and high carbohydrate has been associated with mechanisms that has shown to exacerbate to mechanisms that leads to impaired glucose homeostasis. Impaired glucose homeostasis leads to the development of insulin resistance and diabetes. The use of anti-diabetic agents have been associated with the progression of diabetic complications. Furthermore, treatments require dietary and lifestyle changes for which patients do not comply. There is therefore a need for alternative treatments that will be effective with or without dietary and lifestyle intervention. The WHO however, has proposed the use of medicinal plants as alternative treatment since they possess hypoglycaemic properties and alleviate hyperglycaemia-induced diabetic complications. *Momordica balsamina* (MB) has been shown to possess hypoglycaemic effects in streptozotocin (STZ)-diabetic animals. However, there are limited studies on *Momordica balsamina* and its effects on insulin resistance, prediabetes and type 2 diabetes. This study therefore, evaluated the effects of *Momordica balsamina* on glucose handling in insulin resistance C2C12 skeletal muscle cell lines *in vitro.* Furthermore, the study also evaluated the effects of *Momordica balsamina* on glucose handling in high fat high carbohydrate diet induced prediabetic rat model: effects of selected markers.

Declaration

I, Bongiwe Khumalo (student number : 215080288) hereby declare that the dissertation entitled:

"Effects of *Momordica balsamina* on glucose uptake in insulin resistant C2C12 skeletal muscle cell lines and glucose handling in high fat high carbohydrate induced prediabetic rat model and: Effects on selected metabolic markers" is the result of my own investigation and research that is no been submitted in part or full for any other degree or to any other university. Where use of the work of others was made, it is dully acknowledged in the text. The research done in this study was carried out under the supervision of Dr P.S. Ngubane, Dr N Sibiya and Dr A. Khathi.

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MASTER'S DEGREE IN MEDICAL SCIENCES 2019

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I would firstly like to thank God for my being for giving me courage to continue and strive for the best, without him this would not be possible.

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Abbreviation list

ANOVA	Analysis of variance
CO_2	Carbon dioxide
Con	Control
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
EPR	Electron paramagnetic resonance
FCS	Foetal calf serum
GLUT	Glucose transporter
h	Hours
H_2O	Water
HNMR	Proton nuclear magnetic resonance
KOH	Potassium hydroxide
L	Litre
mL	Millilitre
mmol	Millimol
MRC	Medical Research Council
Na_2SO_4	Sodium sulphate
PBS	Phosphate buffered saline
Pen	Penicillin
SEM	Standard error of means
T1DM	Type 1 Diabetes Mellitus

T2DM	Type 2 Diabetes Mellitus
UV	Ultraviolet
µg	Micrograms
Akt	Protein Kinase B
DMEM	Dulbecco's Modified Eagle Medium
GLUT4	Glucose Transporter 4
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate 1
OGTT	Oral glucose tolerance test
PBS	Phosphate Buffered Saline
PI3-Kinase	Phosphatidylinositol-3-Kinase
KCl	Potassium Chloride
KOH	Potassium Hydroxide
TBS	Tris Buffered Saline
AMPK	Activated Protein Kinase
ATP	Adenosine Triphosphate
ANOVA	one-way analysis of variance
α	alpha
AREC	animal resource ethics committee
β	beta
BRU	Biomedical Research Unit
BHT	Butylated Hydroxytoluene
Ca^{2+}	Calcium ion

CHS	College of Health Sciences
NC	Normal control
DM	Diabetes Mellitus
DAG	Diacylglycerol
DMSO	Dimethyl Sulphoxide
ELISA	Enzyme-linked Immunosorbent Assay
FFA	Free fatty acid
hbA1c	Glycated haemoglobin
Hb	Haemoglobin
OH^{-2}	Hydroperoxyl
OH^{-}	Hydroxyl radical
Kg	Kilogram
LDL	Low-density lipoprotein
Ltd	Limited
MA	Masilinic acid
MDA	Malondialdehyde
GPx	Glutathione peroxidase
G3P	Glyceraldehyde 3-phosphate
GSK-3	Glycogen synthase 3
H_2O_2	Hydrogen peroxide
INS	Insulin
i.p.	Intraperitoneally
JAK	Janus Kinase

Kg	Kilogram
λ	Lambda
LDL	Low-density lipoprotein
MA	Masilinic acid
MDA	Malondialdehyde
Met	Metformin
µ	Micro
µg	Micrograms
µl	Microlitre
m	Milli
mg	Milligram
mmHg	Millimeters of mercury
MAPK	Mitogen Activated Protein Kinase
PDK-1	Phosphoinositide-dependent kinase-1
MB	*Momordica balsamina* methanolic extract
NAD^+	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO_2	Nitrogen Dioxide
NO	Nitric Oxide
NF-κβ	Nuclear factor kappa-beta
PYY	Peptide YY
p.o.	Per os (orally)
PPAR	Peroxisome Proliferator-Activated Receptor

PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol-4, 5-bisphosphate
PS	Phosphatidylserine
3-PK	3-phosphokinase
PUFA	Polyunsaturated Fatty Acids
PKC	Protein Kinase C
ROS	Reactive Oxygen Species
SD	Sprague-Dawley
SOD	Superoxide dismutase
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBARs	Thiobarbituric acid reactive substances
TNF	Tumuor necrosis factor α
T2DM	Type 2 Diabetes Mellitus
UKZN	University of KwaZulu-Natal

Study Outline

The current dissertation is presented in a manuscript format, consisting of 6 sections viz. Chapter 1: abstract, introduction/literature review, chapter 2: manuscript 1, chapter 3: manuscript 2, chapter 4: synthesis. The abstract briefly summarizes the purpose and innovation of the present study in research. Chapter 1 entails a brief background and relevant literature review in problem solving and current gaps, and how the aims of the current study fills the gaps in the literature. Chapter 2 contains the first novel research study in manuscript format that seeks to investigate the effects of *Momordica balsamina* in palmitic induced C2C12 insulin resistant skeletal muscle cell lines. This work is authored by B Khumalo, A Siboto, K Maate, A Khathi and N Sibiya and P.S Ngubane and has been submitted for publication to **Journal of Bioorganic and Medicinal Chemistry**. Chapter 3 contains the second research study in manuscripts format that seeks to investigate the effect of *Momordica balsamina* in a high fat high carbohydrate induced prediabetic model. This work is authored by B Khumalo, A Siboto, A M Akinnuga, A Khathi, N Sibiya and P.S Ngubane and has been submitted for publication to **Journal of Diabetes** according to the author's guidelines. Chapter 4 is the synthesis, which discusses the link between the two studies and highlights the main findings for the specific aims of the current project. Appendices include the letter of ethical clearance, abstract and certificate of CHS presentation and journal's guideline to authors for the first research paper.

Abstract

Background

Increased consumption of fat and high carbohydrate coincided with increased prevalence in type 2 diabetes, a condition whose onset is always preceded by prediabetes. Prediabetes is best characterised by hyperglycaemia, insulin resistance and glucose intolerance. Prolong chronic hyperglycaemia exacerbates complications of increased oxidative stress, advanced glycation end products (AGE), dyslipidaemia, hyperinsulinemia and increased inflammatory markers. Once diagnosed, life style and dietary interventions strategies are one of the cornerstones in management of prediabetes. However, patients do not adhere to these life style changes. Hence the present study investigated the effects of *Momordica balsamina* (MB) on glucose handling in insulin resistance in C2C12 palmitic acid induced insulin resistant cell lines and in high fat high carbohydrate induced prediabetic rat model.

Methods

Briefly, air-dried MB leaves were extracted with methanol to yield methanolic extracts. The study was divided into 2 experimental series *invitro*, first series investigated the effects of MB compounds on cell viability in skeletal muscle cell lines. The second series investigated the effects of MB on glucose uptake in palmitic acid induced insulin resistant skeletal muscle cell lines. Invivo studies encompassed HFHC-induced diabetic rats which were divided into untreated and treated groups. The rats were treated with metformin (500 mg kg^{-1} p.o.) as standard drug and MB (250 mg kg^{-1} p.o.) a test drug. MB (250 mg kg^{-1} p.o.) was administered once every third day. Blood glucose concentration, body weight and calorie intake were monitored every fourth week for a period of 12 weeks. Terminally, animals were sacrificed after which blood, liver and skeletal muscle were collected for biochemical analysis.

Results

MB significantly decreased media glucose concentration whilst glycogen concentration was improved by comparison with insulin resistant cells. Treatment with MB reduced tissue damage which was shown MDA in the plasma while also improving their antioxidant status compared with insulin resistant cells. *In vivo* study, we measured caloric intake, body weights, ghrelin concentration, OGT response, glycogen concentration, GLUT 4, glycogen synthase, HOMA2- IR value and glycated haemoglobin (HbA1c) concentration. Interestingly, *Momordica balsamina* coupled with dietary intervention resulted in decreased fasting glucose concentration, suggesting improvement in insulin sensitivity. Reduced caloric intake and restored a steady constant weight growth, thus preventing obesity. This was associated with decreased plasma ghrelin levels. Additionally, there was a significant decrease in HOMA2-IR value. This was further evidenced by decreased levels of glycated haemoglobin in the MB-treated rats.

Conclusion

The results obtained suggests that *Momordica balsamina* (MB) possesses anti-hyperglycaemic and protective properties in vivo and in vitro, therefore could be potent in the management of prediabetes, impaired glucose homeostasis induced hyperglycaemia. In addition, these findings provide new scope to comprehensively delineate the medicinal plant, *Momordica balsamina* mechanism of activity.

Chapter 1: introduction / literature review

Introduction

Prediabetes is a long lasting state of progression from normal glucose homeostasis to tolerance to intermediate insulin resistance (1). This condition is characterized by impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) which is due to the onset of insulin resistance (2). The reasons for the increasing incidence of prediabetes developing countries include, urbanisation and a shift towards a "Westernized lifestyle" amongst others (3). The westernized lifestyle involves a diet that is generally high in refined carbohydrates and saturated fats (4). The consumption of this diet is associated with the increased prevalence of prediabetes (5). Researchers have estimated that prediabetes is expected to rise from 10.8 million to 18.7 million by 2025 in developing countries (6). Impaired insulin action and secretion are the first pathophysiological changes that result to prediabetes, thus leading to intermediate hyperglycaemia, insulin resistance with subsequent abnormalities in glucose, lipid and protein metabolism (7). Studies indicate that in prediabetes there is an impaired glucose homeostasis and cardiovascular complications which is often accompanied by dyslipidemia (8). Conventional treatments for managing prediabetes are associated with many adverse effects such as lactic acidosis, headaches and diarrhea (9). However, literature has shown that medicinal plant extracts such as *Momordica charantia* can ameliorate diabetes and its associated complications (10). In our laboratory, we have shown that *Mormodica balsamina* possesses hypoglyceamic and kidney dysfunction ameliorative effects in STZ-induced diabetic rats (11, 12). However, the effects of *Momordica balsamina* on glucose handling in high fat high carbohydrate induced prediabetic animals is yet to be established. Accordingly, the aim of the study therefore, was to investigate the effects of *Momordica balsamina* on glucose handling in high fat high carbohydrate induced prediabetic rat model.

1.1 Glucose homeostasis dysregulation

Maintaining normoglycaemic conditions is critical for normal cellular function (13). Various hormones play a crucial role in maintaining glucose homeostasis in the postprandial state, these include insulin, leptin and ghrelin (14, 15). Ghrelin is a stomach derived orexigenic hormone that stimulates feeding and weight gain to regulate energy homeostasis (16). Ghrelin secretion occurs in the small intestine, endocrine pancreas and the hypothalamus (16). Ghrelin plasma concentrations are increased during fasting and this sends the stimulus to the neuronal activation in the arcuate nucleus of the hypothalamus to stimulate food intake (17). A negative energy balance (body stores) translates into increased circulating ghrelin concentration, while surplus energy balance (obesity) is reflected in a decrease in ghrelin concentration (18, 19).

Diets rich in carbohydrates, particularly those with a high glycemic index (GI) and saturated fats, contribute to an increase in postprandial glucose /and insulin concentration, while suppressing ghrelin concentrations (18, 20). Plasma insulin concentration increase upon increasing plasma glucose and directly and indirectly facilitates glucose uptake in various tissues such as the adipose tissue, liver and

skeletal muscle cells which play a significant role in maintaining glucose concentration within normal ranges in a postprandial state (21, 22). Glucose uptake by these tissues is discussed below.

1.2 Hepatic glucose uptake

In the liver, during a postprandial state, glucose is taken up by the hepatocytes through facilitated diffusion via glucose transporter 2 (GLUT 2) (23). Insulin has been shown to stimulate glycogen synthesis via increased expression of glycogen synthase (GS) and GLUT 2 transporters while inhibiting hepatic glucose production (24).

1.3 Skeletal muscle glucose uptake

The skeletal muscle metabolizes 95 % of glucose and is the major site for glucose uptake (25). In the skeletal muscle and adipose tissue, glucose uptake is as a result of an insulin dependent cascade of reactions via the binding of insulin to the insulin receptors as per figure 1 below.

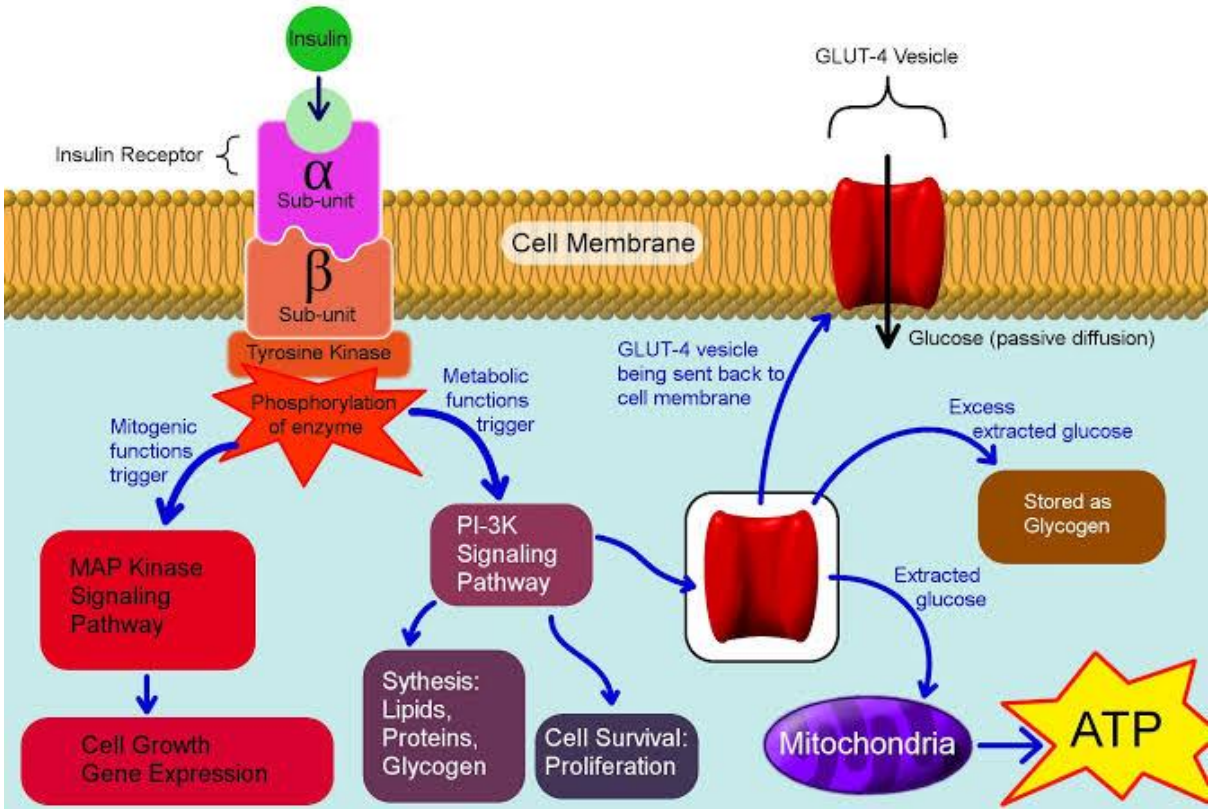

Figure 1. The insulin signalling pathway adapted from Brian, *et al*, 2012 (26)

The insulin receptor is a heterotetrameric membrane protein consisting of two identical α and β subunits (27). Insulin binds to the α subunits of the insulin receptor (IR), thereby activating the intrinsic kinase activity in the β subunit (28). This results in an intramolecular autophosphorylation of the insulin receptor substrate 1 (IRS-1). The insulin receptor substrate (IRS) family of proteins interacts explicitly with the phosphorylated IR through a phosphotyrosine binding, which then facilitates phosphorylation of IRS on several of tyrosine including tyrosine kinase 2 and JAK family residues by the activated IR

(29). These phosphotyrosine residues on IRS proteins provide docking sites for proteins with Src Homology 2 (SH2) domains with p85 being the most important regulatory subunit of the phosphatidylinositol 3' kinase (PI3K). phosphatidylinositol 3’ kinase (PI3K) exists in the cytosol as a dimer of a regulatory p85 subunit and a catalytic p110 subunit (30). Recruitment of the regulatory subunit brings the catalytic p110α subunit to the plasma membrane, where it catalyses the phosphorylation of the 3' position in the inositol ring of phosphoinositide (PI) lipids (31). PI3K catalyses the formation of PI (3,4,5)-trisphosphate from PI(4,5)-bisphosphate, and PI(3,4)-bisphosphate from PI(4)-phosphate (32). The phosphorylation of the 3' position recruits and activates proteins containing pleckstrin homology domains, including the 3' phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase B (Akt) (33). In turn, PDK-1 phosphorylates and activates downstream effectors including Akt and the protein kinase C, Akt phosphorylates many proteins, including glycogen synthase kinase 3b (GSK3B) in the liver and GLUT 4 translocation in the skeletal muscle (34). Translocation of GLUT 4 from the intracellular vesicles to the surface of the membrane results in glucose uptake in the skeletal muscle (35). In addition to glucose uptake, processes such as lipid synthesis and glycogen deposition are modulated by insulin (36).

Urban lifestyle and prolonged intake of high carbohydrate diets, particularly those high in fructose with little to no fibre and high fat diet intake has been shown to increase the circulation of free fatty acids which stimulate lipogenesis (37). This has been associated with obesity, insulin resistance and type 2 diabetes (38). The section below describes insulin resistance and the mechanisms involved in the development of insulin resistance leading to obesity.

1.4 Insulin resistance

Insulin resistance can be described as the reduced response of tissues such as the liver, adipose and skeletal muscle tissues to insulin, consequently resulting in the dysregulation of glucose homeostasis and ultimately, hyperglycaemia (39). The onset of insulin resistance is one of the factors that alters the peripheral glucose uptake including skeletal muscle (40). Studies have shown that several factors and mechanisms are implicated in the development of insulin resistance (41). These include oxidative stress as a result of obesity, sustained hyperglycaemia, saturated fats, inflammation and mitochondrial dysfunction amongst many (42, 43). These mechanisms are further discussed below. In this study, insulin resistance was induced in both *invivo* and *in-vitro*. The induction of insulin resistance *in-vitro* resembles the increased circulating saturated free fatty acid which result into skeletal muscle insulin resistance. Insulin resistance is one of the characteristics of prediabetes, hence this study uses the diet-induced prediabetes rat model to investigate the effects of *Momordica balsamina* on systemic insulin resistance and glucose homeostasis.

1.4.1 Insulin resistance in skeletal muscle tissues

Skeletal muscle metabolizes 95 % of glucose and is the major site for glucose uptake (44). The mechanism of insulin resistance in skeletal muscle tissues, involves the abnormalities in the α subunit

of the insulin receptor at the surface of the cell membrane and impaired phosphorylation of IRS-1 protein prevents the action of insulin (45). Impaired insulin signalling pathway at receptor level or phosphorylation of IRS-1 in the skeletal muscle hinders the translocation of GLUT4 transporters from the intracellular vesicles, inhibiting the entrance of glucose into the cell (46). This has been shown to be associated with low glycogen formation and glycogen synthase activity and expression in the skeletal muscle (47). Elevated plasma free fatty acid (FFA) from high carbohydrate (particularly fructose) and high fat diets have been implicated in the development of skeletal muscle insulin resistance (42, 47). Exposure of insulin sensitive cells to high levels of FFA inhibits the reaction of insulin to insulin receptors thus deactivating the phosphorylation of IRS-1 resulting in the impairment of the insulin signalling pathway which results in reduced glucose uptake (48). For example, the saturated long chain FFA such as palmitic acid and stearic acid are known to cause insulin resistance by inducing the activation of PKCθ and NF-kB, thereby reducing the activity of IRS-1 and IRS-2 at tyrosine residues known to promote insulin-signalling (49). Hence, this study used palmitic fatty acid to induce insulin resistance in muscle cell lines *in-vitro*. Moreover, insulin-signalling effectors such as PI3K and AKT, as well as AMPK, become deactivated leading to the activation of p38 MAP kinase, JNK, PKCs which have been associated with a decrease in glycogen synthesis and glycogen synthase activity and expression in the skeletal muscle (42, 50). Therefore the reduced activity of the insulin receptor α subunit in the phosphorylation of tyrosine kinase results in blockage of downstream signal transduction resulting in increased blood glucose (51).

1.4.2 Insulin resistance in the adipose tissue

Adipose tissue is a highly active metabolic and endocrine organ which is known to express and secrete bioactive peptides, known as adipokines and has also been shown to play a major role in insulin resistance (52). Excessive free fatty acids (FFA) in adipose tissue, contribute to the decreased glucose uptake into peripheral tissues causing lipids to accumulate in other cells such as skeletal muscle, liver and heart resulting in the increased formation of diacylglycerol (DG) and triglycerides leading to lipotoxicity (53, 54) Dietary saturated fatty acids (SFAs) activate toll like receptor (TLR), thereby promoting metabolic syndrome which are interceded by pro-inflammatory signalling, leading to the activation of IκB kinase beta (IKK-β) and c-Jun N-terminal kinase (JNK) (55-57). Inflammation results in the generation of cytokines such as interleukin-1 and interleaukin-6, and tumour necrosis factor (TNF) resulting in decreased sensitivity of the α subunit of the insulin receptor and dephosphorylation of the IRS-1 thus causing insulin resistance (58). Consequently, all these abnormalities and mechanisms result in the impairment of the insulin receptor substrate 1 (IRS-1) which lead to the development of insulin resistance (15, 59). High blood glucose concentration leads to hyperglycaemia from the generation of free radicals resulting into oxidative stress (60, 61). These free radicals reacts with nitrogen ion thereby contributing to lipid peroxidation (62). Lipid peroxidation is an important biomarker for oxidative stress and can be assessed by measuring the MDA concentration (63). Adipose

tissue are significantly damaged during lipid peroxidation which affect their ability to take up glucose hence the development of hyperglycaemia and excess release of insulin (64, 65).

Momordica charantia, a plant of the same genus as *Momordica balsamina* has been shown to improve insulin signalling and insulin sensitivity by increasing skeletal muscle insulin stimulated insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation in STZ-induced diabetic rats and high fat-fed induced animals, thereby stimulating glucose uptake (66, 67). There is also evidence of medicinal plants increasing glucose uptake by activating IRS-1 signaling, and enhancing GLUT4 translocation in C2C12 myotubes *in-vitro* (68, 69). The expression of GLUT 4 transporters has been shown to activate signal transduction cascades required to activate and phosphorylate other proteins such glycogen synthase kinase in order to express the protein glycogen synthase (70). This evidence suggests that medicinal plants may have the ability to ameliorate insulin resistance however, this has not been fully established. As highlighted above *Momordica balsamina* has been shown to lower blood glucose in STZ-induced diabetic animals, however, its effect on insulin resistance has not been demonstrated. In this study, we used palmitic acid, a saturated fat, to induce insulin resistance in C2C12 muscle cell lines to investigate the insulin resistance ameliorative effects of MB (71). High calorie diets that contain high amounts of saturated fats and complex carbohydrates are strongly associated with the development of prediabetes, a condition that is characterized by moderate insulin resistance and precedes the onset of type 2 diabetes.

1.4.3 Insulin resistance in the liver

Fatty acids in the liver come from several different sources such as fatty acids derived from high fat high carbohydrate diet, released from adipocytes via lipolysis, and from hepatic lipogenesis (72). An imbalance of triaclyglycerol delivery and its synthesis often leads to its accumulation in the liver (73). The increased accumulation of these fatty acids has been associated with a decrease in GLUT 2 transporters thereby leading to a decrease in glucose uptake and ultimately decreased glycogen synthesis (74). This disturbance leads to increased concentration of free fatty acids in the skeletal muscle and adipose which often diverge and accumulate in the liver (75, 76). Excess caloric intake and lower adiponectin has also been shown to reduce AMPK activation which then promote stellate cell proliferation and generation of oxygen species leading to conversion of steatosis to steatohepatitis (77, 78). Disturbances in gluconeogenesis and generation of steatosis leads to a decrease expression of GLUT 2 and glycogen synthesis resulting to a decrease glucose uptake (79, 80).

1.5 Prediabetes

Prediabetes is characterized by sustained moderate hyperglycaemia which leads to non-enzymatic glycation of proteins such as haemoglobin and subsequently the formation of advanced glycation end-products and glycosylated haemoglobin (81). In addition, prediabetes and diabetes are associated with lipid profile derangement which makes the cells to be vulnerable to lipid peroxidation (82). Prediabetes

is associated with augmented plasma TG's and lipoprotein derangements including increased LDL-C and reduced HDL-C (83).

Prediabetes has been identified as the leading risk factor for type 2 diabetes and there is increasing evidence that shows that it increases risks of developing cardiovascular disease (84-86). A number of meta-analytic and pathophysiological studies show that more often than not, every onset of type 2 diabetes is preceded by prediabetes (87, 88). Prediabetes is preceded by the simultaneous presence of insulin resistance and or pancreatic β cell dysfunction prior to diagnoses (85, 89). In insulin resistant state, normal insulin concentrations are affected where the pancreatic β cell responds by producing more insulin to overcome the high blood glucose concentrations (90). The progressive loss of pancreatic β cell function due to insulin resistance correlates with a continued increase in blood glucose concentration to above normal range (91). This change in blood glucose concentration begin to manifest when the β cell is unable to secrete sufficient insulin to compensate for insulin resistance, resulting in supra-physiologic glucose concentrations (89, 92, 93). In spite of altered insulin sensitivity and β cell dysfunction, the glucose concentrations are tightly regulated within a normal range for a while before a prominent but gradual increase (93, 94). Progression from normal glucose tolerance to the intermediate hyperglycaemic state of diabetes includes the stages of impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) (85, 95, 96). High fat high carbohydrate diet has been shown to induce insulin resistance by impairing insulin receptor substrate 1 and increasing pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and several interleukins (IL) leading to hyperglycaemia (97). In this study, we envisaged that the administration of MB may attenuate insulin resistance often observed in prediabetes. Studies have shown that the current cut-off or numerical definition also does not cover all individuals at high risk of developing diabetes (98, 99). The following section discusses the diagnosis of prediabetes and highlights the challenges due to lack of observations in regular programmes and unawareness.

1.5.1 Prediabetes pathophysiology

Among factors contributing to T2DM such as, β cell failure, glucotoxicity, insulin resistance IFG and IGT, prediabetes continues to be leading cause of T2DM (100). According to WHO, individuals at risk have one or both prediabetic conditions such as impaired fasting glucose of 5.6 to 6.9 mmol/L and impaired glucose tolerance 7.8 to 11.1 mmol/L (101). IGT and IFG are potential indicators that show an individual has a high risk of developing type 2 diabetes (102). Abnormalities in glucose homeostasis and insulin concentrations occurs continuously in individuals with type 2 diabetes (103). This however may be due to the fact that there is a long period of insulin resistance that is accompanied by a compensatory increase rate of insulin secretion and β cell mass that often goes unnoticed (104). This results in β cells not being able to compensate for the insulin secretion any longer, thereby resulting in changes in its phenotype (105). This leads to hyperinsulinaemia and glucotoxicity, where an individual's glucose concentration and circulating insulin are chronically elevated (106). Although the

liver indirectly converts glycogen to glucose in the stages of low glucose concentration in the blood, the presence of hyperglycaemia that already exists results in the liver converting the excess glucose to free fatty acids (FFA), thereby resulting in the imbalance or impaired glucose homeostasis (107). Free Fatty Acids stimulate gluconeogenesis by increasing ATP and NADH generated from their oxidation in the liver (108). In the skeletal muscle however, defects in muscle glycogen synthesis have been demonstrated in insulin resistant states, with a 50 % reduction observed in type 2 diabetes (100). As glycogen synthesis is known to account for the majority of non-oxidative glucose metabolism, a defect in glucose incorporation into glycogen is an important manifestation of insulin resistance (109, 110). The impairment in skeletal muscle glycogen synthesis has been attributed to defects in glucose transport, GLUT 4 transporter and glycogen synthase (111). Improved insulin signalling pathway has been associated with improved translocation of GLUT 4 from the intracellular vesicle to the surface of the cell membrane thus lowering glucose concentration and increasing glycogen storage (112). Studies suggest that medicinal plants may be helpful in ameliorating prediabetes since some *in-vitro* studies have shown that some medicinal plants have the ability to increase glucose uptake by activating IRS-1 signalling which has been shown to be associated with the enhancement of GLUT 4 in prediabetic animal subjects (113). This could also be in the presence and absence of dietary intervention. However, this has not been fully shown. Hence the investigation of glucose lowering effects of MB in the presence and absence of dietary intervention.

Treatment for diabetes often involves the use of four classes of drugs which include biguanides, alpha glucosidase inhibitors, sulfonylureas, thiazolidinediones. However, type 2 diabetes and its associated complications relies on various treatment strategies which involves the use of antihyperglycaemic agents such as biguanides, thiazolidinediones, exercise, dietary change and insulin injections (114). The following section discusses the diagnosis of prediabetes below.

1.5.3 Diagnosis of Prediabetes

Improper screening and diagnosis of prediabetes has also been a contributing factor to the development of type 2 diabetes (115). Prediabetes encompasses conventional diagnostic categories of impaired fasting glucose and impaired glucose tolerance which overtime can extend from conventional normal glucose tolerance to overt type 2 diabetes (116). Insulin resistance and defective glucose sensing at the β cell are the central pathophysiologic determinants that together cause hyperglycaemia (117). The diagnostic criteria for diabetes ranges from impaired fasting glucose and glucose tolerance test (118, 119). Prediabetes, a condition of blood glucose concentration that is above the norm but below the detectable threshold of diabetes, is mainly marked by either IFG and or IGT (120). While the diagnosis criteria for prediabetes are not uniform among the international professional organizations, it still remains the state of high risk of the development of type 2 diabetes (121). The World Health Organization (WHO) has described the prediabetes criteria to be defined by IFG of 6.1 to 6.9 mmol/L and IGT as 2-h plasma glucose of 7.8 to11.0 mmol/L following load of 75g of oral glucose load based

on a 2-h OGT test (120). Despite American Diabetes Association (ADA) having the same cut-off value of IGT, however, it has lowered the cut-off value for IFG 5.6-6.9 mmol/L (122). ADA additionally included glycosylated hemoglobin (HbA1c) based criteria of 5.7% to 6.4% as a diagnostic criterion for prediabetes (123). There is however, considerable new knowledge regarding the aetiology of different forms of diabetes and the predictable value of different blood glucose values in epidemiology studies (124). Hepatic insulin resistance, stationary β cell dysfunction, chronic low beta cell mass are causative factors in the pathogenesis of IFG, whereas IGT is characterized by reduced peripheral insulin sensitivity, near-normal hepatic insulin sensitivity (125). Studies however have shown that progressive loss of beta cell function does not correlate with the person's IFG detectable levels with those of IGT (126). Previous studies have documented that defective insulin secretion and altered insulin sensitivity already exist when fasting plasma glucose is still within the normal range that is 5.6 mmol/L (127, 128). Oral glucose test has been extensively used as a diagnostic tool to screen for IFG in diabetes (129). The oral glucose tolerance test distinguishes whether an individual has normal glucose tolerance or unknown type 2 diabetes (130). This test potentially reveals whether an individual has IGT even when the fasting plasma glucose is within the normal range and establishes whether an IFG individual has a normal 2-h post-prandial glucose (130). This particularly discloses individuals who have combined IFG and IGT, an advanced stage of impaired glucose homeostasis with pronounced alterations in insulin secretion and sensitivity (131). Identification of prediabetes using IFG and or IGT as diagnostic measures faces a lot of criticism due to poor reproducibility and ineffectiveness to predict predisposition to diabetic complications (132-134). Therefore, glycated hemoglobin (HbA1c) test is emerging as an alternative to plasma glucose in the diagnosis of type 2 diabetes (135). The following section discusses the HbA1c diagnostic test. In this study we used the WHO criteria to diagnose prediabetes.

1.5.4 The use of HbA1c as a diagnostic test

HbA1c has been used as a test for glycaemia in screening and diagnosis of diabetes (136). The privilege of HbA1c test includes measurement of the overall exposure to glucose and probability to predict long-term complications of diabetes. Hence this test has been extensively used in the diagnosis of diabetes to monitor the overall glycemic index of patients and risk of diabetes complications (137). Whether HbA1c can be used as an appropriate approach for diagnosis of prediabetes and prediction of the underlying complications is controversial (138). The WHO expert committee has discouraged the use of HbA1c for the diagnosis of prediabetes, citing lack of sufficient evidence (139, 140). HbA1c represents a haemoglobin that is irreversibly glycated at one or both N-terminal valines of the β chains (141). The HbA1c level provides information about the degree of long-term blood glucose control rather than the exact mean blood glucose (142). The HbA1c intermediary compounds, HbA1a and HbA1b are normally reversed through enzymatic deglycation process to generate free amino groups by phosphorylation of fructose-lysine residue on glycated proteins (143-145). However, this process is easily overwhelmed by sustained increase of blood glucose concentrations in conditions of insulin

resistance or dysfunctional pancreatic β cell as non-enzymatic glycation continues unabated thus forming a stable HbA1c (146).

1.5.5 Animal models of prediabetes

There are various models used to study type 2 diabetes (T2DM) (147). These includes animal model and cell line *in vivo* and *in-vitro*, respectively (148). Animal models has gained a lot of interest in researchers because they show genetic predisposition to the disease (149). The most commonly used-genetic animal models of diabetes are those induced by streptozotocin or alloxan, in addition to diet or models obtained by partial pancreatectomy which leads to insulin deficiency, hyperglycaemia, and ketosis (150-153). However, lifestyle factors linked with incidence of prediabetes includes physical inactivity, dietary habits amongst others (154). The World Health Organization (WHO) has recommended that there should be simple strategies to identify those who are at risk of developing the disease and provide them with early lifestyle interventions (155). Therefore, it is very crucial to establish predictive models using risk factors for interventions relating to the development of prediabetes.

Although animal models described above are useful for the study of diabetes, however, they are not representative of diet-induced human metabolic syndrome and prediabetes. Diet consumption has been considered an important factor in the impairment of insulin activity (156). Literature has shown that inducing prediabetes with a high fat diet (HFD) or high carbohydrates (HCD) and fructose to rats for 2 months is a convenient and fast way to induce prediabetes which was associated with metabolic and oxidative disorders without modulation of glycaemia (153, 157, 158). However, studies have confirmed that daily consumption of sugar, high calorie dense food and food that are high in fat are at great risk of developing prediabetes (159). This modern style of feeding is based on the western-styled diet which incorporates high amounts of saturated fats, trans-fatty acid, refined grains and high sweetened refined sugars (160). Dietary carbohydrates mediate their effects on insulin sensitivity by increasing the free fatty acid concentrations via *de novo* synthesis of fatty acids (161, 162). A diet that promotes chronic consumption of high fructose of sweetened beverages exposes the liver to high fructose amounts thus resulting in rapid stimulation of lipogenesis and accumulation of triglycerides, which in turn contributes to reduced insulin sensitivity and hepatic resistance or glucose intolerance (163-165). Exposure of skeletal muscle to increased concentrations of circulating free fatty acids induces insulin resistance by inhibiting the insulin-mediated glucose uptake (166, 167). Of interest in this study was the diet-induced prediabetic rat model by the combination of high-fat diet (HFD) and high carbohydrate diet (HCD) supplemented with 15 % fructose. This prediabetic model has been shown to dysregulate glucose homeostasis by inducing factors important for glucose homeostasis such as ghrelin concentrations (168). Poor suppression of ghrelin by fructose ingestion suggests that chronic consumption of high-fructose diets result in impairment of hunger-satiety system (169). As it has been shown that there is a reciprocal relationship between ghrelin and insulin which plays a role in hyperglycaemia by increasing food intake evidenced by body weight and weight gain. Therefore this model displays the complications

that arises during intake of high fat carbohydrate consumption which has been further shown with increased triglycerides, insulin resistance which are the aetiology for type 2 diabetes (170, 171). The use of this prediabetic rat model that best describes clinical manifestations and what are humans are exposed to is advantageous in developing therapeutic novel treatments for management of prediabetes.

1.6 Conventional treatments

1.6.1 Thiazolidinediones

Thiazolidinediones(TZDs) are agonists of PPAR-gamma, a transcription factor involved in adipocyte development (172). PPAR receptors are a family of nuclear receptors consisting of three subtypes designated PPARa, PPAR and PPARd or -β. peroxisome proliferator-activated receptor (PPAR) receptors are found in key target tissues for insulin action in adipose tissue, skeletal muscle and liver (173). TZDs reduce plasma triglycerides and free fatty acids by binding to peroxisome proliferator-activated receptor gamma (PPARϒ) in adipocytes to promote adipogenesis and fatty acid uptake (174). This uptake facilitates in reducing circulating fatty acid concentrations and lipid availability in the liver and the skeletal muscle, thereby improving insulin resistance. Common side effects that are associated with this drug however include weight gain, hepatotoxicity and heart failure (175). Therefore, searching for new PPAR ligands without undesirable action has become increasingly important. Several medicinal plants have been reported to be a rich source of ligands for nuclear receptors (176). These plants include *Daphne gnidium*, *Illicium anisatum*, *Juniperus virginiana*, *Terminalia chebula*, and *Thymelaea hirsute* (176). They have been proven to function in similar a fashion to TZD's by activating the PPARϒ receptors hence decreasing blood glucose concentration in diabetic animal subjects (176).

1.6.2 Biguanides

Biguanides are a class of drugs that function as oral antihyperglycaemic agents and are considered as the first line option for the management of diabetes mellitus or prediabetes. Biguanides include metformin, phenformin and buformin (177, 178). Biguanides lower blood glucose by suppressing hepatic glucagon signalling by decreasing the production of cyclic AMP (179, 180). They have also been shown to reduce hepatic glucose production and also increases glucose utilisation in the gut (181). However their use is associated with side effects which include diarrhoea and lactic acidosis (182). Studies have shown that despite the lowering glucose effects, biguanides fail to lower the glycation of haemoglobin (181, 183).

Despite the effects demonstrated above, medicinal plants have been shown to have an impact on various cellular proteins including AMPK along the insulin signalling pathway which highlight some of the mechanisms involved in lowering blood glucose (184). These mechanisms may also be utilised in insulin resistance and prediabetes, however, this has not been fully demonstrated.

Furthermore, the use of conventional treatment has been shown to be not effective unless there is lifestyle modifications and diet adjustments which individuals in developing countries fail, at times, to

adhere to (185). There is therefore a need to establish alternative treatment that will function with or without dietary intervention. The section below highlights some of the mechanisms utilised by medicinal plants in diabetic studies.

1.7 Medicinal plants

Many plants have been reported to be beneficial in managing diabetes mellitus, but only a small number of these have received scientific and medical evaluation to assess their efficacy (186). Traditional treatments have mostly disappeared in occidental societies, but some are prescribed by practitioners of alternative medicine or taken by patients as supplements to conventional therapy (187). However, in developing countries medicinal plants are widely used as main treatment of many diseases (188). A hypoglycaemic action from some treatments has been confirmed in animal models and non-insulin-dependent diabetic patients, and various hypoglycaemic compounds have been identified (188, 189).

Several plant species have been reported to possess antidiabetic properties (190). These include *Momordica charantia*, *Daphne gnidium*, *Illicium anisatum*, *Juniperus virginiana*, *Terminalia chebula*, and *Thymelaea hirsute* (132). These plants have been said to exert their antidiabetic properties via improved insulin sensitivity at the insulin receptor level, increasing insulin production thereby decreasing the amount of glucose in the blood (176, 184). They have been proven to function in a similar manner as TZD's by activating the PPARϒ receptors and hence decreasing blood glucose in type 2 diabetic animal subjects (176). *Cinnamomum zeylaniucm*, has been shown to lower blood glucose and improve hepatic glycogen and increase the expression of GLUT 4 transporter to the peripheral tissues (191) .

Compounds such as glycopeptides and terpenoid which are derived from medicinal plants have been reported to show potential as antidiabetic agents with most promising with biological features including their low toxicity and mechanism of action (192). *Parkia biglobosa* also known as 'afitin' in Nigeria has been shown to initiate insulin secretion from the pancreatic beta cells, however the seeds are often fermented and consumed for nutritional condiment (193). *Cyclopia maculate* (honeybush) which is rich in hesperidin has been shown to possess cardioprotective effects via the PPAR-c pathway in an ischemic heart disease model in diabetic rats (194). The combination of acarbose, a synthetic antidiabetic agent and *Anogeissus leiocarpus* (African birch) extract have also an inhibitory effect on α-amylase and synergistic effect on α-glucosidase enzyme inhibition, in addition to the antidiabetic property it also has antioxidant property (195). The attributed anti-hyperglycaemic effects of these plants are due to their ability to restore the function of pancreatic tissues by causing an increase In insulin output or a decrease in the intestinal absorption of glucose (196). Another plant extract derived from plants such as metformin which was synthesised and developed based on a biguanide compound found in French lilac (*Galega officinalis L.*) (197). However, these plants have not been investigated on the diet intervention studies.

Therefore, it is crucial to further evaluate plant extracts that will improve sensitivity to insulin and with minimal side effects while attain glycaemic control with or without diet intervention.

1.8 Plant toxicity

Herbal remedies are widely used for the treatment and prevention of diabetes in acting as anti-inflammatory agents, lipid lowering activity, hepatotoxicity (198, 199). Many medicinal herbs and pharmaceutical drugs are therapeutic at one dose and toxic at another (200, 201). Toxicity related to traditional medicines is becoming more widely recognized as these remedies become popular in the Mediterranean region as well as worldwide (202). The World Health Organization estimated that perhaps eighty percent of the inhabitants of the world rely chiefly on traditional medicines (203). According to a WHO study, the use of plants for healing purpose is getting increasingly popular as they are believed to be beneficial and free of side effects (204). Toxic substances from plants can affect the entire spectrum of vital human organs while some may affect key functional body systems like the central nervous system (CNS) thereby interfering with the coordination of nerve functions of the body (205). In order to investigate the effects of plant extracts, cell culture model system are used (206). Cell culture model allows for observing whether the plant extract gives cell proliferation or cytotoxic effects of the plant extract (203). However, it lacks the cell–cell and cell–substrate interaction characteristic of whole tissue occurs *in vivo* (207).

It has been shown that the toxicity of a given plant depends on various factors, including the strength of secondary metabolites, the quantity consumed, the time of exposure, different parts of the plant (root, oil, leaves, stem bark and seeds), and genetic differences within the species (208). For example, *Herniaria cinerea* DC which belongs to the family of the Caryophyllaceae, which is used to treat kidney diseases. This plant has been shown to cause abnormal digestive signs such as anorexia when the dose is increased (208). *Lauroside B*, a megastigmane glycoside isolated from *L. nobilis*, induced apoptosis in human melanoma cell lines by inhibiting NF-κB activation. Additionally, an aqueous fraction of *L. nobilis* induced generation of reactive oxygen species, p53-dependent apoptosis (209). In our laboratory, the biosafety of three different concentrations plant extracts were evaluated *in vitro* using the skeletal muscle cell lines. Methyltetrazolium assay (MTT) was used to assess cytotoxicity and cell viability.

1.9 Basis of the study

In our laboratory, medicinal plants such as *Syzygium cordatum* and *Syzygium aromaticum* and their bio-active compounds maslinic acid (MA) and oleanolic acid (OA) respectively, have shown the ability to reduce blood glucose concentrations in STZ- diabetic rats (210-212). These plants have shown the ability to increase the expression of GLUT 4 transporters and activity of the key glycogenic enzymes such as hexokinase (HK), glucokinase (GK) and glycogen synthase (GS) as mechanisms by which these plants lower blood glucose and increase glycogen in STZ induced diabetic rats. *Momordica charantia*,

a medicinal plant of the same genus as *Momordica balsamina* is extensively used and studied for the management of diabetes (213). Studies have shown that it can increase a number of β cells in the pancreas thereby improving the ability of the body to produce insulin (214). Furthermore, extracts from this plant have shown the ability to enhance glucose uptake by the liver and muscle cells, potentiating the effect of insulin (214). MB has also been shown to possess hypoglycaemic effects in STZ induced type 1 diabetic animals (12). However, the effects of MB on glucose handling in high fat high carbohydrate induced prediabetic rat model have not yet been established.

In this study, we used the high fat high carbohydrate induced prediabetic animal model which was established in our laboratory as per Luvuno *et al*, 2017 (215). This prediabetic animal model accurately mimics the dietary consumption and prediabetic conditions observed in humans which therefore allows for control of genetic, and environmental factors that may influence the development of the disease and its secondary complications, therefore gaining useful information on its management and treatment. This preliminary diabetic animal model has been shown to display the complications and aetiology of T2DM, which is insulin resistance, hyperglycaemia and glucose intolerance over time (215).

1.10 AIM

The aim of the study therefore, was to investigate the effects of *Momordica balsamina* on glucose handling in both palmitic acid induced-insulin resistant C2C12 skeletal muscle cell line, and high fat high carbohydrate induced prediabetic rat model.

1.11 Objectives

The objectives of the study were as shown below

Study 1.

To investigate MB in skeletal muscle (C2CI2) *in-vitro* on the following;

- Cell viability studies using MTT assay
- Glucose utilization
- Lipid peroxidation using TBARS
- Glycogen synthesis using the glycogen assay

Study 2.

To investigate the of effect of MB in high fat high carbohydrate induced prediabetes rats on the following;

- Body weight
- Calorie intake
- Blood glucose concentration
- Glycated haemoglobin

- HOMAR-IR index
- Ghrelin
- Hepatic and skeletal muscle glycogen
- Glucose transporters (GLUT 4)
- Hepatic and skeletal muscle glycogen synthase expression

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Chapter 2

Prologue

Manuscript 1

Hyperlipidaemia has been associated with the development of insulin resistance. Some of the risk factors that are implicated in the onset of insulin resistance during hyperlipidaemia include oxidative stress and mitochondrial dysfunction. The exposure of peripheral organs such as the skeletal muscle to reactive oxygen species has been shown to diminish the insulin-dependent stimulation of insulin signalling pathway and subsequently glucose uptake. Insulin sensitizers including metformin, have been used in the management of insulin resistance however, they are associated with contra indications such as lactic acidosis. However, medicinal plant extracts including *Momordica balsamina* (MB) have been reported to have antidiabetic properties in type 1 diabetic rat model. The effects of MB on insulin resistance however, are limited. This study therefore evaluated the effects of *Momordica balsamina* on glucose uptake in insulin resistant C2C12 skeletal muscle cell lines, *Invitro*.

The current manuscript is formatted according to the Journal of **Bioorganic and Medicinal Chemistry** author guidelines.

Bioorganic and Medical Chemistry

Effect of *Momordica balsamina* on palmitic-induced insulin resistant C2C12 skeletal muscle cell lines.

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Abstract

Introduction

Studies have reported that *Momordica balsamina* (MB) attenuates hyperglycaemia. However, the mechanism at which MB attenuates hyperglycaemia still remains unclear. The aim of the study therefore, was to investigate the effect of *Momordica balsamina* on glucose utilisation in palmitic acid induced insulin resistant (C2C12) skeletal muscle cell line, *in-vitro.*

Methods

Cell viability was conducted in skeletal muscle cells to examine the cytotoxic effects of *Momordica balsamina* using Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were trypsinised and seeded into 24-well plates at a seeding density of 1.8 x 104 cells/well and incubated for 24 h to permit attachment and growth of cells to semi-confluency. Media was replaced with the media containing palmitic (500ul) acid then cells were incubated for 4 hours. After 4 h, old media was replaced with new media. Thereafter, Media glucose concentration was measured at 12, 24, and 48 h with One Touch select glucometer. After the experimental period the cells were harvested for glycogen and oxidative malondialdehyde (measurement).

Results

There was no decline in cell viability observed all 3 concentrations of *Momordica balsamina*. The administration of MB significantly decreased media glucose at 48 hours of incubation by comparison to palmitic acid exposed control cells. Interestingly, the combination of MB and insulin decreased media glucose by comparison to MB alone at 48 hours. Furthermore, MB-treated cells showed an increase in glycogen synthesis, while a decrease in MDA was observed

Conclusion

These observations suggest the *Momordica balsamina* increase glucose uptake in the insulin resistant cells, thus encourages further developments towards glycaemic control.

Keywords: *Momordica balsamina*, Insulin resistance, palmitic acid.

1. Introduction

Insulin resistance is a characteristic feature for many metabolic complications that can be defined as the reduced cellular response to endogenous insulin (1). This metabolic complication has been deemed as a strongest risk factor for the development of obesity and type 2 diabetes mellitus (T2DM) (2). The pathogenesis of insulin resistance may be attributed to environmental factors such as unhealthy lifestyle including high caloric diet (3). The endemic of T2DM is expected to rise from 10.8 million to 18.7 million by 2025 in developing countries (4-6). The expected rise may be attributed with factors such as urbanisation and a shift towards unhealthy lifestyle including westernised diets (7, 8).

Saturated fatty acids such as palmitic acid palmitic acid and stearic acid are known to cause insulin resistance by inducing the activation of PKCθ and NF-kB, thereby reducing the activity of IRS-1 and IRS-2 (9). Moreover, excessive intake of saturated fatty acids (SFAs) has been shown to exacerbate insulin resistance (10). Therefore, lifestyle changes such as diet and physical activity has been reported to have positive effects on insulin sensitivity and insulin resistance (11, 12).

Conventional treatments such as biguanides and thiazolidinediones remain the most popular drugs for the management of T2DM as they possess anti-hyperglycaemic effect (13). These drugs facilitate the action of insulin in the skeletal muscle, liver and adipocytes (14, 15). Their mechanism includes improving the sensitivity and phosphorylation of insulin receptor substrate 1(IRS-1) of the skeletal muscle and adipose and improved expression of glucose transporters (GLUT 4) (16). However, these drugs have been shown to possess side effects such as flatulence, diarrhoea and heart failure (17-19). Recently, some medicinal plants have been reported to be useful in treating diabetes. These plants include *Syzygium aromaticum, Syzygium cordatum and Tapinathus nyascius* (1, 2). Some of these plants have been shown to possess anti-hyperglycaemic effects inhibition of absorption of glucose across the small intestine (20, 21).

The plant *Momordica balsamina* commonly known as Balsam apple (English), Intshungu (IsiZulu), Junglee karela (Hindi) has been widely used in tropical regions of Africa, Australia and Central America where leaves and fruits are used as vegetables (22, 23). Previous studies have reported *Momordica balsamina* fruits to possess antioxidant properties (24-26). In our laboratory, we have previously shown the antihyperglycaemic and reno protective properties of MB in STZ-induced diabetic rats (23). However, the mechanism at which *Momordica balsamina* attenuates hyperglycaemia still remains unclear.

The aim of this study therefore was to assess the effect of *Momordica balsamina* crude extract on glucose uptake in palmitic-induced insulin resistant C2C12 skeletal muscle cell line.

2. Materials and Methods

2.1 Drugs and Chemicals

Chemicals and drugs used;

Dimethyl sulphoxide (DMSO), butylated hydroxytoluene (BHT), phosphate buffered saline (PBS), Dulbecco's Modified Essential Medium (DMEM), palmitic acid (≥99%), sodium hydroxide (NaOH), phosphoric acid (BDH, Poole, England), hydrochloric acid (HCl) (Merck, Wadeville, South Africa), Butanol (Saarchem, Krugerdorp, South Africa), Foetal calf serum (FCS) and trypsin - (Highveld Biological, Johannesburg, South Africa), Insulin - (NovoRapid Pen Refill, Novo Nordisk Pty Ltd, Westwood pharmacy, South Africa); ethanol, sodium sulphate (Na_2SO4), potassium hydroxide (KOH) (Merck chemicals, Johannesburg, South Africa); insulin (Novo Rapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, South Africa).

2.2 Methods

2.2.1 Crude extract extraction

Momordica balsamina was identified by a botanist, Baijnath at the University of KwaZulu Natal, Westville. The plant was harvested at the University of KwaZulu Natal in Westville. The leaves were washed three times with water to remove any residual dirt.

2.2.2 Extraction method

The *Momordica balsamina* (MB) leaves extract was obtained using methanol by a well established standard protocol previously validated in our laboratory (27). Briefly, air-dried *Momordica balsamina* leaves were sequentially extracted twice at 24 h intervals at room temperature using methanol (45 mL) on each time interval. The solvent was removed from the crude extract under reduced pressure at 55±1 ^{o}C using a rotary evaporator to yield a methanol crude extract.

2.3 Cell culture

A well-established cell culture protocol by Czifra et al was used to conduct the study (28). DMEM for culturing muscle (C2C12) was supplemented with FCS (10%), pen/strep (1%) and L-glutamine (1%). Frozen muscle cell lines were regenerated in DMEM medium and transferred into 25cm^3 flasks which was incubated at 37 ˚C in the presence of 5% CO_2 in a humidified (89%) incubator (Shel Lab, Cornelius, Oregon, USA). The cells were allowed to grow and attach.

2.3.1 Sub-Culture of cells

When cells reached 70% confluency, media was removed and cells were washed with warm Dulbecco's phosphate buffered saline (DPBS) three times. Cells were then trypsinised with trypsin (1mL) and incubated for 2 minutes to allow the cells to detach from the flask. Microscope was used to confirm that the cells have dislodged and are freely floating. Pre-warm growth media was added to stop the process of trypsin. Fresh growth media was introduced every second day.

2.3.2 Seeding of cells into multi-well plates

Cells were sub-cultured after reaching 80% confluency. Thereafter the cells were seeded in 24 and 96 well plates for experiments. 96 well plates were used for MTT assay and 24 well plates were used for glucose utilization studies. To prepare doses required for the experiment, *Momordica balsamina* (12.5, 25 and 50 µmol/L) was freshly prepared in DMSO (0.1%) and subsequently diluted in fully supplemented cell culture growth medium (DMEM).

2.3.3 Cell viability studies

Cell viability was conducted in muscle cell lines to examine the cytotoxic effects *of M. balsamina*. Cell viability was measured by means of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay originally described by Mosmann (1983). Cells were trypsinised and seeded into 96-well plates (Bibby-Sterilin, Staffordshire, England) at a seeding density of 1.8 x 104 cells/well and incubated for 24 h to permit attachment and growth of cells to semi-confluency. Thereafter, the medium (0.5 ml) was replaced and *M. balsamina* (50, 25 and 12.5 µmol/L) was added to the wells and incubated at 37ºC for 12, 24 and 48 h respectively. After each incubation period, the medium was removed and MTT solution (5 mg/ml in phosphate buffered saline, 200 µL) was added to each well. The cells were incubated for 4 h to allow for the formation of blue formazan crystals. After 4 h incubation DMSO (200 µl/well) was added into each well and absorbance measured at 570 nm in a UV-visible spectrophotometer (Thermoscientific Biomate, Cambridge, UK).

The percentage cell viability was calculated as follows: [A570 treated cells – background] / [A570 control cells – background] x 100.

2.4 Induction of insulin resistance

Insulin resistance was induced in C2C12 skeletal muscle cell line using palmitic acid (1 mM) administered in each wells. Cells were trypsinised from the flask and seeded into 24-well plates (Bibby-Sterilin, Staffordshire, England) at a seeding density of 1.8 x 104 cells/well and incubated for 24 h to permit attachment and growth of cells to semi-confluency. Media was replaced with the media containing palmitic (500ul) acid then these cells were incubated for 4 hours.

After 4 h of insulin resistance induction, an old media was replaced with new media, media glucose concentration was measured at 12, 24, and 48 h with One Touch select glucometer (Lifescan, Mosta, Malta and United Kingdom).

2.5 Glucose utilization studies

The glucose utilization experiments were conducted as described by Ventera et al (2008) with slight modifications (29). Separate 80 % confluent skeletal muscle cells ($1.5\ 10^5$) in 24 well plates were incubated at 37 ^{0}C with DMEM (1 mL) containing 29 mmol/L of glucose in the presence of *Momordica balsamina* (MB) (25 ug/mL). The insulin resistant cells were divided into the following groups, group 1: Control insulin resistant cells (IR); group 2: insulin resistant cells treated with insulin (4 µmol/L) to confirm insulin resistance (IR + INS); group 3: insulin resistant treated with *Momordica balsamina* (MB) (25 ug/mL), to observe whether the plant can enhance glucose utilisation in insulin resistant cells (IR); insulin resistant cells treated with plant combined with insulin (4 µmol/L) to observe whether the plant combined with insulin has positive additional effects on glucose utilisation and insulin sensitivity (MB + INS).

Glucose concentration was measured at time 12, 24, and 48h with Accu-Chek glucometer (Lifescan, Mosta, Malta, and United Kingdom). After 48 h period, cells were trypsinised and harvested for measurements of glycogen and MDA in TBARS assay.

2.6 Glycogen assay

Glycogen analysis was performed in skeletal muscle cells after 48 h. Glycogen assay was conducted using a well-established laboratory protocol. The harvested muscle cells were heated with KOH (30%, 2 mL) at 100 ˚C for 30 min. thereafter, Na_2SO4 (10%, 0.194 mL) was added to cease the reaction and allowed to cool at room temperature. For glycogen precipitation, the cooled mixture (200 µL) was aspirated and mixed with ethanol (95%, 200 µL). The precipitated glycogen was pelleted, washed and resolubilized in H_2O (1 mL). Thereafter, anthrone (0.5g dissolve in 250 ml of sulphuric acid, 4 mL) was added and boiled for 10 min. After cooling the absorbance was read using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Württernberg, Germany) at 620 nm. The glycogen concentrations were calculated from the glycogen standard curve. The standard curve ranges from 200 to 1000 mg/L.

2.7 Thiobarbituric acid reactive substances (TBARS) assay

Thawed skeletal muscle cells were supplemented with 2% phosphoric acid (50µl) and centrifuge at 1000rpm for 10 minutes. 7 % phosphoric acid (200µl) was added into glass tube followed by the addition of 400µl of BHT. To ensure an acidic pH of 1.5, 1M HCl (200 µl) was added to sample. The solution was cooled at room temperature after heating at 100 ^{o} C for 15 minutes. The sample was vortexed after adding Butanol (1.5 ml) the cooled solution, two phases were observed. The butanol

phase (top layer) was transferred to eppendorf tubes and centrifuged at 13200xg for 15 minutes. A 96-well microtiter plate in triplicate was used to aliquot the sample and the absorbance was read at 532nm (reference λ 600nm) on a BioTek μQuant spectrophotometer. The absorbance's from these wavelengths were used to calculate the concentration of MDA using the Beer's Law.

Concentration = Absorbance Final / Absorption coefficient (156mmol-1)

2.8 Total antioxidant capacity

Total antioxidants capacity was analysed in the sample using specific total antioxidants capacity kit (Elabscience and Biotechnology, Wuhan, China) according to the manufacturer's instructions.

2.9 Statistical analysis:

All data is expressed as mean ± standard error of mean (S.E.M.). Statistical analysis performed using GraphPad Instant software (version 5). One-way analysis of variance (ANOVA) followed by Tukey-Kramer was used for analysis of differences between control and experimental groups. Values of $P<0.05$ will be considered significant.

3. Results

3.1 Cell viability

Effects on cell viability

Figure 1 shows the effects of MB on cell viability in C2C12 skeletal muscle cell line using MTT assay. By comparison with the control group, the administration of 3 concentrations of MB (12.5, 25, and 50 mmol/L) showed no significant decline is cell viability at corresponding time intervals of the 48h incubation period.

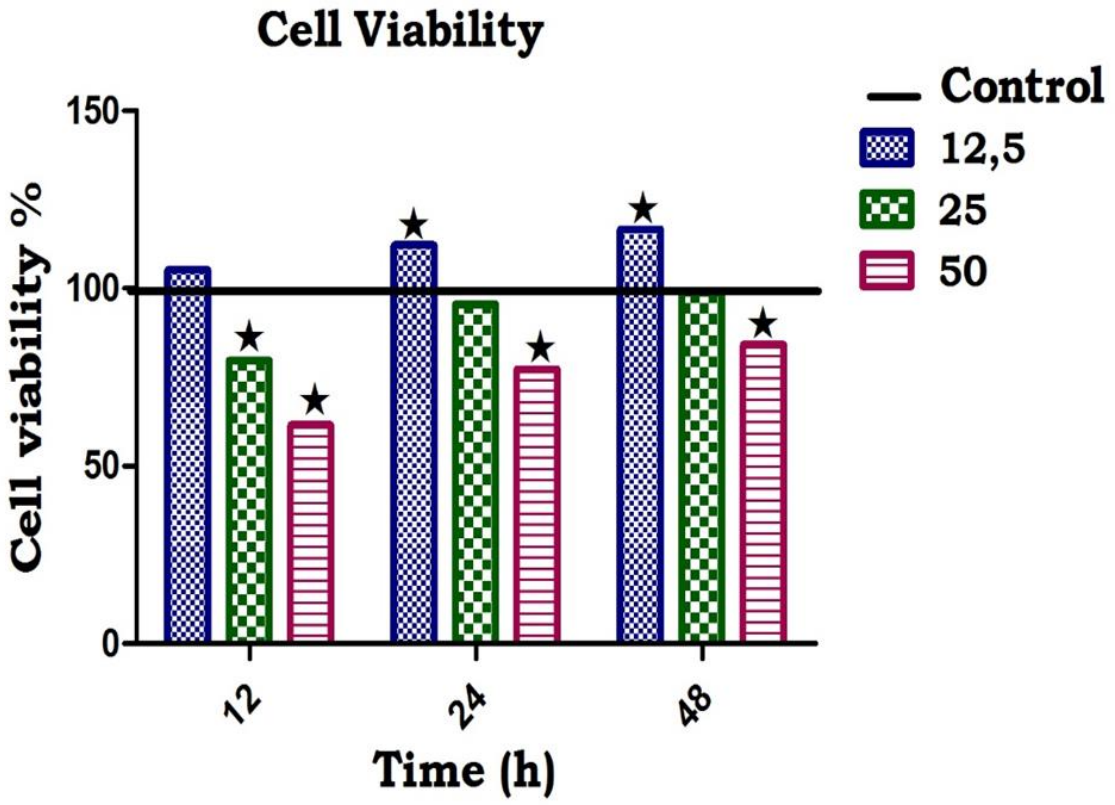


Figure 1. The effects of *Momordica balsamina* on cell viability on C2C12 muscle cell after 48 -hours of treatment period. ★ = P<0.05 in comparison to a control group.

3.2 Glucose utilization

Effects on glucose utilization

Figure 2 Shows the effects of insulin on glucose utilization in normal C2C12 skeletal muscle cell lines where insulin significantly increased glucose uptake after 48 hrs of incubation by comparison to normal untreated C2C12 muscle cell lines.

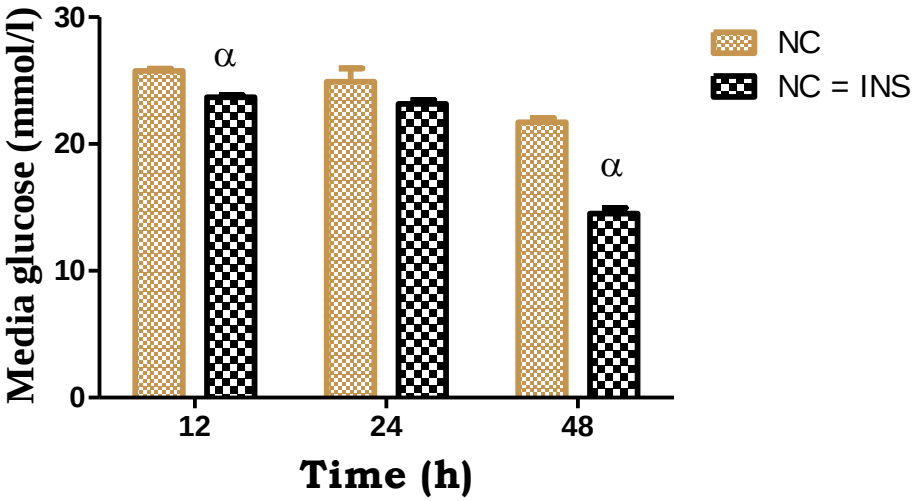


Figure 2. The effects of Insulin on glucose utilization at C2C12 skeletal muscle cell line.

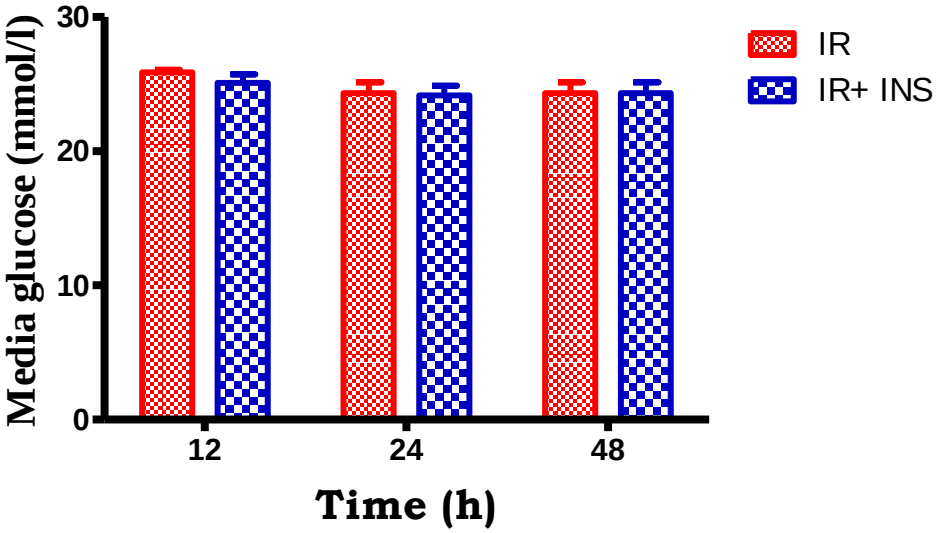
★ = $p<0.05$ by comparison with normal cells groups at each corresponding time.

3.3 Effects on insulin resistance

Figure 3 (a) shows the effects of insulin on glucose utilization in palmitic acid induced insulin resistant C2C12 muscle cell line where insulin showed no effect on glucose utilisation by comparison to control palmitic-induced control muscle cells.

Figure 3 (b) however, shows the effects of MB and insulin on glucose utilization of palmitic acid-induced insulin resistant C2C12 muscle cell lines. MB treated insulin resistant (IR) skeletal muscle cells significantly increased glucose uptake by comparison to both IR-control and insulin treated IR-insulin resistant muscle cell lines after 48 hrs. Interestingly, the combination of MB and insulin significantly increased glucose uptake by comparison to MB alone.

a)



b)

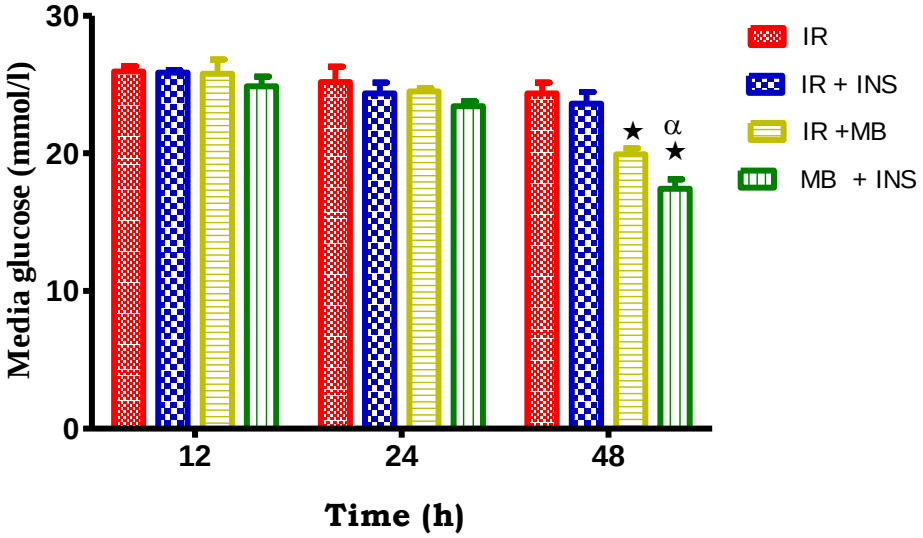


Figure 3a-b. The effects of insulin on glucose utilization in skeletal muscle cells after 12, 24 and 48 h. b) The induction of insulin resistance. Values are expressed as mean ±SEM. ★ = $p<0.05$ by comparison with normal cells groups at each corresponding time.

α = $p<0.05$ by comparison with the IR control group.

3.4 Effects on glycogen concentration

Table 1 shows comparison of glycogen concentrations of Palmitic acid-induced insulin resistant C2C12 skeletal muscle cells treated with MB after 48 h. In comparison to a control, insulin showed a significance increase in glycogen storage. The administration of MB + insulin significantly increased ($p<0.05$) glycogen concentrations in C2C12 muscle cells in comparison to the control (IR). (See table 1)

Table 1. Comparison of glycogen concentrations of palmitate induced insulin resistant C2C12 skeletal muscle cell lines treated with *M. balsamina* after 48 h. Values are expressed as mean ± SEM.

Groups	**Media Glucose(mmol/l)**	**Glycogen (mmol/cells)**
NC	23.68 ± 0.27	0.07 ± 0.02
NC +INS	14.75 ± 0.41 *	0.27 ± 0.13*
IR	22.60 ± 1.12	0.07 ± 0.01
IR+INS	16.70 ± 2.13 #	0.08 ± 0.05
IR + MB	19.93 ± 0.44 #	0.09 ± 0.03
MB + INS	17.43 ± 0.69 #	0.15 ± 0.02*#

★ $P<0.05$ In comparison to control, # $P<0.05$ in comparison with IR.

3.5 MDA and total antioxidant capacity.

Table 2 Shows the effects of MB on MDA levels in C2C12 muscle cell line using TBARS assay after 48h and total antioxidant capacity in insulin resistant skeletal muscle cell line treated with *Momordica balsamina* plant extract. By comparison with the control, insulin resistant cells showed a significant increase in MDA concentration. However, by comparison with the insulin resistant cell, administration of MB (25 mmol/L) showed a decrease in MDA levels in C2C12 muscle treated cell lines (See table 2)

Table 2. Comparison of the effects of MB on C2C12 skeletal muscle cell line malondialdehyde (MDA) concentration and total antioxidants concentration.

Groups	**IR**	**IR + INS**	**MB + INS**	**MB**
MDA (ug/mL)	5.85±0.064	5.79 ± 0.287	3.25±0.089*	3.95±0.288*
Total antioxidant capacity (mmol.mon^{-1}g protein)	0.0019±0.0003	0.00196±0.0004	0.0066±0.0006*	0.0055±0.0004*

★ P<0. 05 in comparison to control

4. Discussion

Insulin resistance is the pathological condition on which tissues such as the skeletal muscle, adipose tissue and the liver fail to respond to insulin (30). This condition may be attributed to the overindulgence of high calorie diet such including high fat diet. The current therapeutic strategies of insulin resistance include the use of insulin sensitizers such as metformin, however medicinal plants such *Momordica balsamina* have been traditionally used in the management of diabetes. In addition, *Momordica balsamina* crude extracts have been reported to possess cardio-protective and reno-protective effects in type1 diabetes (22, 23). However, the effects of *Momordica balsamina* on insulin resistance C2C12 skeletal cell line has not been established. Hence the current study evaluated the effect of *Momordica balsamina* in toxicity, glycogen, antioxidant capacity and glucose uptake in palmitic acid induced insulin resistant C2C12 skeletal muscle cell lines.

Previous studies have reported the use of medicinal plants to be associated with toxicity when taken in high doses (31, 32). In this study, we used MTT assay to investigate cell viability in normal skeletal muscle cell line. The observations in cell viability studies suggested that lower doses of *Momordica balsamina* had no cytotoxic effects in the skeletal muscle cell lines. This is because the plant does not promote cell death and cell proliferation, this suggests that it is safe to be further evaluated in *invivo* studies.

Palmitic acid is a saturated fatty acid that has been shown to induce insulin resistance in muscle, liver and adipocytes, by inducing the activation of PKCθ and NF-kB, thereby reducing the activity of IRS-1 phosphorylation known to promote insulin-signalling (33, 34). Moreover, insulin-signalling effectors such as PI3K and AKT, as well as AMPK, become deactivated by palmitic acid (35). Indeed, in this study the 4 hour exposure of C2C12 skeletal muscle cell lines to palmitic acid successfully induced insulin resistance. This was evidenced by the reduced glucose uptake despite the incubation with insulin for 48 hours. However, treatment with MB showed an improvement in the glucose uptake in the insulin resistant cell lines after 48 hours of incubation. This suggests that *Momordica balsamina* possibly mimics insulin's effects resulting in increased glucose uptake. Furthermore, the administration of MB in combination with insulin showed a significant improvement in glucose uptake. MB may possibly increase insulin sensitivity since studies on medicinal plant extract such as *Momordica Charantia,* plant of the same genus as MB have been shown to improve insulin sensitivity through increasing the activity of tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1) (36). *Momordica Charantia* and *Momordica balsamina* has been shown to possess alkaloids, flavonoids, terpenes and glycosides, which may be attributed to its glucose lowering effects.

Insulin resistance is characterised by reduced glycogen synthesis in the skeletal muscle (37). This may be due to free fatty acid-induced suppression of pyruvate dehydrogenase complex leading to reduced glucose uptake (38). Indeed, this study found that insulin resistant skeletal muscle cell had a reduced glycogen. However, the administration of MB with and without insulin resulted in the improvement of

glycogen synthesis as evidenced by an increase in glycogen storage. Previous studies have shown that *Momordica charantia* improves glycogen storage by potentiating mitochondrial function via signalling pathways including Peroxisome Proliferator-Activated Receptor alpha (PPARα) and Peroxisome Proliferator-Activated Receptor gamma ($PPAR_{\Upsilon}$) (39). MB may also utilise the same mechanism to improve glycogen synthesis in the palmitic induced skeletal muscle cell lines.

Malondialdehyde (MDA), a by product of lipid peroxidation and oxidative stress has been shown to increase during insulin resistance, additionally it also has been associated with a decrease in antioxidants such as glutathione and vitamin E (40). As seen in this study, there was an increase in the MDA concentrations and a decrease in total antioxidant capacity in insulin resistant cells. Notably, however, the administration of MB showed a decrease in MDA concentration which exerted an improved total antioxidant capacity. This suggest that MB possess protective effects against tissue damage that may be induced by reactive oxygen species which often occurs in insulin resistant tissues as a result of saturated fatty acid.

5. Conclusion

Momordica balsamina increased glucose uptake in palmitic acid-induced skeletal muscle cell lines. This suggests that inhibited insulin resistance induced by palmitic acid in C2C12 muscle cell lines perhaps by increasing the activity of tyrosine phosphorylation of the insulin receptor substrate, thereby increasing insulin sensitivity to insulin. Furthermore, *Momordica. balsamina* in combination with Insulin significantly increased glucose uptake in induce insulin resistant muscle cell line. The findings of the study provide evidence in support of the potential health benefits of *Momordica. balsamina* in improving or prevention of insulin resistance**.**

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Diabetes

The effects of *Momordica balsamina* on glucose handling in high fat high carbohydrate Diet induced pre-diabetic rat model

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Abstract

Introduction

Prediabetes is an intermediate state of progression from normoglycemia to overt type 2 diabetes. Literature evidence suggests that managing prediabetes is beneficial in delaying the onset of diabetes. Previous studies have shown that *Momordica balsamina* (MB) possesses antihyperglycaemic effects in STZ-induced diabetic rats; however, its effect on glucose handling in a prediabetic state hasn't been explored. Therefore, the aim of this study was to investigate the effects of MB on glucose handling in a diet-induced prediabetic rat model.

Methodology

Male Sprague-Dawley rats (130-180 g) were divided into six groups of six rats per group; Normal control, Prediabetic (PD), Metformin (500 mg/kg, p.o.) treated PD without dietary intervention (DI), MB (250 mg/kg, p.o.) treated PD without DI, Metformin treated PD with DI and MB treated PD with DI. The animals were treated for 12 weeks of the last 32 weeks. Body weight, blood glucose concentration, oral glucose tolerance and caloric intake were monitored at every 4 weeks of the treatment period. After 12 weeks, the animals were sacrificed, blood and skeletal muscle tissues samples were collected for biochemical analysis.

Results

The untreated prediabetic group showed an increase in body weight, blood glucose concentration, caloric intake and impaired glucose tolerance compared to normal diabetic control rats. However, rats treated with MB showed an improvement in glucose tolerance, blood glucose concentration, caloric intake and body weight. Interestingly, MB treated rats showed an improvement in insulin sensitivity through normalisation of the HOMA-IR index, HbA1c, ghrelin and glycogen when compared to untreated prediabetic animals. The expression of skeletal muscle GLUT 4 and glycogen synthase was increased in MB treated rats with or without diet modification in comparison to the untreated prediabetic rats.

Conclusion

The findings of this study suggest that MB crude extracts improve glucose tolerance in prediabetic rat models. Taken together, the observations imply that MB may delay the progression of prediabetes to type 2 diabetes.

Keywords

Glucose homeostasis, glycated haemoglobin, *Momordica balsamina*, prediabetes

1. Introduction

Pre-diabetes is a chronic metabolic complication defined as glycaemic variables that are higher than normal, but lower than diabetes thresholds (1). This condition of prediabetes is presented by impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and elevated glycated haemoglobin (HbA1c) concentrations (2). Impaired glucose tolerance reflects the progression from normoglycaemic state to pre-diabetic or diabetic state (3). The impaired glucose homeostasis is a result of the defect in insulin response by insulin substrate 1(IRS-1) or action by targeted cells such as the liver and skeletal muscle (4). Prediabetes is associated with numerous metabolic complications and is a prominent risk factor for the onset of type 2 diabetes (5).

The current prediabetes epidemiological findings are of a great concern. Researchers have estimated that prediabetes is expected to rise from 10.8 million to 18.7 million by 2025 in developing countries (6). Literature has shown that unhealthy eating habits which include high carbohydrates and high fats are responsible for the high incidence of prediabetes in developing countries (7-9). Under physiological conditions, tissues such as the adipose, skeletal muscle and the liver play a crucial role in maintaining blood glucose (10). However, in a state of insulin resistance, these tissues lose insulin sensitivity causing slightly elevated blood glucose concentrations (11). Persistent elevated blood glucose concentration results in the development of metabolic complications including dysregulation of the hunger hormone, ghrelin, adipocytes and glycation of proteins such as haemoglobin (12, 13).

The ingestion of high fat high carbohydrate has been a major cause of insulin resistance which also contribute to obesity (14). This results in decreased insulin sensitivity to the insulin receptor substrate 1 (IRS-1) of the target tissue such as the skeletal muscle (15). Chronic ingestion of a high fat high carbohydrate diet has also been shown to compromise the sensitivity of these tissues to insulin resulting in hyperglycaemia and the generation of reactive oxygen species which causes oxidative stress (16). However, a change of diet to a lower-caloric diet has been shown to lower blood glucose concentration (17). Despite the diet change, some individuals cannot strictly adhere to strict diet guidelines, thus posing a challenge in managing the disorder (18). In addition, metformin has been shown to function effectively in the presence of lifestyle modification (19). However, there is a need for alternative treatment strategies that will function in both the presence and absence of dietary intervention and lower blood glucose concentration with minimal undesirable effects.

Literature has shown that medicinal plant extracts can ameliorate glucose impairment in streptozotocin (STZ) diabetic rats (20). Evidently from our laboratory, medicinal plant extracts such as *Syzygium cordatum* and *Syzygium aromaticum*, have been shown to exert anti-hyperglycaemic effects by stimulation of glucose uptake via increased expression of GLUT 4 transporters in a streptozotocin (STZ)-induced rat model (21-23). *Momordica balsamina* has been shown to possess anti-hyperglycaemic and kidney ameliorative effects in STZ-induced diabetic rats (8). However, the anti-hyperglycaemic effects of MB in high fat high carbohydrate induced prediabetic animal models have

not been established. Therefore, in this study, we sought to investigate the effects of *Momordica balsamina* on glucose handling in a high fat high carbohydrate diet induced prediabetic rats.

2. Materials and Methods

2.1 Drugs and Chemicals

All chemicals and reagents were of analytical grade and sourced from the standard pharmaceutical suppliers.

2.1.1 Crude extract extraction

Momordica balsamina was identified by a botanist, Baijnath at the University of KwaZulu Natal, Westville. The plant was harvested at the University of KwaZulu Natal in Westville. The leaves were washed three times with water to remove any residual dirt.

2.1.2 Extraction method

Methanolic extract (ME) was extracted from MB leaves by using a standard protocol that has been validated in our laboratory with minor modifications (24). Briefly, the air-dried MB leaves were sequentially extracted twice at 24 h intervals at room temperature using methanol (45 mL) and deionised water (45 mL) on each occasion. The solvent was removed from the crude extract under reduced pressure at 55±1 ºC using rotatory evaporator to yield dichloromethane solubles (DCMS) and ethyl acetate soluble (EAS).

2.3 Animal studies

Male Sprague-Dawley rats (150-180g) used in this study were bred and housed in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal. The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO_2 content (<5000 p.m.), relative humidity (55±5%) and illumination (12 h light/dark cycle, lights on at 07h00).

The animals were allowed access to food and water ad libitum. The animals were acclimatised to their new environment for one week while consuming standard rat chow and water before exposure to an established experimental diet (HFHC) used in previous studies (25). The composition of HFHC diet is shown in table 1 below. The drinking water of the experimental animals included 15% fructose. All Procedures involving animal care and housing conditions were approved by Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal with ethics number AREC/062/018M.

Table 1: The composition of the HFHC diet

Ingredients	**%g**
Corn starch	31.3
Casein	19.7
Maltodextrin	10.9
Sucrose	7.9
Vegetable shortening	6.1
Milk fat	6.1
Lard	6.1
Soybean oil	1.3
Corn oil	0.5
AIN93G mineral mix/fibre	4.0
AIN93 vitamin mix/fibre	1.2
Cellulose	0.0
L-Cysteine	0.3
Choline bitartrate	0.2
Cholesterol	0.1
t-BHQ	

2.4 Induction of pre-diabetes

Experimental pre-diabetes was induced in male Sprague-Dawley rats using a previously described protocol (26). The experimental animals were exposed to HFHC diet and 15% fructose for 20 weeks while the control group was exposed to standard chow for the equal number of weeks. After 20 weeks, the animals that exhibited impaired fasting blood glucose concentrations of 5.6 to 6.9 mmol/L were considered pre-diabetic. Oral glucose tolerance test was also performed and animals that have blood glucose concentration of 7.0-11.00 mmol/L after 2hours post-load of glucose were also considered prediabetic.

2.5 Experimental design

Post prediabetes, the animals were randomly divided into 6 groups of six animals in each group as follows: Normal control, Prediabetic (PD) without diet intervention (DI), Metformin treated PD without DI, *Momordica balsamina* (MB) treated PD without DI, Metformin plus dietary intervention (DI) treated, MB + DI treated. The normal control rats received vehicle (DMSO 3 ml/kg-1 p.o); Group 2: Pre-diabetic control rats continued with HFHC diet (see table 1) and received vehicle (DMSO 3 ml/kg-p. o); Group 3:Pre-diabetic control (PD) rats continued with standard diet (STD) received metformin (MET) (500 mg/kg p. o); Group 4: Pre-diabetic treated rats continued with STD diet received *Momordica balsamina* (MB) (250 mg/kg p. o) that was suspended in (DMSO 3 ml/kg p. o); Group 5: Pre-diabetic animals continued with HFHC diet received *M. balsamina* (MB) (250 mg/kg p. o) that was suspended in (DMSO 3 ml/kg p. i); and Group 6: Pre-diabetic treated rats continued with STD diet received metformin (500mg/kg p. o) that was suspended in (DMSO 3 ml/kg p. o). The animals were treated orally once every third day at 9h00 am. In each group, parameters such as fasting blood glucose concentration, glucose intolerance test, blood glucose concentration, body weight and food intake were measured after every 4th week at 9h00 am for 12 weeks. The animals were placed on metabolic cages

overnight for monitoring urine output, food intake, and fluid intake, once after 4 weeks and they were placed back to colony cages.

2.6 Blood collection and tissue harvesting

For blood collection, all animals were anaesthetised with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Rooderport, South Africa) via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for 3 minutes. While the rats remained unconscious, blood was collected by cardiac puncture and then injected into individual pre-cooled heparinised containers. The collected blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes to obtain the plasma. The Plasma was collected and stored at -80 °C in a Bio Ultra freezer (Snijers Scientific, Holland) until ready for biochemical analysis. Thereafter, tissues such as the liver and skeletal muscle were rinsed with cold normal saline solution and snap frozen in liquid nitrogen before storage in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C for biochemical analysis.

2.7 Biochemical analysis

2.7.1 HbA1c and ghrelin measurements

HbA1c and ghrelin concentrations were measured using their respective rat ELISA kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) according to the manufacturer's instructions. Plasma insulin concentration was also measured using an ultrasensitive rat insulin ELISA kit (Mercodia AB, Sylveniusgatan 8A, SE-754 50, Uppsala, Sweden) according to the manufacturer's instructions.

2.7.2 Glycogen Assay

Glycogen analysis was performed in skeletal muscle tissues using a well-established laboratory protocol. The harvested skeletal muscle tissues were weighed and heated with potassium hydroxide (KOH) (30%, 2 mL) at 100 C for 30 min. Then immediately, disodium sulphite (Na_2SO4) (10%, 0.194 mL) was added into the mixture to stop the reaction. The mixture was then allowed to cool, and the glycogen precipitate was formed. The cooled mixture with precipitate was aspirated (200 L) and mixed with ethanol (95%, 200 L). Then centrifuged at the speed of 1800 r.p.m. The precipitated glycogen was pelleted, washed and resolubilized in H_2O (1 mL). After that, anthrone (0.5 g dissolved in 250 ml of sulphuric acid, 4 mL) was added and boiled for 10 min. After cooling, the absorbance was read using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, LGBW Germany) at 620 nm. The glycogen concentrations were calculated from the glycogen standard curve. The standard curve ranges from 200 to 1000 mg/L.

2.7.3 Western blot analysis

Skeletal muscle tissues harvested from untreated and treated diet-induced diabetic rats at the end of 32 weeks were analysed for GLUT 4 using Western blotting. The tissues (0.1 g) were homogenized on ice in isolation buffer (0.5 mM Na_2EDTA, 0.1 M KH_2PO_4, 0.1 mM dithiothreitol, 0.25 M sucrose) and then

centrifuged at 400 x g for 10 min (4 °C). The protein content was quantified using the Lowry method. All the samples were standardized to one concentration (1 mg/mL). The proteins were then denatured by boiling in laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulphate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5 min. The denatured proteins were loaded (25 µL) on prepared resolving (10%) and stacking (4 %) polyacrylamide gels along with molecular weight marker (5 µL). The gel was electrophoresed for 1 h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS, pH 8.3). Following electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidene difluoride (PVDF)/ membrane for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCL, 0.05% Tween-20). The membranes were then immuno-probed with antibodies, GLUT 4 and (1:1000 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT). The PVDF membrane was then subjected to 5 washes (10 min each with gentle agitation) with TTBS. The membranes were then incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:10 000; Bio-Rad) for 1 h at RT. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

2.8 Statistical Analysis

All data were expressed as means ± S.E.M. Statistical comparisons were performed with Graph pad Software version 5 by using two-way analysis of variance (ANOVA) to simultaneously determine statistical differences between the means of two independent groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Effects on body weights

The figure below shows the effects of MB on body weight. All experimental groups were monitored every fourth week of the treatment period of 12 weeks. The results showed that the non-treated prediabetic group (HFHC) had significantly increased body weights in comparison to normal control (NC) (HFHC vs. NC) ($p < 0.05$). However, the administration of MB with and without dietary intervention showed a significant decrease in body weights when compared to HFHC ($p < 0.05$) (Table 2). Whereas metformin (Met) showed a significant steady body weight increase from week 0 to week 8, however, with a significant decrease in week 12.

Table 2: Effects of MB on body weight of rats that continued with HFHC diet during treatment and those that changed the diet. Values are presented as mean ±SEM.

Experimental groups	**Week 0**	**Week 4**	**Week 8**	**Week 12**
NC	311.80 ± 7.61	415.80 ± 14.70	411.20 ± 12.82	427.80 ± 17.19
HFHC	496.00 ± 24.41 **α**	498.20 ± 18.91 **α**	536.60 ± 15.43 **α**	526.20 ± 8.93 **α**
ND + MB	411.20 ± 18.41 **α**	418.00 ± 16.33 ★	415.20 ± 13.69 ★	428.60 ± 14.00 ★
HFHC + MB	409.80 ± 10.71 **α** ★	472.00 ± 17.64	457.60 ± 14.11 ★	468.60 ± 20.01 **α** ★
ND + Met	426.80 ± 17.23 **α**	479.00 ± 16.10 **α**	488.40 ± 16.11 **α** ★	476.00 ± 18.96 **α**
HFHC + Met	400.40 ± 8.97 **α**	482.40 ± 20.37 **α**	499.40 ± 4.40 **α**	488.00 ± 14.88 **α**

α =$p < 0.05$ denotes comparison with NC;

★ = $p < 0.05$ denotes comparison with HFHC.

3.2 Caloric intake

Table 3 below presents the caloric intake at week 12 of Both MB-treated groups (that continued with the diet, and that underwent a change in diet) and those treated with metformin (Met). There was a significant decrease in calorie intake in groups that underwent a diet change in both MB and metformin treated groups compared to HFHC control group.

Table 3: Effects of MB on calorie intake (kcal/g) of rats that continued with HFHC diet during treatment and those that changed the diet. Values are presented as the standard error of mean ± SEM and in percentage increase (↑ = increase and ↓ = decrease).

Experimental groups	**Week 0**	**Week 4**	**Week 8**	**Week 12**
NC	109±1.90 (100%)	125.04±2.4 ↑ (14.52%)	165.04±1.61 ↑ (51.16%)	178.40±0.87 ↑ (63.34%)
HFHC	121.47±1.01 (100%)	120.90±0.64* ↑ (0.47%)	206.58±0.84* ↑ (70.07%)	230.01±0.85* ↑ (89.36%)
ND + MB	119.58±0.51* (100%)	119.04±0.86*α ↓ (0.40%)	157.59±2.80*α ↑ (25.52%)	168.85±2.23α ↑ (43.58%)
HFHC + MB	141.35±0.03* (100%)	105.94±2.00*α ↓ (20.26%)	166.09±1.73α ↑ (19.76%)	184.26±1.99*α ↑ (49.03%)
ND + Met	115.02±0.67* (100%)	102.69±1.17*α ↓ (10.72%)	120.51±0.75*α ↑ (4.77%)	144.72±1.64*α ↑ (25.82%)
HFHC + Met	118.09±0.51* (100%)	100.54±0.98*α ↓ (14.86%)	99.51±1.52*α ↓ (15.73%)	151.66±0.69*α ↑ (25.82%)

3.3 Oral Glucose Tolerance (OGTT)

Figure 1 below shows the OGTT results measured at the end of the treatment period (week 12) in all the experimental groups. The results showed that at time 0, the HFHC had a significantly high blood glucose concentration when compared to NC. At 120 minutes both MB-treated groups had a significantly lower blood glucose concentration when compared to HFHC group as shown in figure 1. Metformin showed results that are similar to HFHC control group.

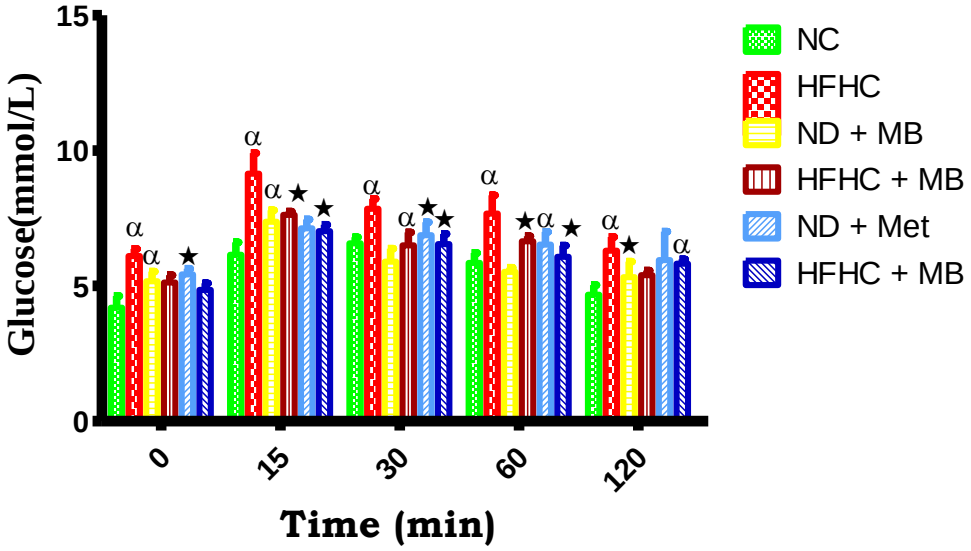


Figure 1: Effects of MB on OGTT of rats that were treated with MB and without diet intervention during the treatment period. Values are presented as mean ± SEM. **α**= $p < 0.05$ denotes comparison with NC; ★ = $p < 0.05$ denotes comparison with HFHC.

3.4 Glycated Haemoglobin Concentration (HbA1c)

Figure 2 below shows the effects of MB on glycated haemoglobin concentrations in rats that continued with HFHC diet and those that changed diet or had diet intervention. All experimental groups were analysed for HbA1c concentration at week 12. The results showed that the blood HbA1c concentration in HFHC was significantly higher in comparison with normal control (NC) ($p < 0.05$). The administration of MB with and without diet intervention resulted in a significant decrease in HbA1c concentration when compared to HFHC as demonstrated in figure 2 ($p < 0.05$).

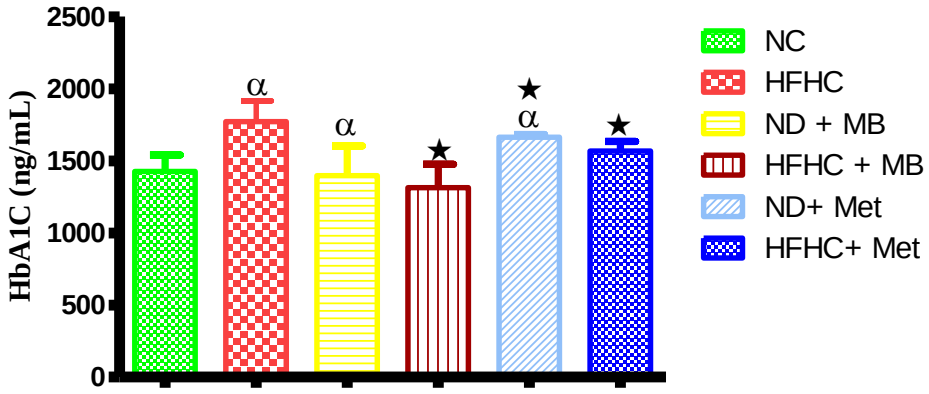


Figure 2: Effects of MB on the glycated haemoglobin concentrations of rats that continued with HFHC diet and those that changed diet or had diet intervention during the treatment period. Values are presented as mean ± SEM. **α**= $p < 0.05$ denotes comparison with NC ★= $p < 0.05$ denotes comparison with HFHC.

3.5 HOMA2-IR index

The HOMA2-IR index of all animals was calculated by using plasma glucose and insulin concentrations at week 12. The results showed that HFHC had a significantly higher HOMA2-IR index when compared to normal control (NC) ($p < 0.05$). Both MB-prediabetic treated groups (that continued with the same diet and that underwent a change in diet) showed a significantly lower HOMA2-IR index in comparison to HFHC ($p < 0.05$) as shown in table 3. Similarly, metformin showed similar effects in both groups that underwent diet intervention and the ones that continued with the high fat high carbohydrate diet.

Table 3: Presents the effects of *Momordica balsamina* in the response to HOMA-IR in high fat high carbohydrate induced pre-diabetic rat. Values are presented as mean ± SEM.

Groups	**Plasma glucose(mmol/L)**	**Plasma insulin (mU/L)**	**HOMAR-IR values**
NC	4.30±0.51	7.84±0.38	0.95±0.16
HFHC	5.68±0.44 α*	9.66±0.55 α	3.95±0.66 α
ND+ MB	4.80±0.29 α	5.89±0.64 α*	1.00±0.11 α*
HCHF +MB	4.65±0.72 α*	4.68±3.08 α*	1.05±0.09 α*
ND + Met	5.28±0.14 α	7.84±0.74 α*	1.00±0.09 α*
HFHC + Met	4.85±0.25α	10.52±1.37 α*	1.05±0.09 α*

α $p<0.05$ by comparison with the normal control group at each corresponding time.

* $p<0.05$ by comparison with the prediabetes control group at each corresponding time.

3.6 Ghrelin

Figure 3 Shows the effects of MB on plasma ghrelin concentration in HFHC diet rat and rats during diet intervention at 12 weeks period. The results showed that HFHC had a significantly higher plasma ghrelin concentration in comparison to NC ($p < 0.05$). However, all MB-treated animals had a significantly lower ghrelin concentration when compared to HFHC.

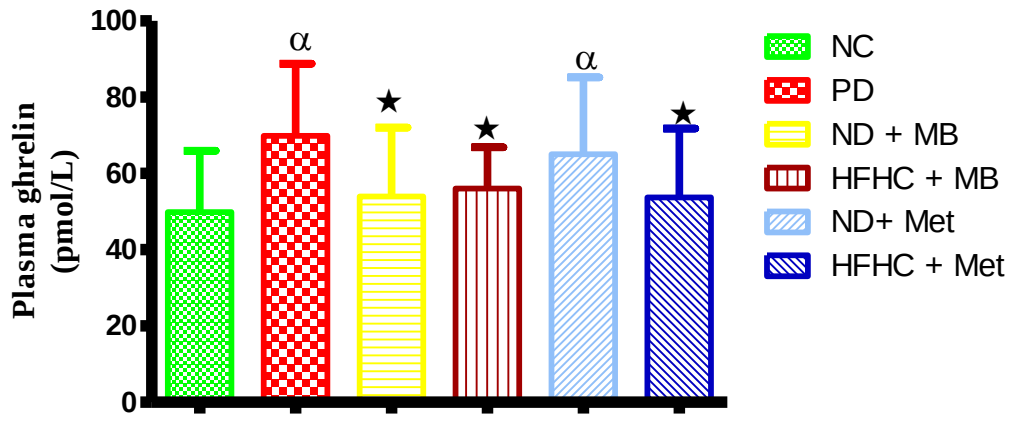
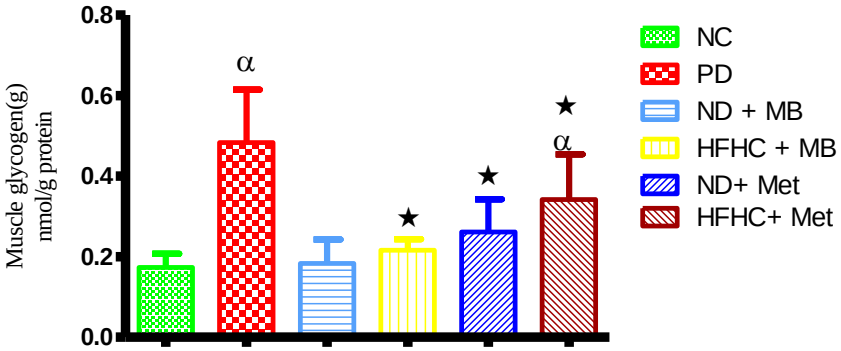


Figure 3: Effects of MB on plasma ghrelin concentrations of HFHC and rats that changed their diet during the treatment period. Values are presented as mean ±SEM. **α**= $p < 0.05$ denotes comparison with NC; ★= $p < 0.05$ denotes comparison to HFHC**.**

3.7 Skeletal Muscle glycogen concentrations

Figure 4 presents the skeletal muscle glycogen concentration in all experimental groups at the 12th week treatment period. The results showed that HFHC had a significantly higher skeletal muscle and liver glycogen concentration in comparison to NC. Treatment with MB resulted in a significant decrease in skeletal muscle compared to HFHC.

a)



b)

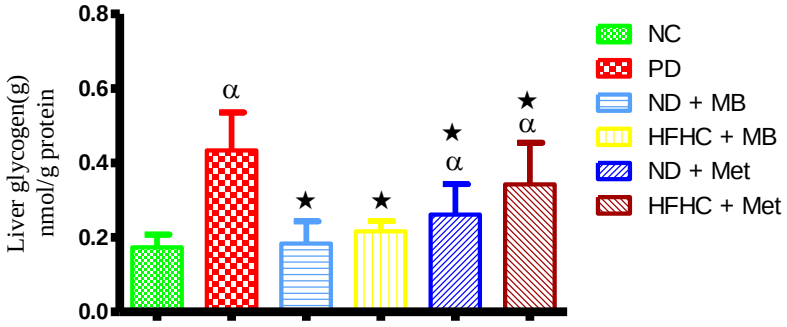


Figure 4: Effects of MB on (a) muscle concentrations in HFHC and rats that changed their diet during the treatment period (b) liver glycogen concentration in HFHC and rats that changed their diet. Values are presented as ± SEM. **α**= $p < 0.05$ denotes comparison with NC; ★ = $p < 0.05$ denotes comparison with HFHC.

3.8 Effects of MB on GLUT 4 expression

Figure 5 below shows GLUT 4 expression in the skeletal muscle of Both MB-treated groups (that continued with the diet and that underwent a change in diet) and those of metformin at the end of experimental period. There was a significant increase of GLUT 4 expression in both MB-treated groups and metformin treated groups compared to HFHC control group.

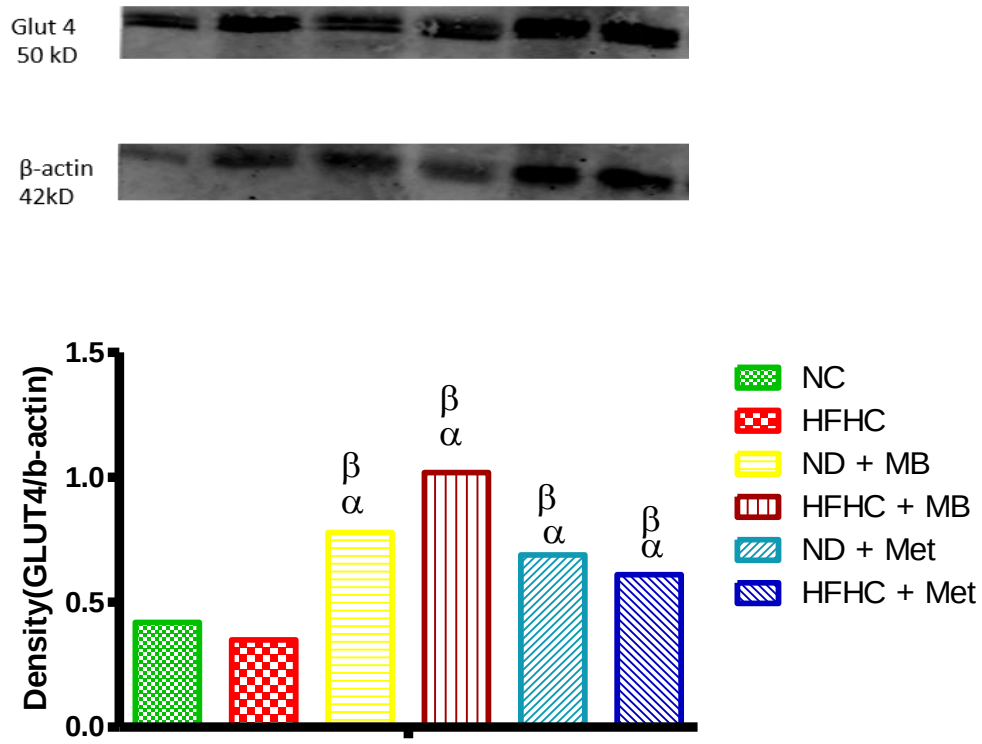


Figure 5: Shows the graph of GLUT 4 expression in high fat induced and the rats that changed the diet. Values are expressed as means ±SEM (n=6). α $p < 0.05$ in comparison to NC. β $p < 0.05$ in comparison to HFHC.

3.9 Effects of MB on the (a) liver and (b) skeletal muscle

Figure 6 below presents the graph for glycogen synthase of Both MB-treated groups (that continued with the diet, and that underwent a change in diet) and those of metformin. There was a significant increase in both MB-treated groups and metformin treated groups compared to HFHC control group.

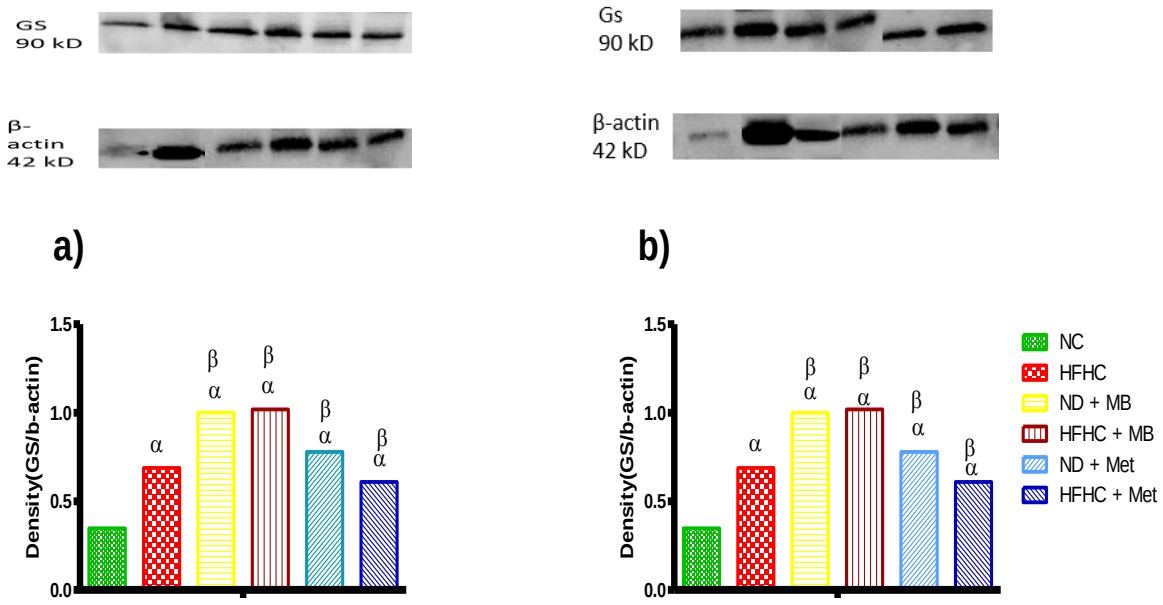


Figure 6 (a) and (b): Shows the graph of Glycogen Synthase expression in the muscle and liver in high fat high carbohydrate induced and the rats that changed the diet. Values are expressed as means ±SEM (n=6). α $p < 0.05$ in comparison to NC. β $p < 0.05$ in comparison to HFHC.

4. Discussion

The study aimed to determine the effects of MB on glucose handling in a high fat high carbohydrate diet-induced (HFHC) pre-diabetic rat model. Literature has reported that MB has medicinal value (27). Studies indicate that MB possesses hypoglycaemic, kidney dysfunction ameliorative effects and hepatoprotective properties in streptozotocin induced diabetic rats (28, 29). However, the glucose handling effects in high fat high carbohydrate induced prediabetes rat model have not been established. This HFHC rat model adopted the world health organisation (WHO) prediabetes criteria to diagnose rodents as prediabetic. The criteria includes glycated haemoglobin (Hb1Ac) as one of the diagnostic features for pre-diabetes (30). In this study, there was a significant increase in the concentration of Hb1Ac in HFHC control group, indicating the presence of hyperglycaemia and prediabetes. However, animals that were treated with MB and metformin showed a significant decrease in Hb1Ac compared to HFHC untreated animals. We speculate that MB was able to decrease Hb1Ac through its known hypoglycaemic effect (8). Several literature observations have stated that Hb1Ac alone is not sufficient to screen or diagnose diabetes as it has been shown to lack sensitivity (31-33). The glycated haemoglobin test is shown to be more effective when coupled with the oral glucose test (OGTT) (34).

The oral glucose tolerance test (OGTT) is considered as a standardised test for the diagnosis of diabetes and impaired glucose tolerance (35). On this basis, we also performed an OGTT of which blood glucose remained high in HFHC diet control animals, suggesting that the animals were prediabetic since hb1Ac and OGTT remained elevated after 2 hours of postprandial glucose feeding. MB treated groups however, showed a decrease in blood glucose concentration in a 2hr postprandial glucose feeding. This may be again due to the hypoglycaemic effects of MB (28). Similarly, animals that were treated with metformin showed a slight decrease in glucose levels. Metformin is known to activate the AMPK activity thereby inhibiting hepatic glucose production (36). To further look into the mechanisms by which MB exerts its hypoglycaemic effects, we measured the energy regulating hormone since it also plays a role in glucose homeostasis.

Ghrelin is a peptide that acts on the growth hormone (GH) secretagogue receptor in the pituitary and hypothalamus and acts to stimulate food intake (37). In diabetes, ghrelin has been shown to be secreted in excess thus the development of polyphagia which was observed in this study (38). Studies have also associated high ghrelin concentration with increased insulin secretion and development of obesity in prediabetes and diabetes which was also shown in HFHC prediabetic rats in this study (39, 40). The administration of both MB and metformin however showed a decrease in ghrelin concentration thus decreasing calorie intake in these animals. Furthermore, MB and metformin maintained steady body weight gain in comparison to HFHC and normal control animals which showed an increase over the 12 week period. The regulation of calory intake is essential in maintaining adequate insulin sensitivity, body weights and managing hyperglycaemia in metabolic disorders (41). Various medicinal plants have

been shown to decrease ghrelin concentration through sensitizing the peripheral cells for insulin thereby suppressing ghrelin secretion, consequently decreasing food intake (42, 43). MB may possibly exert the same mechanism to lower ghrelin concentrations. Interestingly, MB exerted anti-hyperglycaemic effects with and without dietary intervention showed by an improvement in parameters such as ghrelin, body weight and calory intake. This is important since many conventional treatments have been rendered ineffective in the absence of dietary intervention due to poor patient compliance (44-46).

In an insulin resistant state, impairment of insulin receptor substrate-1(IRS-1) results in the inability of GLUT4 transporter to translocate to the surface of the membrane to allow for glucose entry into the cell (47). This was observed in the HFHC diet group as there was a significant decrease in GLUT 4 protein expression. However, the administration of metformin and MB restored the GLUT 4 protein expression in both animals that underwent diet change and those that did not undergo diet intervention. This suggests that MB may increase glucose uptake by activating IRS-1 signalling thus increasing the expression of GLUT 4 transporters, reducing blood glucose levels independent to diet change which has been a challenge with conventional medicine (27).

Glycogen synthase (GS) is an enzyme that is responsible for the conversion of glucose to glycogen during fasting in the liver and skeletal muscle (48, 49). Literature has shown that the overexpression and overactivity of glycogen synthase are associated with an impaired ability of insulin to activate glucose disposal (50). Interestingly, in this study, HFHC diet fed animals demonstrated a slight increase in glycogen storage and an increase in glycogen synthase expression compared to normal control animals. A similar trend was observed in the expression of GLUT 4 transporters. In some cases, Glycogen synthase kinase 3 (GSK-3) activity has been shown to be higher in insulin resistant tissues, thus causing impaired insulin action, further resulting in high expression of glycogen synthase in diabetes (51). Furthermore, this may be due to a high carbohydrate diet causing high levels of circulating free fatty acid which cause an imbalance in the phosphorylation of glycogen synthase kinase (52). Importantly in this study, MB and metformin treatment increased glycogen, protein expression of glycogen synthase and GLUT 4 expression with or without a change of diet compared to HFHC fed animal controls. A plant of the same genus *Momordica charantia* (MC) has been shown to achieve its homeostatic blood glucose by sensitising the insulin receptor substrate 1(IRS-1) at the surface of the membrane leading to an increased GLUT 4 expression thereby promoting blood glucose uptake in animals (53, 54). We speculate that MB may also increase insulin sensitivity via IRS-1 since the protein expression of GLUT 4 was increased. Some studies reported that MC stimulates phosphoinositol-3-kinase which phosphorylates Akt and downregulates p-mTOR to improve insulin resistance (55, 56). Indeed, this study showed that MB in both the presence and absence of diet intervention resulted in the reduction of insulin concentrations which was further evidenced by the decrease in HOMAR-IR index which suggests an improvement in insulin resistance.

5. Conclusion

In conclusion, the study clearly demonstrates that MB improves protein expression of GLUT 4 in the muscle and prevents hyperinsulinaemia and decreases glycogen levels in HFHC fed animals when compared to those that were fed standard diet. Furthermore, this study reveals that medicinal plants such as *Momordica balsamina* can increase insulin sensitivity and improve glycaemic control in HFHC diet fed insulin resistant prediabetic rats. Therefore, *Momordica balsamina* can play a pivotal role decreasing prediabetes incidence and its progression to overt Type 2 Diabetes.

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CHAPTER 4

Synthesis

Type 2 diabetes is preceded by a long lasting stage known as prediabetes (1, 2). The diagnosis of prediabetes includes insulin resistance followed by impaired glucose tolerance and dyslipidaemia (3). Prediabetes is conventionally managed with the combination of lifestyle modification and insulin sensitizers (4). However, insulin sensitizers are reported to have severe side effects such as diarrhoea and headaches. In our laboratory, medicinal plants including *Momordica balsamina* (MB) have been shown to possess anti-hyperglycaemic in managing cardiovascular and renal protective effects in type 1 diabetes (5). However, the effects of MB as an insulin sensitizer agent in type 2 diabetes has not been established. Hence the study therefore was aimed at investigating whether *Momordica balsamina* can improve insulin sensitivity and stimulate glucose uptake in insulin resistant cells *in-vitro*. We further investigated the effects of *Momordica balsamina* on glucose handling in high fat high Carbohydrate (HFHC) induced prediabetic animals.

Some medicinal plant extracts have been deemed toxic (6, 7). Therefore, understanding the toxicity of medicinal plants is essential for them to be further investigated as potential antidiabetic agents. As confirmed by the MTT assay, MB showed no toxic effect on C2C12 skeletal muscle cell lines *in-vitro*. Palmitic acid has been shown to induce insulin resistance in skeletal muscle *in vitro* by inducing pro-inflammatory cytokines such as tumour necrosis factor alpha and interleukins that cause a decrease in the insulin sensitivity of the Insulin receptor substrate 1 (IRS-1) (8). Hence, we induce insulin resistance in C2C12 skeletal muscle cell to investigate insulin sensitizing effects of MB. Indeed, glucose uptake was inhibited in palmitic acid treated skeletal muscle cell lines in the presence of insulin. However, the administration of MB in the palmitic acid induced insulin resistant skeletal muscle lines improved glucose uptake. Furthermore, the combination of MB and insulin had more pronounced glucose uptake. These observations may indicate that MB can function as an insulin sensitizer since there was synergistic effects when administered in combination with insulin had synergistic effect in promoting glucose uptake.

Impaired glucose uptake in skeletal muscle has been associated with a decrease in glycogen synthesis (9). In agreement, palmitic acid induced insulin resistant skeletal muscle cell line showed low glycogen concentration. However, the administration MB alone and in combination with insulin improved glycogen synthesis. This suggests that MB can ameliorate insulin resistance and improved glycogen storage with or without insulin. Furthermore, insulin resistance has been associated with increased production of reactive oxygen species which may be attributed to increased lipid peroxidation (10-12). This is usually accompanied by the low concentrations of antioxidant enzymes including superoxide dismutase, glutathione and vitamin E, resulting in oxidative stress (13, 14). As observed in this study, palmitic acid induced insulin resistant cell had an increased concentration of malonaldehyde (MDA) which is a marker for lipid peroxidation (15). Moreover, these cells had a reduced antioxidant capacity.

However, the administration of MB with or without insulin showed a reduced lipid peroxidation with improved antioxidant capacity. Thus, suggesting that MB has antioxidant properties.

The findings from the *in-vitro* study served as the basis for the investigation of *Momordica balsamina* (MB) *invivo.* Therefore, we further investigated the effect of MB on glucose handling in in a high fat high carbohydrate (HFHC) induced prediabetic rat model. This animal model of prediabetes is characterised by abnormalities including insulin resistance, impaired glucose tolerance, increased glycated haemoglobin, increased calorie intake and hyperinsulinaemia (16). Interestingly, we observed that MB treated animals had a significantly improved glucose tolerance. This could be attributed to the increased GLUT 4 expression on the skeletal muscle. The increase in GLUT 4 was associated with increased glycogen synthase expression and glycogen storage. In addition, the treatment of MB with and without diet intervention reduced HOMAR-IR index. This finding concurs with the results obtained from the *in-vitro* study which revealed that MB can sensitize skeletal muscle cells for insulin, resulting into an improved glucose uptake. The MB treatment with or without diet intervention resulted into a decrease in glycated haemoglobin which suggests that MB had a sustained effect on glucose homeostasis regulation. The dysregulation of calorie intake results into weight gain (17). The study found that calorie intake regulating hormone ghrelin was increased in the prediabetic animals. Consequently, the calorie intake was also increased followed by weight gain. However, MB treatment in the presence and absence of diet intervention showed a decrease in ghrelin concentration, a decrease in calorie intake and a maintained body weight gain. This suggest that MB treatment can delay the onset of type 2 diabetes from prediabetic animals since weight gain was prevented compared to prediabetes as it has been shown to be associated with prediabetes.

Conclusion

The administration of MB protects against hyperglycaemia-induced insulin resistance and prediabetes by attenuating hyperglycaemia, oxidative stress which may reduce the risk of onset of type 2 diabetes and its complications.

Shortfalls

In this study, we didn't explore the insulin signalling pathway in order to identify the mechanism that is utilised by *Momordica balsamina* (MB) to increase glucose uptake. In addition, the effects of *Momordica balsamina* on adipocytes cell lines was not investigated.

Future studies

In future we can explore the insulin signalling pathway components including protein kinase B and PI3K in order to investigate its mechanism.

We will also investigate MB on liver studies and renal studies

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Appendices

Appendix 1: Ethical clearance

Appendix 2: Certificate for LMSS presentations

Appendix 3: Manuscript 1 Journal Guide

Appendix 4: Manuscript 2 Journal Guide