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Effect of *Momordica balsamina* on Insulin Resistant C2C12 Skeletal Muscle Cell lines

[1]Bongiwe Khumalo, [1]Angezwa Siboto, [1]Ntethelelo Sibiya, [1]Andile Khathi and Phikelelani Siphosethu Ngubane

[1]*School of Laboratory Medicine and Medical Science, College of Health Sciences, University of KwaZulu Natal, Durban 4000, South Africa*

[2]*Pharmacology Division, Faculty of Pharmacy, Rhodes University, Grahamstown 6139, South Africa*

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Corresponding Author:
Phikelelani Siphosethu Ngubane
Pharmacology Division, Faculty of Pharmacy, Rhodes University, Grahamstown 6139, South Africa

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Abstract: Studies have reported that *Momordica balsamina* (MB) increases glucose uptake in skeletal muscle cells. The effects of *M. balsamina* on insulin resistant skeletal muscle cells however, are yet to be established. The aim of the study therefore, is to investigate the glucose lowering effects of *M. balsamina* in the palmitic acid induced insulin resistant (C2C12) skeletal muscle cell lines, *in-vitro*. Cell viability was conducted in muscle cells to examine the cytotoxic effects of *M. balsamina*. Cell viability of C2C12 muscle cell lines was measured by means of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Insulin resistance was induced in skeletal muscle cell line (C2C12) using palmitic acid (1 mM) administered in each well. Cells were trypsinised and seeded into 24-well plates at a seeding density of 1.8×104 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 (500 uL) acid then these cells were incubated for 4 h. After 4 h, new media was added. Media glucose concentration was measured at 12, 24 and 48 h with One Touch select glucometer. The lipid peroxidation marker, malanoaldehyde (MDA) was measured and the total antioxidants capacity. Furthermore, glycogen storage was measured using glycogen assay. No toxic effects were demonstrated in all 3 doses *M. balsamina*. The administration of MB significantly increased glucose uptake at 48 h of incubation by comparison to palmitic acid exposed control cells. Interestingly, the combination of MB and insulin significantly increased glucose uptake by comparison to MB alone at 48 h. Furthermore, MB-treated cells had an increase glycogen storage and reduced reactive oxygen production. These

findings may suggest that MB possesses potential health benefit such as insulin sensitising and anti-hyperglycaemia through improving glucose uptake and attenuating oxidative stress in skeletal muscle.

INTRODUCTION

Insulin resistance is a characteristic feature for many metabolic complication 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]. This growth in patients affected by T2DM in developing countries may be attributed with factors such as urbanisation and a shift towards unhealthy lifestyle including westernised diets[5].

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 at tyrosine residues known to promote insulin-signalling[6]. Moreover, excessive intake of Saturated Fatty Acids (SFAs) has been shown to exacerbate insulin resistance[7]. Therefore, lifestyle changes such as diet and physical activity has been reported to have positive effects on insulin sensitivity and insulin resistance[8, 9].

Conventional treatments such as biguanides and thiazolidinediones remain the most popular drugs for the management of T2DM as they possess hypoglyceamic effect[10]. These drugs facilitate the action of insulin in the muscle, liver and adipocytes[11, 12]. Their mechanism includes improving the sensitivity and phosphorylation of insulin receptor substrate 1 (IRS-1) of the muscle and adipose and improved expression of glucose transporters[13]. Consequently, these drugs have been shown to possess side effects such as flatulence, diarrhoea and heart failure[14-16]. Recently, some medicinal plants have been reported to be useful in treating diabetes. These include *Syzygium aromaticum, Syzygium cordatum* and *Tapinathus nyascius*. Furthermore, these plants have been shown to possess hypoglycaemic effects and have been reported to inhibit the absorption of glucose across the small intestine[17, 18].

The plant *M. 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[19, 20]. Previous studies have reported *M. balsamina* fruits to possess antioxidant properties[21-23]. In our laboratory, we have previously shown the antihyperglycaemic and kidney ameliorative properties of MB in STZ-induced diabetic rats[20]. The effects of *Momordica balsamina* on glucose homeostasis in C2C12 induced insulin resistant cells, however has not been established.

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.

MATERIALS AND METHODS

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_2SO_4), potassium hydroxide (KOH) (Merck chemicals, Johannesburg, South Africa); insulin (Novo Rapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, South Africa).

Crude extract extraction: The *Momordica balsamina* (MB) leaves extract was obtained using methanol by a well established standard protocol previously validated in our laboratory[24]. Briefly, air-dried *M. 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°C using a rotary evaporator to yield a methanol crude extract.

Experimental design: The study was divided into 2 series. The first series investigated the effects of *M. balsamina* on cell viability in skeletal muscle cell lines. Second series investigated the effects of *M. balsamina* on glucose uptake ininsulin resistant skeletal muscle cell lines.

Cell culture: A well-established cell culture protocol by Czifra et al was used to conduct the study[25]. 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 25 cm^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.

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 (1 mL) and incubated for 2 min 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.

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. The 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).

Cell viability studies: Cell viability was conducted in muscle cell lines to examine the cytotoxic effects of *M. balsamina*. Cell viability of C2C12 muscle cell lines was measured by means of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay originally described by Mosmann. Cells were trypsinised and seeded into 96-well plates (Bibby-Sterilin, Staffordshire, England) at a seeding density of 1.8×104 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 37°C 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]×100.

Induction of insulin resistance: Insulin resistance was induced in C2C12 skeletal muscle cell lineusing 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 1.8×104 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 (500 uL) acid then these cells were incubated for 4 h.

Glucose utilization studies: After 4 h a new media was added, media glucose concentration was measured at 12, 24 and 48 h with One Touch select glucometer (Lifescan, Mosta, Malta and United Kingdom). The glucose utilization experiments to be conducted as described by Ventura-Clapier *et al*.[26] with slight modifications. Confluent muscle cell lines that were allowed to differentiate in 24 well plates were firstly incubated for 24 h at 37°C with DMEM (500 µL) containing 29 mmol/L of glucose. The plates were then washed with PBS three times. Thereafter, DMEM media (500 µL) and/or insulin (4 µmol/L) to plates with cells that were exposed and not exposed to Palmitic acid. *M. balsamina* (25 mmol/L) was conducted in four separate wells (n = 4) to the cells that were exposed to Palmitic and not exposed to Palmitic acid. Concentration of *M. balsamina* used was determined from MTT assay results and incubated at 12, 24 and 48 h time intervals. The unexposed plates acted as positive control (cells with media and insulin) and negative control (cells with media only). Glucose concentration was measured at time 12, 24 and 48 h with Accu-Chek glucometer (Lifescan, Mosta, Malta and United Kingdom). To examine the effects of combined treatments, doses (25 µmol/L) of *M. balsamina* was combined with insulin (4 µmol/L). These results allowed for the observation of whether the combination with insulin has positive additional effects on glucose utilisation. After 48 h period, cells were trypsinised and harvested for measurements of glycogen and MDA in TBARS assay.

Glycogen assay: Glycogen analysis was performed in 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_2SO_4 (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 200-1000 mg/L.

Thiobarbituric acid reactive substances (TBARS) assay: Thawed muscle cells were supplemented with 2% phosphoric acid (50 µL) and centrifuge at 1000 rpm for 10 min. The 7 % phosphoric acid (200 µL) was added into glass tube followed by the addition of 400 µL 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°C for 15 min. 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 min. A 96-well microtiter plate in triplicate was used to aliquot the sample and the absorbance was read at 532 nm (reference λ 600 nm) on a BioTek µ Quant 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 (156 mmol-1).

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.

Statistical analysis: All data is expressed as mean± standard error of mean (SEM). 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. The $p<0.05$ will be considered significant.

RESULTS

Cell viability

Effects on cell viability: Figure 1 shows the effects of *M. balsamina* on cell viability of C2C12 skeletal muscle cell line using MTT assay. By comparison with the control group, the administration of 3 concentrations of *M. balsamina* (12.5, 25 and 50 mmol/L) showed no toxic effects at corresponding time intervals of the48h incubation period.

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 h of incubation by comparison to normal untreated C2C12 muscle cell lines.

Effects on insulin resistance: Figure 3a shows the effects of insulin on glucose utilization of palmitic acid induced insulin resistant C2C12 muscle cell lines where insulin showed no effect on glucose utilisation by comparison to control pa-induced control muscle cells.

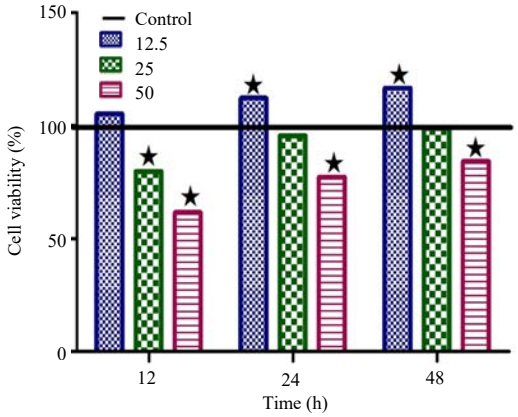

Fig. 1: The effects of *Momordica balsamina* on cell viability on C2C12 muscle cell after 48 h of treatment period; ★ = $p<0.05$ in comparison to a control group

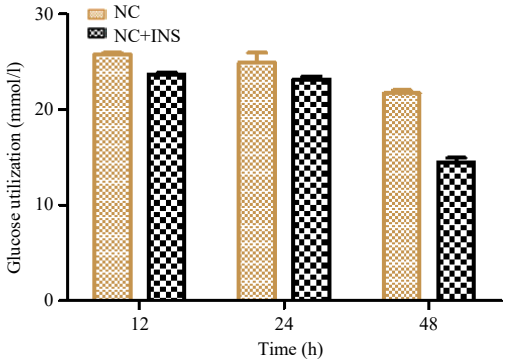

Fig. 2: The effects of insulin on glucose utilization at C2C12 skeletal muscle cell line; ★ = $p<0.05$ by comparison with normal cells groups at each corresponding time

Figure 3b, 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) muscle cell lines significantly increased glucose uptake by comparison to both IR-control and insulin treated IR-insulin resistant muscle cell lines after 48 h. Interestingly, the combination of MB and insulin significantly increased glucose uptake by comparison to MB alone.

Effects on glycogen concentration: Table 1 shows comparison of glycogen concentrations of Palmitic acid-induced insulin resistant C2C12 skeletal muscle cell lines treated with *M. balsamina* after 48 h. In comparison to a control, insulin showed a significance increase in glycogen storage. The administration of MB+insulin concentration significantly increased ($p<0.05$) glycogen concentrations in C2C12 muscle cells in comparison to the control (IR) (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; ★p<0.05 In comparison to control, #p<0.05 in comparison with IR

Groups	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*#

Table 2: Comparison of the effects of MB on skeletal C2C12 skeletal muscle cell line malondiadehyde (MDA) concentration and total antioxidants concentration; ★p<0. 05 in comparison to control

Variables	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*

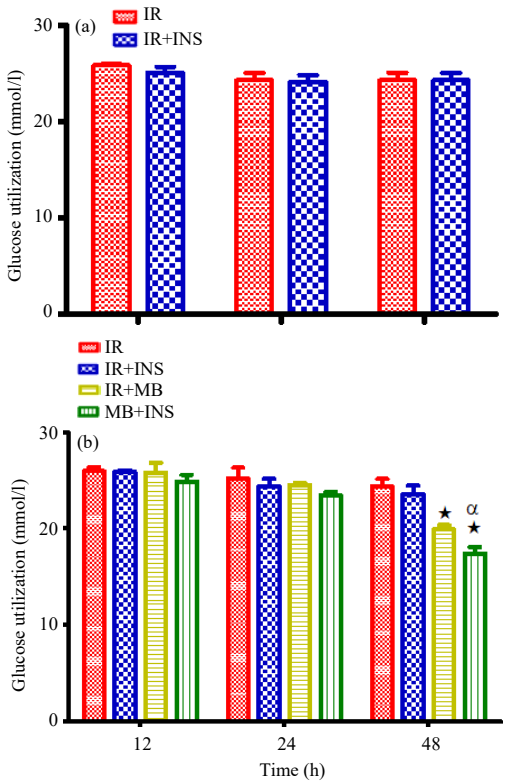

Fig. 3(a, b): The effects of insulin on glucose utilization in skeletal muscle cells after 12, 24 and 48 h, b) The induction of insulin resistance. Values are expressed as mean±SEM; ★ = p<0.05 by comparison with normal cells groups at each corresponding time; α = p<0.05 by comparison with the IR control group

Effects on MDA: Table 2 shows the effects of *M. balsamina* 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 levels. However, by comparison with the insulin resistant cell, administration of *M. balsamina* (25 mmol/L) showed a decrease in MDA levels in C2C12 muscle treated cell lines (Table 2).

DISCUSSION

Insulin resistance is the pathological condition on which cells such as the skeletal muscle, adipose tissue and the liver fail to respond to the hormone insulin. 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[19, 20]. 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[27, 28]. In this study, we used MTT assay to investigate cell viability in normal skeletal muscle cell line. MB showed no cytotoxic effects in C2C12 muscle cell lines with all 3 doses(12.5, 25 and 50 mmol).

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[29, 30]. Moreover, insulin-signalling effectors such as PI3K and AKT as well as AMPK, become deactivated by palmitic acid[31]. Indeed, in this study the 4 h 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 h. However, treatment with MB showed an improvement in the glucose uptake in the insulin resistant cell lines after 48 h 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)[32].

Insulin resistance is characterised by reduced glycogen synthesis in the skeletal muscle[33]. This may be due to free fatty acid-induced suppression of pyruvate dehydrogenase complex leading to reduced glucose uptake[34]. Indeed, this study found that insulin resistant skeletal muscle cell had a reduced glycogen storage. 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 ($PPAR_{\Upsilon}$)[35]. MB may also utilise the same mechanism to improve glycogen synthesis in the palmitic induced skeletal muscle cell lines.

Malonaldehyde (MDA), a byproduct 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[36]. 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.

CONCLUSION

Momordica balsamina increased glucose uptake in palmitic acid-induced skeletal muscle cell lines. This suggests that *M. balsamina* 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, *M. 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 *M. balsamina* in improving or prevention of insulin resistance.

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