American Journal of Biochemistry and Biotechnology

Original Research Paper

Momordica balsamina Fruit Extracts Enhances Selected Aspects of the Insulin Synthesis/Secretion Pathway

[1*]Ananias Hodi Kgopa, [2]Leshweni Jerry Shai and [1]Motetelo Alfred Mogale

[1]Department of Biochemistry, Sefako Makgatho Health Sciences University, PO Box 236, Medunsa, 0204. Pretoria, South Africa
[2]Department of Biomedical Sciences, Faculty of Science, Tshwane University of Technology, Private Bag X680, Pretoria, 0001, South Africa

Article history
Received: 20-08-2020
Revised: 12-12-2020
Accepted: 15-12-2020

Corresponding Author:
Ananias Hodi Kgopa
Department of Biochemistry, Sefako Makgatho Health Sciences University, PO Box 236, Medunsa, 0204. Pretoria, South Africa
Email: ananias.kgopa@smu.ac.za

Abstract: Previous studies have suggested that crude plant extracts of the anti-diabetic medicinal plant, *Momordica balsamina* (*MB*) may exert their anti-diabetic effect through either enhancement of insulin sensitivity or inhibition of intestinal glucose absorption. However, the effects of these *MB* extracts on insulin synthesis and secretion are not well documented in the literature. Therefore, a main purpose of the current research was to examine *in vitro* effects of *MB* crude fruit extracts on selected aspects of the insulin synthesis and secretion pathway. The study results indicated that ethanol, ethyl acetate and hexane *MB* crude extracts had the significantly enhanced the uptake of glucose by RIN-m5F β-cells in a manner depending on concentration. Furthermore, these same *MB* fruit extracts significantly increased the quantities of synthesized and secreted insulin by RIN-m5F β-cells. In addition, significant upregulation of the pre-proinsulin gene levels by the same *MB* fruit extracts was detected by the conventional end-point Polymerase Chain Reaction (RT-PCR), whereas a significant upregulation of gene expression levels of the two pancreatic glucose sensors (glucokinase enzyme and Glucose Transporter 2 (GLUT2)) as well as the two insulin gene transcription factors Musculoaponeurotic Fibrosarcoma homolog A (MafA) and Pancreatic Duodenal Homeobox-1 (PDX-1) were detected by means of quantitative reverse transcriptase PCR. In conclusion, the outcomes of this research propose that *MB* fruit extracts may, in addition to their effects on sensitivity of insulin and inhibition of intestinal glucose absorption, exert their anti-diabetic effects through stimulation of both the insulin synthesis and secretion.

Keywords: RIN-m5F Cells, Synthesized and Secreted Insulin, Glucose Uptake, Anti-Diabetic, *Momordica balsamina*

Introduction

Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder characterized by sustained hyperglycaemia owing to the incapability of the pancreatic β-cells to complement for peripheral insulin resistance (Prentki and Nolan, 2006; Cerf, 2013). The global prevalence of diabetes, approximately 90% of which is T2DM was projected to be 9.3%, which amount to 463 million individuals in 2019 and it is also expected to escalate to 10.2%, which is 578 million individuals by 2030 and to 700 million people (10.9%) by the end of 2045 (Saeedi *et al*., 2019). Current anti-diabetic treatments (Nkansah *et al*., 2018) for T2DM include lifestyle intervention strategies and pharmacological management with oral hypoglycaemic agents like sulphonylureas, biguanides, dipeptidyl peptidase-4 inhibitors and sometimes injections with different insulin analogues either as monotherapy or in combination therapy (Chaudhury *et al*., 2017). However, these conventional anti-diabetic agents are often associated with undesirable side effects, are expensive and often, do not meet their therapeutic targets (Chaudhury *et al*., 2017; Davies *et al*., 2018). In light of the increasing prevalence of T2DM, its long-term consequences in terms of morbidity, mortality and economic costs, as well as the problems associated with

conventional anti-diabetic agents, there is an urgent need for the discovery and/or development of therapeutic alternatives for diabetes that are safe, efficacious and affordable. For the effective use of these alternative anti-diabetic agents, especially in combination therapy, it is imperative that their discovery and development be accompanied by knowledge and understanding of their hypoglycaemic mechanisms of action.

Medicinal plants have been used cost-effectively in traditional folk medical systems of many countries to manage an assortment of human illnesses, including diabetes mellitus (Govindappa, 2015; Salehi *et al*., 2019). Actually, in numerous regions of the globe, particularly in underprivileged nation state, this has been the solely means of treatment accessible to manage diabetes (Joseph and Jini, 2013). *Momordica balsamina* Linn, (*MB*). Cucurbitaceae family of plants, commonly known as African pumpkin or Balsam apple, a therapeutic plant that has been used for decades in folk medication to manage diabetes and associated complications (Thakur *et al*., 2009). The hypoglycaemic effects of *MB* crude leaf, fruit and seed extracts have been confirmed in zoological simulations of diabetes (Otimenyin *et al*., 2008; Bhardwaj *et al*., 2010). *MB* plant extracts as other well-documented antidiabetic plant extracts may exert their hypoglycaemic effects through wide range of mechanisms including stimulation of insulin synthesis, enhancement of the secretion of insulin by pancreatic β-cells, enhancement of the sensitivity of insulin and/or intestinal glucose digestion and absorption inhibition (Govindappa, 2015; Wajid *et al*., 2019). Whereas *MB* plant extracts are reported to possess an ability to improve insulin sensitivity in insulin unresponsive tissues (Thakur *et al*., 2009) and to inhibit the intestinal absorption of glucose (Bhardwaj *et al*., 2010; Santa Cruz and Sfara, 2018), there are no studies in the literature that have investigated and reported on the effect of *MB* plant extracts on the insulin synthesis and secretion. Thus, the objective of the present study was to explore the effects of *MB* crude fruit extracts on selected aspects of the pathways that involve synthesis and secretion of insulin in RIN-m5F β-cells and this include the uptake of glucose, secretion of insulin stimulated by glucose and the total amount of synthesized insulin in addition to effects on the mRNA levels of designated gene whose yields of protein are involved in production of insulin.

In this study, different concentrations of the crude fruit extracts of *Momordica balsamina* were prepared and the extracts effect on the uptake of glucose, mRNA levels of the pre-proinsulin gene, pathways in which insulin is synthesized and secreted as well as the determination of MafA, PDX-1, Glucokinase and GLUT2 mRNA levels in RIN-m5F were investigated To the greatest of our knowledge, this study is one of its kind to explore and report on the effect of *Momordica balsamina* crude fruit extracts which is likely to provide valuable information about the exact mechanisms whereby these plant extracts exert their blood glucose lowering effect. This information can then be used to avoid the undesirable herb-drug interactions when these plant extracts are used alongside conventional synthetic anti-diabetic drugs.

Materials and Methods

Research Design

The flow diagram of the research approach in this current study presented in the Fig. 1 and the large indices nomenclature as used in the article is presented in Table 4

Plant Material Collection, Identification and Extraction

The unripe fruits of *MB* were collected in Mentz-Segoreng village, Ga-Mamabolo, Limpopo Province, South Africa (23°53'54.3"S, 29°46'44.3"E). The taxonomic distinctiveness of this plant validated in Pretoria by a taxonomist at the South African National Biodiversity Institute (SANBI) and the plant sample representing the verified species of the plant were deposited with the sole identification code PRE09964130-0. The collected *MB* fruits were rinsed with running tap water, sliced into portions and frozen at -80°C awaiting investigations. Upon usage, frosted fruit pieces were freeze-dried and ground into fine powder. Fifty grams of the dried fruit powder extracted consecutively with hundred and fifty milliliters each of water, ethanol, ethyl acetate and hexane following the technique mentioned by (Mogale *et al*., 2011). Each of the resultant crude extraction was filtered by means of Whatman no. 1 filter paper and the ethanol, ethyl acetate and hexane extract was concentrated under *vacuum* by means of a rotating Büchi evaporator from Lasec (SA) at 37°C, while extract from water was prepared by freeze-drying through the Vacutec lyophilizer from Lasec (SA). Each crude extract was dissolved separately in 100% DMSO to prepare stock solution of 20 mg/mL concentration.

Culturing of Liver and Pancreatic Cells

The RPMI-1640 culture media enriched with 0.1 g/L streptomycin and 10 000 units/mL penicillin (1% (v/v) concoction solution an antibiotic as well as 10% (v/v) Foetal Bovine Serum (FBS) was used to maintain the growth of liver cells (H-4-II-E (ATCC® CRL1548™)) at 37°C in 5% CO_2 incubator. This same RPMI-1640 media also improved with addition of 1 mM sodium pyruvate, 10% FBS and 10 mM HEPES to maintain the pancreatic RIN-m5F beta cells at 37°C in a moistened thermosphere of 5% CO_2 incubator. Culture media in these cells were changed three to four times a week and cells were seeded for testing when confluence was at eighty-ninety percent.

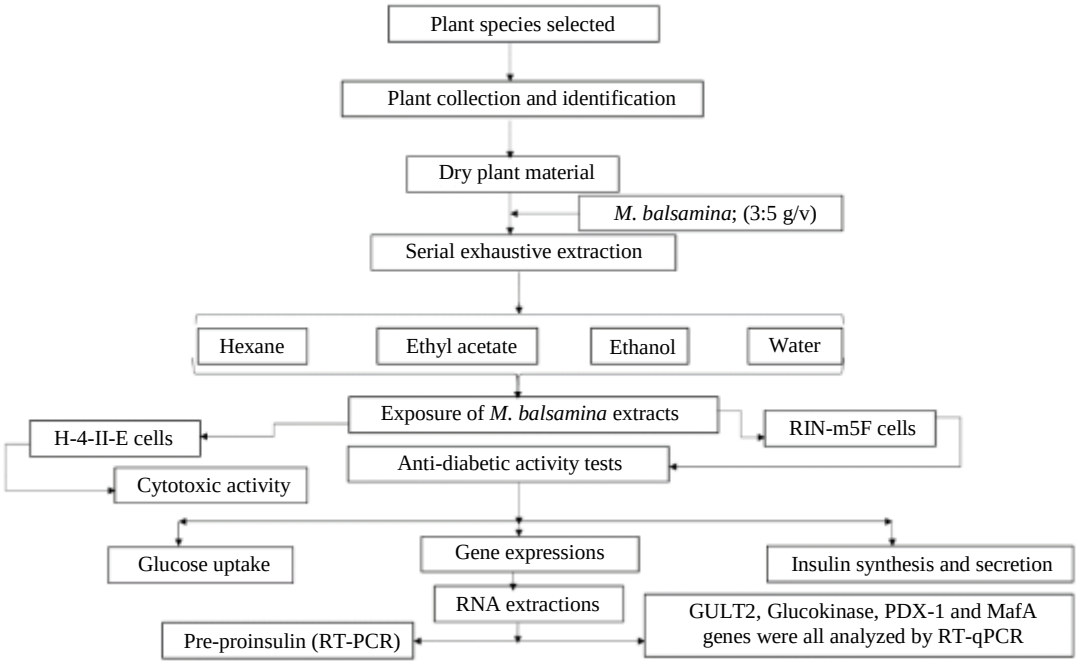


Fig. 1: Flow chart displaying research approach

Cytotoxicity Test

The effect of *MB* fruit extracts on liver cell viability was examined by means of the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay protocol described by (Bahuguna *et al*., 2017), with modifications. In summary, liver cells cultured at a density of 1×10^5 cells/well were harvested and seeded in 96-well microtiter plates (Lasec, SA) and then grown in an incubator with 5% CO_2 at 37°C for 24 h. Cells cultured were then incubated for 72 h with varied doses (100 µL) of extracts of *MB* fruit ranging between 3.125 and 200 µg/mL. Hydrogen peroxide (1% v/v) as well as DMSO (1% v/v) were positive and vehicle controls, respectively. Subsequently, the medium in the plates were removed and each substituted with 5 mg/mL MTT solution at a volume not exceeding 200 µL. The formed formazan crystals in these plates were then dissolved by adding 100% pre-warmed DMSO to individual plates. The plates were then incubated for 4 h in the dark, after which, the absorbance were measured at 570 nm using an ELISA plate reader (ThermoFisher Scientific, SA). The cytotoxic effect of the *MB* fruit extracts was expressed as percentage of the untreated control using the formula presented here:

$$\%Cell\ death = \frac{\left(A_{treated\ cells} - A_{blank}\right)}{\left(A_{untreated\ control\ cells} - A_{blank}\right)} \times 100$$

The LC50 (lowest concentration of plant extract causing 50% cell death) was calculated from a percentage cell death plot versus of plant extracts concentration (Sagbo *et al*., 2018).

Stimulation of Glucose Uptake by RIN-m5F Cells

The uptake of glucose by RIN-m5F cells was examined by means of a revised technique detailed by (Roffey *et al*., 2007), with amendments. Briefly, cultured RIN-m5F cells maintained previously as reported were seeded at a 2×10^5 cells/well in a 96-well microtiter plates and incubated at 37°C in 5% CO_2 for 72 h. Following attachment post incubation, cells were washed once with RPMI-1640 medium without serum and then incubated for 2 h at 37°C in the same medium (100 µl/well). Afterwards, cells were rinsed twice in 1X glucose-free Krebs-Ringer Bicarbonate HEPES (KRBH) buffer (4.7 mM KCl, 128 mM NaCl, 1.25 mM $CaCl_2$, 5 mM $NaHCO_3$, 10.5 mM NaH_2PO_4, 1.25 mM $MgSO_2$, 20 mM HEPES, pH 7.4) enriched with 0.1% Bovine Serum Albumin (BSA) and incubated for 30 min at 37°C in 100 µL/well 1X glucose-free KRBH buffer. Afterwards, media in the wells was taken out and substituted by 1X KRBH buffer (100 µl/well) enriched with glucose (15 mmol/L) and 0.1% BSA and cells were then incubated in fruit extracts of *MB* in an increasing concentrations ranging between 1.6 µg/mL and 50 µg/mL. The cell concoctions then incubated at 37°C in a 5% CO_2 incubator for 60 min. Subsequently, the quantity of the remaining glucose in the media was confirmed via glucose oxidase-based assay kit (KAT Medical Laboratory, SA) according to the manufactures's procedure. The quantity of the effected intake of glucose

by four *MB* fruit extracts was calculated as the difference amongst the preliminary 15 mmol/L glucose and the remaining glucose in the growth media. All the experiments were performed in triplicate (n = 9).

Extracellular and Intracellular Insulin Assays

The RIN-m5F cells were stimulated by treatment with varying doses of MB fruit extracts for the insulin synthesis and secretions assays following method detailed by (Zhang *et al*., 2014), with modifications. Subsequently, the sum of intracellular and extracellular insulin quantities was summed to calculate the overall quantity of synthesized insulin. Cultured RIN-m5F cells maintained as previously indicated in the cell culture section were harvested and seeded at a density of 2.5×10^5 cells/well in a 24-well plate in pre-warmed RPMI-1640 media and then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 48 h for attachment. Following attachment, cells were washed in 0.1% BSA and then incubated for 20 min in 5% CO_2 at 37°C. Additionally, cells were washed two times with 1X KRBH buffer free-of-glucose and incubated at 37°C in 5% CO_2 incubator for 30 min with 12.5, 25 and 50 µg/mL *MB* fruit extracts each containing 15 mmol/L glucose. Thereafter, plates were shared into two portions, with one-part used for assaying insulin secretions and another part used for assaying quantities of intracellular insulin.

The sum of both the secreted insulin stimulated in the presence of glucose and internal quantities of insulin were measured by means of rat insulin enzyme-linked immunosorbent assay (Sigma-Aldrich, SA), following manufacturers procedure. Both the secreted and the internal quantity of insulin was normalized by dividing them with the corresponding protein quantities measured by means of Lowry protein assay kit (ThermoFisher Scientific, South Africa).

RNA Extraction and End-Point Polymerase Chain Reaction

Effect of *MB* fruit extracts on mRNA levels of the pre-proinsulin gene was studied by means of the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) following procedure reported by (Kgopa *et al*., 2020). Briefly, the RIN-m5F cells grown in a RPMI-1640 media enriched with 10 mM HEPES, 10% (v/v) FBS, 100 µM β-mercaptoethanol and 1 mM sodium pyruvate were seeded in 6-well plates and incubated at 37°C in a 5% CO_2 incubator for 48 h. Afterwards, cells were then washed and incubated for 16 h at 37°C in a 5% CO_2 with *MB* fruit extracts (1.25, 25 and 50 µg/mL) using the same medium comprising of 15 mmol/L glucose concentration. Negative control were the non-treated cells while vehicle control were the cells treated with DMSO.

The entire ribonucleic acid was isolated from the RIN-m5F cells treated with *MB* fruit extracts and non-treated control cells using GeneJet RNA Kit (ThermoFisher Scientific, SA), following manufacturers protocol. The cDNA was transcribed in a reverse direction by means of RevertAid Moloney-Murine Leukemia Virus Reverse Transcriptase kit (ThermoFisher Scientific, SA) with oligo(dT)12 primers and isolated RNA (0.1 µg) according to the manufacturer's instructions. The KAPATaq ReadyMix DNA polymerase kit (Lasec, SA) was used to amplify the pre-proinsulin gene and the PCR cycles was performed in My Cycler (Bio-Rad, SA). The forward (5´-TGCCCAGGCTTTTGTCAAAC-3´) and reverse (5´-CTCCAGTGCCAAGGTCTGAA-3´) primer pairs were used at optimum combinations of 0.4 µM for the amplification of this gene. PCR conditions for each 32 cycles were as follows: Denaturation at 95°C for 4 min, 95°C for 30 sec, 59°C for 30 sec and 72°C for 2 min and to a final extension at 72°C for 4 min. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the housekeeping control gene and was co-amplified using the following primer pairs: Forward (5´-ACTTTGGCATTGTGGAAGG-3´) and reverse (5´-ACACATTGGGGGTAGGAACA-3´). Agarose gel of 1.25% was used in this study to separate the products amplified by PCR and the amplicons were then visualized via Chemiluminescence Image System (Bio-Rad, SA). Thereafter, an ImageLab software version 5.2.1 program (Bio-Rad, SA) was performed to quantify the amount of mRNA levels of the pre-proinsulin gene.

Quantitative Polymerase Chain Reaction Analysis

The effect of *MB* fruit extracts based on mRNA levels of MafA, PDX-1, Glucokinase and GLUT2 genes was quantified by means of reverse-transcriptase-quantitative polymerase chain reaction (RT-qPCR). In brief, cultured RIN-m5F cells reported earlier in the cell culture section were treated with *MB* fruit extracts (25 and 50 µg/mL) concentrations and incubated at 37°C in a 5% CO_2 incubator for 16 h. Total RNA isolation and the synthesis of cDNA were performed as described earlier in the RNA extraction section. RT-qPCR was carried out using the 2X SensiFAST SYBR Hi-ROX PCR Mix (Bioline Celtic Diagnostics, SA) following the company's procedure and the genes amplification was achieved by means of the StepOnePlus™ Real-Time PCR Detection System (AB Applied Biosystems, Bio-Rad, SA), with some modification. Pairs of primer used in this analysis were reported by (Kgopa *et al*., 2020) and are listed in Table 1. mRNA levels of MafA, PDX-1, Glucokinase and GLUT2 gene were measured based on comparative CT technique and were standardized to the comparative mRNA levels of the GAPDH gene using a StepOnePlus software v2.1 (AB Applied Biosystems, Bio-Rad, SA).

Table 1: Primers used in gene expression analysis

Primer sequences	
Forward primer	Reverse primer
GAPDH 5´-ACCACACAGTCCATGCCATCAC-3´	5´-TCCACCACCCTGTTGCTGA-3´
MafA 5´-AGGCCTTCCGGGGTCAGAG-3´	5´-TGGAGCTGGCACTTCTCGCT-3´
PDX-1 5´-AACCCGAGGAAAACAAGAGG-3´	5´-GTTCAACATCACTGCCAGCTC-3´
Rat glucokinase 5´-TGACAGAGCCAGGATGGAG-3´	5´-TCTTCACGCTCCACTGCC-3´
Rat GLUT2 5´-TGGGTTCCTTCCAGTTCG-3´	5´-AGGCGTCTGGTGTCGTATG-3´

Primers used for the respective genes presented

Data Analysis

All tests were conducted in three independent experiments (n = 9) and data are expressed as mean ± Standard Error of the Mean (SEM). Student's *t*-test with the assumption of equal variance was used to compare experiments and controls. Data was considered statistically significant when a two-tailed p-value were less than 0.001 and 0.05. The analysis of data was executed by means of Microsoft 16 Excel software.

Results

Percentage Extract Yield and LC_{50} Cytotoxicity Values

The percentage extracts yield and the LC_{50} values of the different *MB* crude fruit extracts obtained by means of the MTT assay are tabulated in Table 2.

As shown in Table 2, the maximum percentage of extract yield was attained with water extraction and the extract with the least percentage yield was achieved with hexane extraction. The *MB* crude ethanol fruit extract was the most toxic of the four extract (lowest LC_{50}) while the ethyl acetate fruit extract was the least toxic. The LC_{50} values in Table 2 were used to inform the *MB* crude fruit extracts concentration used in all the performed experiments in the current study (i.e., in all experiments carried out in the study, each extract was used at concentration below its LC_{50} in order to limit cell death due to extract toxicity).

Uptake of Glucose by RIN-m5F Cells

Effect of increasing concentrations *of MB* crude fruit extracts on consumption of glucose by RIN-m5F cells were explored as described in the methodology section and the result are shown in Fig. 2.

As shown in Fig. 2, the *MB* fruit hexane and ethyl acetate extracts showed a significant enhancement in uptake of glucose by RIN-m5F cells that were treated compared with non-treated RIN-m5F cells in a manner that depends on extract dose from the 3.1 µg/mL concentration up to the 25 µg/mL concentration ($p<0.05$). However, in both extracts, the effect of the extract seemed to drop unexpectedly as the extract concentration increased beyond 25 to 50 µg/mL. *MB* fruit water extract at 1.6, 3.1 and 50 µg/mL also demonstrated a significant enhancement of consumption of glucose by RIN-m5F cells when evaluated against RIN-m5F cells that were not treated ($p<0.05$). However, in this case, the effect was not dose-dependent. In contrast, the *MB* fruit ethanol extract at all investigated concentrations in the current study non-significantly inhibited the consumption of glucose when compared with non-treated RIN-m5F cells.

Effects of Extracts on Synthesis and Secretion of Insulin

Effect of *MB* crude fruit extracts on the glucose-stimulated insulin secretions, intracellular amounts of insulin and the total quantity of synthesized insulin by RIN-m5F cells are presented in Fig. 3.

With exception of the water extract, a significant increase in intracellular insulin content in all other concentrations of *MB* fruit extracts tested in this study was demonstrated in RIN-m5F cells once compared with both the non-treated RIN-m5F cells and the glibenclamide (0.1 µM) control ($p<0.05$) (Fig. 3A). Similarly, with the exemption of the water extract, a substantial increase in secretion of insulin stimulated by glucose in tested concentrations and overall quantity of synthesized insulin by RIN-m5F cells of all other *MB* fruit extracts was detected in comparison with non-treated RIN-m5F control cells ($p<0.05$) (Fig. 3B and 3C, respectively). However, only *MB* fruit hexane and water extracts at 50 µg/mL significantly enhanced secretion of insulin stimulated by glucose and increased the overall quantity of synthesized insulin by RIN-m5F cells when comapred with the control drug glibenclamide (0.1 µM,) ($p<0.05$) (Fig. 3B and 3C, respectively).

Effects of Extracts on Pre-Proinsulin mRNA Levels

Effect of *MB* fruit extracts at 25, 50 and 100 µg/mL concentrations were examined by means of RT-PCR as reported in the methodology section. The pre-proinsulin mRNA level profile in the RIN-m5F cells treated with *MB* fruit extracts in comparison with the solvent treated control cells is presented in Fig. 4.

MB crude fruit hexane and ethanol extracts significantly increased mRNA levels of the pre-proinsulin gene in a dose-dependent manner in RIN-m5F

cells that were treated when compared with the mRNA levels of the very similar genetic material in non-treated cells ($p<0.001$). *MB* crude fruit ethyl acetate extract also significantly increased mRNA levels of the pre-proinsulin gene in RIN-m5F cells that were treated comparing it with mRNA levels of the very similar RNA component in non-treated cells ($p<0.001$). However, in this case, the effect of the extract was not dose-dependent. In addition, *MB* crude fruit extract of water at 25 and 50 µg/mL concentrations showed a significant increase in mRNA levels of the gene in RIN-m5F cells treated with extracts compared with non-treated control cells ($p<0.001$). In contrast, the water extract of *MB* fruit at 12.5 µg/mL unexpectedly significantly decreased the mRNA levels of this gene in RIN-m5F cells that were treated compared to the mRNA levels of the very similar gene in non-treated cells ($p<0.001$).

Effects of Extracts on MafA, PDX-1, Glucokinase and GLUT2 mRNA Levels

Effect of *MB* crude fruit extracts on the PDX-1, MafA glucokinase and GLUT2 mRNA levels in folds are graphically presented in Fig. 5 and in percentage expressions are presented in Table 3. *MB* fruit ethanol, hexane and ethyl acetate extracts at 25 µg/mL significantly enhanced the increase in mRNA levels of GLUT2 gene 1.6-fold, 2.0-fold and 1.2-fold, respectively in RIN-m5F cells that were treated when compared with the mRNA levels of the very similar gene in non-treated RIN-m5F cells ($p<0.05$) (Fig. 5A). In contrast, *MB* fruit ethanol, ethyl acetate and hexane extracts, all at 50 µg/mL concentration, as well as the *MB* fruit water extract at both 25 and 50 µg/mL concentrations, significantly decreased mRNA levels of the GLUT2 gene 3-fold, 3-fold, 1.86-fold, 2.3-fold and 1.3-fold, respectively, in RIN-m5F cells that were treated when compared with the mRNA level of the very similar gene in non-treated RIN-m5F cells ($p<0.05$) (Fig. 5A). *MB* fruit ethanol, ethyl acetate and hexane extracts, all at 50 µg/mL concentration significantly improved mRNA levels of the glucokinase gene 646-fold, 662-fold and 997-fold respectively in RIN-m5F cells that were treated comparing it with mRNA levels of the similar gene in non-treated RIN-m5F control cells ($p<0.05$) (Fig. 5B). As presented in Fig. 5C, *MB* fruit hexane extract, *MB* fruit ethyl acetate extract, *MB* fruit ethanol extract and *MB* fruit water extract, all at 25 µg/mL but not at 50 µg/mL significantly upregulated the expression of PDX-1 gene 9.9-fold, 7.3, 6.2-fold and 5.7-fold respectively in RIN-m5F cells that were treated when compared with non-treated RIN-m5F cells ($p<0.05$). Similarly, *MB* fruit hexane extract, *MB* fruit ethyl acetate extract, *MB* fruit ethanol extract and *MB* fruit water extract at 25 µg/mL significantly enhanced the increase in mRNA levels of MafA gene 17.5-fold, 9-fold, 12-fold and 9-fold respectively in RIN-m5F cells that were treated when compared with mRNA levels of the similar gene in non-treated RIN-m5F cells ($p<0.05$) (Fig. 5D). These same *MB* fruit extracts, at 50 µg/mL, significantly increased mRNA levels of the MafA gene 3.5-fold, 2.5-fold, 13.2-fold and 3.1-fold respectively compared with the mRNA levels of the similar gene in non-treated RIN-m5F control cells ($p<0.05$) (Fig. 5D).

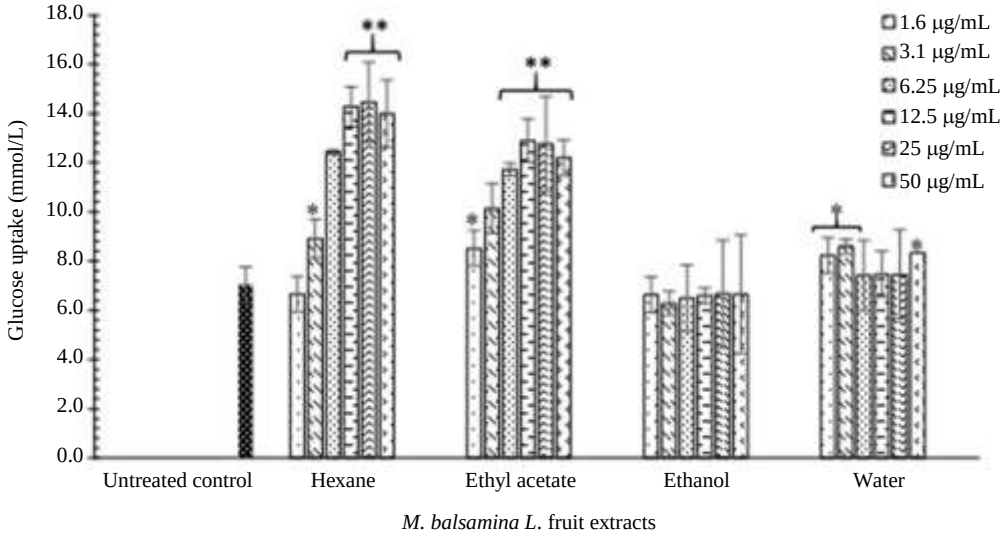
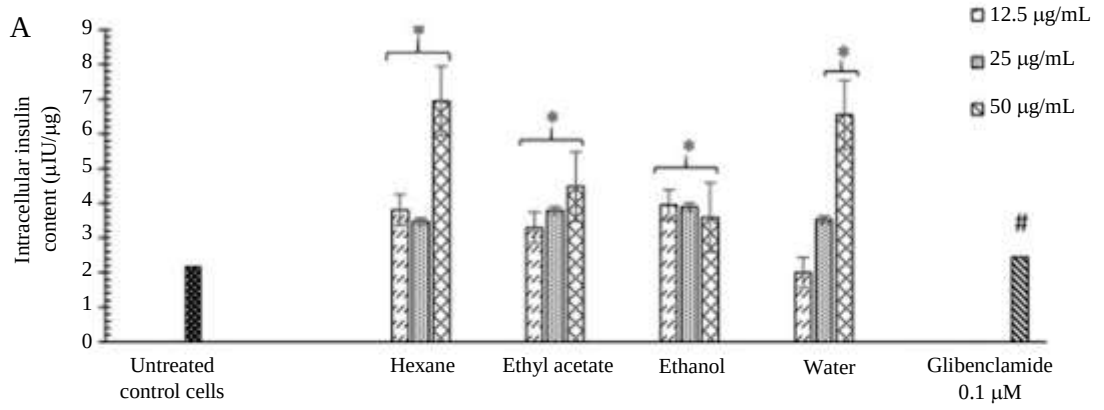
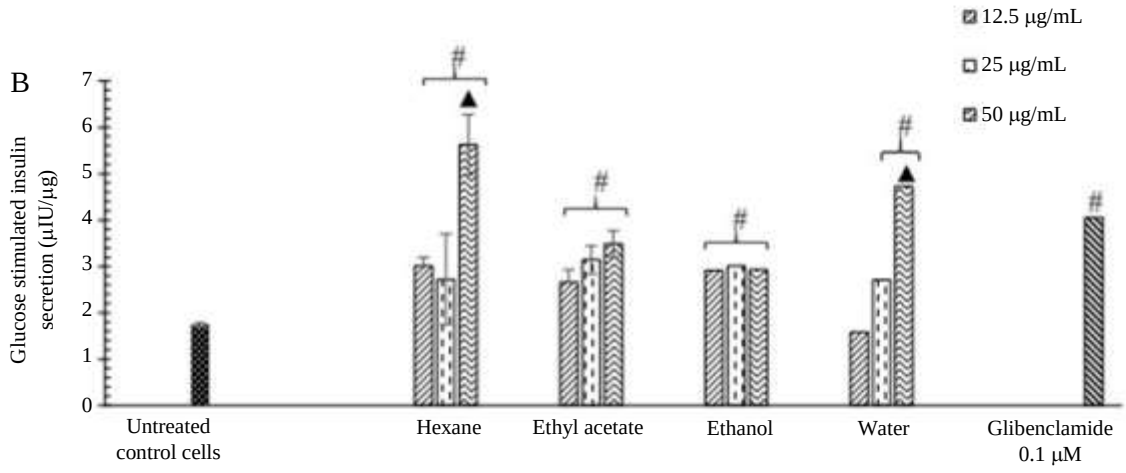


Fig. 2: Effect of *MB* fruit extracts on the consumption of glucose by RIN-m5F β-cells grown in glucose concentration (15 mmol/l) for 60 min. Data are mean consumption of glucose ± SEM of three independent experiments (n = 9). ***** indicate statistical significance at $p<0.05$ compared with non-treated control cells and ****** indicates statistical significance at $p<0.001$ compared with non-treated control cells

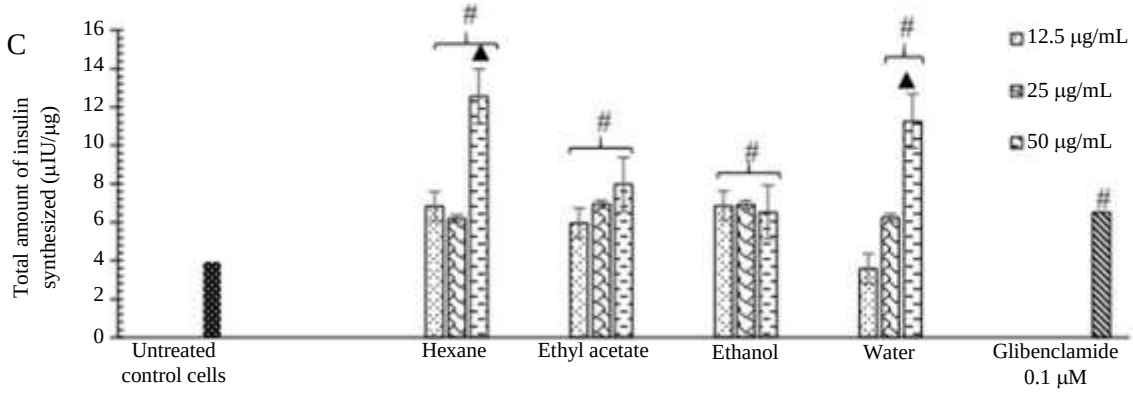
DOI: 10.3844/ajbbsp.2020.549.560



M. balsamina L. fruit extracts



M. balsamina L. fruit extracts



M. balsamina L. fruit extracts

Fig. 3: Effect of *MB* fruit extracts on (A) intracellular insulin amount, (B) glucose-stimulated insulin secretion and (C) synthesis of insulin by RIN-m5F β-cells. Data are mean of amounts of insulin ± standard error of the mean of triplicate experiments (n = 9). * indicates statistical significance compared with both non-treated control cells and glibenclamide treated cells ($p<0.05$) # designates statistical significance when compared with non-treated control cells ($p<0.05$) and ▲ indicate statistical significance at $p<0.05$ when compared with 0.1 µM glibenclamide

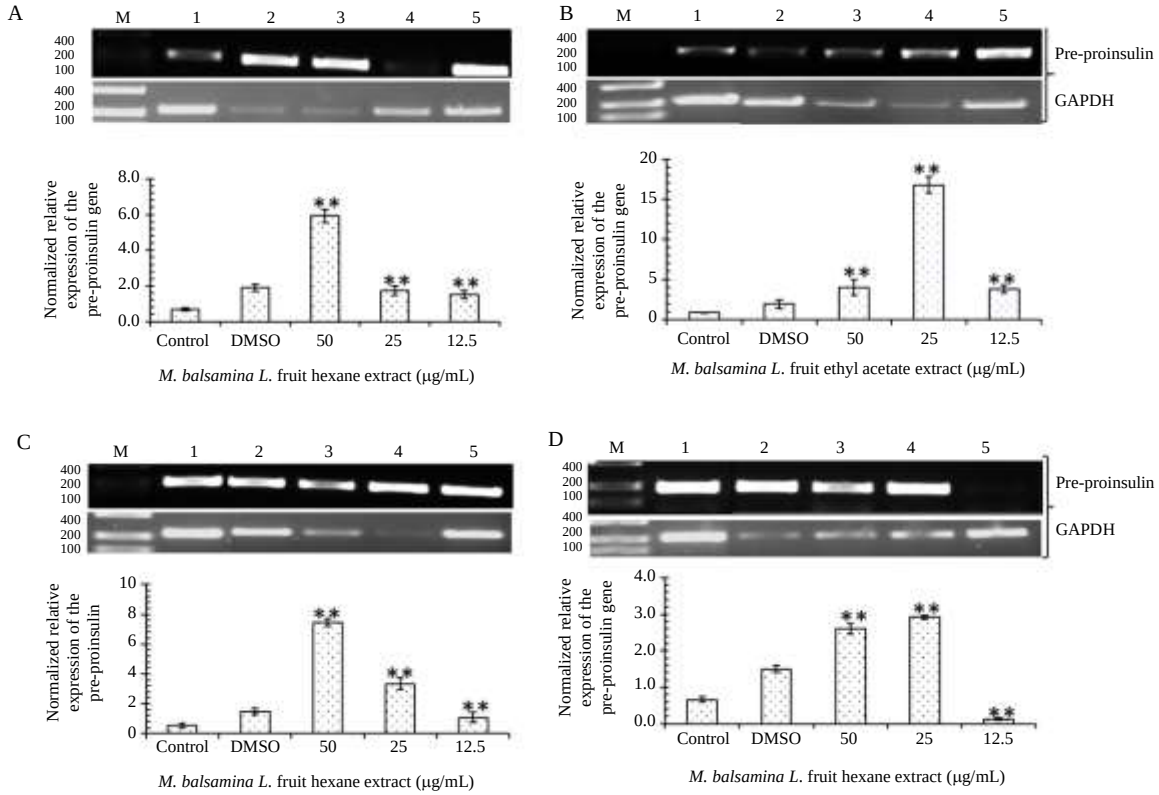


Fig. 4: Effect of *MB* fruit extracts on mRNA levels of the pre-proinsulin gene. (A) hexane, (B) ethyl acetate, (C) ethanol and (D) water extract. Data expressed as the -fold change in gene mRNA levels normalized to the GAPDH housekeeping gene. ** indicates statistical significance at $p<0.001$ when compared with the DMSO control. Lanes: (M) DNA marker, (1) non-treated RIN-m5F control, (2) DMSO and (3-5) Pre-proinsulin gene

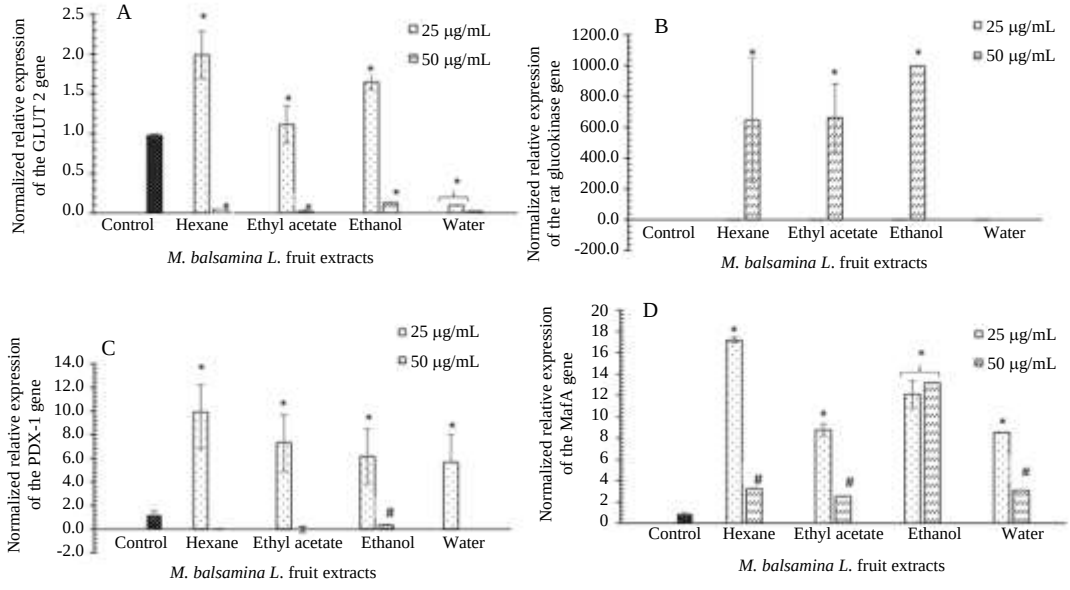


Fig. 5: Effects of *MB* fruit extracts on the relative expression of (A) GLUT2, (B) glucokinase (C) PDX-1 and (D) MafA mRNAs in RIN-m5F pancreatic beta cells. Data are expressed as the relative fold change in mRNA expression normalized to the GAPDH housekeeping reference gene. * (25 µg/mL) and # (50 µg/mL) indicates statistical significance at $p<0.05$ when compared with the untreated RIN-m5F pancreatic beta control cells, respectively

Table 2: Cytotoxic effects of *M. balsamina* fruit extracts by H-4-II-E cells

Extract	Yield (%)	LC_{50} (µg/mL)
Hexane	1.12	61.4±5.02
Ethyl acetate	1.26	255.2±0.01
Ethanol	1.24	49.8±11.2
Water	7.86	240±13.0

LC_{50} = Dose of extracts inhibiting cell growth by 50% presented as means ± SEM (n = 9)

Table 3: Percentage gene expression by RT-qPCR

	% GLUT2 (µg/mL)		% Glucokinase (µg/mL)		% PDX-1 (µg/mL)		% MafA (µg/mL)	
Extract	25	50	25	50	25	50	25	50
Hexane	50.8	<0.0	14.8	99.9	88.0	n.d	95.0	73.6
Ethyl acetate	12.0	<0.0	45.6	99.9	83.8	n.d	90.2	66.0
Ethanol	40.6	<0.0	37.8	99.9	80.7	<0.0	92.9	93.5
Water	<0.0	<0.0	<0.0	n.d	79.0	n.d	89.9	72.2

Note: <0.0% indicate mRNA level below control levels; n.d indicate genes not detected

Table 4: Large indices nomenclature as used in the article

Index name	Description
GLUT2	Glucose transporter 2
PDX-1	Pancreatic duodenal homeobox-1
MafA	Musculoaponeurotic fibrosarcoma homolog A
NeuroD1	B-2/Neurogenic differentiation 1
RIN)-m5F	Rat insulinoma pancreatic beta cells
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
T2DM	Type 2 diabetes mellitus
MB	*Momordica balsamina*
SEM	Standard error of the mean
ELISA	Enzyme-linked immunosorbent assay
GSIS	Glucose-stimulated insulin secretion
SANBI	South African National Biodiversity Institute
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
LC_{50}	Lethal concentration at 50%
KRBH	Krebs-Ringer-Bicarbonate HEPES
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid
RT-PCR	Transcriptase-polymerase chain reaction
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
BSA	Bovine serum albumin
RPMI-1640	Roswell Park Memorial Institute,
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
PBS	Phosphate buffered saline
cDNA	complementary DNA

Discussion

The present study investigated the effects of crude fruit extracts of the anti-diabetic medicinal plant, *Momordica balsamina*, on selected aspects of the insulin synthesis and secretion pathway. In this study, dried *MB* fruit powder as subjected to a successive extraction with increasing polarity of solvents (hexane, ethyl acetate, ethanol and water) to obtain and examine the effects of phytochemicals of different polarity (non-polar, moderate polar and polar) on the study parameters. Also, in order to limit the toxicity of the plant extracts on RIN-m5F β-cells, all extracts used in this study were used at their sub-toxic concentrations (i.e., at concentrations much lower than their corresponding LC_{50} values) when tested in liver cells.

The glucose entry into β-cells is essential in both the control of the synthesis and secretion of insulin (Fu *et al*., 2013; Komatsu *et al*., 2013; Ren *et al*., 2007). Hence, the current study investigated among other things, the effect of the different *MB* fruit extracts on the uptake of glucose by RIN-m5F β-cells. The results obtained revealed that both the relatively non-polar *MB* fruits extracts (hexane and ethyl acetate extracts) have the capacity to enhance the uptake of glucose by RIN-m5F β-cells in a manner that depends on extract dose. This

capacity could most probably be attributed to the presence of non-polar Cucurbitane-type triterpenoids or their saponins, which are also known to enhance glucose uptake by insulin sensitive tissues (Kaushik *et al*., 2017) and are reported to be more abundant in *MB* plant extracts (Thakur *et al*., 2009). A search of literature revealed that there are no similar previous studies that have investigated and reported on the effects of crude plant extracts on the uptake of glucose by the RIN-m5F cells that do not depend on insulin. Hence, the outcomes of our glucose uptake experiments using RIN-m5F beta cells cannot be compared with results of other studies.

Glucose entry into pancreatic cells is non-insulin dependent, rather, it is controlled by two notable pancreatic beta cell sensors, namely the GLUT2 transporter and the glucokinase (Thorens, 2015). Thus, in order to determine whether the observed enhancement of uptake of glucose by RIN-m5F cells is associated with the upregulation of expressions of either glucokinase, GLUT2 or both genes, the effect of the different *MB* crude fruit extracts on the mRNA levels of glucokinase and GLUT2 genes were also explored in the current study. The results obtained suggest that both the *MB* fruit extracts of hexane and ethyl acetate enhanced the uptake of glucose by RIN-m5F cells in a manner that depend on concentration, have the capacity to upregulate the expressions of both GLUT2 and glucokinase at least at the mRNA level. However, in this study, the upregulation of GLUT2 mRNA levels was observed only at 25 µg/mL and not at 50 µg/mL concentration. On the other hand, the upregulation of glucokinase mRNA levels was observed only at 50 µg/mL concentration. The observation that a crude plant extract may have a biological effect at a lower concentration than at a higher concentration have been stated in previous researches (Karthikeyan *et al*., 2010; Sowemimo *et al*., 2015; Zandi *et al*., 2016), however, no suitable explanation has been brought forward to account for this observation. Interestingly, it was also detected in this aspect of the study that the crude *MB* fruit ethanol extract which did not enhance the uptake of glucose in concentration dependent manner by RIN-m5F cells, significantly upregulated the expression of both glucokinase and GLUT2 genes. Taken together, the results of the uptake and the GLUT2, glucokinase experiments suggest that both the hexane and ethyl acetate *MB* fruit crude extracts may improve the uptake of glucose by RIN-m5F cells through upregulation of the glucokinase and GLUT2 glucose sensors.

Some medicinal plant extracts reported to exert their glucose-lowering effect through the glucose stimulated insulin synthesis and/or insulin secretion (Ajabnoor, 1990; Mukesh and Namita, 2013; Govindappa, 2015; Noor *et al*., 2017). Based on these reports, the current study has also investigated the effect of *MB* fruit extracts on the total quantities of the synthesized and secreted insulin by glucose stimulated RIN-m5F cells. The results obtained from this study suggest that *MB* fruit hexane, ethyl acetate and ethanol has the ability to stimulate both insulin synthesis and secretion by RIN-m5F cells and hence, the hypoglycaemic mechanism of action for these crude extracts may in part, resemble that of the crude extracts of *Biophytum sensitivum* (Mukesh and Namita, 2013) and crude extracts of *Aloe* sp (Ajabnoor, 1990; Noor *et al*., 2017). In addition, the finding that some crude fruit extracts of *MB* significantly stimulated GSIS in comparison with the standard insulin secretagoque drug, glibenclamide imply that these extracts may have phytochemicals, most probably saponins that are reported to be more abundant in extracts of the related plant species, *Momordica charantia* (Keller *et al*., 2011).

Insulin synthesis is controlled at both the transcriptional and translational level (Fu *et al*., 2013; Ren *et al*., 2007; Vanderford *et al*., 2007). At the transcriptional level, insulin synthesis is regulated by the binding of specific trans-activators including the MafA, B-2/Neurogenic Differentiation 1 (NeuroD1) and the Pancreatic and Duodenal homeobox-1 (PDX-1) to the cis-acting sequences within the 5′ flanking region of the insulin gene (Fu *et al*., 2013; Ren *et al*., 2007). High blood glucose levels within the pancreatic beta cells induce the mRNA levels of the MafA and PDX-1 genes which in turn, stimulate the pre-proinsulin gene levels (Ren *et al*., 2007; Vanderford *et al*., 2007). Thus, in addition to the effects of *MB* fruit extracts on the uptake of glucose and expressions of the two pancreatic glucose sensors, the current study investigated the influence of *MB* fruit extracts on the pre-proinsulin expressions levels, PDX-1 and MafA mRNA levels. Results obtained indicate that *MB* fruit ethanol, ethyl acetate and hexane extracts have the ability to upregulate pre-proinsulin expression levels, enhance the increase in MafA and PDX-1 mRNA levels in RIN-m5F cells. These findings, which could most probably due to the occurrence of Cucurbitane-type triterpenoids in *MB* fruit extracts (Kaushik *et al*., 2017), imply that indeed, upon its entry into the beta cells, glucose triggered the transcription and translation of the transcription factors MafA and PDX-1 which then resulted in the increase in mRNA levels of the pre-proinsulin gene (Fu *et al*., 2013; Vanderford *et al*., 2007).

Limitations and Strengths of the Study

The main drawback in this study is that information that is more valued might have been acquired if this research was supplemented by studies of the effect of the *MB* fruit extracts on the enzyme or/or protein activities of MafA, PDX-1, glucokinase and GLUT2 genes. Regardless of these constraints, numerous strengths associated to the present study can be outlined. Primarily, *MB* extract of diverse polarities acquired over

and done with the successive extraction of individual fruit material of this plant under research were examined in the current study. Furthermore, the current study investigated the concurrent effect of *MB* fruit extracts on numerous factors that are part of the synthesis and secretory pathway of insulin. Thirdly, the effect of individual extract was explored at diverse concentrations in order to disclose the existence or the absence of a concentration-dependent outcome.

Conclusion

In this *in vitro* study, effect of crude *MB* fruit extracts on selected aspects of the insulin synthesis and secretion by RIN-m5F β-cells were explored. The outcomes of this study showed that ethyl acetate, hexane and ethanol *MB* fruit extracts have the capacity to enhance glucose uptake and to stimulate insulin synthesis and secretion by cultured RIN-m5F pancreatic beta cells. Furthermore, these same three *MB* fruit extracts were able to upregulate gene expression levels of GLUT2, glucokinase enzyme, MafA and PDX-1 in cultured beta cells. It can be speculated and concluded on the basis of these results that *MB* fruit extracts may in addition to their effects on inhibition of absorption of the intestinal glucose and sensitivity of insulin, exert their anti-diabetic effect through stimulation of both the synthesis and secretion of insulin. Future research in our department will focus on the isolation, identification and characterization of the bioactive principles responsible for the different anti-diabetic mechanisms of this plant, *Momordica balsamina* crude fruit extracts.

Acknowledgement

The authors would like to acknowledge Dr OO Olaokun of the University of Pretoria for the contribution of RIN-m5F cells and Dr Mapula Razwinani of the Tshwane University of Technology for assisting with the Real-Time PCR instrument.

Funding

The Research Development Grant of the Sefako Makgatho Health Sciences University supported this work.

Author Contribution

Ananias Hodi Kgopa: Carried out the experimental work and writing of the manuscript.

Motetelo Alfred Mogale: Contributed in the design, supervision of the study, analysis of the data and editing of the manuscript.

Leshweni Jerry Shai: Contributed with the co-supervision of the study, modification of methodologies and analysis of results.

All authors have proofread and approved the final manuscript.

Declaration of Conflict of Interest

The authors declare no conflict of interest with respect to the research, authorship and publication of this article.

References

Ajabnoor, M. A. (1990). Effect of aloes on blood glucose levels in normal and alloxan diabetic mice. Journal of ethnopharmacology, 28(2), 215-220.

Bahuguna, A., Khan, I., Bajpai, V. K., & Kang, S. C. (2017). MTT assay to evaluate the cytotoxic potential of a drug. Bangladesh Journal of Pharmacology, 12(2), Online-Apr.

Bhardwaj, N., Gauttam, V., & Kalia, A. (2010). Evaluation of antidiabetic activity of Momordica balsamina Linn seeds in experimentally-induced diabetes. Journal of Chemical and Pharmaceutical Research, 2(5), 701-707.

Cerf, M. E. (2013). Beta cell dysfunction and insulin resistance. Frontiers in endocrinology, 4, 37.

Chaudhury, A., Duvoor, C., Reddy Dendi, V. S., Kraleti, S., Chada, A., Ravilla, R., ... & Sasapu, A. (2017). Clinical review of antidiabetic drugs: implications for type 2 diabetes mellitus management. Frontiers in endocrinology, 8, 6.

Davies, M. J., D'Alessio, D. A., Fradkin, J., Kernan, W. N., Mathieu, C., Mingrone, G., ... & Buse, J. B. (2018). Management of hyperglycaemia in type 2 diabetes, 2018. A consensus report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetologia, 61(12), 2461-2498.

Fu, Z., R Gilbert, E., & Liu, D. (2013). Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. Current diabetes reviews, 9(1), 25-53.

Govindappa, M. (2015). A review on role of plant (s) extracts and its phytochemicals for the management of diabetes. J Diabetes Metab, 6(7), 1-38.

Joseph, B., & Jini, D. (2013). Antidiabetic effects of Momordica charantia (bitter melon) and its medicinal potency. Asian Pacific Journal of Tropical Disease, 3(2), 93-102.

Karthikeyan, R., Somasundaram, S. T., Manivasagam, T., Balasubramanian, T., & Anantharaman, P. (2010). Hepatoprotective activity of brown alga Padina boergesenii against CCl4 induced oxidative damage in Wistar rats. Asian Pacific Journal of Tropical Medicine, 3(9), 696-701.

Kaushik, U., Aeri, V., Showkat, R. M., & Ali, M. (2017). Cucurbitane-type triterpenoids from the blood glucose-lowering extracts of Coccinia indica and Momordica balsamina fruits. Pharmacognosy Magazine, 13(Suppl 1), S115.

Keller, A. C., Ma, J., Kavalier, A., He, K., Brillantes, A. M. B., & Kennelly, E. J. (2011). Saponins from the traditional medicinal plant Momordica charantia stimulate insulin secretion in vitro. Phytomedicine, 19(1), 32-37.

Kgopa, A. H., Shai, L. J., & Mogale, M. A. (2020). Effects of Sclerocarya birrea Stem-Bark Extracts on Glucose Uptake, Insulin Synthesis and Expression of Selected Genes Involved in the Synthesis and Secretion of Insulin in Rat Insulinoma Pancreatic Beta Cells. Asian Journal of Chemistry, 32(9), 2195-2202.

Komatsu, M., Takei, M., Ishii, H., & Sato, Y. (2013). Glucose-stimulated insulin secretion: A newer perspective. Journal of diabetes investigation, 4(6), 511-516.

Mogale, M. A., Lebelo, S. L., Thovhogi, N., De Freitas, A. N., & Shai, L. J. (2011). α-Amylase and α-glucosidase inhibitory effects of Sclerocarya birrea [(A. Rich.) Hochst.] subspecies caffra (Sond) Kokwaro (Anacardiaceae) stem-bark extracts. African Journal of Biotechnology, 10(66), 15033-15039.

Mukesh, R., & Namita, P. (2013). Medicinal plants with antidiabetic potential-A review. American-Eurasian J Agric Environ Sci, 13(1), 81-94.

Nkansah, M. A., Shamsu–Deen, M., & Opoku, F. (2018). Phytocompounds, Heavy Metal and Mineral Contents in honey Samples from Selected Markets in the Kumasi Metropolis. Emerging Science Journal, 2(5), 287-294.

Noor, A., Gunasekaran, S., & Vijayalakshmi, M. A. (2017). Improvement of insulin secretion and pancreatic β-cell function in streptozotocin-induced diabetic rats treated with Aloe vera extract. Pharmacognosy research, 9(Suppl 1), S99.

Otimenyin, S. O., Uguru, M. O., & Ogbonna, A. (2008). Antimicrobial and hypoglycemic effects of Momordica balsamina. Linn. J Nat Prod, 1, 3-9.

Prentki, M., & Nolan, C. J. (2006). Islet β cell failure in type 2 diabetes. The Journal of clinical investigation, 116(7), 1802-1812.

Ren, J., Jin, P., Wang, E., Liu, E., Harlan, D. M., Li, X., & Stroncek, D. F. (2007). Pancreatic islet cell therapy for type I diabetes: understanding the effects of glucose stimulation on islets in order to produce better islets for transplantation. Journal of Translational Medicine, 5(1), 1-15.

Roffey, B. W., Atwal, A. S., Johns, T., & Kubow, S. (2007). Water extracts from Momordica charantia increase glucose uptake and adiponectin secretion in 3T3-L1 adipose cells. Journal of Ethnopharmacology, 112(1), 77-84.

Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., ... & Shaw, J. E. (2019). Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas. Diabetes research and clinical practice, 157, 107843.

Sagbo, I. J., van de Venter, M., Koekemoer, T., & Bradley, G. (2018). In vitro antidiabetic activity and mechanism of action of Brachylaena elliptica (Thunb.) DC. Evidence-Based Complementary and Alternative Medicine, 2018.

Salehi, B., Ata, A., V Anil Kumar, N., Sharopov, F., Ramírez-Alarcón, K., Ruiz-Ortega, A., ... & Iriti, M. (2019). Antidiabetic potential of medicinal plants and their active components. Biomolecules, 9(10), 551.

Santa Cruz, X. M., & Sfara, V. (2018). Chemical Signals are Involved in the Detection and Preference of Food Sources in Blattella Germanica. Emerging Science Journal, 2(5), 261-271.

Sowemimo, A., Okwuchuku, E., Samuel, F. M., Ayoola, O., & Mutiat, I. (2015). Musanga cecropioides leaf extract exhibits anti-inflammatory and anti-nociceptive activities in animal models. Revista Brasileira de Farmacognosia, 25(5), 506-512.

Thakur, G. S., Bag, M., Sanodiya, B. S., Bhadauriya, P., Debnath, M., Prasad, G. B. K. S., & Bisen, P. S. (2009). Momordica balsamina: a medicinal and neutraceutical plant for health care management. Current pharmaceutical biotechnology, 10(7), 667-682.

Thorens, B. (2015). GLUT2, glucose sensing and glucose homeostasis. Diabetologia, 58(2), 221-232.

Vanderford, N. L., Andrali, S. S., & Özcan, S. (2007). Glucose induces MafA expression in pancreatic beta cell lines via the hexosamine biosynthetic pathway. Journal of Biological Chemistry, 282(3), 1577-1584.

Wajid, S., Menaka, M., Ahmed, F., & Samreen, S. (2019). A literature review on oral hypoglycemic drugs – mechanistic aspects. Asian J. Pharm. Clin. Res, 12(11), 5-10.

Zandi, M., Masoumian, M., Shariatinia, A., & Sanjabi, M. R. (2016). Optimal concentrations and synergistic effects of some herbal extracts on viability of dermal fibroblasts. Gene Cell Tissue, 3(4), 1-7.

Zhang, X., Han, W., Jiang, X., Li, M., Gao, L., & Zhao, J. J. (2014). Chronic leucine exposure results in reduced but reversible glucose-stimulated insulin secretion in INS-1 cells. Molecular medicine reports, 9(6), 2554-2558.