Insulinotropic actions of *Moringa oleifera* involves the induction of membrane depolarization and enhancement of intracellular calcium concentration in BRIN-BD11 clonal pancreatic cells

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ABSTRACT

Objective: *Moringaoleifera* extracts have been widely reported for its insulinotropic and other antidiabetic effects. However, mechanisms behind the insulinotropic actions of *M. oleifera*extracts are not well understood. This study investigated the mechanism underlying the insulinotropic actions of acetone extract of *M. oleifera*

Method: Phytochemical composition of *M. oleifera* extracts was determined using standard procedures. Total flavonoid and total phenolic compounds in the extract were also quantified. Effects of the extracts on glucose stimulated insulin secretion (GSIS), membrane depolarization and intracellular calcium concentration using BRIN-BD11 clonal pancreatic beta cells.

Results: Resultsobtained showed the preponderance of alkaloids, flavonoids, glycosides, phenols, saponins and tannins in the extract. Total flavonoids and phenolic contents of the extract were estimated as 25.23±0.57mgQE/g and 54.26±1.89 mgGE/g respectively. The glucose dependent insulinotropic effects of the extract were significantly inhibited in the presence of diazoxide (48%, P<0.001) or verapamil (35%, P<0.001) and in the absence of extracellular calcium (47%, P<0.001). Co-incubation of cells with the extract and IBMX or tolbutamide increased insulin secretion by 2-fold while a 1.2-fold (P<0.05) increase was observed in cells depolarized with KCl (30mM)in the presence of the plant extract. The extract significantly induced membrane depolarization (7.1-fold, P<0.001) and enhanced intracellular calcium concentration (2.6-fold, P<0.01) in BRIN-BD11 cells.

Conclusion: These observations suggest that the insulinotropic actions of acetone extract of *M. oleifera* may be mediated via the K_{ATP}-dependent pathway of insulin release.

Keywords: Glucose stimulated insulin secretion, K_{ATP}-dependent pathway, *Moringaoleifera*, BRIN-BD11 cells, membrane potential, intracellular calcium

INTRODUCTION

Type 2 diabetes is characterized by relative insulin insufficiency or insulin resistance and accounts for about 90% of all cases of diabetes globally [1]. This has made the development of agents that could either improve insulin secretion or insulin sensitivity an important approach towards the treatment of the disease. Sulphonyurea is a well-known class of molecules with the ability to stimulate insulin release by pancreatic beta cells via the inhibition of the K_{ATP} channel [2]. However, side effects such as the risk of hypoglycaemia and promotion of weight gain have limited their use in the treatment of type 2 diabetes; resulting in increased interests in research targeted at the identification of novel insulinotropic agents with better therapeutic potential.

Insulinotropic effects of several agents, including novel endogenous peptides or their analogues [3,4], exogenous peptides such as amphibian host-defence peptides [5] and several plant compounds [6-8] have been reported. In addition to several other antidiabetic effects of *Moringaoleifera*[9-11], insulinotropic actions of various extracts of the plant were recently reported [12]. Our previous study reported significant concentration-dependent stimulation of insulin release from BRIN BD11 clonal pancreatic beta cells by aqueous and acetone extracts of *M. oleifera*. However, mechanisms underlying the insulinotropic actions of these extracts are not yet understood. *Moringaoleifera* (commonly known as drumstick tree) is indigenous to India but is widely cultivated in many tropical and subtropical parts of the world [10]. In addition to many medicinal traditional uses which include treatment of diabetes [9-12],*M. oleifera* leaves and pods are usually consumed as vegetables. The present study screened acetone extracts of *M. oleifera* for key phytochemicals, assessed the effects of the extracton glucose stimulated insulin secretion and monitored changes in the insulinotropic actions of the extract in the presence of modulators of cellular insulin secretion. The study also

investigated the involvement of extracellular calcium in the insulin-releasing effects of acetone extracts of *M. oleifera*and examined the effect of the extract on membrane depolarization and intracellular calcium concentration. Membrane depolarization and increased intracellular calcium concentration are two key events in the well-known K_{ATP}-dependent insulin secretion pathway in BRIN-BD11 clonal pancreatic cells[13]. Agents, such as diazoxide and verapamil, are known to modulate the activities of membrane receptors involved in the K_{ATP}-dependent insulin secretion pathway while KCl is a known membrane depolarizing agent, hence their inclusion in this study [13].

MATERIALS AND METHODS

Plant material preparation

Preparation of the plant materials has been described in details elsewhere [12]. Briefly, botanically identified fresh leaves of *M. oleifera* (voucher specimen No: OO2013-04), collected in Yola, Nigeria, were dried at room temperature and pulverized using a pestle and mortal. Pulverized plant material was sequentially extracted with dichloromethane, acetone, ethylacetate and water as described previously [12].Dried acetone fraction, redissolved in Krebs Ringers Bicarbonate buffer, was used for this study.

Phytochemical screening

Screening of the acetone fraction of *Moringa oleifera* leaves extracts for the presence of saponins, tannins, triterpines, alkaloids and flavonoidswas carried out as previously described [14].

Measurement of total phenolic and total flavonoid contents

Total phenolic content of the plant extract was estimated by Folin-Ciocalteu'smethod as described by Kim *et al*[15]. The plant extract or standard solution (200 µl) was mixed with 1

ml of Folin-Ciocalteu's reagent and incubated for 5 min at room temperature prior to the addition of 10ml of $NaCO_3$ (7%) and 13ml of distilled water. The mixture was incubated at 23^{o}C in the dark for 90min prior to measuring the absorbance at 750nm. Total phenolic content was extrapolated from a standard curve made with gallic acid (0 - 1000µg) and was expressed as gallic equivalent per gram of sample (GE/g). Estimation of total flavonoid content was conducted as described by Park *et al*. (2008). Plant sample (0.3ml) or standard was mixed with 3.4ml of methanol (30%), 0.15ml of $NaNO_2$(0.5M) and 0.15ml of $AlCl_3.6H_2O$ (0.3M) and incubated for 5min before the addition of 1ml NaOH (1M) to stop the reaction. Absorbance was measured at 506nm. Flavonoid content was extrapolated from a standard curve made with quercetin(0 - 1000µg) and expressed asquercetin equivalents per gram sample (QE/g).

In vitro insulin-releasing studies

Invitro insulin-releasing effects of the various extracts of *M. oleifera* were assessed using BRIN-BD11 rat clonal β-cells maintained at 37^{o}C in an atmosphere of 5% CO_2 and 95% air in RPMI-1640 supplemented with 10% (v/v) foetal calf serum, antibiotics (100 U/ml penicillin, 0.1mg/ml streptomycin) and 11.1mM glucose. Cells, seeded into 24-well plates (10^5 cells per well)and allowed to attach overnight as previously described [12, 13].After an overnight culture, culture media containing foetal calf serum were poured off and cells were pre-incubated for 40 min in Krebs Ringers Bicarbonate (KRB) buffer (pH 7.4) supplemented with 1.1mM glucose prior to test incubations performed for 20 min in the presence of 5.6 or 16.7mMglucoseand different modulators of insulin release in the absence or presence of the plant extract (100µg/ml).In another set of experiments, cells were incubated with the plant extract for 20 min using calcium free KRB buffer. After incubation, aliquots of cell

supernatants were removed for insulin measurement by radioimmunoassay as described previously [12, 16].

Intracellular calcium ($[Ca^{2+}]_i$) and membrane potential assays

Effects of *M. oleifera* extracts on membrane depolarization and $[Ca^{2+}]_i$ were determined fluorimetricallyin BRIN-BD11 cells as previously described [13] using a membrane potential assay kit or a Ca^{2+} assay kit from Molecular Devices (Sunnyvale, CA, USA) according to the manufacturer's protocols. Briefly, cells were seeded at a density of 1 million cells per well and allowed to attach overnight. Following a 10 min pre-incubation at 5.6mM glucose with KRB buffer (for membrane potential) or KRB buffer supplemented with 5µMprobenecid and 12.7mM $CaCl_2$ (for intracellular calcium), cells were incubated with the appropriate dye for 1 h at 37^{o}C. Plant sample (10µg/ml) or appropriate positive controls were added to the solution at data were acquired using a FlexStation scanning fluorimeter with integrated fluid transfer workstation (Molecular Devices, USA) every 1.52s for 5 min.

Statistical Analysis

Results are expressed as mean± SEM with n=8. Values were compared using two-way ANOVA followed by Newman-Keuls post-hoc test. Groups of data were considered to be significantly different if $P<0.05$.

RESULTS

Phytochemical composition and total flavonoid and phenolic contents of *M. oleifera*acetone extract

Phytochemical screening of revealed the presence of alkaloids, flavonoids, glycosides, phenols, saponins and tannins. In addition, total flavonoids and phenolic content of theextract

were estimated as 25.23±0.57mgQE/g and 54.26±1.89 mgGE/g respectively. Values were interpolated using standard curves with R^2= 0.949 or 0.964 for flavonoids or phenolic compounds respectively.

Effects of *M. oleifera* extracts on glucose stimulated insulin secretion

Basal insulin secretion from BRIN-BD11 cells in the absence of the plant extract increased by 1.82-fold as the concentrationof glucose increased from 1.1mM to 5.6mM (Figure 1). This stimulation index was increased to 1.98-fold in the presence of acetone extracts of *M. oleifera*. Similarly, the stimulation index increased from 1.59-fold to 1.69-fold in the presence of *M. oleifera* extract as the concentration of glucose increased from 5.6mM to 16.7mM.

The role of the K-ATP channel and membrane depolarization in the insulinotropic effects of *M. oleifera* extracts

Diazoxide (300mM),a K-ATP-channel activator, reduced basal insulin release from BRIN-BD11 cells at 5.6mM glucose by 31% (P<0.001) in the absence of the plant extract and produced a higher insulin-release inhibitory effects (48%, P<0.001) in the presence of the plant extract (Figure 2). Conversely, insulin-release increased by 2-fold (P<0.001) in the presence of the plant extract and 200µM tolbutamide (Figure 2). Similarly, an increase of 1.2-fold (P<0.05) was observed in cells depolarized with KCl (30mM) and high glucose concentration (16.7mM). No augmentation of insulin release was observed in the presence of metformin (100mM) and acetone extracts of *M. oleifera.*

Observation of changes in membrane potentials in BRIN-BD 11 cells showed that at a glucose concentration of 5.6 mM, acetone extract of *M. oleifera* elicited a sustained

membrane depolarization in BRIN-BD11 cells (Figure 3A). Though the depolarization produced by the extract was less than that produced by 30 mMKCl (data not shown), it was significantly ($P< 0.001$) greater than the depolarization produced by glucose alone. The integrated responses (area under the curve) for the extract showed a 7.1-fold increase in membrane depolarization compared with glucose alone (Figure 3B).

The role of the voltage dependent calcium channel and calcium concentrations in the insulinotropic effects of *M. oleifera* extracts

Verapamil (50mM), an inhibitor of the voltage dependent calcium channel, reduced basal insulin release from BRIN-BD11 cells at 5.6mM glucose by 33% ($P<0.001$) in the absence of the plant extract and by (35%, $P<0.001$) in the presence of the plant extract (Figure 4A). On the other hand, insulin release from cells co-incubated with IBMX (200µM) and acetone extracts of *M. oleifera*(100µg/ml) was 2.0-fold ($P<0.001$) higher compared with insulin release from cells incubated with IBMX alone (Figure 4A). However, in the absence of extracellular calcium, basal insulin release from BRIN-BD11 cells at 5.6mM glucose reduced by 28% ($P<0.01$) compared with 47% ($P<0.001$) (Figure 1B) reduction observed in the presence of acetone extract of *M. oleifera*.

Effects of the plant extract or alanine (a positive control) on the influx of extracellular calcium was investigated by monitoring the rise in intracellular calcium concentrations in BRIN-BD11 cells. While alanine produced a sharp rise in the concentration of intracellular Ca^{2+} that was significantly greater than the increase produced by 5.6mM glucose alone (data not shown), acetone extract of *M. oleifera* produced only a mild increase in intracellular concentration with a significantly higher effect observed in the first 30s of incubation (Figure

5A). A graph of area under the curve values showed an increase of 2.6-fold ($P<0.01$) in the presence of the extract compared with glucose alone (Figure 5B).

DISCUSSION

Insulinotropic actions of many plant extracts have been linked to their phytochemical constituents [17]. In addition, purified plant compounds such as rutin and apigenin have been reported to stimulate insulin release from isolated rat islets [18]. Moreover, isolation of bioactive compounds such as methyl 2-[4-(α-L-rhamnopyranosyl)phenyl]acetate, N-[4-(β-L-rhamno-pyranosyl)benzyl]-1-O-α-D-glucopyranosylthiocarboxamide, 1-O-phenyl-α-D-rhamnopyra-noside, 4-[(β-D-glucopyranosyl)-(1→3)-(α-L-rhamnopyranosyl)]-phenyl-acetonitrile, 4-(α-L-rhamnopyranosyl)-phenylacetonitrile, methyl-N-{4-[(4'-O-acetyl-α-L-rhamnopyranosyl)-benzyl]}-thiocarbamate, methyl-N-{4-[(α-L-rhamnopyranosyl)benzyl]}-carbamate and methyl-N-{4-[(4'-Oacetyl-α-L-rhamnopyranosyl)benzyl]}carbamate from *M. oleifera* extracts has been reported [12]. These compounds together with high contents of flavonoid and phenolic compounds in the acetone extracts of *M. oleifera* may play a significant role in its previously reported insulinotropic actions and effects observed in this study. Results obtained in the present study indicated that the insulinotropic effect of the extract is glucose-dependent and its stimulation index (fold-increase as glucose concentration changes) increases with increasing glucose concentration. Glucose level in normal individuals is around 4mM while non-fasting plasma glucose levels greater than 9mM is indicative of type 2 diabetic condition in humans. Moreover,glucose stimulated insulin secretion is usually activated at glucose concentrations greater than 5.6mM. Therefore, effects of the plant extract on insulin-secretion was examined at 5.6mMas well as at 16.7mM glucose concentrations with the later mimicking glucose concentrations observed in people with type 2 diabetes.No significant stimulation of insulin release is usually observed at low

glucose concentrations such as 1.1mM used in Figure 1 while higher glucose concentrations such as 16.7mM could result in hyper-polarization which may mask other effects investigated in this study. Our previous studies have shown that 5.6mM glucose produces mild stimulation of insulin-release which is sufficient to study effects of insulinotropic agents such as plant phytochemicals and peptides [12,13].

Most insulinotropic drugs, particularly sulphonyureas, act via the blockage of the ATP-dependent K^+ channel to stimulate insulin release [19]. To study the involvement of the K_{ATP}-dependent pathway in the insulinotropic actions of acetone extracts of *M. oleifera*, changes in the insulin secretion responses to the plant extract in the presence of inhibitors such as diazoxide, a K_{ATP}-channel activator [20]or verapamil which blocks the L-type voltage dependent calcium channelwere examined. The significant reduction in the stimulatory effects of the plant extract in the presence of verapamil or diazoxide strongly suggests that the insulin-releasing effects of the plant extract are mediated partly via the K_{ATP}-dependent pathway. Similar inhibition of insulinotropic effects have been reported for some plant extracts [21] and some recently identified novel insulin-releasing amphibian host defence peptides [15, 22].

Increased membrane potential is a key feature of the K_{ATP}-dependent pathway of insulin secretion [23]. The binding of an insulinotropic agent (such as glibenclamide, a sulphonyureas) to the ATP-dependent K^+ channel on beta cell membranes inhibits the hyperpolarizing outflow of potassium, resulting in membrane depolarization [24]. Though the identification of the specific binding site of the active component of the acetone extract of *M. oleifera* is beyond the scope of the present study, investigations conducted in this study revealed that the plant extract significantly induced membrane depolarization in BRIN-BD11 cells. Moreover, the combination of high glucose concentration (16.7mM) and KCl (30mM)

was used to induce extensive cell depolarization with a view to examining the effect of the plant extracts in depolarized cells. In the presence of a depolarizing concentration of KCl, more potassium ion moves into the cell leading to increased positively charged ions within the cell and subsequent membrane depolarization.Effects of the plant extracts on membrane depolarization in normal cells and in cells depolarized with KCl, coupled with the reduced insulin-releasing effects observed in the presence of diazoxide, further implicates the K_{ATP}-channel in the insulinotropic actions of acetone extracts of *M. oleifera*.

The opening of the voltage dependent calcium channel (VDCC) and the subsequent increase in intracellular calcium are attendant effects of increased membrane depolarization in beta cells [23,24]. This increase in intracellular calcium culminates in the fusion of insulin containing vesicles with the cell membrane and the eventual insulin secretion by exocytosis. Acetone extracts of *M. oleifera* elicited a mild increase in intracellular calcium in this study. Moreover, the insulinotropic effect of the plant extract was significantly reduced in the absence of extracellular calcium (Figure 1) and in the presence of verapamil, a known L-type calcium channel blocker (Figure 3A).

No stimulation of insulin secretion was observed in cells depolarized with KCl but additional stimulation of insulin secretion was observed in the presence of IBMX. This observation presupposes that the insulinotropic effects of the extract may not only be restricted to the K_{ATP}-dependent pathway. IBMX stimulates insulin release through the activation of adenylate cyclase leading to increased cAMP production and the recruitment of calcium from the endoplasmic reticulum [25]. The involvement of this pathway in the action of acetone extracts of *M. oleifera* is not yet fully understood and will form the focus of future studies.

In conclusion, this study has revealed that the previously reported insulinotropic effects of acetone extracts of *M. oleifera* is glucose-dependent and involve the stimulation of membrane depolarization and elevation of intracellular calcium concentration. These observations provide preliminary explanations for insulinotropic and antidiabetic effects previously reported for *M. oleifera* extracts.

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Not applicable

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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LEGEND TO FIGURES

Figure 1: Effects of acetone extracts of *Moringaoleifera* on glucose stimulated insulin secretion by BRIN BD11 cells. Values are mean ± SEM with n=8. *P<0.05, **P<0.01, ***P<0.001 compared with 5.6mM glucose without *M. oleifera* extract. $^{\Delta\Delta}$P<0.01, $^{\Delta\Delta\Delta}$P<0.001 compared with 5.6mM glucose with *M. oleifera* extract. $^{++}$P<0.01, $^{+++}$P<0.001 compared with respective incubations in the absence of *M. oleifera* extracts.

Figure 2: Effects of acetone extracts of *Moringaoleifera* on insulin secretion by BRIN BD11 cells in the presence of modulators of K-ATP channel activities. Values are mean ± SEM with n=8. *P<0.05, **P<0.01, ***P<0.001 compared with 5.6mM glucose without *M. oleifera* extract. $^{\Delta\Delta\Delta}$P<0.001 compared with 5.6mM glucose with *M. oleifera* extract. $^{+}$P<0.05, $^{++}$P<0.01 compared with respective incubations in the absence of *M. oleifera* extracts.

Figure 3: Figure 3: Effects of acetone extracts of *Moringaoleifera* on membrane depolarization in BRIN BD11 cells. Values are mean ± SEM with n=8. ***P<0.001 compared with 5.6mM glucose.

Figure 4: Effects of acetone extracts of *Moringaoleifera* on insulin secretion by BRIN BD11 cells in the presence of agents that increase intracellular calcium concentration (A) and in the absence of extracellular calcium (B). Values are mean ± SEM with n=8. *P<0.05, **P<0.01, ***P<0.001 compared with 5.6mM glucose without *M. oleifera* extract. $^{\Delta\Delta\Delta}$P<0.001 compared with 5.6mM glucose with *M. oleifera* extract. $^{+}$P<0.05, $^{++}$P<0.01 compared with respective incubations in the absence of *M. oleifera* extracts.

Figure 5: Effects of acetone extracts of *Moringaoleifera* on intracellular calcium concentration in BRIN BD11 cells.Values are mean ± SEM with n=8. ***P<0.001, compared with 5.6mM glucose.