



# Insulinotropic actions of *Moringa oleifera* involves the induction of membrane depolarization and enhancement of intracellular calcium concentration

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## ABSTRACT

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

**Materials and Methods:** Phytochemical composition of *M. oleifera* extract 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, membrane depolarization, and intracellular calcium concentration were investigated using BRIN-BD11 clonal pancreatic beta-cells. **Results:** Results obtained showed the preponderance of alkaloids, flavonoids, glycosides, phenols, saponins and tannins in the extract. The glucose-dependent insulinotropic effects of the extract were significantly inhibited in the presence of diazoxide (48%) or verapamil (35%) and in the absence of extracellular calcium (47%). Co-incubation of cells with the extract and 3-isobutyl-1-methylxanthine or tolbutamide increased insulin secretion by 2-fold while a 1.2-fold increase was observed in cells depolarized with 30 mM KCl in the presence of the plant extract. The extract significantly-induced membrane depolarization (7.1-fold) and enhanced intracellular calcium concentration (2.6-fold) 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.

**KEY WORDS:** BRIN-BD11 cells, glucose-stimulated insulin secretion, intracellular calcium, KATP channel, membrane potential, *Moringa oleifera*

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## 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. Sulphonylurea 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 hypoglycemia 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-defense peptides [5] and several plant compounds [6-8] have been reported. In addition to several other anti-diabetic effects of *Moringa oleifera* [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. *M. oleifera* (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 extract on 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. 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 mortar. Pulverized plant material was sequentially extracted with dichloromethane, acetone, ethyl acetate and water as described previously [12]. Dried acetone fraction, re-dissolved in Krebs-Ringer Bicarbonate (KRB) buffer, was used for this study.

### Phytochemical Screening

Screening of the acetone fraction of *M. oleifera* leaves extracts for the presence of saponins, tannins, triterpenes, alkaloids, and flavonoids was 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's method as described by Kim *et al.* [15]. The plant extract or standard solution (200  $\mu$ l) was mixed with 1 ml of Folin-Ciocalteu's reagent and incubated for 5 min at room temperature prior to the addition of 10 ml of  $NaCO_3$  (7%) and 13 ml of distilled water. The mixture was incubated at 23°C in the dark for 90 min prior to measuring the absorbance at 750 nm. Total phenolic content was extrapolated from a standard curve made with gallic acid (0-1000  $\mu$ g) 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.* [16]. Plant sample (0.3 ml) or

standard was mixed with 3.4 ml of methanol (30%), 0.15 ml of  $NaNO_2$  (0.5 M) and 0.15 ml of  $AlCl_3 \cdot 6H_2O$  (0.3 M), and incubated for 5 min before the addition of 1 ml NaOH (1 M) to stop the reaction. Absorbance was measured at 506 nm. Flavonoid content was extrapolated from a standard curve made with quercetin (0-1000  $\mu$ g) and expressed as quercetin equivalents per gram sample (QE/g).

### *In vitro* insulin-releasing studies

*In vitro* insulin-releasing effects of the various extracts of *M. oleifera* were assessed using BRIN-BD11 rat clonal  $\beta$ -cells maintained at 37°C in an atmosphere of 5%  $CO_2$  and 95% air in RPMI-1640 supplemented with 10% (v/v) fetal calf serum, antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. Cells seeded into 24-well plates (10<sup>5</sup> cells per well) and allowed to attach overnight as previously described [12,13]. After an overnight culture, culture media containing fetal calf serum were poured off and cells were pre-incubated for 40 min in KRB buffer (pH 7.4), supplemented with 1.1 mM glucose prior to test incubations performed for 20 min in the presence of 5.6 or 16.7 mM glucose and different modulators of insulin release in the absence or presence of the plant extract (100  $\mu$ g/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 [12,17].

### Intracellular Calcium ( $[Ca^{2+}]_i$ ) and Membrane Potential Assays

Effects of *M. oleifera* extracts on membrane depolarization and  $[Ca^{2+}]_i$  were determined fluorimetrically in 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 5.6 mM glucose with KRB buffer (for membrane potential) or KRB buffer supplemented with 5  $\mu$ M probenecid and 12.7 mM  $CaCl_2$  (for intracellular calcium), cells were incubated with the appropriate dye for 1 h at 37°C. Plant sample (10  $\mu$ g/ml) or appropriate positive controls were added to the solution and data were acquired using a FlexStation scanning fluorimeter with integrated fluid transfer workstation (Molecular Devices) every 1.52 s for 5 min.

### Statistical Analysis

Results are expressed as mean  $\pm$  standard error of the mean 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**

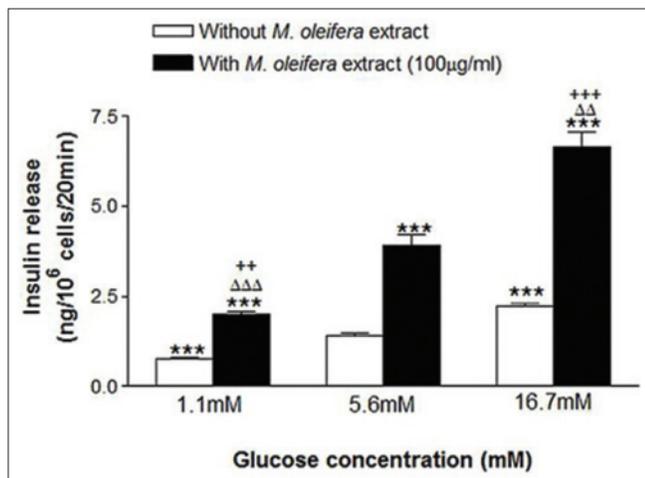
Phytochemical screening of *M. oleifera* acetone extract revealed the presence of alkaloids, flavonoids, glycosides, phenols, saponins, and tannins. In addition, total flavonoids and phenolic content of the extract were estimated to be  $25.23 \pm 0.57$  mgQE/g and  $54.26 \pm 1.89$  mgGE/g, respectively. 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 concentration of glucose increased from 1.1 mM to 5.6 mM [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 5.6 mM to 16.7 mM.

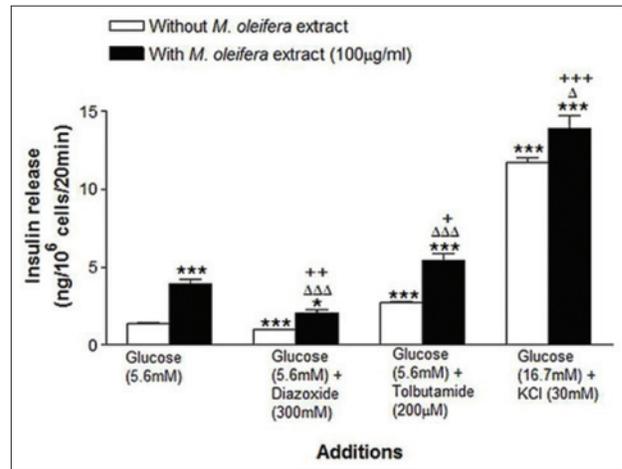
**The Role of the  $K_{ATP}$  Channel and Membrane Depolarization in the Insulinotropic Effects of *M. oleifera* Extracts**

Diazoxide (300 mM), a  $K_{ATP}$  channel activator, reduced basal insulin release from BRIN-BD11 cells at 5.6 mM glucose by 31% ( $P < 0.001$ ) in the absence of the plant extract and produced a higher insulin-release inhibitory effect (48%,  $P < 0.001$ ) in the presence of the plant extract [Figure-2]. Conversely, insulin-

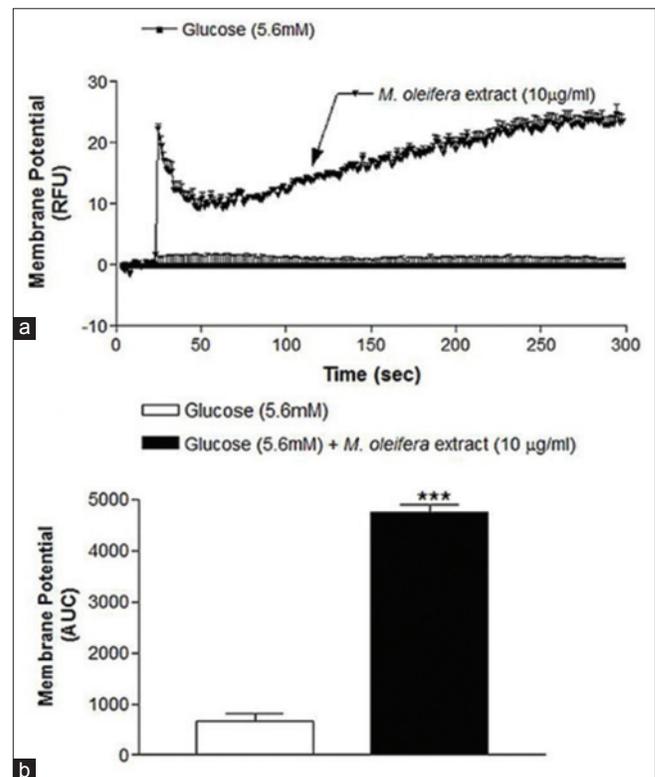


**Figure 1:** Effects of acetone extracts of *Moringa oleifera* on glucose-stimulated insulin secretion by BRIN-BD11 cells. Values are mean  $\pm$  standard error of mean with  $n = 8$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with 5.6 mM glucose without *M. oleifera* extract.  $\Delta\Delta P < 0.01$ ,  $\Delta\Delta\Delta P < 0.001$  compared with 5.6 mM glucose with *M. oleifera* extract. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with respective incubations in the absence of *M. oleifera* extracts

release increased by 2-fold ( $P < 0.001$ ) in the presence of the plant extract and 200  $\mu$ M tolbutamide [Figure-2]. Similarly, an increase of 1.2-fold ( $P < 0.05$ ) was observed in cells depolarized with KCl (30 mM) and high glucose concentration (16.7 mM). No augmentation of insulin release was observed in the presence of metformin (100 mM) and acetone extracts of *M. oleifera*.



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

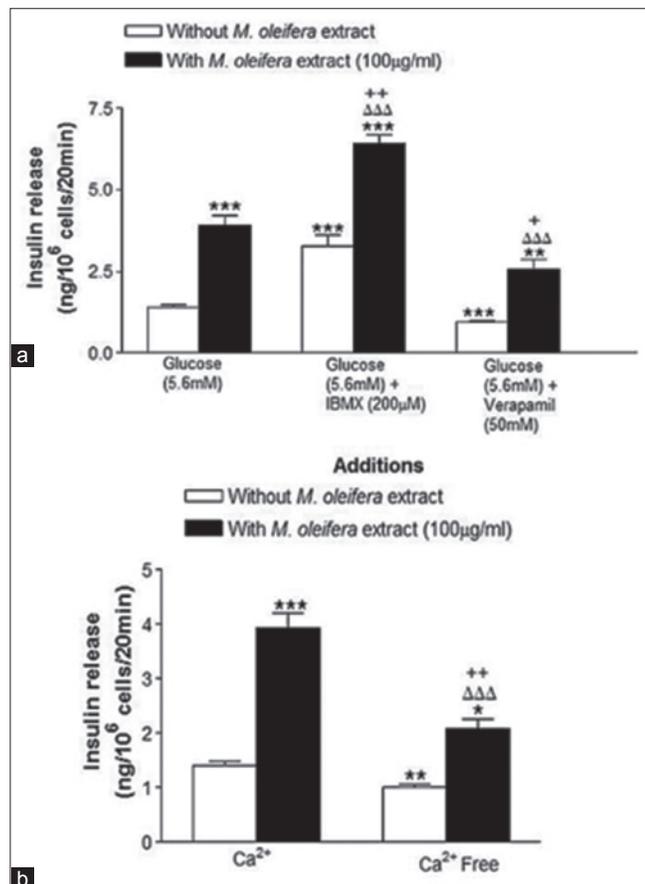


**Figure 3:** Effects of acetone extracts of *Moringa oleifera* on membrane depolarization in BRIN-BD11 cells. Values are mean  $\pm$  standard error of the mean with  $n = 8$ . \*\*\* $P < 0.001$  compared with 5.6 mM glucose

Observation of changes in membrane potentials in BRIN-BD11 cells showed that at a glucose concentration of 5.6 mM, acetone extract of *M. oleifera* elicited a sustained membrane depolarization [Figure 3a]. Though the depolarization produced by the extract was less than that produced by 30 mM KCl (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 (50 mM), an inhibitor of the voltage-dependent calcium channel, reduced basal insulin release from BRIN-BD11 cells at 5.6 mM 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



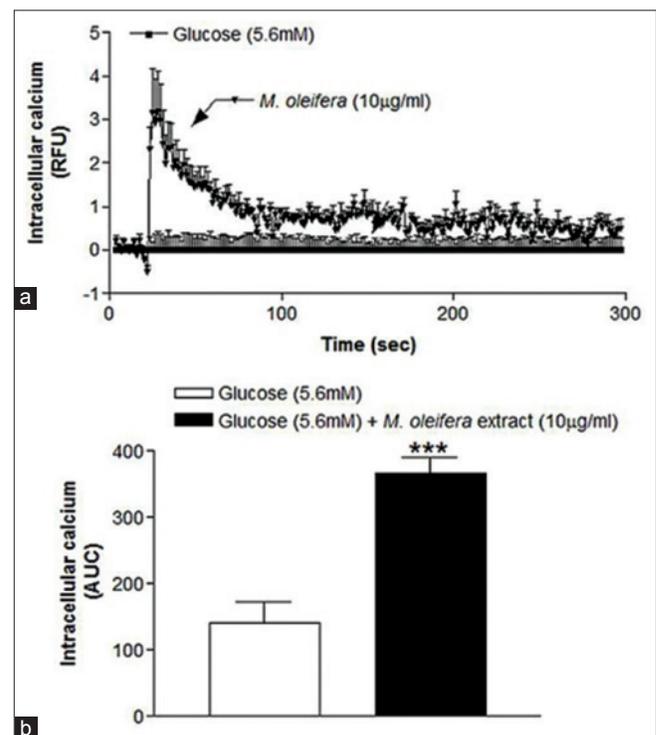
**Figure 4:** Effects of acetone extracts of *Moringa oleifera* on insulin secretion by BRIN-BD11 cells in the presence of agents that increase intracellular calcium concentration (a) and in the absence of extracellular calcium (b). Values are mean  $\pm$  standard error of mean with  $n = 8$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with 5.6 mM glucose without *M. oleifera* extract.  $\Delta\Delta\Delta P < 0.001$  compared with 5.6 mM glucose with *M. oleifera* extract.  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$  compared with respective incubations in the absence of *M. oleifera* extracts

from cells co-incubated with 3-isobutyl-1-methylxanthine (IBMX, 200 µM) and acetone extracts of *M. oleifera* (100 µg/ml) was 2-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 5.6 mM glucose reduced by 28% ( $P < 0.01$ ) compared with 47% reduction ( $P < 0.001$ , Figure 4b) 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 were investigated by monitoring the rise in  $[Ca^{2+}]_i$  concentrations in BRIN-BD11 cells. While alanine produced a sharp rise in the concentration of  $[Ca^{2+}]_i$  that was significantly greater than the increase produced by 5.6 mM 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 30 s 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 [18]. In addition, purified plant compounds such as rutin and apigenin have been reported to stimulate insulin release from isolated rat islets [19]. Moreover, isolation of bioactive compounds such as methyl 2-[4-( $\alpha$ -L-rhamnopyranosyl) phenyl] acetate, N-[4-( $\beta$ -L-rhamno-pyranosyl) benzyl]-1-O- $\alpha$ -D-glucopyranosylthiocarboxamide, 1-O-phenyl-



**Figure 5:** Effects of acetone extracts of *Moringa oleifera* on intracellular calcium concentration in BRIN-BD11 cells. Values are mean  $\pm$  standard error of the mean with  $n = 8$ . \*\*\* $P < 0.001$ , compared with 5.6 mM glucose

$\alpha$ -D-rhamnopyranoside, 4-[( $\beta$ -D-glucopyranosyl)-(1 $\rightarrow$ 3)-( $\alpha$ -L-rhamnopyranosyl)]-phenyl-acetonitrile, 4-( $\alpha$ -L-rhamnopyranosyl)-phenyl-acetonitrile, methyl-N-{4-[(4'-O-acetyl- $\alpha$ -L-rhamnopyranosyl)-benzyl]}-thiocarbamate, methyl-N-{4-[( $\alpha$ -L-rhamnopyranosyl) benzyl]}-carbamate and methyl-N-{4-[(4'-O-acetyl- $\alpha$ -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 4 mM while non-fasting plasma glucose levels  $>9$  mM is indicative of type 2 diabetic condition in humans. Moreover, glucose-stimulated insulin secretion is usually activated at glucose concentrations  $>5.6$  mM. Therefore, effects of the plant extract on insulin-secretion were examined at 5.6 mM as well as at 16.7 mM 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 like 1.1 mM [Figure 1] while higher glucose concentrations such as 16.7 mM could result in hyper-polarization, which may mask other effects investigated in this study. Our previous studies have shown that 5.6 mM glucose produces a 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 sulphonylureas, act via the blockage of the ATP-dependent  $K^+$  channels to stimulate insulin release [20]. 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 [21] or verapamil which blocks the L-type voltage-dependent calcium channel were 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 [22] and some recently identified novel insulin-releasing amphibian host defense peptides [15,23].

Increased membrane potential is a key feature of the  $K_{ATP}$ -dependent pathway of insulin secretion [24]. The binding of an insulinotropic agent (such as glibenclamide, a sulphonylurea) to the ATP-dependent  $K^+$  channel on beta-cell membranes inhibits the hyperpolarizing outflow of potassium, resulting in membrane depolarization [25]. 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 (16.7 mM) and KCl (30 mM) 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 and the subsequent increase in intracellular calcium are attendant effects of increased membrane depolarization in beta cells [24,25]. 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 [26]. 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 anti-diabetic effects previously reported for *M. oleifera* extracts.

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