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In vitro insulinotropic actions of various extracts of *Moringa oleifera* leaves

Ojo, O. Opeolu[1,2]
[1]Chevron Biotechnology Centre, Modibbo Adama University of Technology, PMB 2076, Yola, Nigeria
[2]SAAD Centre for Diabetes and Pharmacy, School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA, UK

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Abstract

This study investigated the *in vitro* insulin-releasing and cytotoxic effects of various extracts of *Moringa oleifera leaves.* Pulverized *Moringa oleifera* leaves were sequentially extracted with dichloromethane, acetone, ethylacetate and water. Clonal pancreatic beta cells, BRIN-BD11, were grown in an atmosphere of 5% CO_2 and 95% air in RPMI-1640. Insulin and LDH assays were carried out using Krebs Ringers Bicarbonate buffer. Insulin concentration was measured by radioimmunoassay and LDH release was assessed using a commercially available non-radioactive assay kit (Promega, UK). Aqueous extraction produced the best yield (361 mg/20kg powder). Acute incubation of BRIN-BD11 cells with different extracts of *M. Oleifera* produced a concentration-dependent stimulation of insulin-release at concentrations ≥10 µg/ml. The aqueous extract produced the best insulinotropic effect, with a maximum stimulation of 347% (P<0.001) of basal rate at 100µg/ml and the lowest stimulating concentration of 0.1 µg/ml. Dichloromethane and acetone extracts produced similar stimulatory effects (130% of basal rate, 100µg/ml, P<0.01). Insulin-releasing effects of the four extract were not associated with beta cell cytotoxicity. These results showed that extracts of *M. oleifera* possess insulinotropic effects and encourage further studies to uncover the physiological mechanism involved.

Keywords: Insulin-releasing, *Moringaoleifera*, BRIN-BD11 cells, cytotoxicity, anti-diabetic

Correspondence: *o.ojo@mautech.edu.ng*

Introduction

Records abound of the folkloric use of various plant-based preparations in the management of many diseases worldwide (Newman and Craig, 2007). Advances in science and technology exemplified by improved separation and purification techniques have significantly aided scientific investigations of the efficacy of many therapeutic natural products (Conlon *et al*, 2007). In addition, the development of various cell-lines for *in vitro* screening of drug candidates (McClenaghan *et al*, 1996) and various animal models of human diseases (King, 2012) has further encouraged bio-prospecting for novel agents that could cater for unmet needs in the management of many human diseases including diabetes mellitus. Increasing incidence of diabetes (Wild *et al*, 2004) and several challenges facing its management have motivated the search for novel agents with anti-diabetic effects.

Moringa oleifera is a tropical tree commonly found in Africa, the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan (Fahey, 2005). In addition to its several other uses, many recent studies have investigated nutritional and medicinal properties of *M. oleifera*, reporting its antibiotic (Caceres et al, 1991; Dahot, 1998), antitrypanosoma l(Mekonnen et al, 1999), hypotensive (Faizi et al, 1994), anti-cancer (Costa-Lutofo et al, 2005), anti-inflammatory (Ezeamuzie et al, 1996), hypocholesterolemic (Mehta et al, 2003) and hypoglycemic activities (Kar et al, 2003). Several bioactive compounds, including carbamates and thiocarbamates (Francis et al, 2004) (Table 1), isothiocyanates and several peptides (Fahey, 2005) have been isolated from the pod, leaves and roots of *M. oleifera*. This study investigated insulin-releasing effects of various extracts of *M. oleifera*

using clonal pancreatic cell-line, BRIN-BD11. We also assessed cytotoxic effects of the extracts, using the release of the cytosolic enzyme, lactate dehydrogenase (LDH) as a bio-marker.

Materials and Methods

Plant material preparation: Fresh leaves of *M. oleifera* were collected in August 2013 around Sangere Village in Girei Local Government Area of Adamawa State, Nigeria. After botanical identification, a voucher specimen (No: OO2013-04) was deposited in the herbarium of Chevron Biotechnology Centre, Modibbo Adama University of Technology, Yola. *M. oleifera* leaves were dried at room temperature and pulverized using a pestle and mortar into fine powder. Ground plant material was sequentially extracted with dichloromethane, acetone, ethylacetate and water. Briefly, dichloromethane extract was prepared by soaking 20g of the ground powder in 50ml of the solvent with intermittent shaking for 3 days. The mixture was first filtered with a double-layer of muslin cloth and then Whatman No.1 filter paper. The resultant residue was rinsed twice with dichloromethane and all the filtrates were pooled together. The solvent was removed from the extract at 40^{o}C in an oven. After complete drying, the yield of the extract was ascertained as presented in Table 2. The residue was subjected to further extraction sequentially with ethylacetate, acetone and water following the same extraction procedure described for dichloromethane.

In vitro insulin-releasing studies: Invitro insulin-releasing actions of the various extracts of *M. oleifera* were assessed using BRIN-BD11 rat clonal β-cells seeded into 24-well plates (10^5 cells per well). Cells were grown 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 as previously described (Abdel-Wahab et al, 2008; Mechkarska et al, 2010; Ojo et al, 2011). Prior to acute tests, cells were pre-incubated for 40 min at 37^{o}C in Krebs Ringers Bicarbonate (KRB) buffer (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1mM glucose. Test incubations were performed for 20 min at 37^{o}C in the presence of 5.6mM glucose and graded doses of different *M. oleifera* extracts (0 - 100µg/ml). After incubation, aliquots of cell supernatants were removed for insulin measurement by radioimmunoassay.

Measurement of insulin concentration: Measurement of insulin concentration was carried out as described by Flatt and Bailey (1981) using sodium phosphate (40 mM) supplemented with thiomerosal(0.02% w/v) and BSA (1% w/v). Insulin standard curve was prepared using crystalline rat insulin over a concentration range of 0.039 to 20.0 ng/ml. Insulin antibody (100µl, 1:40,000 dilution) and ^{125}I-labelled tracer (100µl, 10,000 cpm per tube) were added in triplicate to insulin standard and unknown samples and incubated for 48 hours at 4^{o}C. Following the addition of a suspension of 5% (v/v) dextran coated charcoal (5% w/v), the mixture was further incubated for 20 min, centrifuged (2500 rpm, 4^{o}C) and decanted for the measurement of free ^{125}I-insulin (now bound to charcoal) using a gamma counter. Insulin concentration were interpolated from the standard curve plotted using a spline curve-fitting algorithm using known concentrations of rats insulin.

Assessment of cytotoxicity: To determine cytotoxicity of plant extracts, BRIN-BD11 cells were seeded into 24-multiwell plates as previously described (Ojo et al, 2011). Following overnight culture in RPMI 1640, cells were pre-incubated in KRB buffer containing 1.1 mM glucose (1.0 ml) for 40 min at 37 °C. This was followed by a 20-min incubation with KRB buffer containing 5.6mM glucose in the presence or absence of the various extracts of *M. oleifera* or DMSO (40%). Concentrations of lactate dehydrogenase (LDH) in the cell assay supernatants were measured using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical Analysis: Results are expressed as mean ± S.E.M. and values were compared using two-way ANOVA followed by Newman-Keul's post-hoc test. Groups of data were considered to be significantly different if $P<0.05$.

Results

Extraction yield: Figure1 presents yield obtained from the sequential extraction of *M. oleifera* leaf powder with the different solvents. Extraction with ethylacetate produced the lowest yield while the highest yield was obtained for aqueous extraction.

Insulin- releasing effects of M. oleifera extracts: The basal rate of release of insulin from BRIN-BD11 cells in the presence of 5.6mM glucose alone was 0.91 ± 0.04 ng/10^6 cells/20 min. Addition of alanine (10mM) to the assay buffer increased this rate to 3.14 ± 1.14 ng/10^6 cells/20 min ($P < 0.001$; $n = 8$). Effects of the various extracts of *Moringa oleifera* on insulin-release from BRIN-BD11 cells at 5.6mM glucose is shown in Fig. 2. It showed that all the extracts produced a significant

(P<0.05) and concentration-dependent stimulations of insulin secretion at concentrations ≥ 10µg/ml. The aqueous extract exhibited the most potent effects, producing a significant stimulation of insulin release (154% of basal rate, P < 0.05) from BRIN-BD11 cells at a concentration of 0.1µg/ml, with a maximum response (347% of basal rate, P<0.001) at a concentration of 100µg/ml. Maximum insulin-releasing response (130% of basal rate, 100µg/ml, P<0.01) to acetone and dichloromethane extracts was moderate while a poor stimulatory effect was observed for incubation with ethylacetate extract (120%, 100µg/ml, P<0.05).

Cytotoxic effects of M. oleifera extracts: The release of the cytosolic enzyme, lactate dehydrogenase (LDH), due to damaged plasma membrane was used as a marker of cytotoxicity in this study. Significant (P<0.001) release of the enzyme was observed in incubations involving DMSO (40% v/v) (Fig 3). However, incubation with the different extracts of *M. oleifera* produced no significant release of LDH.

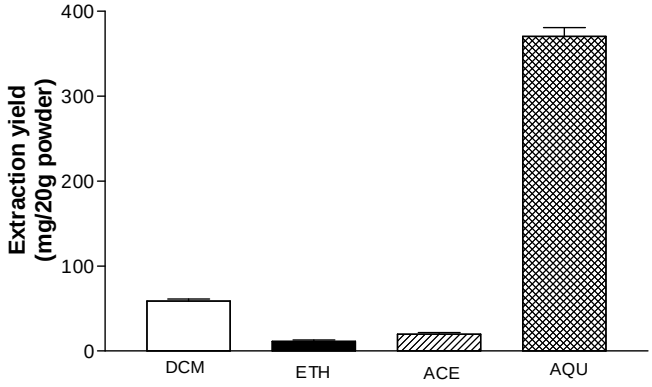

Fig. 1:*M. oleifera* extraction with different solvents.
DCM = Dichloromethane, ETH = Ethylacetate, ACE = Acetone and AQU = Aqueous

Table 1: Selected bioactive compounds isolated from *Moringa oleifera*

S/No	Name
1	Methyl 2-[4-(α-L-rhamnopyranosyl)phenyl]acetate
2	N-[4-(β-L-rhamnopyranosyl)benzyl]-1-O-α-D-
3	glucopyranosylthiocarboxamide
4	1-O-phenyl-α-D-rhamnopyranoside
5	4-[(β-D-glucopyranosyl)-(1→3)-(α-L-rhamnopyranosyl)]phenylacetonitrile
6	4-(α-L-rhamnopyranosyl)phenylacetonitrile
7	Methyl N-{4-[(4'-O-acetyl-α-L-rhamnopyranosyl)benzyl]}thiocarbamate
8	Methyl N-{4-[(α-L-rhamnopyranosyl)benzyl]}carbamate
	Methyl N-{4-[(4'-Oacetyl-α-L-rhamnopyranosyl)benzyl]}carbamate

Francis *et al.*, 2004; Fahey, 2005

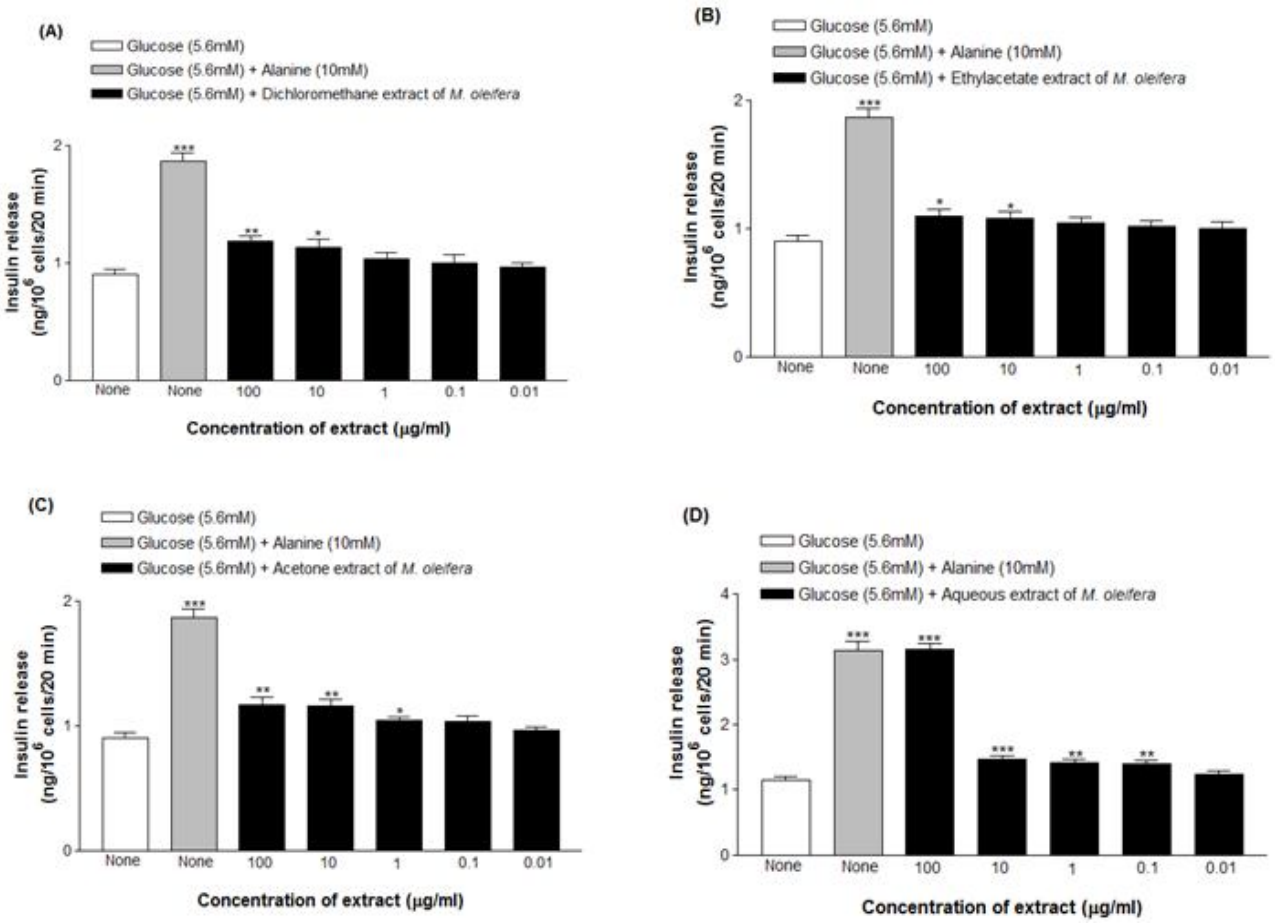

Fig. 2: Effects of various extracts of *M. oleifera* on the release of insulin from BRIN-BD11 clonal β-cells in the presence of 5.6 mM glucose. (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to glucose; n = 8).

Discussion

Pharmacological effects of several phytochemicals have been reported and studies providing scientific explanations for the ethnopharmacological use of many plants are increasing. Activities such as prevention of hyperglyceamia (Kar et al, 2003), inhibition of oxidative stress caused by streptozotocin in rats (Yassa and Tokamy, 2014) and prevention of the formation of advanced glycated end-products (Sangkitikomol et al, 2014) have been previously reported for *M. oleifera* extracts. An earlier study has also reported that N-benzyl thiocarbamates, N-benzyl carbamates, benzyl nitriles, and benzyl esterextracted from *M. oleifera* pod stimulated insulin secretion from the insulin secreting cell-line, INS-1 (Francis et al, 2004). Consistent with these studies, our results indicated significant stimulation of insulin-release by the aqueous and acetone extracts of *M. oleifera* leaves from BRIN-BD11 cells. BRIN-BD11 cell-line was generated by electrofusion of RINm5F cells with New England Deaconess Hospital rat pancreatic islet cells (McClenaghan et al, 1996) and has been used extensively to characterize insulinotropic effects of several agents, including crude plant extracts (Hannan et al, 2007), amphibian skin peptides (Ojo et al, 2011) and synthetic analogues of various endogenous peptides such as GIP and GLP-1 (Irwin et al, 2010).

Compared to other extracts, the superior insulinotropic actions of the aqueous extract coupled with significantly higher amount of yield generated following aqueous extraction suggests that the active ingredient in *M. oleifera* leaves might be water soluble. While the aqueous extract of *M. oleifera* powdered leaves (100mg/ml) has been reported to down-regulate the expression of some pro-inflammatory genes and increase cytotoxic effect of chemotherapy in pancreatic cancer cells

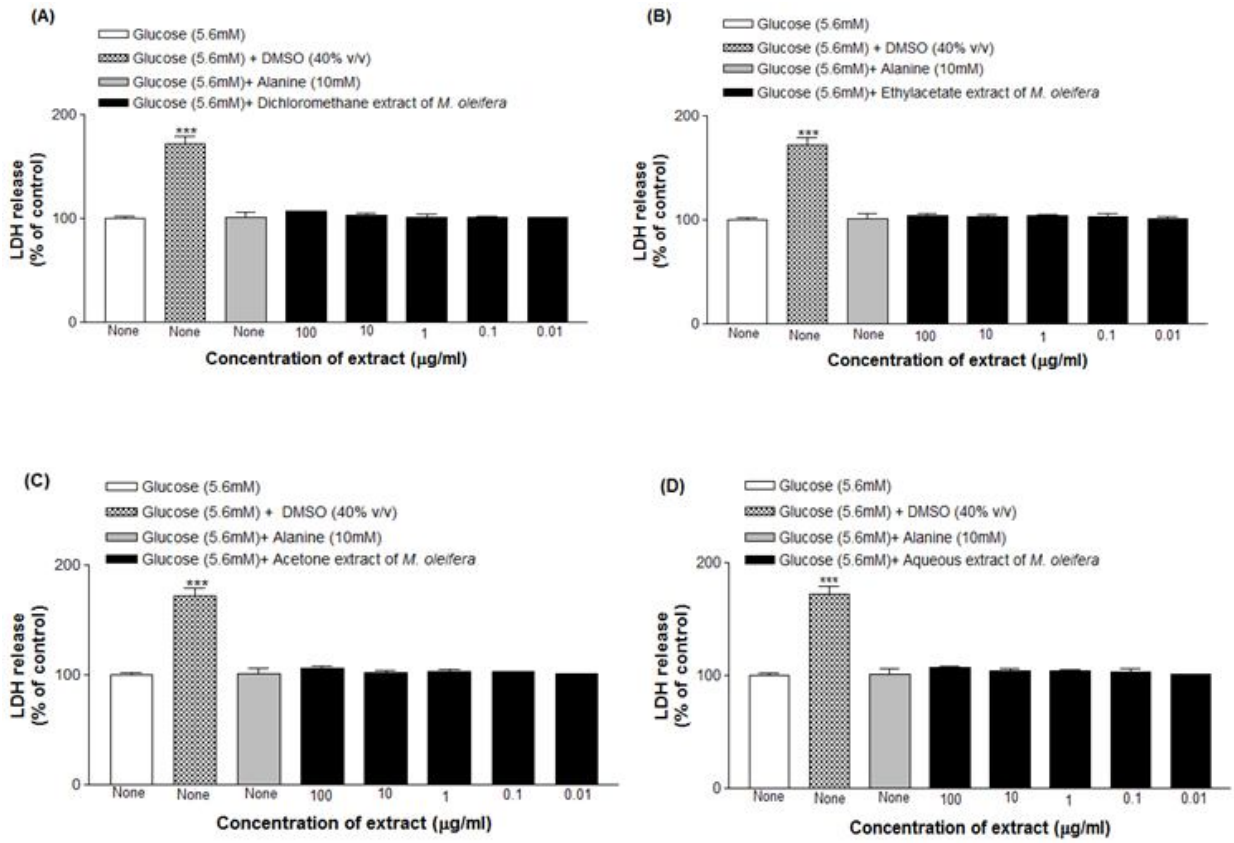

Fig. 3: Effects of various extracts of *M. oleifera* on lactose dehydrogenase from BRIN-BD11 clonal β-cells in the presence of 5.6 mM glucose.

(Berkovich et al, 2013), no cytotoxicity was observed following the incubation all four extracts of *M. oleifera* with clonal pancreatic beta cell, BRIN-BD11. Significant release of LDH in the presence of DMSO (40% v/v) observed in this study is consistent with previous reports of its cytotoxic actions at concentrations above 20% (v/v) (Violante et al, 2002). In addition, this result indicates that the integrity of the plasma membrane had been preserved and the increased insulin secretion observed in the presence of *M. oleifera* extracts reported is not a consequence of damaged plasma membrane, leading to uncontrolled release of cytosolic enzymes.

Conclusion

The insulin-releasing effects reported for extracts of *M. oleifera* leaves in this study partly provide preliminary explanation for the anti-diabetic effects previously reported for the plant. However, further studies to understand the molecular mechanisms underlying its insulinotropic actions are needed.

Conflict of Interest

The author declare no conflict of interests

Ethical Approval

Not applicable

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