In vitro insulinotropic actions of various extracts of Moringa oleifera leaves

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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% CO₂ 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/20 kg 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

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 (McLennaghan 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 (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, 1996) and hypoglycemic activities (Kar et al, 2003). Several bioactive compounds, including carbanates and thiocarbamates (Francis et al, 2004) (Table 1), iso-thiocyanates 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: O02013-04) 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, aceton, 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°C in an oven. After complete drying, the yield of the extract was ascertained as presented in Table 2. Prior to acute tests, cells were pre-seeded into 24-well plates as previously described (Ojo et al, 2011). Following overnight culture, cells were pre-incubated for 40 min at 37°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°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.

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°C in an atmosphere of 5% CO₂ 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°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°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.

Assessment of cytotoxicity: To determine the 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: Figure 1 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/10⁶ cells/20 min. Addition of alanine (10mM) to the assay buffer increased this rate to 3.14 ± 1.14 ng/10⁶ 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 BRN-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.

![Extraction yield](image)

**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**

<table>
<thead>
<tr>
<th>S/ No</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl 2-{4-(α-L-rhamnopyranosyl)phenyl]acetate</td>
</tr>
<tr>
<td>2</td>
<td>N-{4-{β-L-rhamnopyranosyl}benzyl]-1-O-α-D-glucopyranosylthiocarboxamide</td>
</tr>
<tr>
<td>3</td>
<td>1-O-phenyl-α-D-rhamnopyranoside</td>
</tr>
<tr>
<td>4</td>
<td>4-[[β-D-glucopyranosyl]-{(1→3)-(α-L-rhamnopyranosyl)]phenylacetonitrile</td>
</tr>
<tr>
<td>5</td>
<td>4-(α-L-rhamnopyranosyl)phenylacetonitrile</td>
</tr>
<tr>
<td>6</td>
<td>Methyl N-{4-[[4'-O-acetyl-α-L-rhamnopyranosyl benzyl]thiocarbamate</td>
</tr>
<tr>
<td>7</td>
<td>Methyl N-{4-[[α-L-rhamnopyranosyl]benzyl]carbamate</td>
</tr>
<tr>
<td>8</td>
<td>Methyl N-{4-[[4'-Oacetyl-α-L-rhamnopyranosyl]benzyl]carbamate</td>
</tr>
</tbody>
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Francis et al., 2004; Fahey, 2005
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 hyperglycemia (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

References


