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Moringa oleifera SEED EXTRACT INHIBITS OXIDATIVE STRESS AND CYTOTOXICITY VIA INTRINSIC AND EXTRINSIC PATHWAYS IN HIGH GLUCOSE-INDUCED RINM5F β-CELLS

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Abstract

Hyperglycemia is one of the hallmarks of diabetes mellitus and is known to cause apoptosis in pancreatic islets. A reduction in pancreatic β-cell number and function results in impaired insulin production and diabetes. *Moringa oleifera* has substantial evidence of anti-diabetic and anti-hyperglycemic activity. However, there is a lack of study on the anti-cytotoxicity mechanism of *Moringa oleifera* seed extract in high glucose-induced RINm5F β-cells. In this study, we investigate the effect of *Moringa oleifera* seed extract on cytotoxicity, oxidative stress, and caspases pathway in high glucose-induced RINm5F β-cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results showed that *Moringa oleifera* seed extract inhibits high glucose-induced cytotoxicity in RINm5F β-cells. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay demonstrated that *Moringa oleifera* seed extract inhibits oxidative stress in high glucose-induced RINm5F β-cells. Moreover, *Moringa oleifera seed* extract inhibits high glucose-induced cytotoxicity via intrinsic and extrinsic pathways in RINm5F β-cells.

INTRODUCTION

Diabetes mellitus is a public health concern with a significant impact on the global population. Diabetes, cancer, cardiovascular disease, and respiratory disease account for more than 80% of premature non-communicable disease mortality [1]. Diabetes mellitus is a chronic metabolic condition characterized by hyperglycemia that results from insulin deficiency or insensitivity to insulin action [2]. Diabetes mellitus is mainly classified into type 1 diabetes and type 2 diabetes. Other rare forms of diabetes include monogenic diabetes and gestational diabetes. Among the main types, type 1 diabetes mellitus is the less common form caused by autoimmune destruction of pancreatic β-cells [3]. On the other hand, type 2 diabetes is caused by impaired insulin signalling resulting in insulin resistance.

Diabetic patients with poor glycemic control experience persistent hyperglycemia that leads to severe complications. Chronic exposure to glucose can trigger various damaging effects mediated by glucotoxicity that results in β-cell deterioration and apoptosis [3,4,5]. In the presence of hyperglycemia and increased insulin secretory demand, β-cells experience stress in various form such as chronic endoplasmic reticulum stress, mitochondrial dysfunction, and excessive oxidative stress due to the overwhelming metabolic flux. These phenomena lead to activation of pro-apoptotic signals and subsequently promote β-cell apoptosis [6]. β-cell apoptosis is initiated by a series of signalling cascade to activate caspase family proteases, namely the caspase-9 via the mitochondrial pathway (intrinsic), and caspase 8 via the death receptor pathway (extrinsic). The activation of Caspase-8 and -9, in turn activate executioner Caspase3-7 to induce apoptotic event. Hence, developing

intervention to target hyperglycemia-induced apoptosis pathways in pancreatic cell is essential to prevent β-cell dysfunction and β-cell mass decline

Moringa oleifera is a consumable cruciferous plant popular across different countries due to its nutritional value and medicinal properties. The seeds, leaves, oil, bark, flower, sap, and roots have been reported to have multiple pharmacological functions such as anti-diabetic, anti-bacterial, anti-inflammatory, anti-cancer, anti-infertility, diuretic, neuroprotection, and thyroid regulation [7]. *Moringa oleifera* has been used as a traditional remedy for diabetes, and many scientific reports support its anti-glycaemic property [7-9]. Most studies on the anti-hyperglycaemic properties of the *Moringa oleifera* utilises either the aqueous extract or ethanol extract of the leaves [9]. *Moringa oleifera* leaves methanol extract has shown antioxidant, anti-apoptotic, and anti-inflammatory properties in streptozotocin (STZ)-induced diabetic mice [10]. In addition, *Moringa oleifera* decreased caspase-3 and -9 expression in streptozotocin (STZ)-induced diabetic mice. Studies proved that *Moringa oleifera* seed powder has anti-diabetic effects such as amelioration of fasting blood glucose level and restoration of kidney and pancreas functions [11]. However, the protective mechanism of *Moringa oleifera* seed extract has not yet been studied in high glucose-induced RINm5F β-cells. RINm5f is an insulin secreting pancreatic beta cell line originated from rat insulinoma, making it an excellent model for studies of the pathogenesis and potential treatments for diabetes mellitus [12-14]. Thus, in this study, we investigate the effect of *Moringa oleifera* seed extract on cell viability, reactive oxygen species (ROS) production, and apoptosis in high glucose-induced RINm5F β-cells.

MATERIALS AND METHODS

Preparation of 30mM Glucose and *Moringa oleifera* Seed Aqueous Extract

30mM glucose solution was prepared from the D-(+)-Glucose solution (Sigma-Aldrich). The glucose solution was freshly prepared and used to induce hyperglycemia. The *Moringa oleifera* seed extract was prepared from freeze-dried *Moringa oleifera* seed powder by dissolved in C-DMEM and supplemented with 10% foetal bovine serum (FBS) prior to treatment. A stock solution of 1mg/mL *Moringa oleifera* seed extract was prepared and used to create a working solution of different concentrations (5, 10, 20, 30, 40, 50 µg/mL) for the treatment regimen.

Cell Culture

Rat insulinoma cell line, RINm5F β-cells was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained as a monolayer in in C-DMEM supplemented with 10% FBS, and 100 µg/mL penicillin/streptomycin. The cell line was maintained in an incubator at 37 °C and 5% CO_2 atmosphere. The RINm5F β-cells were trypsinised and isolated for treatment once the confluency reached 70-80%.

Cell Viability Assay

The toxicity of *Moringa oleifera* seed extract and the effect of *Moringa oleifera* seed extract on high glucose-induced RINm5F β-cells was determined by MTT assay (Signa-Aldrich). RINm5F β-cells were seeded at a density of 2.0×10^4 cells/mL in 96-well plates and incubated for 24 hours. The cells were treated with different treatment regimen to measure the toxicity of *Moringa oleifera* seed extract in RINm5F β-cells, and the effect of *Moringa oleifera* seed extract in cell viability of high glucose-induced RINm5F β-cells. To measure toxicity and cell viability, the cells were incubated with or without 30mM glucose for 12 hours followed by treatment with *Moringa oleifera* seed aqueous extract (0, 5, 10, 20, 30, 40, 50 µg/mL) for 12 hours. After treatments, the cells were incubated with 40 µL MTT solution (5mg/mL) for 4 hours at 37 °C and 5% CO_2 atmosphere. The supernatant was removed from each well and 80 µL Dimethyl sulfoxide (DMSO) was added. The absorbance value was measured at 570nm and at a reference wavelength at 630nm (SpectraMax® M3 microplate reader, Software: SoftMax Pro).

DCFH-DA Assay

The effect of *Moringa oleifera* seed extract on ROS generation in high glucose-induced RINm5F β-cells was measured using DCFH-DA assay. DCFH-DA assay is a widely used assay for ROS detection such as hydroxyl radicals (•OH) and nitrogen dioxide (•NO2) [15]. ROS mediate the conversion of DCFH into DFH, which emit fluorescence signal that corresponds to the level of ROS. RINm5F β-cells were seeded into a transparent, black-walled 96-well plate at 1.5×10^4 cells/mL and incubated for 24 hours. The cells were treated with 30mM glucose for 12 hours, and different concentrations of *Moringa oleifera* seed extract was added and incubated for 12 hours. Then, the supernatant was removed from each well and replaced with 100 µL of PBS containing DCFH-DA solution (10µM). The plates were tapped gently, and fluorescence emission was detected at the excitation wavelength of 485nm and emission wavelength of 535nm (SpectraMax® M3 microplate reader, Software: SoftMax Pro).

Caspase-8, -9 and -3/7 Activities Assay

The effect of *Moringa oleifera seed* extract on caspase-8, -9, and -3/7 activities in high glucose-induced RINm5F β-cells were measured using the Caspase-Glo® 8 assay, Caspase-Glo® 9 assay, and Caspase-Glo® 3/7 assay kits, respectively (Promega Corporation). RINm5F β-cells were seeded into white-walled 96-well plates at 1.5×10^4 cells/mL and

incubated for 24 hours. The cells were treated with 30mM glucose for 12 hours and followed by different concentrations of *Moringa oleifera* seed extract for 12 hours. The respective caspase-Glo reagents were prepared as per product's instruction and added into each well at a ratio of 1:1 reagent to sample volume. Luminescence signal is produced as the pro-luminescent caspase substrate in the reagent is cleaved by the caspases in the cells. Luminescence signals were measured with SpectraMax® M3 microplate reader and processed by SoftMax Pro software. The intensity of luminescence generated is proportional to the amount of caspase activity. The fluorescence values were normalised using a corresponding MTT assay data, and the values were presented as fold change of caspase activity in glucose treated RINm5F β-cells as compared to control.

Statistical Analysis

Statistical analysis was performed by paired Student's t-test. All data were presented as mean ± standard deviation and the differences is considered as statistically significant at p-value < 0.05.

RESULTS AND DISCUSSION

Effect of *Moringa oleifera* Seed Extract on Cell Viability

The toxicity of *Moringa oleifera* seed extract on RINm5F β-cell viability is shown in Figure 1. Notably, RINm5F cells treated by 10 µg/mL *Moringa oleifera* seed extract had the highest cell viability (112.92% ± 9.09) compared to control. The toxicity increased significantly as the concentration of *Moringa oleifera* seed extract increased to 40 µg/mL. This can be seen as cell viability (%) decreased significantly as the concentration of *Moringa oleifera* seed extract increased, especially at higher concentration of 40 µg/mL (55.24% ± 13.16, $p< 0.05$) and 50 µg/mL (59.63% ± 9.09, $p< 0.05$). In conclusion, *Moringa oleifera* seed extract treatment at 5-20 µg/mL had increased RINm5F β-cell viability, while toxicity occurred at concentration higher than 40 µg/mL.

In Figure 2, our study demonstrated that cell viability of high glucose-treated RINm5F β-cells (91.38% ± 1.20, $p<0.05$) reduced when compared to control, which indicate high glucose-induced cytotoxicity in RINm5F β-cells. However, after being treated with *Moringa oleifera* seed extract (5, 10, and 20 g/mL), the cell viability increased to 95.88% ± 4.05, 98.48% ± 5.19, and 95.91% ± 3.30, respectively. Similarly, *Moringa oleifera* leaves extract was observed to significantly increased human dermal fibroblasts cell viability and proliferation [16].

Effect of *Moringa oleifera* Seed Extract on ROS production

The effect of *Moringa oleifera* seed extract on the ROS production in high glucose-induced RINm5F β-cells was investigated via DCFH-DA assay as shown in Figure 3. ROS levels were significantly reduced after 5, 10, and 20 µg/mL of *Moringa oleifera* seed extract treatment in high glucose-induced RINm5F β-cells, with ROS fold change of 0.59 ± 0.10, 0.45 ± 0.12, and 0.35 ± 0.09 respectively, indicating antioxidant activity. This result is consistent with cell viability data (Fig 1 and Fig. 2), indicating that a low concentration of *Moringa oleifera* seed extract (5 to 20 µg/mL) increases cell viability and reduces ROS levels; hence, cytoprotective effects. Researchers have found that *Moringa oleifera* has a wide range of anti-oxidative properties, largely because of the presence of flavonoids (221.76 ± 0.221 mg), phenolic (90.97 ± 0.134 mg), and tannin (21.74 ± 0.086 mg) [17]. Ogbunugafor et al. demonstrated that *Moringa oleifera* seed consists of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability and reductive capacity [18]. Another study also showed the presence of chlorogenic acid, gallic acid, quercetin, ellagic acid, ferulic acid, kaempferol, and vanillin in *Moringa oleifera* aqueous leave, seed, and fruit extract. All these can prevent ROS-induced DNA damage [19].

Effect of *Moringa oleifera* Seed Extract on Caspase-8, -9, -3/7 Activities

The effect of *Moringa oleifera* seed extract on caspase-8, -9, and -3/7 activities in high glucose-induced RINm5F β-cells was showed in Figure 4a, 4b, 4c, respectively. The fold change of caspase-8, -9, and -3/7 activities in high glucose-induced RINm5F β-cells increased from 1 to 1.05, 1.02 and 1.06, respectively as compared to control. In consistence with this study, Kim et al. showed that chronic exposure to 33.3mM glucose increased pancreatic β- cell apoptosis by downregulation of glucokinase (GCK) expression, leading to increased Bax oligomerisation, cytochrome c release and caspse-3 activation [14]. Treatment with *Moringa oleifera* seed aqueous extracts in high glucose-induced RINm5F β-cells significantly reduced the activity of caspase-8, -9, and -3/7. After treatment with 5 µg/mL, 10 µg/mL, and 20 µg/mL *Moringa oleifera* seed extract, caspase-8 activity had significant reduction to 0.80 ± 0.05, 0.60 ± 0.06, and 0.45 ± 0.06, respectively (Figure 4a); caspase-9 activity reduced to 0.798±0.04, 0.854±0.06, and 0.66±0.06, respectively (Figure 4b); caspase-3/7 activity reduced to 0.92 ± 0.02, 0.63 ± 0.03 and 0.58 ± 0.05, respectively, as compared to control group (Figure 4c). The reduction of caspase-3/7 activity in the *Moringa oleifera*-treated high glucose-induced RINm5F β-cells correlate with the reduction of initiator caspase-8 and caspase-9 activities. There is evidence that caspase-8 is critical in maintaining β-cell mass and initiation of β-cell apoptosis in the presence of apoptotic stimuli [20]. Other studies showed that high glucose modulate BCL family gene, increase proapoptotic Bad, Bid, Bik protein expressions, decrease anti-apoptotic Bcl-2 and Bcl-xl protein expressions and resulting β-cell death [21]. The balance between proapoptotic and anti-apoptotic BCL family

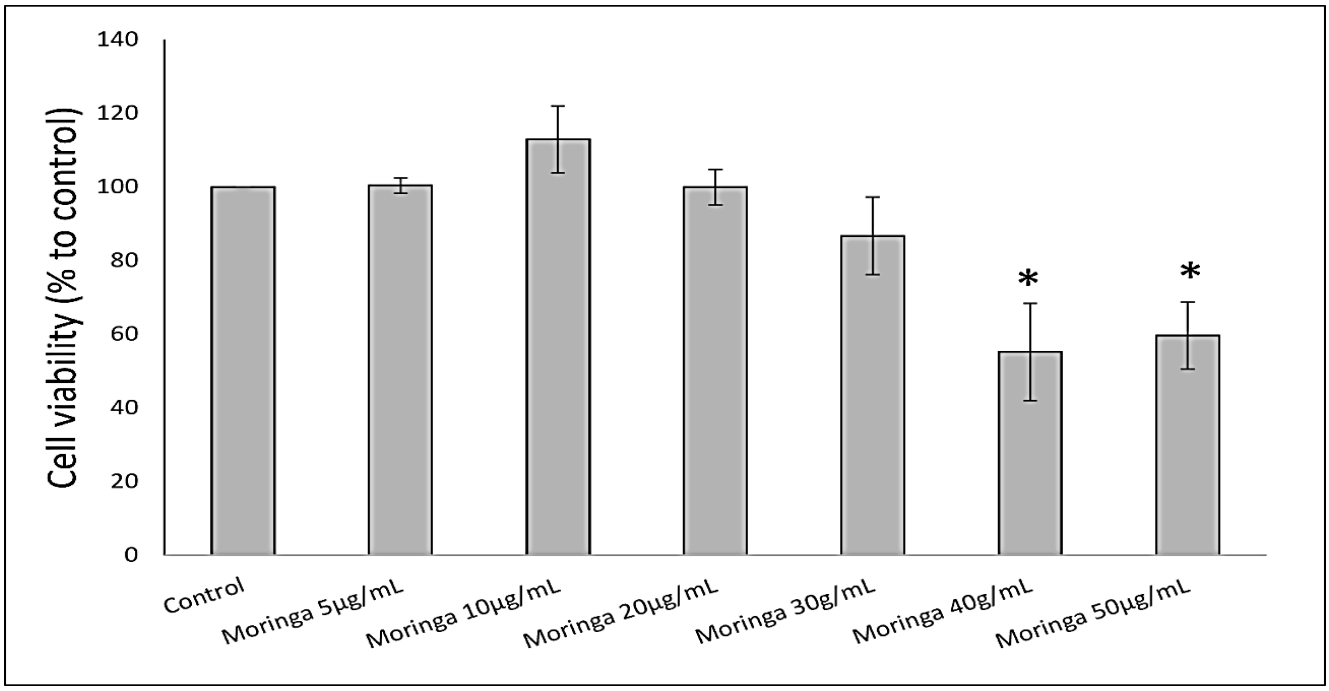

Figure 1. Effect of *Moringa oleifera* seed extract on cell viability of RINm5F β-cells. Percentage of cell viability (% vs control) of RINm5F β-cells after treatment with *Moringa oleifera* seed extract (5, 10, 20, 30, 40, 50 µg/mL) was measured with MTT assay. The data is presented in mean ± SD. The control group is RINm5F β-cells without *Moringa oleifera* seed extract treatment. * indicates statistical significance ($p < 0.05$) as compared to control group

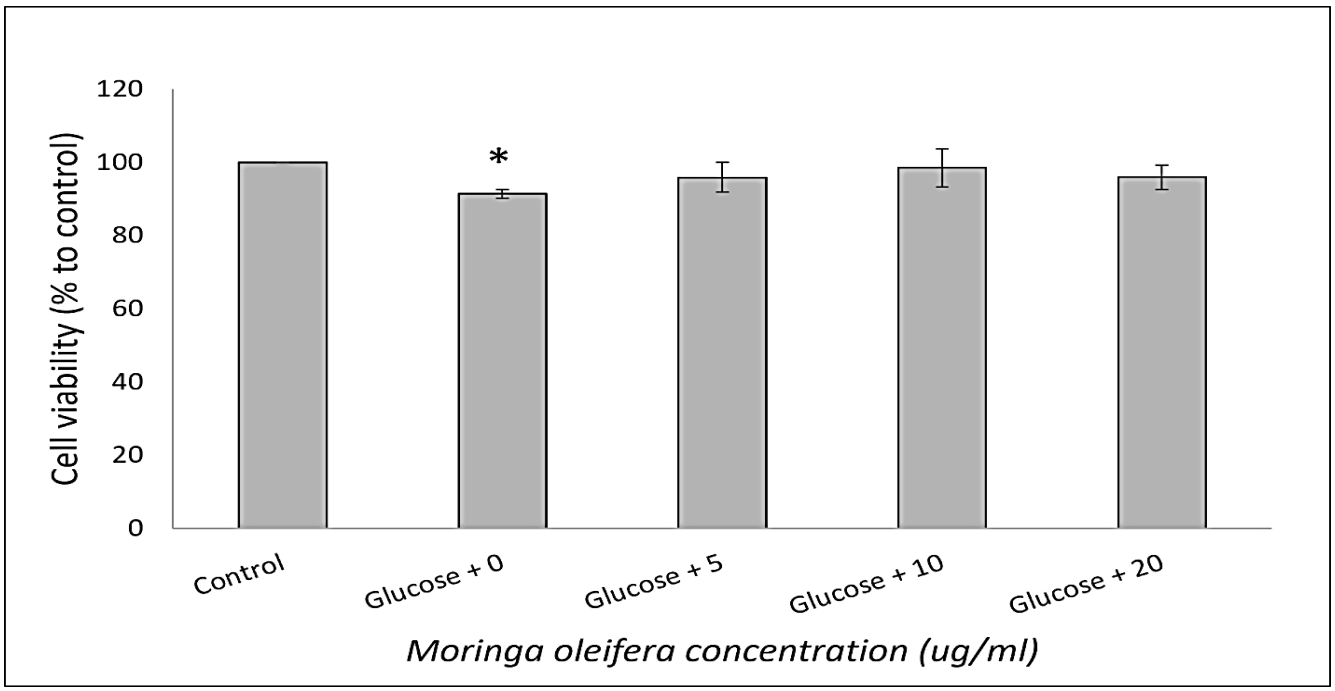

Figure 2. Effect of *Moringa oleifera* seed extract on cell viability of high glucose-induced RINm5F β-cells. Percentage of cell viability (% vs control) of RINm5F β-cells after treatment with glucose and with or without *Moringa oleifera* seed extract (5, 10, 20, 30 µg/mL) was measured with MTT assay. The data is presented in mean ± SD. The control group is RINm5F β-cells without glucose and *Moringa oleifera* seed extract treatment. *represents statistical significance ($p < 0.05$) compared to control. # represents statistical significance ($p < 0.05$) compared to untreated cells

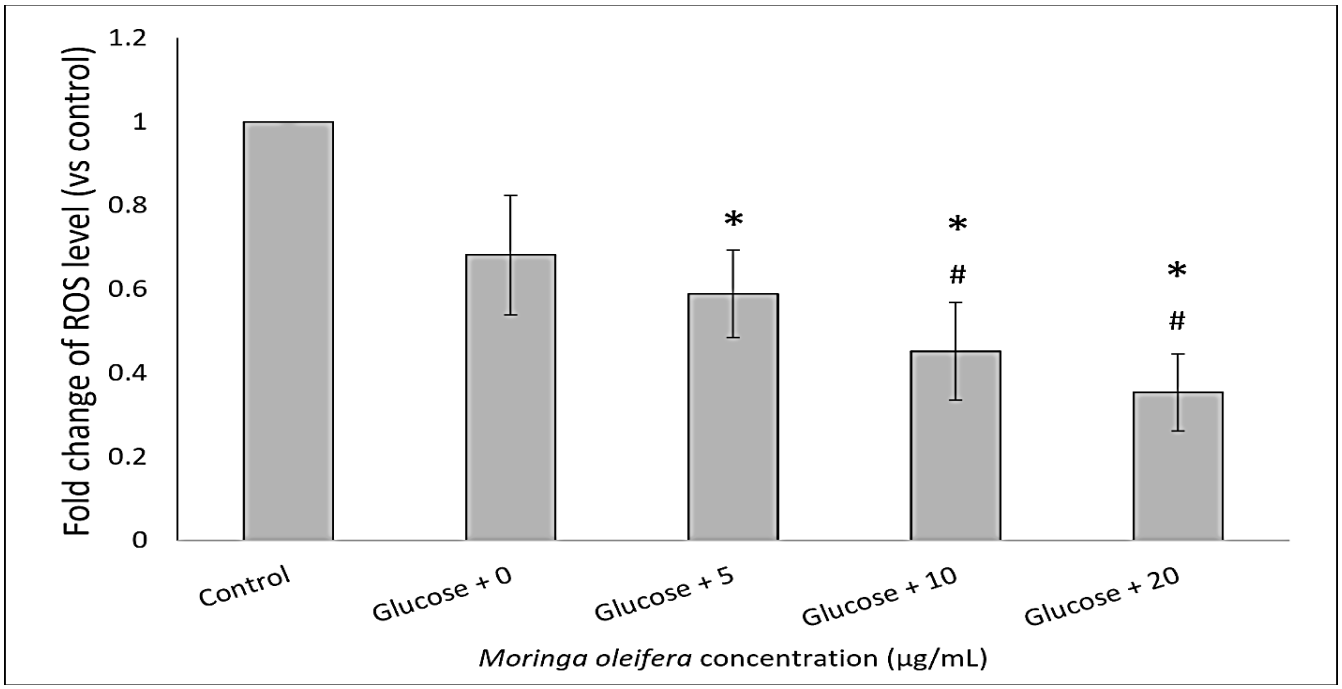

Figure 3. Effect of *Moringa oleifera* seed extract on ROS generation. Fold change of ROS generation of high glucose-induced RINm5F β-cells were measured via DCFH-DA assay. The data is presented in mean ± SD. The control group is RINm5F β-cells without glucose and *Moringa oleifera* seed extract treatment. * represents statistical significance ($p < 0.05$) compared to control. # represents statistical significance ($p < 0.05$) compared to untreated cells

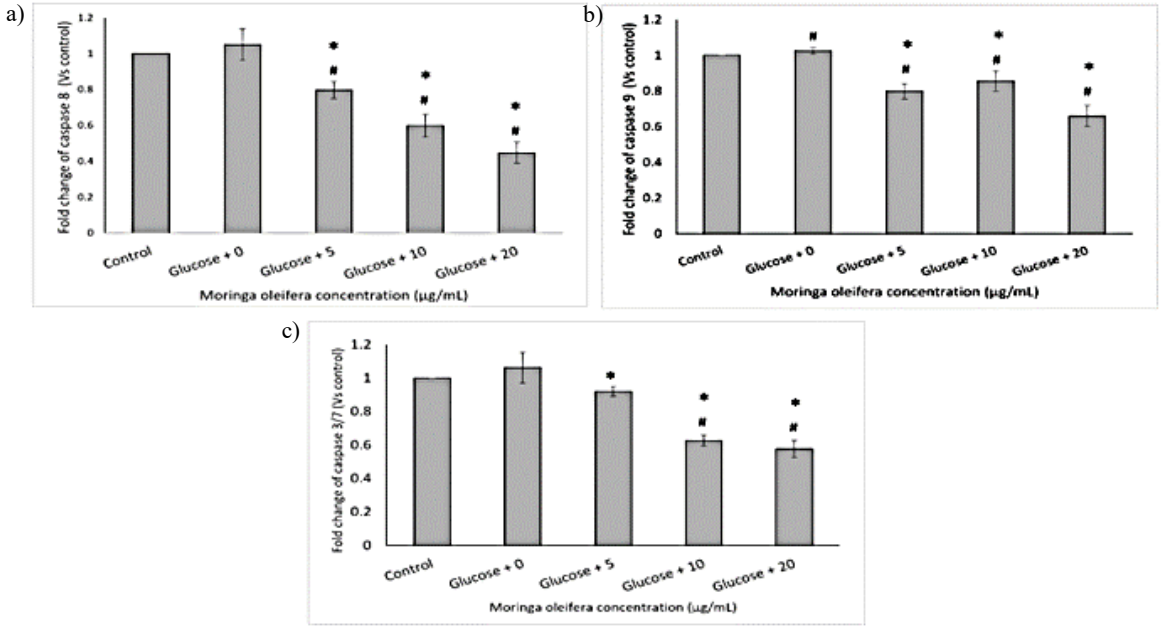

Figure 4. Effect of *Moringa oleifera* seed extract on Caspase-8, -9, -3/7 activities. Fold change of caspases activities in high glucose-induced RINm5F β-cells after treatment with *Moringa oleifera* seed extract: (a) caspase-8, (b) caspase-9, (c) caspase-3/7. The data is represented in mean ± SD. The control group is RINm5F β-cells without glucose and *Moringa oleifera* seed extract treatment. * represents statistical significance ($p < 0.05$) compared to control. # represents statistical significance ($p < 0.05$) compared to untreated cells

proteins is critical in the regulation of inner-mitochondrial permeability transition pore and subsequent activation of intrinsic apoptotic pathway [22].

Moringa oleifera seed extract exhibited regulatory effects and ameliorated the caspase activities of high glucose-induced RINm5F β-cells, possibly by inhibiting ROS formation in the cells. In hyperglycemic environment, oxidation of glucose-derived pyruvate occurs, thus increasing the flux of electron donors (NADH and FADH2) into the electron transport chain, which increases the voltage gradient across the mitochondrial membrane and leads to the generation of superoxide and ROS [23,24]. Overproduction of superoxide by the mitochondria leads to over-activation of polyol, advanced glycation end-products, protein kinase C and hexosamine pathways. Through these pathways, stress-sensitive signalling increased cytokines production and, eventually, cell apoptosis [24]. Chronic oxidative stress due to ROS overproduction is the central mechanism for glucotoxicity in pancreatic islet cells, causing impaired insulin gene expression, insulin secretion, and increased apoptosis [25]. In the current study, *Moringa oleifera* seed extract effectively reduced ROS levels in a dose-dependent manner corresponding to the reduction of intrinsic and extrinsic pathways. This data is consistent with the well-documented anti-oxidative, anti-inflammatory, and anti-apoptotic activity of *Moringa oleifera* leaves extract in kidney, liver and spleen of various diabetic rat and mice models [19,26,27].

CONCLUSION

Moringa oleifera seed extract is a promising therapeutic compound as it has well-documented anti-diabetic properties. This study further supports its effect by investigating its anti-cytotoxicity, anti-oxidative and pathway involvement. Our results revealed a positive correlation between the ROS inhibitory activity of *Moringa oleifera* seed extract and the reduction of caspase activity in high glucose-induced RINm5F β-cells. Key apoptosis mediators, including caspase-8, caspase-9, and Caspase 3/7 activities were reduced after being treated with *Moringa oleifera* seed extract, suggesting that *Moringa oleifera* seed extract treatment has a role in ameliorating intrinsic and extrinsic-dependent apoptosis pathways.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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