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Gymnema montanum improves endothelial function *via* inhibition of endoplasmic reticulum stress by activating Nrf2 signaling

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ABSTRACT

Objective: To investigate the effects of *Gymnema montanum* leaf extract against endoplasmic reticulum (ER) stress-induced toxicity in endothelial cells.

Methods: The immortalized endothelial hybrid cell, EA.hy926 was treated with different concentrations of *Gymnema montanum* leaf extract (0–100 μg/mL) and the ER stress inducer, tunicamycin. The cytotoxicity was assessed by MTT as well as lactate dehydrogenase and malondialdehyde levels were determined. The levels of ER stress markers, GRP78 and CHOP were analysed by Western blot assay. The *Gymnema montanum* leaf extract-mediated activation of nuclear factor erythroid 2-related factor 2 (Nrf2) was assessed by cell-based luciferase enzyme fragment complementation assay and antioxidant responsive element driven luciferase reporter assay. The levels of phosphoproteins of the MAPK pathway were analyzed using the Bioplex system.

Results: A dose-dependent cytoprotective effect of *Gymnema montanum* leaf extract was observed in tunicamycin-induced toxicity. *Gymnema montanum* leaf extract significantly reduced lactate dehydrogenase activity and malondialdehyde levels in ER stress-induced endothelial cells. It also suppressed ER stress markers dose dependently and inhibited the phosphorylation of JNK, ERK, MEK and p38 MAPK in tunicamycin-induced endothelial cells. Moreover, *Gymnema montanum* leaf extract increased the expression of Nrf2 and its downstream targets in endothelial cells.

Conclusions: *Gymnema montanum* leaf extract attenuates ER stress by increasing the expression of Nrf2 and its downstream genes.

KEYWORDS: ER stress; *Gymnema montanum*; Nrf2; Endothelial cells

1. Introduction

Diabetes mellitus is a pandemic multifactorial disorder, characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. Epidemiological studies report that chronic hyperglycemia is associated with an incremental risk of cardiovascular diseases. Endothelial dysfunction is a crucial factor in the development and progression of microvascular diseases associated with diabetes mellitus including atherosclerosis[1,2]. Apoptosis of endothelial cells can be induced by a number of stimuli such as high glucose, oxidative stress, and cytokine stress, leading to endothelial dysfunction in part through the activation of endoplasmic reticulum (ER) stress[3,4]. However, the mechanisms of ER stress-induced endothelial dysfunction and clinical complications remain unclear.

The endomembrane component, ER is the site for protein synthesis, protein folding and trafficking, lipid synthesis, maintenance of calcium homeostasis, and few other processes critical to metabolism[5]. Under physiological conditions, cells maintain ER homeostasis by balancing the demand for protein synthesis and protein folding, whereas, disturbances in ER homeostasis due to elevated protein synthesis result in the accumulation of misfolded and unfolded proteins, imbalance in calcium flux, all adding up to elevated ER stress initiating unfolded protein response (UPR)[6–9].

UPR pathway restores protein homeostasis or modulates the unfolded proteins either by halting protein translation or by producing molecular chaperones, which aid in proper protein folding. The three-major UPR signaling cascades activated by

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ER-resident transmembrane proteins are activating transcription factor 6 (ATF6), inositol requiring kinase 1 (IRE1) and dsRNA-activated protein kinase (PKR)-like ER kinase (PERK). A 78-kDa glucose-regulated protein (GRP78) is a chaperone that regulates these molecules. Under ER stress conditions, GRP78 dissociates from the sensing molecules to activate them. In adverse pathological conditions, the adaptive responses fail to rescue cells from ER stress and eventually apoptosis is triggered in these cells. These unresolved UPR pathways are associated with various metabolic disorders, including diabetes[6,8,9].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is the regulator of an array of detoxifying and antioxidant defense gene expressions. Under physiological conditions, Kelch-like ECH-associated protein-1 (Keap1) ubiquitinates and eventually degrades Nrf2. On the other hand, under pathological conditions, Nrf2 is released from Keap1, translocates to the nucleus and binds to antioxidant responsive element (ARE), triggering a battery of genes including glutathione S-transferase (GST) heme oxygenase 1 (HO1), NAD(P)H quinone oxidoreductase 1 (NQO1) and superoxide dismutase (SOD). Hence, it is plausible to target ER stress through Nrf2 signaling cascade during the onset of diabetes[10,11].

Counteracting ER stress represents a promising therapeutic approach to prevent vascular damage thereby hindering the progression of endothelial dysfunction. Recently, plant and plant derivatives have shown a promising effect in preventing ER stress under hyperglycemia[12]. Numerous studies demonstrated that natural products, as components of the daily diet or phytomedicine preparations aid in improving endothelial function[4,13]. Several medicinal plants are reported to prevent the onset of diabetes-mediated endothelial dysfunction including *Cymbopogon citrates*[14], *Syzygium cumini*[15], *Portulaca oleracea* L[16], *Erigeron multiradiatus*[17], and *Phyllanthus emblica*[18]. Our earlier studies reported that quercetin, a natural polyphenol, attenuates ER stress in endothelial cells both *in vitro* and *in vivo*[19,20].

Gymnema montanum (*G. montanum*) Hook, an Asclepiadaceae member, is extensively used in traditional medicine against various diseases including diabetes. Antidiabetic, anti-hyperlipidemic and anti-apoptotic properties of *G. montanum*, have been earlier reported from our laboratory[21-23]. In the current study, we investigated the effect of *G. montanum* leaf extract against ER stress in endothelial cells and its underlying molecular mechanism.

2. Materials and methods

2.1. Collection of plant material and preparation of leaf extract

Fresh leaves of *G. montanum* were collected from Western Ghats, Nilgiris, identified by Dr. C. Murugan with the Herbarium of Botanical Survey of India, Southern Circle, Coimbatore, India (Voucher number 32561-65). The collected leaves were air-dried in shade and made into a fine powder using a blender. Ethyl acetate extract of *G. montanum* (GMEt) leaves was prepared[24]. For solvent extraction, 500 g of powdered leaves were loaded to the thimble and extracted with ethyl acetate solvent using a Soxhlet extractor for 16 h in the Soxhlet apparatus. The extract was then concentrated using a rotary evaporator below 50 ℃, yielded 47 g final residue and preserved at 4 ℃ in an airtight bottle until further use.

2.2. Cell culture

EA.hy926 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 μg/mL streptomycin and 100 U/mL penicillin. The cells were maintained at 37 ℃ in a CO_2 incubator under a humidified atmosphere.

2.3. Determination of cytotoxicity of GMEt

The cytotoxicity of GMEt and tunicamycin (Sigma-Aldrich, USA) to EA.hy926 cells (1×10^4 cells/well) was assessed using MTT assay. Cells were seeded in 96-well plates and subjected to treatment with different concentrations of either GMEt (0–100 μg/mL) or tunicamycin (TUN, 0–10 μg/mL) and placed in a CO_2 incubator at 37 ℃ for 24 h. The media were then aspirated, and MTT (5 mg/mL) was added, and further incubated for 3 h. The formazan crystals formed were completely dissolved in DMSO and OD was recorded at 570 nm using ELISA reader (Tecan, Switzerland). All experiments were performed in triplicate, and the relative cell viability (%) was measured as a fraction of control and expressed as percentage relative fold. Fifty percent of cytotoxicity was also recorded for cells.

2.4. Cytoprotective effect of GMEt against ER stress in endothelial cells

In order to study the cytoprotective role of GMEt against TUN-induced cytotoxicity, endothelial cells were seeded in a 96-well microplate, treated with different concentrations of GMEt (0–100 μg/mL) and TUN (5 μg/mL) for 24 h at 37 ℃. After treatment, MTT assay was carried out and cell viability calculated as described above. All experiments were performed in triplicate, and the relative cell viability (%) was calculated as a fraction of control.

2.5. Measurement of lactate dehydrogenase (LDH) activity in TUN-induced ER stress

Endothelial cells were seeded at 1×10^6 cells/well in 6-well plate and treated with GMEt (10 or 25 μg/mL) and TUN (5 μg/mL) for 24 h at 37 ℃. After treatment, the cells were trypsinized, and the cell pellet was collected, homogenized using ice-cold assay buffer and centrifuged at 4 ℃. To the supernatant, the reaction mixture (LDH assay buffer and LDH substrate mix) was added and the absorbance was recorded at 450 nm for 1 h at every 5 min interval. LDH activity was calculated as per the manufacturer's instructions (Abcam, UK).

2.6. Determination of malondialdehyde (MDA) levels in TUN-induced ER stress

Endothelial cells were treated with GMEt (10 or 25 μg/mL) and TUN (5 μg/mL) for 24 h at 37 ℃. After treatment, the cell pellet was collected by centrifugation. The pellet was homogenized using PBS and assessed for MDA as per the manufacturer's instructions (Cayman chemicals, UK). Briefly, the samples and standards were boiled for 1 h along with sodium dodecyl sulfate and colour reagent (provided). After incubation, the tubes were placed for 10

min on ice-cold conditions and centrifuged at 1 600×*g* at 4 ℃. The supernatant was collected and used to measure the MDA levels by reading absorbance at 540 nm. MDA levels were calculated by obtaining the equation against the standard curve.

2.7. Expression analysis of GRP78 and CHOP markers using Western blot assay

In order to study the effect of GMEt against TUN-induced ER stress, the levels of ER stress markers GRP78 and CHOP were assessed by Western blot. Briefly, the cells were treated with GMEt (10 and 25 μg/mL) and TUN (5 μg/mL) for 24 h at 37 ℃. The effect of GMEt on normal endothelial cells was also studied at the fixed higher dose of 25 μg/mL. After treatment, cells were harvested, washed twice with PBS, and lyzed in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) freshly added with 0.01% protease inhibitor cocktail (Biorad, PA, USA) for 30 min. Cell lysates were subjected to centrifugation at 4 ℃, 1 000×*g* for 10 min. The protein in the supernatant (20–30 μg) was separated on 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Biorad, PA, USA). The blots were blocked with 3% bovine serum albumin for 1 h, followed by incubation at 4 ℃ with anti-GRP78, anti-CHOP and anti-GAPDH antibodies purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Blots were then incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies (Santa Cruz, USA) for 1 h at 37 ℃. Enhanced chemiluminescence (ECL; Biorad, PA, USA) detection system was used to visualize the target proteins with the GBOX documentation system (Syngene, UK).

2.8. Nrf2 activation potential of GMEt by cell-based luciferase enzyme fragment complementation assay

A complementation system of Nrf2 Keap1 was designed to investigate the activation of Nrf2 in EA.hy926 cells in the presence of GMEt. CLuc-Nrf2 and NLuc-Keap1 fusion proteins[25] were transiently transfected (500 ng) using lipofectamine in EA.hy926 cells. After 6 h, the transfection media were replaced with GMEt (0, 5, 10 and 25 μg/mL) for 24 h. Cell homogenate was prepared using cell lysis reagent (Promega, USA), and dropped in the signal *i.e.* the activity of luciferase due to dissociation of Nrf2 from Nrf2-Keap1 complex upon GMEt treatment was measured by a luminometer (Promega, USA). The results were expressed in percentage as the relative change in the complementation system. The decreased luciferase signal was directly proportional to the activation of Nrf2.

2.9. Validation of Nrf2 activation by ARE-driven luciferase reporter assay

The effect of GMEt on Nrf2 mediated ARE downstream gene activation was assessed using cell-based reporter assay using hGST1-ARE-Luc or hNQO1-ARE-Luc reporter gene constructs. Transfection of EA.hy926 cells with the respective vector constructs was done using Lipofectamine2000 reagent. After 6 h, the medium was aspirated and fresh media were added with different concentrations of GMEt (0, 5, 10 and 25 μg/mL) and incubated for 24 h. Cell homogenate was then prepared using cell lysis reagent (Promega, USA), and luciferase activity was measured using a luminometer (Promega, USA) and expressed as percent change relative to the untreated control. Results were normalized with protein as well as with scrambled control.

2.10. Analysis of Nrf2 and its target genes expression using qPCR

The levels of Nrf2 downstream target genes were studied using qPCR. Briefly, mRNA was extracted from endothelial cells using mRNA isolation kit (Qiagen, Germany), following the manufacturer's instructions. Two μg of total RNA from each sample was subjected to treatment with DNase Ⅰ, and then was subjected to reverse transcription, using cDNA conversion kit (Qiagen, Germany). All samples were reverse transcribed under the same conditions with the same master mix. Triplicate qPCR reactions for each sample were performed (Biorad cfx connect systems, USA). To exclude the possibility of the presence of contaminating template molecules and to identify potential primer-dimer products, no template reactions were also carried out. Amplification plots were analyzed using CFX Manager Software (Biorad, USA) and expressed as relative mRNA expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalizing the gene expression. Table 1 depicts the sequences of primers for the target genes.

2.11. Phosphoprotein assay using BioPlex

Phosphoproteins were analyzed using Bio-Plex Pro™ Cell Signaling MAPK Panel, developed based on multiplex sandwich bead immunoassays (Bio-Rad, Hercules, CA). After treatment, proteins were extracted using the cell factor QG (provided in the kit) supplemented with protease inhibitor cocktail (Thermo Scientific, USA), and used for assay based on manufacturer's instructions. Briefly, 50 μg/mL of control and test protein samples were incubated overnight along with fluorescent capturing beads coupled to phospho antibodies (phospho-ERK1/2, phospho-JNK, phospho-MAPK, phospho-MEK) in a Bio-Plex Pro™ (BioRad, CA, USA) on a platform shaker at 300 rpm at room temperature. The wells were vacuum filtered and washed, and 1 μL of biotinylated detection antibodies were added, vortexed and incubated for 30 min. After additional vacuum filtration and washing of the wells, 0.5 μL streptavidin-phycoerythrin (100×) was added to each well and allowed to incubate for 10 min. The wells were again vacuum filtered and washed, and 125 μL of re-suspension buffer was added and incubated for 30 s. Finally, the data was acquired by cytometric imaging using Bio-plex 200 system (BioRad, CA, USA) and analysed using Bio-Plex Manager™ software 6.1 (Bio-Rad, CA, USA). Phosphoprotein levels were determined from standard curves of each analyte and recorded as mean fluorescence intensities.

Table 1. Primer sequences used in the study.

Gene	Forward	Reverse
GPX	TTCCCGTGCAACCAGTTTG	TTCACCTCGCACTTCTCGAA
SOD	GAAGGTGTGGGGAAGCATTA	ACATTGCCCAAGTCTCCAAC
NQO1	AGGATGGAAGAAACGCCTGG	TCAGTTGGGATGGACTTGCC
HO1	GGGAATTCTCTTGGCTGGCT	AACTGAGGATGCTGAAGGGC
GAPDH	AAGAAGGTGGTGAAGCAGGC	GTCAAAGGTGGAGGAGTGGG

2.12. Statistical analyses

All studies were carried out in three independent experiments and results were represented as mean ± SD. Statistical significance was calculated based on one-way ANOVA, followed by Tukey's *post hoc* test; $P < 0.05$ was considered significant between groups.

3. Results

3.1. Effect of GMEt on TUN-induced cytotoxicity in EA.hy926 cells

Preliminary cytotoxic effect of GMEt (0-100 μg/mL) and TUN (0-10 μg/mL) on endothelial cells, EA.hy926, was assessed using MTT assay. LD_{50} of TUN was recorded as 5 μg/mL (Figure 1A) and GMEt did not cause any significant cytotoxicity up to 50 μg/mL

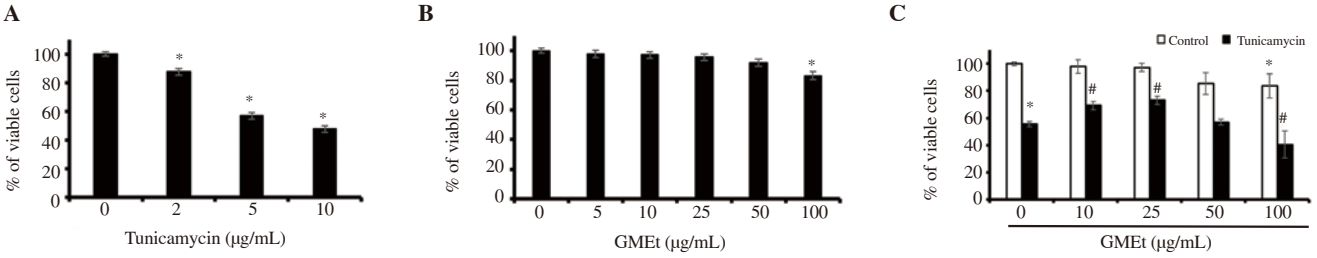

Figure 1. Cytotoxicity of EA.hy926 cells against (A) tunicamycin, (B) GMEt, and (C) effect of GMEt on tunicamycin-induced cytotoxicity assessed by MTT assay. Data are presented as mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post hoc* test. $^{*}P<0.05$, compared with the untreated control; $^{\#}P<0.05$, compared with the tunicamycin-exposed control. GMEt: Ethyl acetate extract of *Gymnema montanum*.

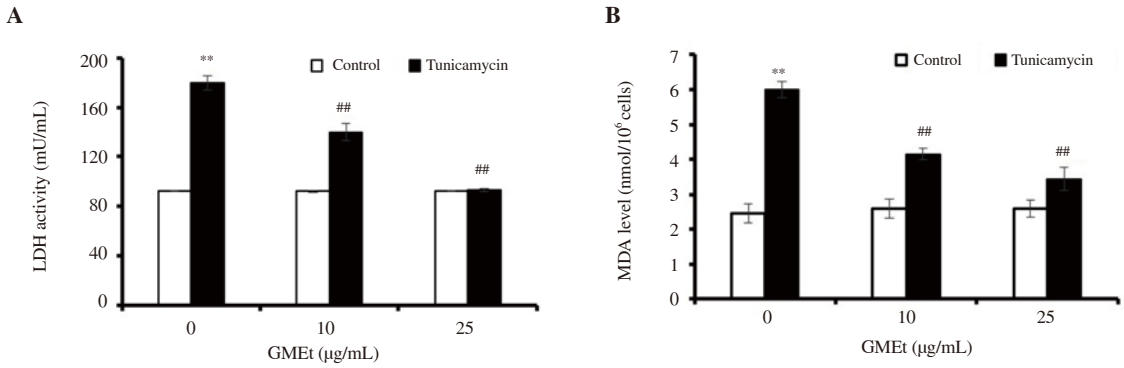

Figure 2. Effect of GMEt on the levels of (A) LDH and (B) MDA on tunicamycin-induced cells. Data are presented as mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post hoc* test. **Significance compared with the untreated control; $^{\#\#}$Significance compared with the tunicamycin-exposed control; ** & $^{\#\#}P<0.01$.

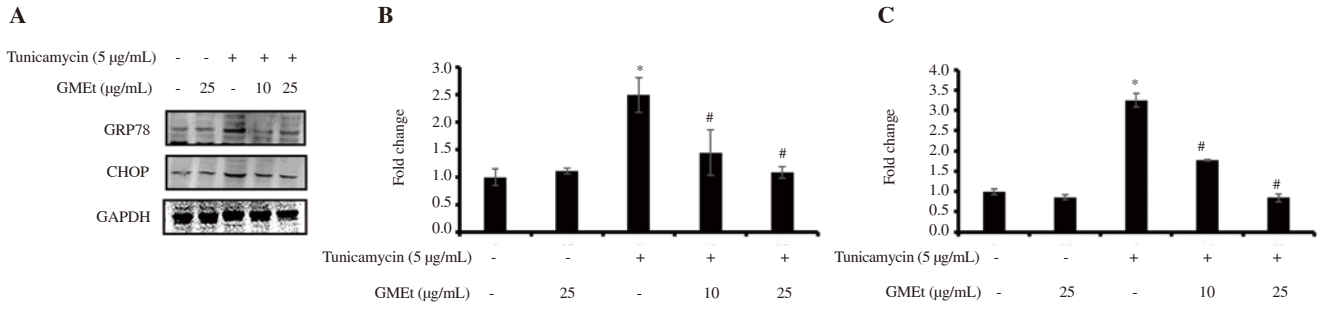

Figure 3. Effect of GMEt against tunicamycin-induced ER stress in endothelial cells using Western blot analysis. (A) Representative blots of GRP78 and CHOP; (B) Level of GRP78; (C) Level of CHOP. Data are presented as mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post hoc* test. *Significance compared the untreated control; $^{\#}$Significance compared with the tunicamycin-exposed control; $P<0.05$. ER: endoplasmic reticulum.

(Figure 1B). To evaluate the cytoprotective effect of GMEt against TUN-induced toxicity, endothelial cells were exposed to different concentrations of GMEt (0-100 μg/mL) and TUN (5 μg/mL) for 24 h. The viability of cells decreased in the presence of TUN to 57%, but in the presence of GMEt, the viability increased to 71% and 75% at 10 μg/mL and 25 μg/mL, respectively, (Figure 1C) showing the protective effect of GMEt against ER stress. From this result, the GMEt concentrations for further experiments were taken as 10 and 25 μg/mL.

3.2. Effect of GMEt on LDH and MDA levels in TUN-induced EA.hy926 cells

In order to understand the involvement of free radicals in ER stress, the levels of LDH and MDA were analyzed. Figure 2 shows that a significant elevation in levels of LDH and MDA was observed upon TUN treatment compared with the untreated control, whereas GMEt treatment significantly reduced the levels of both LDH and MDA.

3.3. Effect of GMEt on ER stress-induced protein expression in EA.hy926 cells

TUN exposed cells showed an increase in the levels of ER stress markers including GRP78 (2.5 fold, $P<0.05$) and CHOP (3.24 fold, $P<0.05$) when compared to the control cells, confirming the induction of ER stress by TUN exposure (Figure 3). Treatment with GMEt (10 and 25 μg/mL) resulted in reduced levels of GRP78 and CHOP compared with TUN treated cells, indicating dose-dependent protection of GMEt against TUN. The GMEt (25 μg/mL) alone did not result in

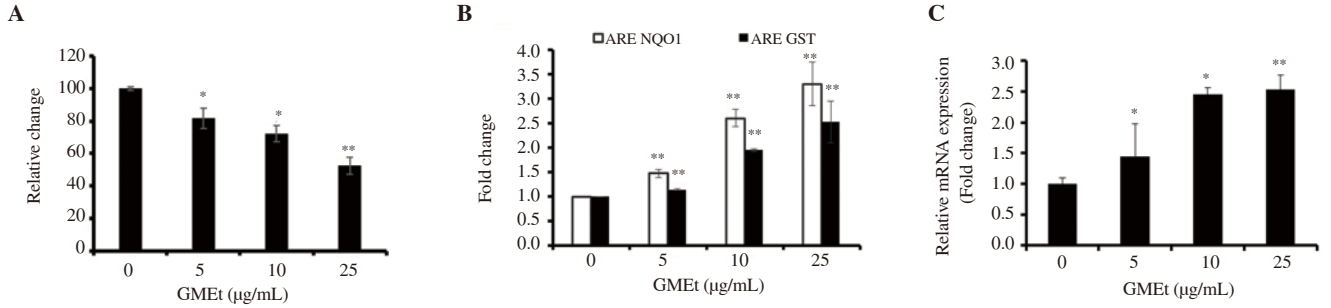

Figure 4. Effect of GMEt on (A) activation of Nrf2 using cell-based luciferase enzyme fragment complementation assay, (B) ARE driven hNQO1-Luc and hGST-Luc gene constructs for its downstream genes and (C) activation of Nrf2 using qPCR. Data are presented as mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post hoc* test. *Significance compared with the untreated control, *$P<0.05$; **$P<0.01$.

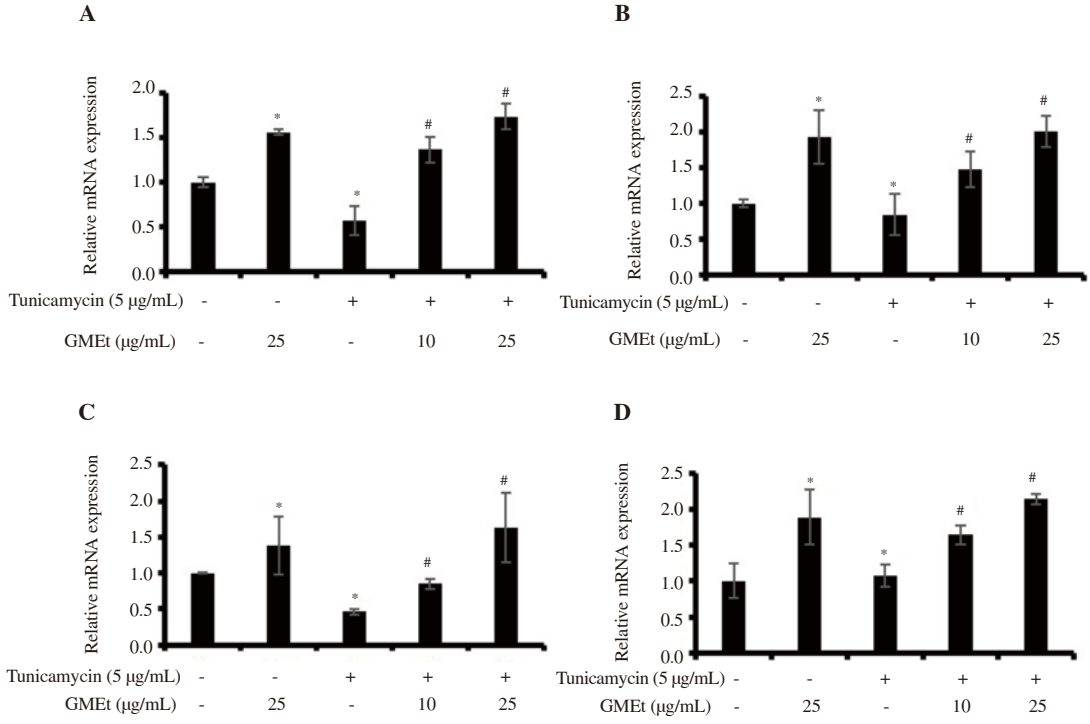

Figure 5. Effect of GMEt on Nrf2 downstream genes including (A) *GPx*, (B) *SOD*, (C) *NQO1*, and (D) *HO-1* assessed by qPCR in tunicamycin-induced cells. Data are presented as mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post hoc* test. *Significance compared with the untreated control; #Significance compared with the tunicamycin-exposed control; $P<0.05$.

any significant change (Figure 3).

3.4. Effect of GMEt on Nrf2 activation in endothelial cells

In order to confirm the Nrf2 activation potential of GMEt, cell-based luciferase enzyme fragment complementation assay and ARE-driven luciferase reporter assay were used. GMEt treatment resulted in a dose-dependent decrease in the luciferase signal in the complementation system, reflecting the activation of Nrf2 (Figure 4A). The ARE-dependent transcriptional activation of Nrf2 was studied using hNQO1-ARE-Luc and hGST-ARE-Luc reporter gene constructs. As shown in Figure 4B, GST and NQO1 reporter luciferase signals increased in a dose-dependent manner in GMEt treated cells, indicating that GMEt stimulated the activation of Nrf2. GMEt treatment also significantly increased the expression of Nrf2 as assessed by qPCR (Figure 4C).

3.5. Effect of GMEt on Nrf2 downstream target genes by qPCR analysis

GMEt treatment increased the expression of downstream target genes including *GPx*, *HO-1*, *NQO1*, and *SOD*, thereby showing protective effect against ER stress induced by TUN exposure (Figure 5).

3.6. Effect of GMEt on phosphoproteins in ER stress-induced EA.hy926 cells

In order to analyze the effect of TUN on upstream kinases and modulatory effect of GMEt in the induction of apoptosis, the levels of phosphoproteins were determined using Cell Signaling MAPK Panel. Exposure of the EA.hy926 cells to TUN led to a significant (P<0.05) increase in the levels of p-ERK1/2, p-JNK, p-MAPK, and p-MEK expression. No significant changes in the levels of phosphoproteins were observed in endothelial cells treated with GMEt alone except p-ERK1/2. GMEt treatment significantly decreased the expressions of all phosphoproteins induced by TUN (Figure 6).

4. Discussion

One of the early events in the development of various vascular diseases, including diabetes, hypertension, coronary and peripheral artery diseases is endothelial dysfunction[26,27]. Numerous factors assault the functioning of endothelial cells including hyperglycemia, accumulation of advanced glycation end products, oxidative stress, production of vasodilators such as NO, endothelin, *etc*[4]. All these factors affect ER homeostasis, leading to ER stress. Several clinical and preclinical studies emphasize the association of diabetes and ER stress, and this occupies the center stage in the progression of diabetes and its complications. Hence, restoring ER homeostasis appears to be a plausible therapeutic approach to impede the progression of diabetes. The current study was aimed at understanding the role of GMEt in TUN-induced ER stress in endothelial cells, and also to explore the possible molecular mechanism of GMEt. Currently, several treatment strategies are being explored to enhance endothelial function from various attacks. Over the past decade, attention was focused on the attenuation of ER stress using naturally available resources through a "mechanism-based approach" that can target various damages induced by oxidative, ER and inflammatory stress.

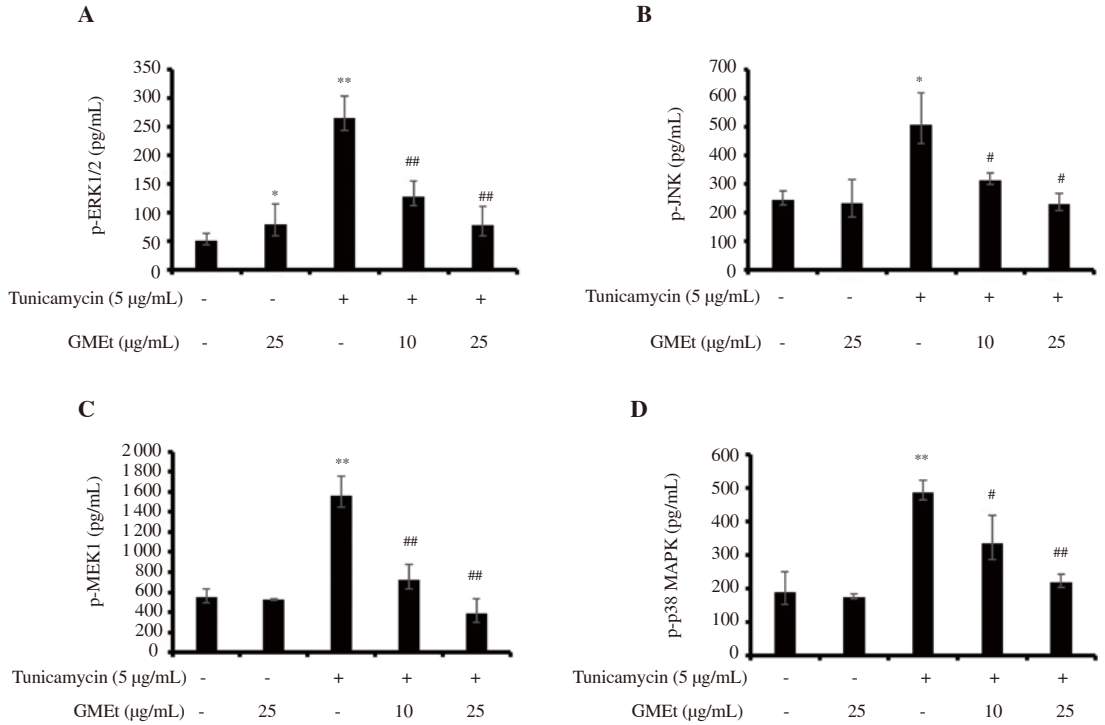

Figure 6. Effect of GMEt on upstream phospo-kinases including (A) p-ERK1/2 (B) p-JNK (C) p-MEK1 and (D) p-p38 MAPK against ER stress. Data are presented as mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's *post hoc* test. *Significance compared with the untreated control; #Significance compared with the tunicamycin-exposed control; *&#P<0.05; **&##P<0.01.

It is well known that sustained or uncontrolled ER stress leads to inflammation, cell injury and apoptosis, resulting in endothelial dysfunction mediated vascular diseases in diabetes. The signaling cascade involves various molecules, including MAP kinases, caspases, chaperones, and ER stress markers *viz.*, CHOP and GRP78, which play a critical role in ER stress-induced apoptosis[28,29]. MAPKs primarily activate and trigger the expression of various cellular response proteins that regulate multiple functions; hence the degree of phosphorylation of MAP kinases including ERK, JNK, MEK, and p38 is important in TUN-induced ER stress[27,29]. Our data is in line with Dai *et al.*, who reported cell death mediated by activation of ERK during chronic ER stress[30]. Activation of MAPK/ERK and MEK/ERK pathways by TUN that causes induction of GRP78 and in turn leads to cell death has been previously reported[31]. Results obtained in the present study are consistent with these reports. According to the previous study, U0126-induced inhibition of MEK reduced the levels of GRP78 and blocked ER stress[31]. The finding of the present study which showed GMEt treatment suppressed the levels of GRP78 induced by TUN is also in agreement with these findings.

It has been reported that, during ER stress, activation of JNK and p38 results in cell death[28]. Hao *et al.* revealed that berberine treatment suppressed the levels of apoptosis markers such as JNK, caspase-3 under ER stress environment[32]. TUN induced ER stress, in the present study, resulted in the upregulation of p-JNK and p-p38, however, GMEt treatment counteracted this effect by reducing the levels of p-JNK. Based on the aforementioned data, it can be ascertained that GMEt protects cells by modulating the MAP kinases during ER stress.

In vitro studies on endothelial cells revealed that GMEt protected endothelial cells from TUN-induced ER stress *via* the suppression of ER stress markers including GRP78 and CHOP. Few other studies also highlighted that plant extracts and their derivatives such as purple perilla extracts, bitter melon extract, propolis extract, and quercetin attenuate ER stress-induced damages[33,34]. Our earlier *in vivo* studies on GMEt confirmed that administration of the extract maintains the glucose homeostasis, protects pancreatic beta cells and also attenuates oxidative stress-mediated disturbances in the pancreas[21,35,36]. Few studies on extracts of *Euphorbia pekinensis*, *Glychirrhiza uralensis*, *Magnolia officinalis*, *Pueraria lobata, etc*., have proven their protective effects on the pancreatic beta-cell *via* suppression of ER stress markers[37].

To understand the molecular mechanisms involved in GMEt mediated protection, the expression of Nrf2 was analyzed. Nrf2 has been reported to be a promising therapeutic target for diabetes and its complication. GMEt treatment resulted in increased expression of Nrf2 in a dose-dependent manner as assessed using enzyme split complementation assay. Furthermore, it also enhanced the expressions of ARE mediated downstream target genes including *Nrf2*, *GST* and *NQO1*. The activation of Nrf2 is critical for the protection of cells against various types of oxidative stress factors. These findings are consistent with results for *Rubia philippinensis*[38], *Garcinia vilersiana*[39], *Psidium guajava pomífera* L.[40], which reported improvement of the antioxidant status through Nrf2 activation. Increased expressions of Nrf2 target genes, such as *NQO1*, *HO-1*, *GPX* and *SOD* were observed upon GMEt treatment to endothelial cells.

We have previously reported the chemical constituents of leaves of *G. montanum* which contain 11.57% w/w of carvacrol, 6.77% of erythritol, 4.58% of gallic acid and 3.09% of quercetin[35,41]. The presence of these constituents may be responsible for the beneficial activity of the extract. Moreover, many polyphenols present in plant extracts have been reported for their Nrf2 activation potential[10]. The cytoprotective effect of *G. montanum* reported in the present study is due to the activation of Nrf2, thereby triggering its downstream targets including antioxidant and detoxifying enzymes.

In conclusion, our results demonstrate that *G. montanum* inhibited ER stress-induced endothelial apoptosis through the activation of the Nrf2 signaling pathway. Further studies will be required in different cell types and in animal models to explore the efficacy of *G. montanum* in the development and progression of conditions associated with ER stress.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

DS, NS, SC and KMR designed the work. DS and NS conducted the work, collected and analyzed the data. DS, NS, SC and KMR drafted the manuscript and revised it critically. All authors agree to be accountable for all aspects of the work.

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