Justicia gendarussa Burm. f. leaf extract protects against glucotoxicity-induced pancreatic β-cell dysfunction and apoptosis

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Abstract. The present study aimed to develop a *Justicia gendarussa* Burm.f. leaf extract enriched with flavonoids and to investigate its antidiabetic mechanisms, particularly its ability to enhance pancreatic β-cell function and prevent β-cell death under hyperglycemic conditions. The extract, prepared using ethanol via the Soxhlet extraction method, yielded 15.06% dry extract by weight and exhibited a dark green, viscous appearance. Phytochemical analysis revealed a high flavonoid content (172.10 mg quercetin equivalents/g extract) and a moderate phenolic content (23.47 mg gallic acid equivalents/g extract). Antioxidant activity, evaluated using 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid assays, demonstrated potent radical scavenging capacity, with IC_{50} values of 1.15±0.14 and 0.42±0.07 mg/ml, respectively. High-performance liquid chromatography analysis identified vitexin as the one bioactive flavonoid in the extract with amount of 0.19±0.02 mg/g extract. *In vitro* studies using INS-1 pancreatic β-cells exposed to high glucose concentrations showed that the extract at concentrations of 50 and 100 μg/ml markedly increased cell viability and reduced intracellular reactive oxygen species levels comparable with those of normal-glucose controls. The antioxidant effect of the JG extract was mediated through the upregulation of key antioxidant genes, Nrf2 and Trx. Furthermore, the extract reduced apoptosis by 25.59%, an effect comparable with that of 10 μM vitexin. Gene expression analysis revealed that the extract markedly upregulated pancreatic and duodenal homeobox 1 mRNA levels compared with the high-glucose group, suggesting enhanced insulin synthesis via activation of key transcriptional regulators. In conclusion, the cytoprotective effects of JG extract are mediated through antioxidant activity, inhibition of apoptosis and stimulation of insulin gene expression. Further molecular and *in vivo* studies, along with clinical validation, are warranted to support the development of this herbal extract as a complementary therapeutic strategy for managing type 2 diabetes.

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Introduction

Type 2 diabetes is the most common type of diabetes. It is one of the non-communicable diseases with an increasing incidence worldwide; global data indicates that approximately 589 million adults (aged 20-79) are living with diabetes (1). The pathophysiology of diabetes is caused by insulin resistance and/or insulin deficiency. Contributing factors include eating a diet high in fat and carbohydrates, lack of exercise, stress and obesity. The result of prolonged high blood sugar levels causes complications in various organs, such as neuropathy, retinopathy and nephropathy (2).

Normally, the progression of clinical diabetes 2 will be insulin resistance or decreased insulin function first due to various contributing factors such as daily behavior, eating foods high in starch and fat, stress and lack of exercise. In addition, there are abnormalities of some genes related to insulin signaling in target cells, resulting in high blood sugar levels, which will stimulate pancreatic β-cells to respond by working

more to produce insulin sufficient for the body's needs. If blood sugar levels are continuously high, the cells will work hard until exhaustion occurs, producing less insulin, resulting in continuous high blood sugar levels, which will create free radicals reactive oxygen species (ROS) and stimulate endoplasmic reticulum (ER) stress (3) and mitochondrial stress (4), which are toxic to pancreatic β-cells, called glucotoxicity, resulting in apoptosis and insulin deficiency in the later stages and eventually diabetes.

Currently, medicinal plants that have medicinal properties for treating diabetes are another alternative that has gained much interest. In the present study, the researchers were interested in the medicinal properties of *Justicia gendarussa* Burm.f. (JG; Acanthaceae) leaf extract. JG is a local medicinal plant that is easy to find and is popularly grown in the eastern region of Thailand. The important substances found in leaves that have antioxidant properties include phenolic compounds and flavonoids, which are found in high quantities (5,6). Anti-inflammatory studies have shown that flavonoids (vitexin and apigenin) can reduce inflammation as well as nonsteroidal anti-inflammatory drugs (7). Studies have demonstrated that JG extracts possess significant anti-inflammatory and antioxidant activities, primarily through the inhibition of pro-inflammatory cytokines and the scavenging of free radicals (8,9), while their potential in treating asthma and hyperuricemia conditions has been explored (10,11). Another interesting property is the anti-diabetic effect. It was found that the JG leaf extract at concentrations of 50 and 100 mg/kg/bw can reduce blood sugar and lipid levels and reduce urine sugar in diabetic rats (12). Moreover, the 12 h treatment period of the methanolic leaf extract of JG at 400 mg/kg bw reduced blood glucose level to 52.75% (12). However, the mechanism used to reduce the blood sugar of JG leaf extract is still unknown. Therefore, the aims of the present study are to develop an extract from JG leaf and to test the anti-diabetic mechanism of JG leaf extract through preventing the death of pancreatic β-cells and increasing the efficiency of pancreatic β-cells in producing and secreting insulin in INS-1 rat insulinoma cell line.

Materials and methods

Plant collection and extraction. JG leaves were collected from Ban Bueng District, Chonburi Province, Thailand. The plant was identified by Associated Professor Dr Watchara Damjuti (Faculty of Integrative Medicine, Rajamangala University of Technology Thanyaburi (RMUTT), Pathumtani, Thailand) and the voucher specimen (identification no. CR0011/25) is kept at herbarium of the Faculty of Integrative Medicine, RMUTT. Plants were cleaned and dried in an oven at 50-60˚C. A total of 366 g of dried plant powder was subjected to extraction using ethanol as the solvent via the Soxhlet extraction method, with a plant-to-solvent ratio of 20 g:300 ml. The extraction was continued until the active compounds were completely extracted from the plant material (~6 h). The resulting extract solution was then concentrated and dried at 50-55˚C under vacuum conditions using a rotary evaporator.

Determination of flavonoid content in the extract. The JG extract has been previously reported to possess a high content of flavonoids. To quantify this, the total flavonoid content was determined using the aluminium chloride colorimetric method, employing quercetin as a reference standard. For each standard solution, 200 μl was mixed with 100 μl of 10% (w/v) aluminium chloride and 100 μl of 0.1 mM potassium acetate in a test tube. The mixtures were incubated at room temperature for 30 min to allow for color development. Absorbance was subsequently measured at 415 nm using a microplate reader and a standard calibration curve was constructed by plotting absorbance values against quercetin concentrations. The JG extract was prepared at a single concentration and subjected to the same assay conditions as the standard solutions. All measurements were performed in triplicate. The absorbance of the extract was interpolated from the standard curve to calculate the flavonoid content, which was expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g).

Determination of total phenolic content (TPC). The total phenolic content of the JG extract was measured using the Folin-Ciocalteu colorimetric method, as described by Singleton *et al* in 1999 (13). Briefly, 25 μl of the JG extract (at concentrations ranging from 0.0156-1 mg/ml) was mixed with 25 μl of 0.2 N Folin-Ciocalteu reagent. The plate was incubated in the dark at room temperature for 30 min. Then, 100 μl of a 7% sodium carbonate solution was added and the reaction mixture was incubated for 5 min in the dark at room temperature. The absorbance was measured at 730 nm using a microplate reader (FLUOstar Omega®; BMG Labtech GmbH). The total phenolic content was calculated as gallic acid equivalents (GAE) in mg per gram of extract (mg GAE/g dry wt.), using a standard curve prepared with gallic acid. The experiment was conducted in triplicate.

Phytochemical analysis by high-performance liquid chromatography (HPLC). According to a previously report, vitexin, naringenin, apigenin and kaempferol are found in JG leaves (14). In the present study, vitexin was used as a chemical marker and its quantitative analysis in JG extract was measured using the HPLC following a previous report (15). HPLC analysis was performed on the Agilent-1260 Infinity system (Agilent Technologies, Inc.) coupled with a photodiode array detector. In the separation condition, reverse phase C18 column (Kromasil 100-5; 250x4.6 mm; Agilent Technologies, Inc.) was used as a stationary phase and acetonitrile:0.1% ortho-phosphoric acid (20:80, v/v) was used as a mobile phase. The flow rate was 1 ml/min. The vitexin signal was detected at 335 nm. The calibration curve of vitexin was conducted from vitexin concentrations and obtained area under the curve. Vitexin content in JG extract was calculated from the calibration curve.

Examination of antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The ability to scavenge DPPH radicals of JG extract was assessed by using a modified version of the Clarke method from 2013 (16). Briefly, a 96-well plate was filled with 50 μl of JG extract in 5% DMSO to the final concentration range of 0.0156-1 mg/ml, followed by 150 μl of 0.3 M DPPH reagent in absolute ethanol. After shaking for two min, the plate was kept in the dark at room temperature for 30 min. At the end of incubation, the absorbance was measured by a spectrophotometer (FLUOstar

Omega®; BMG Labtech GmbH) at a wavelength 517 nm. The ability of the JG extract to scavenge free radicals was compared with standard gallic acid. The experiment was conducted in 3 dependent experiments. % DPPH scavenging potential was calculated by the following formula:

% DPPH scavenging potential=[(Control absorbance-Sample absorbance)/Control absorbance] x100

Examination of antioxidant activity by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay. The ABTS radical scavenging assay was conducted using a modified version by a previous study (17). ABTS•+ radical cations were generated by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate in distilled water and allowing the mixture to stand for 12-16 h in the dark at room temperature. The resulting ABTS•+ solution was diluted with methanol to an absorbance of 0.7±0.02 at 734 nm. To 100 μl of the JG extract at various concentrations (10, 50, 100 and 200 μg/ml), 100 μl of ABTS•+ solution was added and the mixture was incubated for 6 min at room temperature. Absorbance was then measured at 734 nm using a microplate reader (FLUOstar Omega®; BMG Labtech GmbH). % ABTS scavenging potential was calculated by the following formula:

% ABTS scavenging potential=[(Control absorbance-Sample absorbance)/Control absorbance] x100

INS-1-cell culture. INS-1 cells (INS1 832/13 rat insulinoma cell line; MilliporeSigma) were cultivated in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (FCS) (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 g/ml streptomycin at 37˚C in humidified air containing 5% CO_2. The culture medium was changed every 2 days. INS-1 cells were maintained normally in a solution containing 11.1 mM glucose (their basal level) and they were cultured in an approximately fourfold (40 mM) glucose to induce glucotoxicity.

Cytotoxicity and cytoprotective effect assessment by MTT assay. The MTT assay was performed to determine toxicity as previously described (18). Briefly, INS-1 cells, ~10,000 cells per well, were seeded into 96-well plates. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 0.05 mM β-mercaptoethanol at 37˚C with 5% CO_2 incubator. To examine cytotoxicity of JG extract, cells were exposed to varying concentrations of the JG extract ranging from 31.2-2,000 μg/ml for 72 h. To evaluate the cytoprotective effect of the JG extract, cells were divided into several groups: a negative control (11.1 mM glucose), a glucotoxic/positive control (40 mM glucose) and treatment groups (40 mM glucose + 50 or 100 μg/ml JG extract, as well as 10, 20, or 40 μM vitexin). After 72 h of incubation, a 100 μl of MTT solution (final concentration 0.5 mg/ml) was added to each well. After incubation at 37˚C with 5% CO_2 for 90 min, MTT solution were removed, then, the formazan crystals were dissolved in 150 μl DMSO. The absorbance was determined using a microplate scanning spectrophotometer (FLUOstar Omega®; BMG Labtech GmbH) at 570 nm. All experiments were conducted in triplicate in three independent experiments. The following calculation was used to calculate the proportion of viable cells using the averaged 570-nm absorbance values.

Cell viability (%)=Absorbance of sample/Absorbance of control x100 (1).

Measurement of intracellular ROS production by 2'-7'dichlorofluorescin diacetate (DCFH-DA) assay. The DCFH-DA probe was used to assay the ROS in cells. The cells were cultured with 40 mM glucose alone or with 40 mM glucose plus various concentrations of JG leaf extract or vitexin. The cells were grown in a 5% CO_2 incubator at 37˚C for 48 h. The cells were then aspirated from 96-well plates and the cells were washed with 100 μl of Sterile PBS. 100 μM of DCFH-DA solution was added. The cells were incubated in 5% CO_2 incubator at 37˚C for 30 min. The fluorescence absorbance was measured at the wavelengths of Excitation 495 nm and Emission 529 nm using a spectrophotometer (FLUOstar Omega®; BMG Labtech GmbH). All experiments were conducted in triplicate in three independent experiments.

Analysis of cell apoptosis by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining, followed by flow cytometry. Apoptosis was assessed using FITC/PI staining followed by flow cytometric analysis, in accordance with the manufacturer's protocol and the MIFlowCyt guidelines for flow cytometry experiments by Lee *et al* 2008 (19). INS-1 pancreatic β-cells were cultured under standard conditions and exposed to high-glucose stress (40 mM glucose) in the presence or absence of JG leaf extract (50 μg/ml) or vitexin (10 μM). Untreated cells cultured under normal glucose conditions served as controls. Cells were incubated for 72 h prior to analysis. After treatment, cells were harvested, washed twice with cold phosphate-buffered saline (PBS) and resuspended in binding buffer. Cells were then stained with 5 μl of Annexin V-FITC and 5 μl of PI for 15 min at 4˚C in the dark. Following incubation, samples were immediately analyzed by flow cytometry using a FACSort flow cytometer (Becton, Dickinson and Company). Forward scatter and side scatter parameters were used to exclude debris and cell aggregates. A minimum of 10,000 events per sample were collected. Apoptotic cells were quantified as the percentage of Annexin V-positive cells within the gated population. Data analysis was performed using appropriate flow cytometry analysis software (CellQuest Pro software version 5.2.1; BD Biosciences). Axis labels in all flow cytometry plots indicate the fluorochrome-marker combinations, with Annexin V-FITC displayed on the X-axis and PI on the Y-axis. Cells were gated and classified into four distinct quadrants representing different cellular states: Viable cells (Annexin V negative + PI negative), Early apoptotic cells (Annexin V Positive + PI negative), Late apoptotic cells (Annexin V positive + PI positive), and Necrotic cells (Annexin V positive + PI positive). The total apoptotic rate was calculated using the following equation:

Total Apoptotic Rate (%)=[Percentage of Early Apoptotic Cells] + [Percentage of Late Apoptotic Cells]. All experiments were conducted in triplicate in three independent experiments.

Table I. Yield of *Justicia gendarussa* leaf extract and contents of phenolic and flavonoid compounds in the extract.

Plant dry weight	Extract weight	% Yield	Total phenolic content (quercetin equivalents/g extract)	Total flavonoid content (gallic acid equivalents/g extract)
386 g	58.14 g	15.06	172.10±71.50 mg	23.47±4.51 mg

Total phenolic content and total flavonoid content in JG leaf extracts were measured by Folin-Ciocalteu colorimetric and aluminium chloride colorimetric methods, respectively. Data are expressed as mean ± SEM (n=3).

Measurement of insulin and insulin transcription factors mRNA expression by reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from 1x10^6 cells using the PureDireX® Total RNA Isolation Kit (Bio-Helix Co., Ltd.) following a modified manufacturer's protocol. Briefly, harvested cells were transferred to a sterile microcentrifuge tube and lysed in 100 μl of Lysis Buffer supplemented with 4 μl of β-mercaptoethanol. The mixture was homogenized by gentle pipetting and incubated at room temperature for 5 min. The cell lysate was then centrifuged at 16,000 x g for 10 min at 4˚C to pellet cellular debris. The supernatant was carefully collected, transferred to a new microcentrifuge tube, and mixed with 500 μl of 70% ethanol prepared in RNase- and DNase-free water. The total volume was loaded onto a spin column assembled with a collection tube and centrifuged at 14,000 x g for 1 min at 4˚C. The flow-through was discarded, and the column was placed into a new collection tube. Next, the column was washed by adding 400 μl of Wash Buffer 1 and centrifuged at 14,000 x g for 30 sec at 4˚C, followed by a second wash step with 600 μl of Wash Buffer 2 and centrifugation at 14,000 x g for 2 min at 4˚C. To elute the bound RNA, the column was placed into a fresh microcentrifuge tube, and a 50 μl of Elution Buffer was applied directly to the membrane. After incubating at room temperature for 2 min, the purified total RNA was recovered by centrifugation at 14,000 x g for 2 min at 4˚C. The concentration and purity of the isolated total RNA were determined using a NanoDrop spectrophotometer. cDNA synthesis and qPCR complementary DNA (cDNA) synthesis was performed using the RScript cDNA Synthesis Kit (Bio-Helix Co., Ltd.) according to the manufacturer's instructions. The resulting cDNA was subsequently used as a template to measure the mRNA expression level of the target genes. RT-qPCR was employed to assess the mRNA expression levels of the insulin, Nrf2, and Trx genes. The reaction was performed using a ready-to-use 5X HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne OÜ). Each PCR reaction contained 450 ng of cDNA template, 0.5 μl of 10 ng/μl forward and reverse primers and 5 μl of the qPCR master mix (Bio-Helix Co., Ltd.). The thermal cycling conditions were as follows: Initial polymerase activation at 95˚C for 15 min, followed by 45 cycles of denaturation at 95˚C for 15 sec, annealing at 58˚C for 60 sec and extension at 72˚C for 60 sec. β-actin was used as the endogenous reference gene and the untreated control group was used as the calibrator. Relative gene expression levels were calculated using the comparative $2^{-\Delta\Delta Cq}$ method based on fluorescence signal detection. A melting curve analysis was performed at the end of the amplification cycles to confirm the specificity and presence of a single amplicon. All reactions were conducted in triplicate. Primer sequences are following; Forward primer for insulin: 5'-CTGCCCAGGCTT TTGTCAAA-3' Reverse primer for insulin: 5'-CTTCCACCA AGTGAGAACCACA-3' Forward primer for pancreatic and duodenal homeobox 1 (Pdx1): 5'-GGTGCCAGAGTTCAGTGC TAA-3' Reverse primer for Pdx1: 5'-CCAGTCTCGGTTCCA TTCG-3' Forward primer for Nrf2: 5'-AGGACATGGAGCAAG TTTGG-3' Reverse primer for Nrf2: 5'-TTCTTTTTCCAGCGA GGAGA-3' Forward primer for Trx: 5'-CCTTCTTTCATTCCC TCTGTGA-3' Reverse primer for Trx: 5'-CCCAACCTTTTG ACCCTTTTTA-3'. Forward primer for β-actin: 5'-ATCCTG GCCTCACTGTCCAC-3' Reverse primer for β-actin: 5'-CTA GAAGCATTTGCGGTGCA-3'. RT-qPCR was performed to analyze relative gene expression levels. The relative quantification of target genes was calculated using the comparative $2^{-\Delta\Delta Cq}$ method (20). Target gene expression levels were normalized to the reference gene (β-actin) and expressed relative to the control group (untreated cells). All experiments were conducted in triplicate in three independent experiments.

Statistical analysis. All data are expressed as mean ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and Dunnett multiple comparison test. All analyses were performed using GraphPad Prism 6 (Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Extraction and quantification of phenolic and flavonoid compounds in JG leaf extract. The leaves of JG were extracted using ethanol as the solvent via Soxhlet extraction. The process yielded 56.14 grams of crude extract, representing an extraction yield of 15.06% based on the dry powder weight. The resulting extract exhibited a viscous consistency and a deep green color. Phytochemical analysis revealed the presence of key bioactive constituents, particularly phenolic and flavonoid compounds. Quantitative determination indicated that flavonoids were the predominant group, present at a high concentration of 172.10 mg QE/g of extract. By contrast, the total phenolic content was measured at 23.47 mg GAE/g of extract (Table I), substantially lower than the flavonoid content. These findings highlighted the potential of JG leaf as a rich natural source of antioxidant flavonoids and support their application in the development of herbal therapeutics and functional health products.

Analysis of bioactive compounds by HPLC. The vitexin content in JG extract was quantified by HPLC. The calibration curve was conducted by measuring six different concentrations of

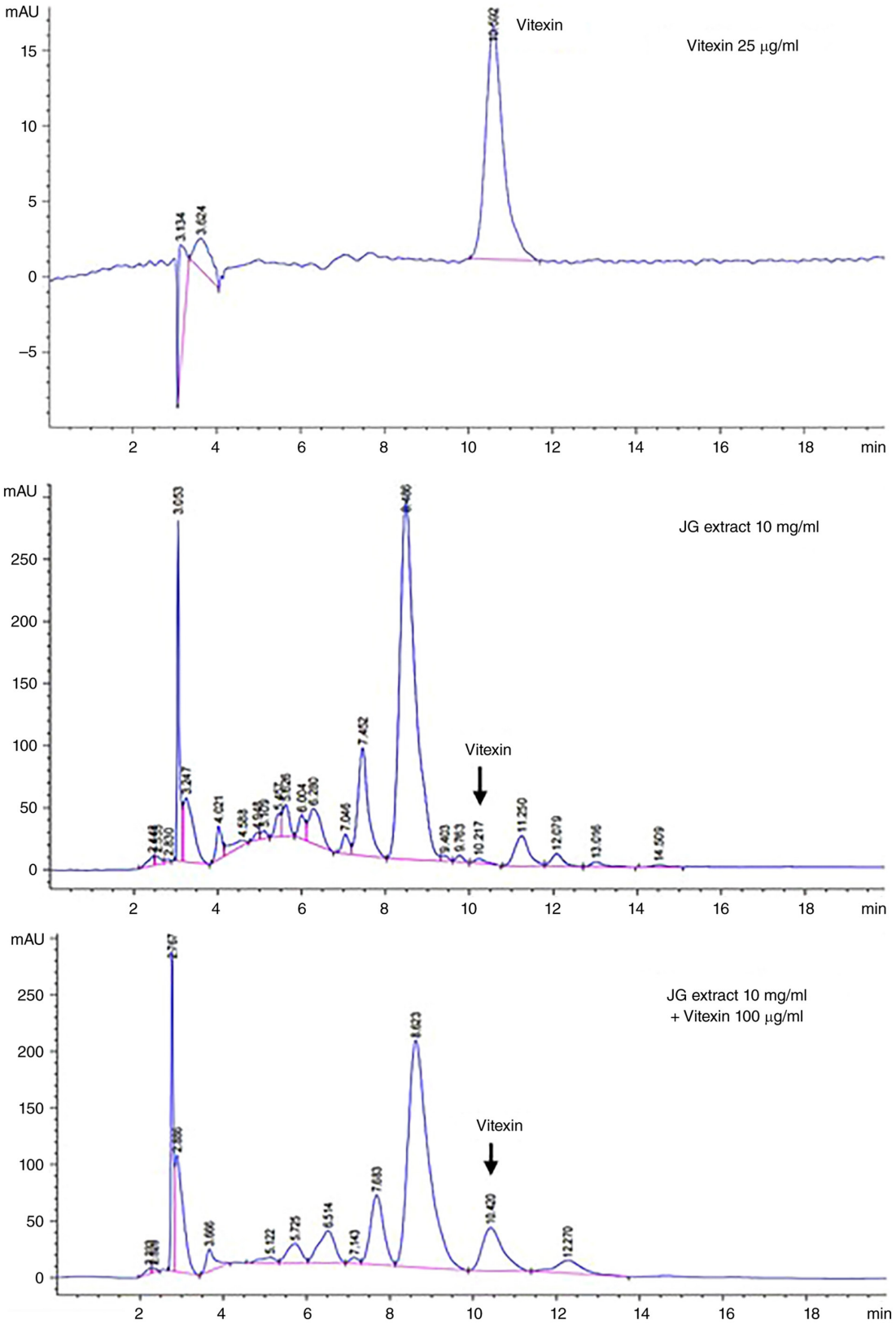

Figure 1. HPLC chromatogram of JG leaf extract and vitexin standard. HPLC, high-performance liquid chromatography; JG, *Justicia gendarussa*.

standard solution in the range of 6.25-200 μg/ml. The obtained linear equation was y=17.024x + 40.949, with a coefficient of determination (R^2) of 0.9997. The chromatograms of vitexin standard and JG extract are shown in Fig. 1. The vitexin content was found to be 0.19±0.02 mg/g extract.

In vitro evaluation of the antioxidant activity of JG extract. The antioxidant activity of JG leaf extract was evaluated *in vitro* using DPPH and ABTS radical scavenging assays. Gallic acid was employed as a standard reference antioxidant. As presented in Table II, the extract exhibited radical scavenging

Table II. *In vitro* DPPH and ABTS•+ antioxidant activity of JG leaf extracts.

Sample	DPPH IC_{50} (mg/ml)	ABTS•+ IC_{50} (mg/ml)
JG leaf extract	1.15±0.14	0.42±0.07
Gallic acid	0.006±0.01	0.0019±0.02

Antioxidant activity of JG leaf extracts compared with standard antioxidants (gallic acid) by DPPH and ABTS assay. Data are expressed as mean ± SEM (n=3). DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; JG, *Justicia gendarussa;* IC_{50}, half-maximal inhibitory concentration.

capacity against both DPPH and ABTS•+ radicals. The extract demonstrated IC_{50} values of 1.15±0.14 mg/ml for the DPPH assay and 0.42±0.07 mg/ml for the ABTS assay, respectively. These results indicated that JG leaf extract possesses notable antioxidant potential, suggesting its utility as a natural source of free radical scavengers for pharmaceutical or nutraceutical applications.

Cytotoxicity assessment of JG leaf extract on pancreatic β-cells. The cytotoxicity of JG leaf extract was evaluated *in vitro* using INS-1 cells, a rat-derived pancreatic β-cell line. Cells were exposed to varying concentrations of the JG extract ranging from 31.2-2,000 μg/ml for 72 h. The results demonstrated that concentrations between 31.2-1,000 μg/ml did not exert cytotoxic effects on INS-1 cells. The percentage of cell viability in these treatment groups was not markedly different from that of the untreated control group. However, at the highest tested concentration (2,000 μg/ml), cell viability decreased markedly to ~70% compared with the control (Fig. 2). These findings indicated that JG leaf extract was non-cytotoxic to pancreatic β-cells at concentrations up to 1,000 μg/ml and therefore, this concentration range was selected for subsequent biological assays.

Protective effect of JG leaf extract on pancreatic β-cell viability under high glucose-induced glucotoxicity. Chronic exposure to high glucose concentrations induces glucotoxicity, leading to pancreatic β-cell dysfunction and reduced cell survival (18,21). In the present study, the protective effect of JG leaf extract against glucotoxicity-induced cytotoxicity in β-cells was evaluated using the MTT assay. INS-1 cells cultured under high-glucose conditions for 72 h exhibited a marked decrease in viability to ≤50% compared with the normoglycemic control group. However, co-treatment with JG extract at concentrations of 50 and 100 μg/ml markedly improved cell viability, restoring it to levels comparable to the normoglycemic control. Additionally, vitexin, a flavonoid compound identified in the extract, was evaluated independently at concentrations of 10, 20 and 40 μM. Vitexin markedly enhanced β-cell survival under high-glucose conditions, with effects comparable to those observed with the crude extract (Fig. 3). These findings suggested that vitexin may play a key role in the cytoprotective activity of JG leaf extract under glucotoxic stress.

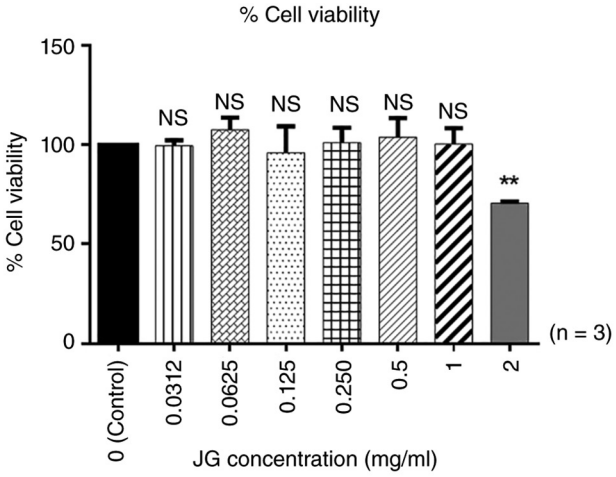

Figure 2. Cytotoxicity test of JG leaf extract in pancreatic β-cells by MTT assay at concentrations ranging from 31.2-1,000 μg/ml for 72 h. The percentage of cell survival was expressed as mean ± SEM (n=3) and analyzed by one-way ANOVA followed by Dunnett multiple comparison (**P<0.01 compared with the control group; NS, Non-significant between test and control (P>0.05). JG, *Justicia gendarussa*.

Antioxidant effect of JG leaf extract on pancreatic β-cells under high glucose-induced oxidative stress is mediated through the upregulation of key antioxidant genes, Nrf2 and Trx. The intracellular antioxidant activity of JG leaf extract was evaluated in pancreatic β-cells subjected to high glucose-induced oxidative stress. The level of intracellular ROS was measured using the DCFH-DA assay after 48 h of treatment. The results demonstrated that treatment with JG extract at concentrations of 50 and 100 μg/ml markedly reduced intracellular ROS levels compared with the high-glucose-only group. Notably, ROS levels in extract-treated groups were comparable to those in the normoglycemic control group. Furthermore, vitexin was tested at concentrations of 10, 20 and 40 μM and showed a similar reduction in intracellular ROS levels (Fig. 4A). High glucose induces oxidative stress by increasing ROS production. Which corresponds to suppressing key antioxidant regulators (Trx and Nrf2) (Fig. 4B and C). JG extract and vitexin effectively counteract these effects by upregulating antioxidant-related gene expression, supporting their protective effect against high glucose-induced oxidative damage.

Anti-apoptotic effect of JG leaf extract on pancreatic β-cells under high glucose conditions. Based on prior findings, the most effective concentrations for cellular protection and intracellular antioxidant activity were determined to be 50 μg/ml for JG leaf extract and 10 μM for the flavonoid standard, vitexin. These concentrations were subsequently used to evaluate the extract's effect on apoptosis in pancreatic β-cells under high-glucose conditions over a 72-h period. Apoptotic cell death was assessed using Annexin V/propidium iodide (PI) staining followed by flow cytometric analysis. As shown in Fig. 5, cells exposed to high-glucose conditions exhibited a significant increase in apoptosis, with 37.39% of cells undergoing programmed cell death compared with the normoglycemic control. Treatment with JG leaf extract at 50 μg/ml markedly reduced the percentage of apoptotic cells to 25.59%, while vitexin at 10 μM achieved a comparable reduction to

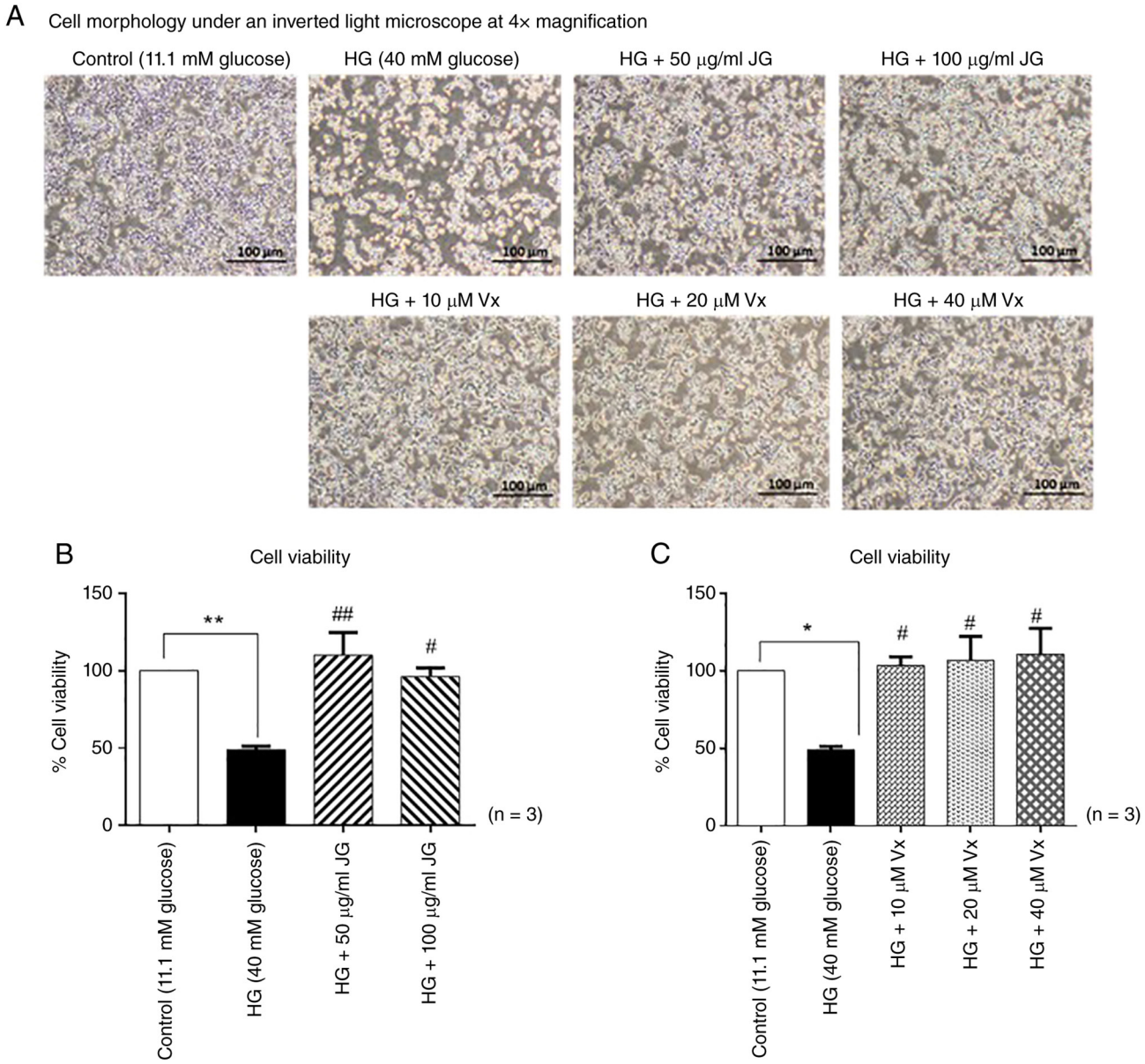

Figure 3. Protective effect of JG leaf extract and Vitexin on pancreatic β-cells against glucose toxicity. INS-1 cells were cultured in 40 mM glucose with or without JG leaf extract or Vitexin for 48 h. (A) Cells morphology in different groups under an inverted light microscope at 4x magnification. Cell viability of INS-1 cell-treated with JG leaf extract (B) or treated with vitexin (C) were measured by MTT assay. Data are expressed as mean ± SEM (n=3) and analyzed by One-way ANOVA with Tukey's multiple comparison test. *P<0.05 vs. control; **P<0.01 vs. control; $^{\#}$P<0.05 vs. HG group; $^{\#\#}$P<0.01 vs. HG group. JG, *Justicia gendarussa*; HG, high glucose.

23.59%. These reductions were statistically significant when compared with the high-glucose-only group. These results highlight the potential of JG leaf extract and vitexin to mitigate pancreatic β-cell apoptosis induced by hyperglycemia. This cytoprotective effect suggests a promising therapeutic role in preserving β-cell function under diabetic conditions.

Effect of JG leaf extract on the expression of insulin and the insulin transcription factor Pdx1 under high-glucose conditions. The effect of JG leaf extract on insulin biosynthesis in pancreatic β-cells under high-glucose-induced stress was investigated by measuring the mRNA expression levels of the insulin gene and its key transcription factor, Pdx1. INS-1 cells were cultured under high-glucose conditions and treated with either the extract (50 μg/ml) or the reference flavonoid compound, vitexin (10 μM), for 48 h. Gene expression was quantified using RTqPCR. The results demonstrated a marked downregulation of insulin mRNA in the high-glucose-only group, with expression reduced to 0.109-fold relative to the normoglycemic control. Treatment with JG leaf extract markedly restored insulin mRNA expression to 0.59-fold, while vitexin increased expression to 0.33-fold, both compared with the high-glucose group. These data suggested that the extract at 50 μg/ml was more effective in restoring insulin gene expression than vitexin at 10 μM (Fig. 6A). Similarly, the expression pattern of Pdx1 mRNA mirrored that of insulin, with significant restoration following treatment with the extract (Fig. 6B). These findings indicated that JG leaf extract enhanced insulin gene expression under hyperglycemic conditions, at least in part, through the upregulation of Pdx1, a master regulator of

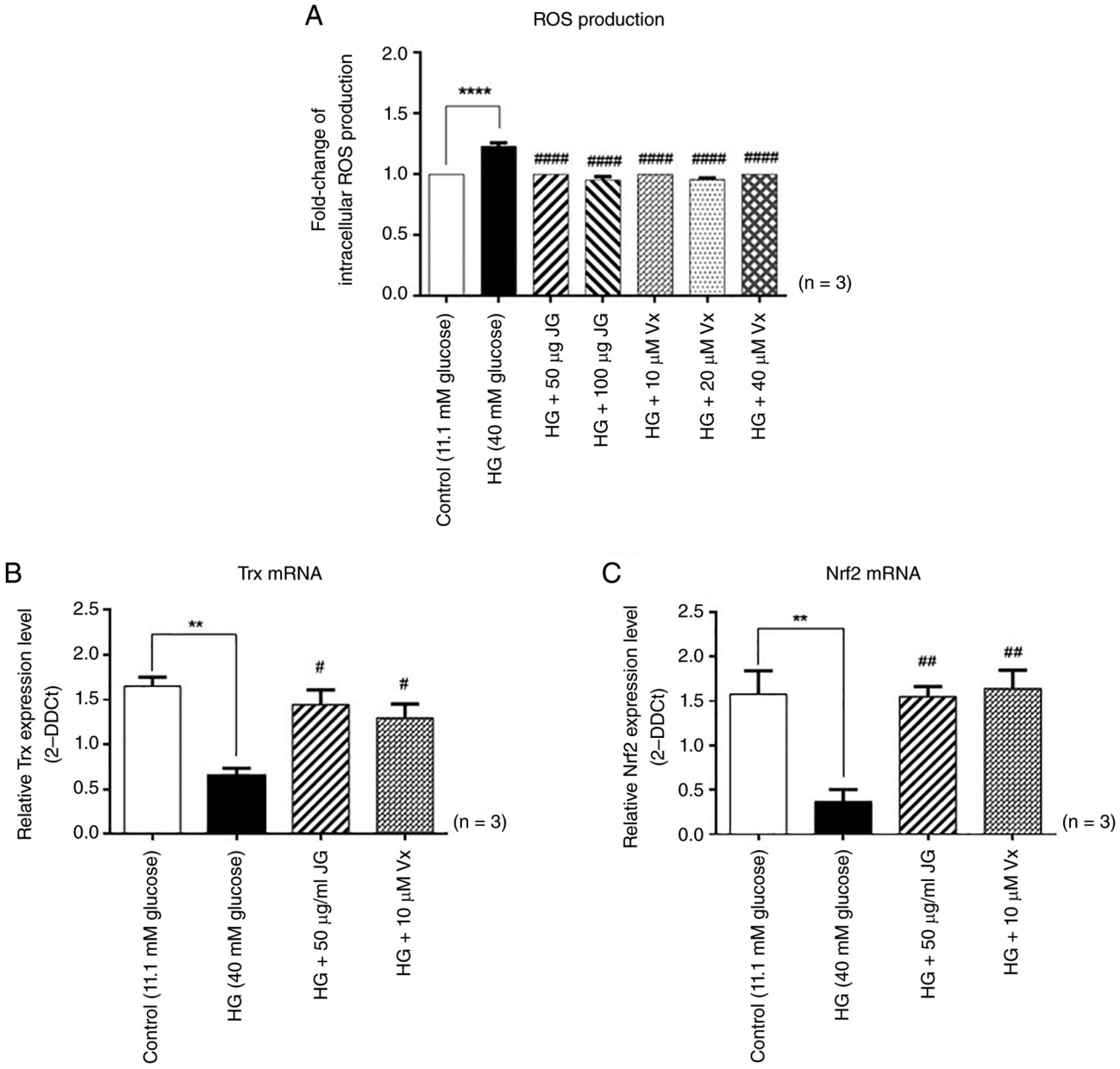

Figure 4. Antioxidant activity of JG leaf extract and vitexin against glucose toxicity. INS-1 cells were cultured in 40 mM glucose with or without JG leaf extract or Vitexin for 48 h. (A) Intracellular ROS levels were measured by DCFH-DA assay. Effect of JG extract on antioxidant regulators (B) Trx and (C) Nrf2 mRNA expression by measuring mRNA levels at 48 h by reverse transcription-quantitative PCR. Data are expressed as mean ± SEM (n=3) and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. **P<0.01 vs. control group; ****P<0.0001 vs. control group; $^{\#}$P<0.05 vs. HG group; $^{\#\#}$P<0.01 vs. HG group; $^{\#\#\#\#}$P<0.0001 vs. HG group. JG, *Justicia gendarussa*; HG, high glucose; ROS, reactive oxygen species; DCFH, 2'-7'dichlorofluorescin diacetate.

insulin transcription. This suggested a potential mechanism by which the extract may help preserve pancreatic β-cell function in diabetes.

Discussion

The present study demonstrated that the extraction of JG leaf using ethanol as a solvent via Soxhlet extraction yielded a crude extract equivalent to 15.06% of the dry leaf powder weight. The extract was characterized by a viscous consistency and dark green coloration. Phytochemical analysis revealed that the total flavonoid content (172.10 mg QE/g extract) was markedly higher than the total phenolic content (23.47 mg GAE/g extract). These findings are consistent with the report by Kuber *et al* (22) in 2021, which documented high flavonoid content (97.6±0.0342 mg Rutin equivalent/g) in ethanolic extracts of JG leaf, while the total phenolic content was relatively low (9.47±0.0216 mg GAE/g). However, the results differ from those reported by Marliani *et al* (5) in 2022, who found that extraction using varying ratios of water, ethanol and hexane yielded higher phenolic content (112.076 mg GAE/g) than flavonoids (34.926 mg QE/g). Such discrepancies may be attributed to differences in the choice of extraction solvents, sample preparation techniques and environmental factors affecting the plant materials used, such as leaf maturity, seasonal variation and geographical origin. These variables are known to influence the phytochemical composition of herbal extracts.

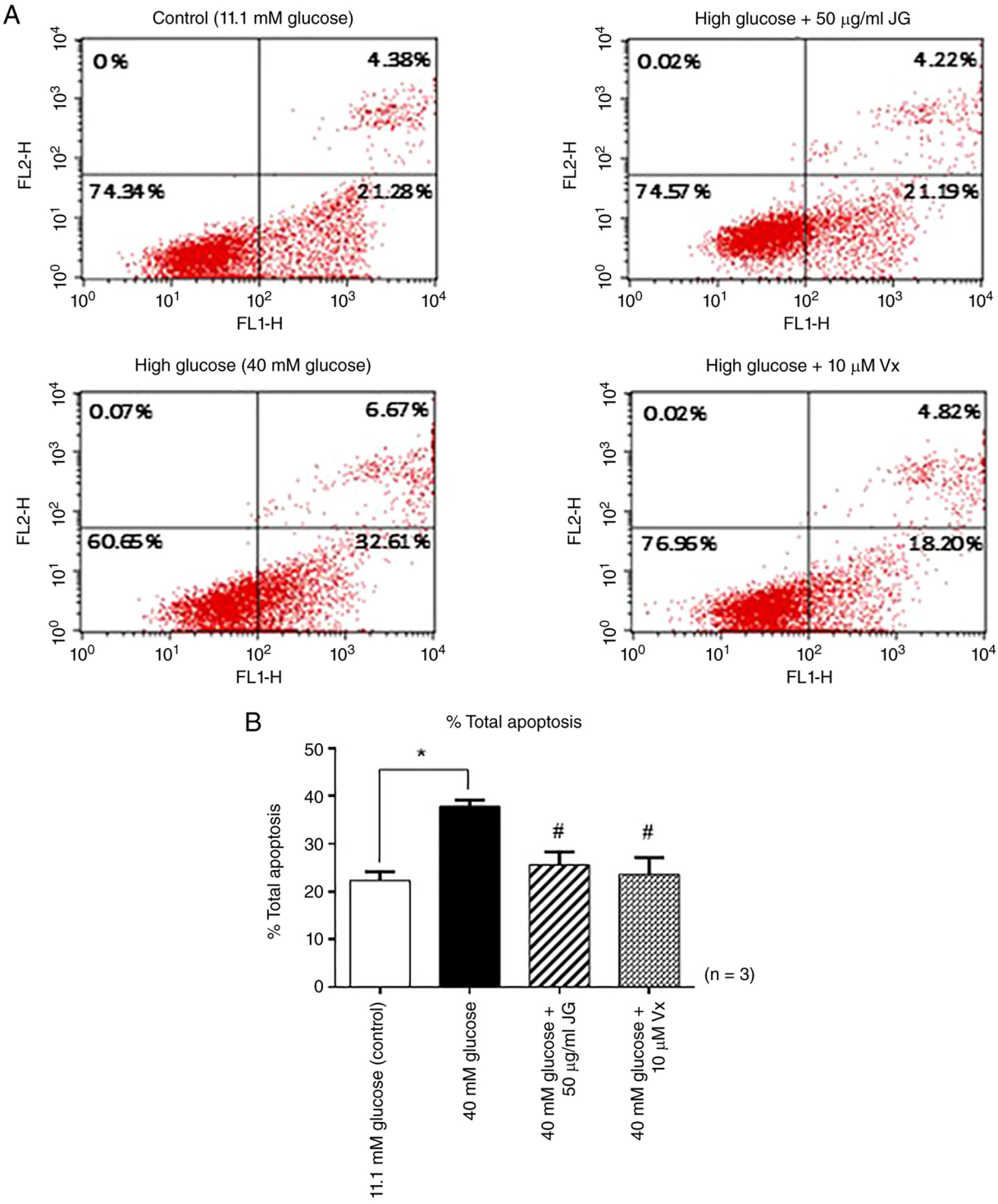

Figure 5. Anti-apoptotic effect of JG leaf extract and Vitexin against glucotoxicity in pancreatic β-cells at 72 h. (A) Dot plots of Annexin V/PI staining analyzed by flow cytometry. (B) The percentage of apoptotic cells was expressed as mean ± SEM (n=3) and analyzed by One-way ANOVA with Tukey's multiple comparison test. *P<0.05 vs. control group; #P<0.05 vs. High glucose (HG) group. JG, *Justicia gendarussa*; PI, propidium iodide; HG, high glucose.

The antioxidant capacity of JG leaf extract was evaluated using DPPH and ABTS radical scavenging assays. The extract demonstrated significant free radical scavenging activity, with IC_{50} values of 1.15±0.14 mg/ml for the DPPH assay and 0.42±0.07 mg/ml for the ABTS assay, indicating prominent antioxidant potential. These findings are consistent with previous studies reporting potent antioxidant properties of JG ethanol extracts. For instance, a prior investigation found that the ethanolic leaf extract exhibited strong DPPH radical scavenging activity, with an IC_{50} value as low as 0.032 mg/ml (22). Together, these results support the conclusion that JG leaf possess substantial antioxidant activity and may serve as a promising natural source of free radical scavengers.

Vitexin and apigenin were shown to be the two major flavonoids present in high content in the JG leaf extract (14,15). However, vitexin was present in the extract at a relatively low

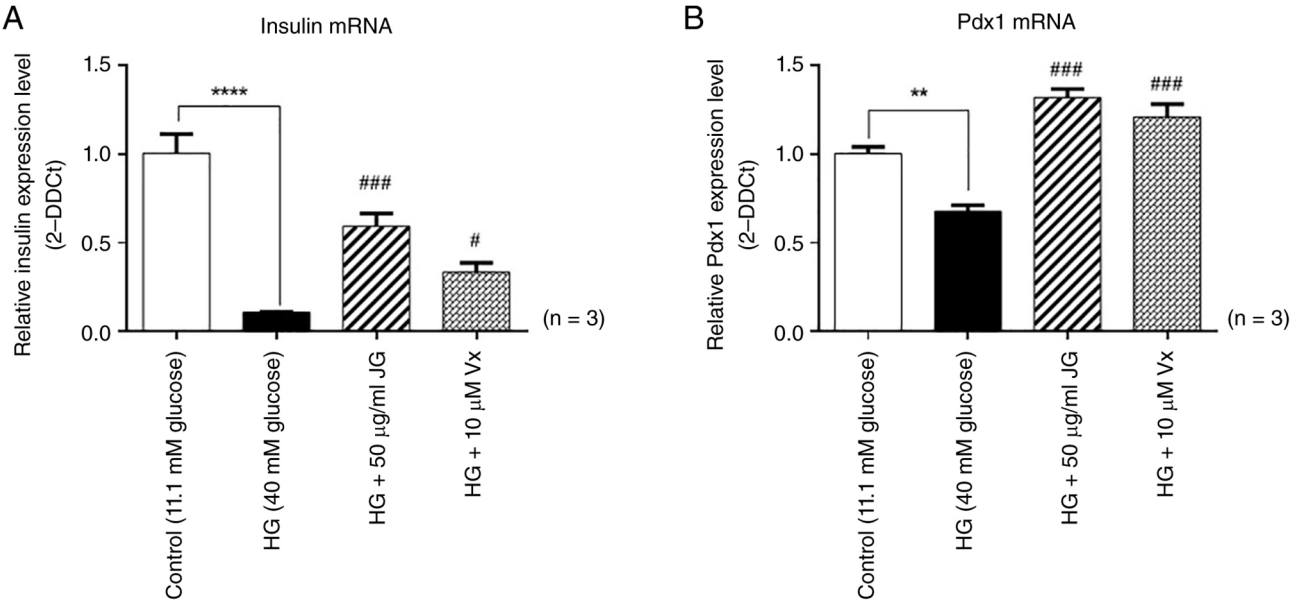

Figure 6. Effects of JG leaf extract on insulin biosynthesis genes. The expression of (A) insulin and (B) insulin transcription factor (Pdx1) by measuring mRNA levels at 48 h reverse transcription-quantitative PCR. Data are expressed as relative insulin mRNA expression levels ($2^{-\Delta\Delta Cq}$) as mean ± SEM (n=3) and analyzed statistically using one-way ANOVA followed by Tukey's multiple comparison test. **P<0.01 vs. control group; ****P<0.0001 vs. control group; $^{\#\#\#}$P<0.001 vs. HG group; $^{\#}$P<0.05 vs. HG group. JG, *Justicia gendarussa*; HG, high glucose.

level (0.19±0.02 mg/g extract) in the present study. Despite its low content, vitexin may still contribute to the observed biological activity of the extract. This assumption is supported by the result of vitexin standard that exhibits significant cytoprotective effects against high glucose-induced pancreatic β-cell apoptosis. Vitexin is a C-glycosylated flavone commonly found in various plant species and is well documented for its diverse biological activities, particularly antioxidant, anti-inflammatory and anticancer properties (23). The cytoprotective effect of vitexin was supported by Zhang *et al* (24) in 2017 and Raghu and Agrawal (25) in 2016 reported that vitexin plays a major role in the extract's antioxidant activity. Nevertheless, the biological activity of JG extract is likely to result from the combined effects of vitexin and other component in the extract.

The cytotoxic effects of JG leaf extract on INS-1 cells, a rat pancreatic β-cell line, were evaluated across a concentration range of 31.2-2,000 μg/ml. The extract exhibited no statistically significant cytotoxicity on INS-1 cells at concentrations between 31.2-1,000 μg/ml, with cell viability percentages remaining comparable to the untreated control group. However, at the highest tested concentration of 2,000 μg/ml, a marked reduction in cell viability was observed, with survival decreasing to ~70%, indicating cytotoxicity at this dose. These findings corroborate previous reports demonstrating that ethanol extracts of JG leaf at concentrations up to 1,000 μg/ml did not induce toxicity in monkey kidney (Vero) cells or human breast cancer (MCF-7) cells (26). Additionally, no cytotoxic effects were noted in the mouse macrophage cell line (RAW264) when exposed to 200 μg/ml of the extract (8). Collectively, these data supported that JG leaf extract concentrations ranging from 31.2-1,000 μg/ml are safe and appropriate for further bioactivity evaluation. The observed decrease in cell viability at 2,000 μg/ml may be attributable to the saturation of bioactive compounds exerting intracellular homeostatic disturbances or the induction of oxidative stress and apoptosis at high concentrations. A previous study (27) reported that elevated flavonoid levels can induce cell cycle arrest and apoptosis via activation of the p53 signaling pathway, as demonstrated in bladder cancer cell lines (T24 and EJ).

Prolonged exposure of cells to elevated glucose levels induces oxidative stress, leading to glucotoxicity, a critical mechanism underlying β-cell dysfunction and apoptosis. The present study evaluated the protective effect of JG leaf extract against high-glucose-induced damage in pancreatic β-cells. INS-1 cells cultured under hyperglycemic conditions (40 mM glucose) for 72 h exhibited a significant reduction in cell viability to <50% compared with normoglycemic controls. Co-treatment with JG leaf extract at concentrations of 50-100 μg/ml markedly improved cell viability, restoring survival rates to levels comparable to the normal glucose group. Furthermore, vitexin demonstrated a dose-dependent protective effect on INS-1 cells under hyperglycemic stress. Treatment with vitexin at 10, 20 and 40 μM concentrations increased cell viability to levels comparable to those observed with the crude extract, suggesting that vitexin may serve as a principal bioactive constituent mediating a cytoprotective mechanism against glucotoxicity. These findings are consistent with previous reports by Ganesan *et al* (28) in 2020, which documented significant enhancement of INS-1 cell survival under high glucose conditions following treatment with vitexin at concentrations ranging from 20-40 μM.

The present study demonstrated that JG leaf extract exhibits potent intracellular antioxidant activity in INS-1 pancreatic β-cells exposed to high glucose conditions. Treatment with the extract at concentrations of 50 and 100 μg/ml markedly reduced intracellular ROS levels compared with the high glucose control group, with ROS levels approaching those observed under normoglycemic conditions. Similarly, vitexin at concentrations of 10, 20 and 40 μM effectively attenuated

ROS generation to levels comparable to the crude extract. These findings are consistent with previous reports indicating that vitexin restores antioxidant system homeostasis by activating the Nrf2 signaling pathway, which upregulates intracellular antioxidant molecules, while concurrently inhibiting the NF-κB pathway involved in inflammation and oxidative stress-induced apoptosis (28). Supporting this, Tao *et al* (29) in 2023 reported that vitexin at 10-80 μM markedly reduced ROS accumulation and lipid peroxidation under hyperglycemic stress. The result of intracellular antioxidant activity showed that JG extract and vitexin upregulated antioxidant-related gene, Nrf2 and Trx expression, supporting their protective effect against high glucose-induced oxidative damage. These findings are consistent with previous reports indicating that vitexin restores antioxidant system homeostasis by activating the Nrf2 signaling pathway, which upregulates intracellular antioxidant molecules, while concurrently inhibiting the NF-κB pathway involved in inflammation and oxidative stress-induced apoptosis (28). The activation of Nrf2 and Trx pathway by JG extract is strongly supported by the known activities of its bioactive components, vitexin and isovitexin. A previous study (29) demonstrated that these vitexin and isovitexin function as potent Nrf2 activators by directly binding to the Keap1 protein pocket, thereby preventing Nrf2 degradation and facilitating its translocation to the nucleus. Furthermore, in *C. elegans* models, these compounds were witnessed to increase the nuclear accumulation of SKN-1 (the mammalian homolog of Nrf2), leading to enhanced stress resistance and longevity. The present findings of upregulated Nrf2 and Trx mRNA levels are highly consistent with these established protein-level mechanisms, suggesting that the observed antioxidant effects are indeed driven by the activation and translocation of the Nrf2 signaling cascade (30). Beyond its antioxidant properties, the present study further revealed that JG leaf extract at 50 μg/ml and vitexin at 10 μM markedly inhibited apoptosis in pancreatic β-cells under high glucose conditions, compared with untreated hyperglycemic controls. This protective effect corroborates previous evidence that vitexin downregulates the pro-apoptotic protein Bax, upregulates the anti-apoptotic protein Bcl-2 and mitigates mitochondrial stress in pancreatic β-cells (30). Collectively, these results substantiate the cytoprotective role of JG leaf extract against glucotoxicity via attenuation of oxidative stress and apoptosis, with vitexin likely serving as a key bioactive constituent mediating these effects.

Prolonged exposure to high glucose levels is a critical factor contributing to pancreatic β-cell dysfunction, characterized by impaired insulin secretion and downregulation of genes involved in insulin synthesis. A key mechanism underlying glucotoxicity is the suppression of insulin gene expression and the Pdx1 gene, the latter serving as a crucial insulin transcription factor regulating insulin gene transcription in pancreatic β-cells. The present study demonstrated that JG leaf extract at a concentration of 50 μg/ml markedly upregulated the expression of insulin and Pdx1 genes in INS-1 pancreatic β-cells cultured under hyperglycemic conditions. Specifically, insulin mRNA levels, which were reduced to 0.109-fold in the high glucose control group, increased to 0.59-fold following treatment with the JG leaf extract and to 0.33-fold with vitexin at 10 μM, indicating a greater potential of the JG leaf extract compared with vitexin alone in enhancing insulin synthesis. Similarly, Pdx1 mRNA expression showed a parallel increase, reflecting a coordinated regulatory mechanism. This finding aligns with the established role of Pdx1 as a direct regulator of insulin gene transcription and a primary target inhibited during glucotoxicity, resulting in diminished insulin production. These results are consistent with previous reports by Zhang *et al* (30) in 2023, who demonstrated that vitexin prevents the decline in Pdx1 and insulin mRNA expression induced by high glucose in INS-1 cells via mechanisms involving the reduction of oxidative stress and mitigation of mitochondrial dysfunction. Activation of Pdx1 thereby restores the insulin-synthesizing capacity of β-cells under oxidative stress (29). At present, there is no evidence on the effect of JG leaf extract on insulin and Pdx1 gene expression in glucotoxic conditions, particularly in INS-1 cells. A previous study demonstrated that decreased protein levels of PDX1 and MafA in pancreatic β-cell under glucolipotoxicity results in the reduction of glucose-stimulated insulin secretion (GSIS) (31). Even though the present study did not perform the GSIS assay, it demonstrated the regenerative and biosynthetic potential of the extract on pancreatic β-cells, for which mRNA levels of Insulin and Pdx1 are established primary indicators.

The protective effects of JG extract against glucotoxicity in INS-1 cells compare favorably with other established herbal antidiabetics. While a number of extracts, such as those from apigenin, a plant-derived flavonoid, primarily act through general ROS scavenging (32), JG extract demonstrated a specialized capacity to upregulate the Pdx1/Insulin axis alongside the Nrf2/Trx pathway. This dual regulatory role is critical for the improvement of pancreatic β-cell mass and function. The present study identified vitexin as the major bioactive in our JG extract, which further distinguished JG from broader extracts. Specifically, vitexin has the ability to bind directly to Keap1, thereby disrupting the Keap1-Nrf2 interaction and subsequently promoting Nrf2 activation. The present study showed that the flavonoid concentration (29). The present study showed that the flavonoid concentration in the extract (172.10 mg QE/g) was markedly higher than that reported for several other tropical medicinal plants, such as *Curcuma longa* and *Centella asiatica* (33,34). While *Curcuma longa* is known as a potent Nrf2 activator (35), the markedly higher flavonoid content in the present JG extract suggested a more robust therapeutic potential for activation of the Nrf2 pathway, a comprehensive shield for pancreatic β-cells against oxidative and inflammatory insult in diabetes.

Regarding the translational potential of the present findings, although *in vivo* experiments of JG extract were not conducted in the present study, the physiological relevance of the *in vitro* findings is supported by established pharmacokinetic data for vitexin in rat models (36). Oral administration of vitexin at 30 mg/kg achieved a peak plasma concentration (C_{max}) of 0.51±0.015 μg/ml or 1.18 μM. This concentration was reached rapidly, with a T_{max} of ~15.82±0.172 min. Although the concentration of 10 μM (4.32 μg/ml) used in the *in vitro* model exceeds C_{max} typically observed in rat pharmacokinetic studies, the findings demonstrated significant biological activity without inducing cytotoxicity. The specific *in vivo* pharmacokinetic study of the JG extract is a necessary next step to confirm these effective dosages. In a clinical context,

such an extract could serve as a natural complementary strategy to slow the progression of Type 2 Diabetes by maintaining β-cell viability and insulin biosynthetic capacity. By upregulating Pdx1 and Nrf2, the JG extract targets the root causes of pancreatic β-cell failure; oxidative stress and transcriptional exhaustion.

While these results are promising, the mechanistic evidence in the present study was limited to mRNA expression. Future studies incorporating western blotting for Nrf2 nuclear translocation and GSIS assays are warranted to confirm these molecular observations. Additionally, the long-term safety, bioavailability and clinical evaluation of the JG extract in human subjects remain to be established through standardized clinical trials.

The present study provided novel and valuable evidence demonstrating the potential of JG leaf extract to protect pancreatic β-cells from apoptosis and enhance their functional capacity to increase insulin production under glucotoxic conditions through antioxidant mechanisms and inhibition of apoptotic pathways. The observed effects are likely mediated by flavonoid compounds, particularly vitexin, which was found in the extract and demonstrated a cytoprotective effect similar to that of the extract. Nevertheless, further investigations at the molecular level are warranted to elucidate the precise mechanisms underlying these bioactivities. In addition, comprehensive *in vivo* studies assessing the antidiabetic efficacy of the extract in animal models, followed by clinical evaluations in patients with type 2 diabetes mellitus, are essential. Such research will support the development of this traditional herbal extract as a promising alternative therapeutic agent for the management of type 2 diabetes.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KS conceptualized and administered the project, performed the *in vitro* experiment and wrote the original draft of the manuscript. SC performed plant extraction, analyzed data, prepared the figure and wrote the original draft of the manuscript. PC performed gene expression analysis. NS and PM performed annexin V-FITC/PI staining and flow cytometry analysis. LY and AJ performed acquisition of data. PS and SP performed the *in vitro* experiment and interpretation of data. NM performed data analysis and prepared the figure. SK supervised the project and edited the manuscript. KS and SC confirm the authenticity of all the raw data. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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