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Optimization of phenolic extraction from *Syzygium samarangense* fruit and its protective properties against glucotoxicity-induced pancreatic β-cell death

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ABSTRACT: Long-term exposure to high glucose levels causes glucotoxicity in pancreatic β-cells associated with diabetes mellitus. The presence of natural antioxidant phenolic compounds, including the compounds from *Syzygium samarangense*, may help mitigate this metabolic disorder. This study aimed to optimize microwave-assisted extraction (MAE) for maximum phenolic recovery and assess the protective properties of *Syzygium samarangense* extract (SSE) against pancreatic β-cell mortality caused by glucotoxicity. We demonstrated that the MAE-based response surface methodology provided higher concentrations of total phenolics than the conventional reflux method (10.21 ± 0.22 mg GAE/g and 6.44 ± 0.13 mg GAE/g, respectively). According to *in vitro* studies, the percentage of viable rat insulinoma cell lines (INS-1 cells) was significantly lower when cultured in high glucose (HG) medium (40 mM) than in normal glucose medium (11.1 mM). Upon treatment of INS-1 cells with SSE (10, 50, and 100 μg/ml) in combination with HG, SSE exhibited a protective effect on the cell viability and inhibited cell apoptosis. In addition, SSE reduced intracellular superoxide ion concentrations in a dose-dependent manner. We conclude that the phenolics of SSE could function as antioxidants, thereby protecting pancreatic β-cells against glucotoxicity-induced apoptosis.

KEYWORDS: diabetes, glucotoxicity, pancreatic β-cell, phenolic compounds, *Syzygium samarangense*

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is defined by an increase in plasma glucose levels because of insulin action and/or secretion impairment [1]. Hyperglycemia results from insufficiency of insulin production from pancreatic β-cells to compensate for insulin resistance [2]. Hyperglycemia, in turn, can induce pancreatic β-cell apoptosis through the overproduction of reactive oxygen species (ROS), including superoxide (O_2^-), hydroxyl radical ($OH^\cdot$), hydroxyl ion (OH^-), and hydrogen peroxide (H_2O_2), which results in decreased pancreatic β-cell mass and function [3, 4]. In both animals and humans with T2DM, a decrease in pancreatic β-cell mass has been reported [1, 5]. The mitochondrial electron transport chain (ETC) is a source of endogenous ROS. Hyperglycemia augments ROS production by overloading the ETC and causing electron leakage that reacts with molecular oxygen to form $O_2^{\cdot-}$, which is quickly converted to H_2O_2 [6]. Furthermore, hyperglycemia has also been shown to increase ROS production through the activation of NADPH oxidases (NOX) [7]. Hyperglycemia not only promotes ROS generation but also impairs the antioxidant defense system in pancreatic β-cells. Several studies have shown that chronic high glucose exposure reduces antioxidant capacity in pancreatic β-cells, including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and thioredoxin (TXN) [8]. High glucose-induced aggravation of ROS is associated with endoplasmic reticulum (ER) stress and mitochondrial stress-induced β-cell apoptosis [3, 4].

Recent research on alternative diabetes treatments has concentrated on polyphenols derived from edible plants and their antioxidant qualities in alleviating the pathophysiology of T2DM [9, 10]. Numerous studies indicate that consuming foods rich in polyphenols may protect pancreatic β-cells from oxidative damage [11, 12]. Gallic acid and quercetin have been shown to increase insulin secretion and cell viability in injured pancreatic β-cells that have been damaged by excessive glucose [8, 12]. Several polyphenol-rich plants have been shown to display antidiabetic properties by enhancing antioxidant or anti-inflammatory status [13] and inhibiting the α-glucosidase enzyme [14, 15].

Syzygium samarangense (Blume) Merr. & L.M. Perry, more popularly referred to as the rose apple, is a member of the Myrtaceae family found throughout Southeast Asia. Rose apple is rich in polyphenol compounds, including phenols, flavonoids, anthocyanins, and ellagitannins [16, 17]. Several studies have demonstrated the pharmacological properties of

rose apple extracts such as free radical scavenging, antioxidant, anticancer, anti-inflammatory, and antibacterial activities [18]. Previous research in streptozotocin (STZ)-induced diabetic rats discovered that unprocessed rose apple powder lowers hyperglycemia while preserving pancreatic β-cell mass [19]. Moreover, rose apple powder could reduce insulin resistance in hepatic rats by inhibiting inflammatory pathways and activating insulin signaling [20]. The oxidative stress-mediated β-cell apoptosis and the protective effect of phenolic compounds on pancreatic β-cells are illustrated in Fig. S1.

Although the antidiabetic effect of unprocessed rose apple powder has previously been studied in diabetic rats [19], it is more convenient to convert the powder into an extract with a high concentration of polyphenols. Processing the fruit into extracts also makes it easier for the industrial sector to produce it as a nutraceutical ingredient. On the other hand, using these fruit powders as active ingredients in health products may present a number of obstacles, the most prominent of which are fruit availability and compatibility with other formulations of ingredients. Incorporating extracts into those products, however, can be a more promising strategy with greater commercial potential. It is likely that the abundant polyphenolic chemicals in rose apples have antioxidant properties, protecting pancreatic β-cells from apoptosis caused by glucotoxicity. Therefore, this study aimed to determine the cytoprotective and antioxidant effects of rose apple extract in the context of high glucose-induced pancreatic apoptosis. MAE in combination with response surface methodology was used to optimize the extraction conditions for rose apple fruit extract. Prior to investigating the pharmacological activity of the extract in a rat insulinoma cell line (INS-1 832/13), its phytochemical profiles were determined using ultrahigh-performance liquid chromatography (UHPLC)-microtime of flight (micrOTOF) Q II-tandem mass spectrometry (MS/MS) analysis.

MATERIALS AND METHODS

Chemicals

Absolute ethanol (⩾ 99%, v/v) (412704), chromatographic acetonitrile (LA198620F), and Folin-Ciocalteu's phenol reagent (E463562) were purchased from Carlo Erba Reagents S.A.S. (Val-de-Reuil, France). An Annexin V-FITC apoptosis detection kit and propidium iodide were purchased from BD Pharmingen (San Diego, CA, USA) whereas β-mercaptoethanol, bovine serum albumin, calcium chloride, diethyl pyrocarbonate (DEPC), dimethyl sulfoxide (DMSO), ethidium bromide, potassium hydroxide, trypsin-EDTA, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), nitroblue tetrazolium chloride (NBT), RPMI-1640 medium, sodium pyruvate, testosterone enanthate, thiazolyl blue tetrazolium bromide, tween-20, trizma-base, quercetin (Q4951), gallic acid (G7384), and formic acid (F0507) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal calf serum (FCS) was from Invitrogen (Thermo Fisher Scientific, MA, USA). All the chemicals were of analytical and cell culture grade.

Plant material

The mature fruits of *S. samarangense* were stochastically sampled in May 2020 from a commercial field in Buriram Province, Thailand. The plant was identified by Associate Professor Bancha Yingngam, and its voucher specimen (BCY UBU no. Myrtaceae/20200001) was deposited at the Herbarium of the Faculty of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Thailand. The fruits were sliced into small pieces, dried at 50 °C for 72 h, and ground before being sieved through a 40-mesh sieve.

Microwave-assisted extraction

In this experiment, a laboratory microwave extraction apparatus (UBU01, Ubon Ratchathani University, Ubon Ratchathani, Thailand) was fitted with an extraction vessel and a condenser. At atmospheric pressure, the extraction device was operated at a frequency of 2.45 GHz with microwave power ranging from 0 W to 800 W. The interior dimensions of the microwave oven were 215 mm × 350 mm × 330 mm.

Reference extraction methods for polyphenols

The reference procedures for *S. samarangense* fruit polyphenol extraction were reflux extraction in a 63% v/v aqueous ethanolic solution, which was carried out at 100 °C for 4 h in a digital heating mantle. The SSE was kept at −20 °C. The morphological alterations in the samples following MAE treatment were compared to those following reflux extraction and the untreated sample using a scanning electron microscope. Mounted on stubs, the samples were sputtered with a thin layer of gold in a sputter coater. The pictures were acquired using a JEOL JSM-6010LV scanning electron microscope (JEOL Ltd., Japan) operated at a 20 kV accelerating voltage.

Determination of total phenolic content

The total phenolic content of the obtained extracts was spectrophotometrically measured using Folin-Ciocalteu reagent [21]. The absorbance was measured at 725 nm using a UV-Vis spectrophotometer (Epoch2 microplate reader, BioTek Instruments Inc., Winooski, VT, USA). The standard calibration curve was produced using gallic acid solutions ranging from 25–200 μg/ml, and the results are presented in mg gallic acid equivalents (GAE) per dry matter basis of the sample (mg GAE/g dry basis). All measurements were performed in triplicate to ensure accuracy.

Identification of individual phenolic constituents in SSE

The profiling of phenolic composition in the SSE was performed using a Dionex Ultimate 3000 UHPLC system (Dionex, Thermo Fisher Scientific) coupled with an electrospray ionization (ESI) tandem mass spectrometer (micrOTOF-Q II) (Bruker, Germany). The sample (5 mg/ml, 20 µl) was injected into a Zorbax SB-C_{18} (250 mm × 4.6 mm, 3.5 µm particle size; Agilent Technologies, USA) by controlling the column temperature at 35 ± 1 °C. The elution solvents were 0.1% v/v formic acid in water (solvent A) and 0.1% v/v formic acid in acetonitrile (solvent B) with a 0.8 ml/min flow rate. Chromatographic separation of the sample acquired from the optimized MAE conditions was accomplished using the following stepwise gradient: 0–2 min, 5% B; 2–5 min, 5-10% B; 5–15 min, 10–15% B; 15–20 min, 15–17% B; 20–23 min, 17–19% B; 23–27 min, 19–28% B; 27–30 min, 28–30% B; 30–35 min, 30–35% B; 35–37 min, 35% B; 37–42 min, 35–100% B; 42–47 min, 100–5% B; and 47–60 min, 5% B. The eluted components were ionized using an ESI source and detected in the mass scanning range of 50–1500 m/z (mass-to-charge ratio) in negative ion mode. Sodium formate solution (10 mM) was used as an external standard for accurate mass calibration and injected into the ESI source before each sample injection by a syringe pump at a flow rate of 60 µl/min. The nebulizer gas (N_2) pressure was adjusted to 2 bar, the drying gas flow rate was 8 l/min, the dry heater temperature was 180 °C, and the capillary voltage was 4.5 kV. The LC-micrOTOF-Q II data were collected in MS/MS scan mode and analyzed using Compass 1.3 software (Bruker).

In vitro pharmacological properties of SSE

INS-1-cell culture

At 37 °C in humidified air containing 5% CO_2, INS-1 cells (rat insulinoma cell line) were cultivated in RPMI 1640 media supplemented with 10% FCS, 100 U/ml penicillin, and 100 g/ml streptomycin. 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 approximately fourfold (40 mM) glucose to induce glucotoxicity.

Measurement of cell viability by MTT assay

The MTT assay was performed to determine toxicity as previously described [22]. Briefly, INS-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with test samples at concentrations ranging from 1 to 500 µg/ml in a 96-well plate. In the experiment of the cytoprotective effects, INS-1 cells, approximately 10,000 cells per well, were seeded into 96-well plates. Cells were grown in glucose solutions of 11.1 mM or 40 mM with or without test substances (SSE, gallic acid, or quercetin). DMSO (0.5% v/v) was used as a final concentration. This concentration is not toxic to cells when compared to RPMI media alone. After 72 h, cell viability was measured using a colorimetric MTT test by applying MTT solution to each culture and incubating at 37 °C with 5% CO_2 for 4 h. After removing the medium and adding a stop solution to each well, the cells were incubated at 37 °C for 1 h. The absorbance was determined using a PowerWave microplate scanning spectrophotometer (BioTek Instruments, Inc.) at 570 nm and 650 nm. The following calculation was used to compute the proportion of viable cells using the averaged 570-nm absorbance values (Eq. (1)).

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

Analysis of cell apoptosis by Annexin V-FITC staining

The assay was performed based on the manufacturer's instructions. Briefly, INS-1 cells were treated with 40 mM glucose in the presence or absence of SSE at doses of 10, 50, and 100 µg/ml or with gallic acid or quercetin at 5 µg/ml concentration. To verify that the antiapoptotic effect of SSE was mediated by its antioxidant properties, 50 µM of the antioxidant *N*-acetylcysteine (NAC) was added to compare the antiapoptotic ability. After 72 h of incubation, the cells were harvested and stained with Annexin V-FITC. Apoptotic cells were then evaluated with a fluorescence microplate reader (CLARIOstar®, BMG Labtech, Germany). Annexin V-FITC was used to detect apoptotic cells by using a filter designed to detect fluorescein with excitation at 485 nm and emission at 535 nm.

Measurement of intracellular superoxide generation

The generation of superoxide was determined using the NBT assay as described [23]. Briefly, INS-1 cells were treated with 40 mM glucose in the absence or presence of SSE at concentrations of 10, 50, and 100 µg/ml for 48 h. Following the incubation, the cells were treated with NBT for 90 min and then lysed in a potassium hydroxide solution. The released insoluble formazan was dissolved in DMSO. The absorbance of superoxide produced was determined using a PowerWave Microplate Scanning Spectrophotometer at 630 nm.

Statistical analysis

The RSM algorithm for maximizing total phenolic yield was run using Statistica software version 12 (TIBCO Software Inc., CA, USA). Concerning the cell-based model system, all experiments were performed in at least triplicate, and the data are expressed as the mean ± standard error of the mean (SEM). Differences

between datasets were determined by one-way analysis of variance (ANOVA) followed by post hoc analysis (GraphPad Prism 6 software, GraphPad Software, USA). A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Model fitting

The central composite design (CCD) was used to find the ideal combination of extraction factors for extracting total phenolics from *S. samarangense* fruits based on the results of the aforementioned one-factor experiment. Twenty experimental runs were carried out to determine the MAE parameter effects and optimize the extraction processes. The results indicated that total polyphenols were extracted at a rate ranging from 7.10 ± 0.03 mg GAE/g to 10.92 ± 0.21 mg GAE/g dry basis (Table 1). According to the ANOVA results in Table S1, the F value of 15.81 ($p < 0.0001$) indicates that the response of the model to the extraction yield of total phenolic compounds was highly significant. The p value of the model for lack of fit was 0.1478 ($p > 0.05$), which means that the model fits effectively. Thus, the reliability of the model was established. Additionally, the coefficient of determination ($R^2 = 0.9343$) and adjusted coefficient value ($R^2_{\text{adj}} = 0.8752$) revealed that it was adequate. The coefficient of variation (CV% = 3.44) showed that the experimental and predicted values were consistent. The ANOVA findings suggested that X_1 and X_2 were very significant ($p < 0.001$), while X_1X_2, X_1X_3, and X_2X_3 were not. Additionally, X_1 had the greatest effect on the recovery of total phenolics of the three independent variables. The final regression equation of total polyphenol content in terms of actual factors is depicted in Eq. (2).

$$\begin{aligned}\text{Total phenolics (mg GAE/g dry powder)} &= 15.21 + 0.08X_1 - 0.02X_2 + 0.0003X_2X_3 \\ &\quad - 0.0012X_1^2 + 0.0001X_2^2. \end{aligned} \tag{2}$$

Residual analysis was used to verify that the resulting model suited the experimental data. The result illustrated the correlation between the experimental and anticipated values obtained, and each point was near the 45° line, indicating that the predicted values are reasonably close to the actual values, and the model accurately captures the association between process variables and response values. The likelihood of the internally studentized residual had a normal distribution. This result indicates that the variance is negligible when the distribution of points is reasonably close to a straight line. As a result, the generated model was accurate in navigating the experimental data within the investigated ranges.

Response surface analysis

Estimates of the performance of the process can be derived via statistical analysis. When analyzing the statistical significance and relative magnitude of the main impacts and their interactions with one another, one might use a Pareto chart, also known as a Pareto distribution diagram, a bar graph that shows the relative significance of events or values in descending order from most important to least important. The most significant variables can easily be seen by looking at the longest bars on the graph. With a model that incorporates an error term, a reference line (red) is drawn on the graph, and any effect that extends beyond this line (assuming a real relationship with 95% accuracy) is statistically significant (Fig. 1a). From this Pareto chart, the most significant effect in the recovery of total phenolics is the microwave power, followed by aqueous ethanolic solution and irradiation time.

Three-dimensional response surfaces and contour plots anticipated the interaction of two test variables by fixing one of the independent variables at the center level, and the response of total phenolics was constructed according to Eq. (2). The edge of the independent variables is flat if it has a weak influence on the yield of the total phenolic content. In contrast, an ellipse-shaped contour graph indicates little or no interaction between the two independent variables, while a circle-shaped contour graph indicates a significant amount of interaction. As illustrated in Fig. 1b-d, the influence of independent factors on the response value was graded as microwave power > aqueous ethanolic solution > irradiation time.

Optimization and model verification

The MAE protocols for polyphenols from *S. samarangense* fruits were optimized using the desired ability function (Table 2) [21]. The maximum yield of polyphenol content in the plant extract was selected, while other independent parameters within the experimental limits were chosen. The desired ability value was determined to be 0.82. The proper extraction parameters for the model were 63% v/v (X_1), 800 W (X_2), and 5 min (X_3). Under these conditions, the model predicted total polyphenols of 10.21 ± 0.22 mg GAE/g dry basis which was in agreement ($p > 0.05$) with the value from experimental data (10.20 ± 0.14 mg GAE/g dry powder). The amount of polyphenols obtained from MAE was significantly greater than that obtained after 4 h of heat-reflux extraction in a 68% v/v aqueous ethanolic solution (total polyphenols = 6.71 ± 0.32 mg GAE/g dry powder) or hot water (total polyphenols = 3.18 ± 0.41 mg GAE/g dry powder) ($p < 0.05$).

Microwave energy could have generated the high temperature and pressure required to rupture the cell wall, allowing the polyphenols to dissolve more easily. In microwave extraction, the conversion of

Table 1 The central composite design matrix layout used to extract total polyphenols from *Syzygium samarangense* fruit powder and their responses.

No.	Order	Independent variable			Response†
		Aqueous ethanolic solution (X_1, % v/v)	Microwave power (X_2, W)	Irradiation time (X_3, min)	Total polyphenol (Y, mg GAE/g dry powder)
1	1	60.00 (−1)	480.00 (−1)	2.50 (−1)	8.96 ± 0.14
2	17	72.50 (0)	640.00 (0)	3.75 (0)	8.72 ± 0.10
3	12	72.50 (0)	909.00 (1.68)	3.75 (0)	9.92 ± 0.21
4	4	85.00 (1)	800.00 (1)	2.50 (−1)	9.52 ± 0.10
5	2	85.00 (1)	480.00 (−1)	2.50 (−1)	7.94 ± 0.07
6	10	93.52 (1.68)	640.00 (0)	3.75 (0)	7.68 ± 0.07
7	15	72.50 (0)	640.00 (0)	3.75 (0)	9.22 ± 0.18
8	3	60.00 (−1)	800.00 (1)	2.50 (−1)	9.42 ± 0.08
9	20	72.50 (0)	640.00 (0)	3.75 (0)	8.52 ± 0.03
10	5	60.00 (−1)	480.00 (−1)	5.00 (1)	9.06 ± 0.10
11	8	85.00 (1)	800.00 (1)	5.00 (1)	9.78 ± 0.14
12	9	51.48 (−1.68)	640.00 (0)	3.75 (0)	9.10 ± 0.12
13	19	72.50 (0)	640.00 (0)	3.75 (0)	8.84 ± 0.14
14	7	60.00 (−1)	800.00 (1)	5.00 (1)	10.04 ± 0.10
15	13	72.50 (0)	640.00 (0)	1.65 (−1.68)	9.40 ± 0.15
16	16	72.50 (0)	640.00 (0)	3.75 (0)	8.86 ± 0.07
17	11	72.50 (0)	370.00 (−1.68)	3.75 (0)	9.00 ± 0.06
18	14	72.50 (0)	640.00 (0)	5.58 (1.68)	9.40 ± 0.39
19	6	85.00 (1)	480.00 (−1)	5.00 (1)	7.10 ± 0.03
20	18	72.50 (0)	640.00 (0)	3.75 (0)	8.84 ± 0.14

† The data are presented as the means ± standard deviations of three independent experiments.

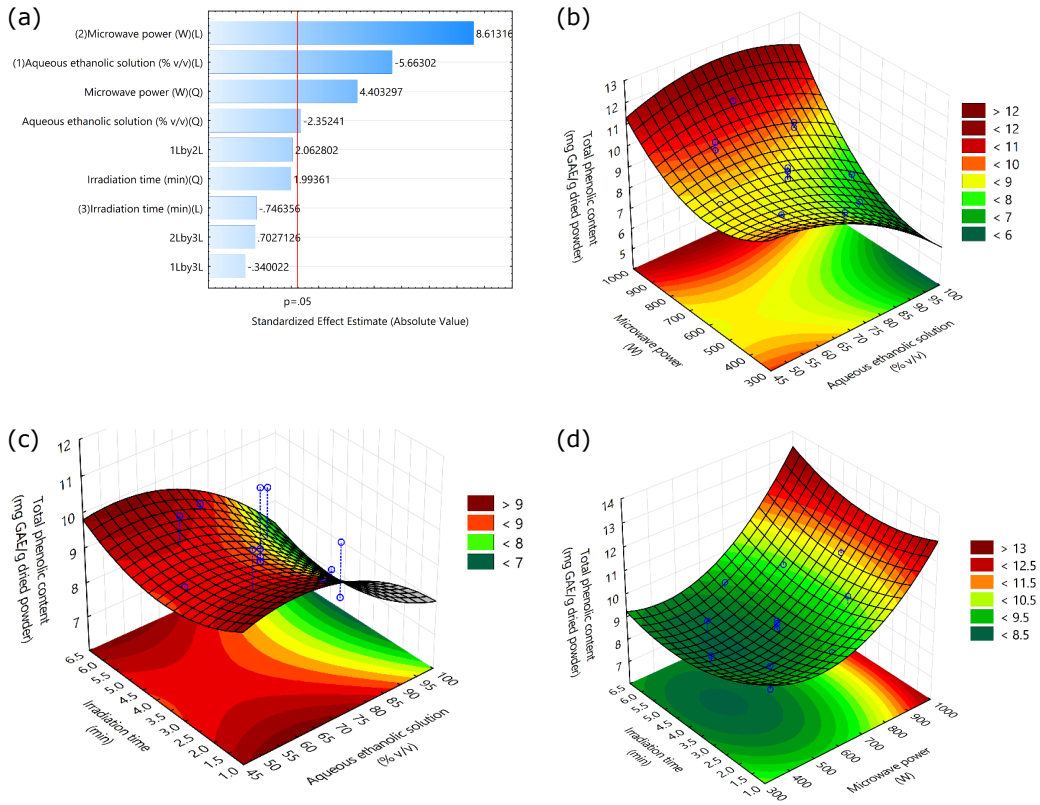

Fig. 1 Pareto charts and 3D plots showing independent variable effects on the response of total phenolic recovery from *S. samarangense* fruit powders.

Table 2 The optimal conditions of MAE and the results of the predicted and validated values of total polyphenols using the response surface methodology approach.

Optimized MAE condition	Predicted value	Validated value
$X_1 = 63\%$ v/v $X_2 = 800$ W $X_3 = 5$ min	Total polyphenols = 10.21 ± 0.22 mg GAE/g dry powder (95% confidence interval low = 9.71 mg GAE/g dry powder; 95% confidence interval high = 10.71 mg GAE/g dry powder)	Total polyphenols = 10.20 ± 0.14 mg GAE/g dry basis[nd]

The ratio of solid to liquid was fixed constant at 1:30 (g/ml). [nd] not statistically significant difference ($p > 0.05$) compared with the predicted value.

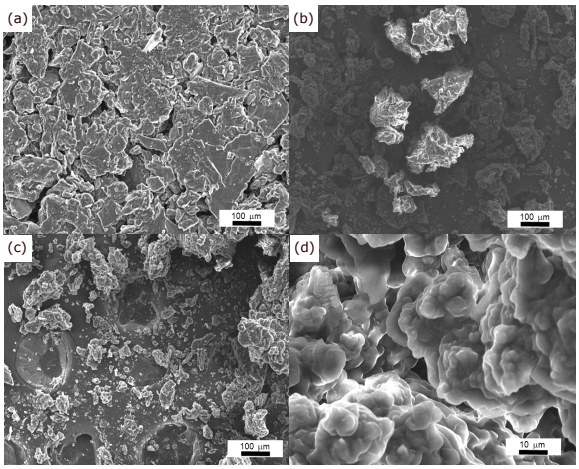

Fig. 2 Scanning electron micrographs of various *S. samarangense* fruit powders: (a) untreated samples; (b) samples treated with reflux extraction at 100 °C for 4 h in a 63% v/v aqueous ethanolic solution; (c) samples treated with MAE under optimal conditions (aqueous ethanolic solution = 63% v/v, microwave power = 800 W, irradiation time = 5 min); and (d) a zoomed-in version of samples treated with MAE under optimal conditions.

high-frequency electromagnetic energy to heat energy enhances the extraction process [24]. As a result of the microwave capabilities, MAE has better efficiency than traditional solvent extraction and appears to be good for scaling up from laboratory to industrial-scale production. The MAE process utilizes a mixture of water and ethanol as the reagent, establishing a reliable foundation for developing and utilizing *S. samarangense* fruit extract, a raw material for functional food products. In terms of commercial feasibility, employing the extract has advantages over using the fruit powder that has been processed solely by grinding, as this fruit cannot be kept for a lengthy period and is available only approximately three to four times each year. In addition, processing the fruit, as opposed to using isolated pure chemicals, makes it easier for the industrial sector to manufacture it as a nutraceutical ingredient. Crude extracts could be superior to pure compounds in several ways such as pharmacology (through synergistic or additive effects) or production cost (by a cheaper and faster preparation procedure). Using isolated pure molecules in health products, on the other hand, may have various drawbacks, the most noteworthy of which is greater production costs.

Scanning electron microscopy was used to examine the microstructures of plant material in *S. samarangense* powders before and after they were subjected to optimized MAE conditions and reflux extraction (total phenolics = 6.44 ± 0.13 mg GAE/g dry basis). As illustrated in Fig. 2a, the cell walls of the untreated plant powders were smooth and unaltered. Although grooves were produced and the cell wall integrity was destroyed by shrinking following reflux extraction, the degree of rupture was rather superficial (Fig. 2b). Cracks and loss of cellular structures and cell wall components were observed in the sample treated with the optimized MAE conditions (Fig. 2c-d). This phenomenon confirms the enhanced phenolic release into the extraction solvent. Our result was consistent with the recent findings by İşçimen and Hayta [25] which also revealed the degradation of cellular structures in MAEs. Subsequently, this procedure could aid the release and extraction of secondary metabolites such as phenolics.

Profiling of polyphenol components by UHPLC-ESI-micrOTOF-Q II-MS/MS

Phenolics are naturally occurring chemicals with a high degree of structural variety; many of them are essential components of human diets and diabetic prevention. Numerous edible fruit extracts have already been analyzed for their polyphenols. However, reports of the phenolics associated with the rose apple fruit extract are scarce in the literature. UHPLC-micrOTOF Q II-MS/MS analysis showed that the secondary metabolites were identified as cinnamic acid, glucaric acid, maloyl-hexose, citric acid, monocaffeoylquinic acid, gallic acid, 2-methylcitric acid, ellagic acid, naringin-*O*-glucoside, benzyl-diglycoside, and matairesinol (Fig. 3 and Table S2).

Peak 1 (t_R = 0.20 min) displayed an $[M\text{-}H]^-$ ion at m/z 147. Its MS/MS spectrum revealed an $[M\text{-}H\text{-}CO_2]^-$ fragment at m/z 103. This molecule was identified as cinnamic acid. Peak 2 (t_R = 4.11 min) was proposed as glucaric acid ($C_6H_{10}O_8$) because it exhibited an $[M\text{-}H]^-$ ion at m/z 209 and MS/MS fragments at m/z 191 and m/z 173 [26]. Peak 3 (t_R

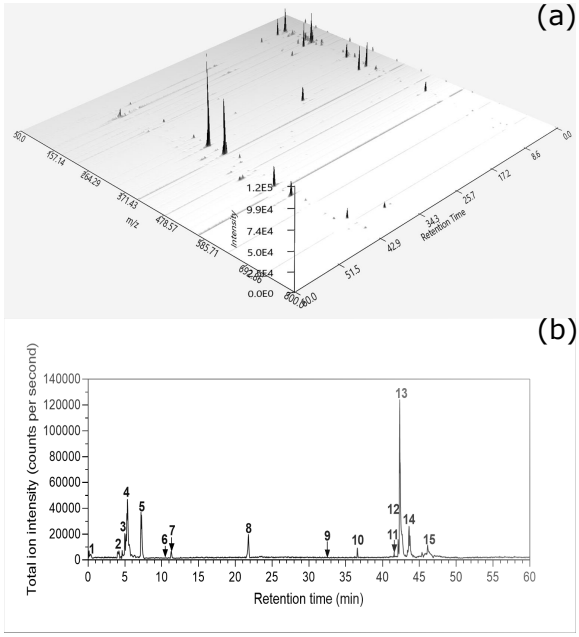

Fig. 3 Chromatograms of phenolic compounds extracted from *S. samarangense* under the optimized extraction conditions using UHPLC-ESI-micrOTOF-Q II-MS/MS. Sample data obtained by the negative ion mode include (a) a 3D intensity matrix and (b) total ion chromatograms.

= 4.41 min) exhibited protonated ions at m/z 295; it emitted MS fragment ions at m/z 179 and m/z 133, corresponding to the loss of maloyl residue ($C_4H_4O_4$, 116 Da) and hexosyl (162 Da) moieties, respectively. Additionally, the MS/MS fragmentation patterns of these two ions (m/z 179 and 133) were consistent with the fragmentation patterns of hexose and malic acid, respectively. Thus, Peak 3 was identified as maloyl-hexose on a provisional basis [27].

Peak 4 (t_R = 4.53 min) displayed an $[M\text{-}H]^-$ ion at m/z 191. The MS/MS fragmentation showed m/z at 85 and 111 $[M\text{-}H_2O\text{-}COOH\text{-}OH]^-$. This organic acid was identified as citric acid [28]. Peak 5 (t_R = 7.24 min) displayed an $[M\text{-}H]^-$ ion at m/z 353 and an MS/MS fragmentation peak at m/z 191, followed by peaks at m/z 179 and 161. Based on its chromatographic retention time and mass spectrum, this acid could be monocaffeoylquinic acid. Peak 6 (t_R = 10.39 min) showed a deprotonated ion at m/z $[M\text{-}H]^-$ 169 and MS/MS fragmentation at m/z 151 $[M\text{-}H\text{-}H_2O]^-$ and m/z 125 $[M\text{-}H\text{-}CO_2]^-$. This molecule was identified as gallic acid [29]. The compound was also reported in the fruit extract of *S. cumini* (L.) Skeels [29] and leaf extract of *S. samarangense* [30]. The parent ion of peak 7 (t_R = 11.31 min) showed an $[M\text{-}H]^-$ ion at m/z 205 and fragment ions at m/z 143, 111, and 87. The fragment ion at m/z 111 suggested the presence of a citric acid derivative, whereas that at m/z 143 indicated a methyl substitution in comparison to the citric acid fragment at m/z 129. Peak 7 was tentatively identified as a methyl derivative of citric acid (2-methylcitric acid) compared with the literature. Peak 8 (t_R = 21.78 min) was an unknown compound that showed a deprotonated ion at m/z 361 ($C_{15}H_{22}O_{10}$). This parent ion yielded MS/MS fragmentation at m/z 329, 281, 253, 226, 196, 179, 165, 121, and 97.

Peaks 8, 14, and 15, which exhibited deprotonated ions at m/z 361, 593, and 489, respectively, were unidentified. Based on its UV spectra and MS data compared with an authentic standard, peak 9 was identified as ellagic acid. Its identity was confirmed by the diagnostic mass ion at m/z 301 $[M\text{-}H]^-$ ion, which underwent dissociation to yield m/z 257 and 229 ions. Ellagic acid was also previously identified in *S. samarangense* fruits [31].

Peak 10 (parent ion at m/z 741) was identified as naringin-*O*-glucoside [32]. This compound was previously found in the leaf extract of the rose apple species [32]. Peak 11 (t_R = 41.55 min) displayed a deprotonated ion at m/z 431 and MS/MS fragmentation of kaempferol at m/z 285 due to the neutral loss of deoxyhexose (146 Da). This compound was identified as kaempferol-*O*-deoxyhexoside [28]. Peak 12 (t_R = 42.13 min) exhibited $[M\text{-}H]^-$ at m/z 401 ($C_{18}H_{25}O_{10}$), $[M\text{-}H\text{-}133]^-$ at m/z 269 ($C_{16}H_{17}O_6$), $[M\text{-}H\text{-}271]^-$ at m/z 131, and $[M\text{-}H\text{-}301]^-$ at m/z 101 as a result of the loss of formic acid, and the alpha-*L*-arabinopyranosyl group successively formed the precursor ion. Thus, this molecule was tentatively identified as benzyl-diglycoside. This molecule was also reported in the fruit extract of *Prunus mume* (Rosaceae family) [33] and in leaf extracts of *Crataegus monogyna* and *Crataegus laevigata* fruits (Rosaceae family) [34].

Peak 13 (t_R = 42.33 min) produced $[M\text{-}H]^-$ at m/z 357. The MS/MS spectra of the ion at m/z 357 revealed a substantial daughter ion at m/z 313, indicating that the structure contains a lactone ring. The subsequent removal of CH_3 from the precursor ion at m/z 313, corresponding to m/z 298 and 283, indicated the presence of two methoxyl groups. Due to benzyl group cleavage, the ion at m/z 161 was detected. This compound was identified as matairesinol compared with the literature [35].

Effect of SSE on pancreatic β-cells

SSE and phenolic substance controls (gallic acid and quercetin) were used to determine their effect on the viability of INS-1 cells using an MTT assay. MTT in its reduced form can be used to determine the activity of mitochondrial dehydrogenase in living cells. Seventy-two hours after treatment, SSE did not induce any cytotoxicity in INS-1 cells at any of the tested concentrations (Fig. S2a). Gallic acid and quercetin had no detectable cytotoxic impact at concentrations of 1, 5,

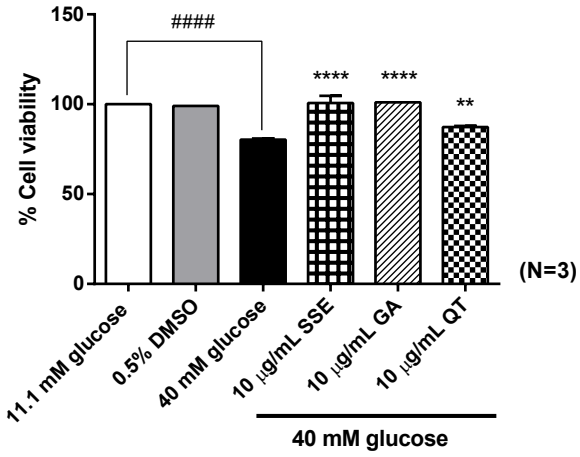

Fig. 4 Comparison of the protective effects of SSE, gallic acid (GA), and quercetin (QT) on high glucose-induced toxicity to pancreatic β-cells. Representative percent cell viability of INS-1 cells after exposure to 40 mM glucose in the presence or absence of 10 μg/ml SSE, GA, or QT. After treatment for 72 h, cell viability was determined by MTT assay. Data are expressed as the mean ± SEM of three independent experiments. $^{\#\#\#\#}$, $p < 0.0001$ vs. control; ****, $p < 0.0001$ vs. 40 mM glucose; and **, $p < 0.01$ vs. 40 mM glucose.

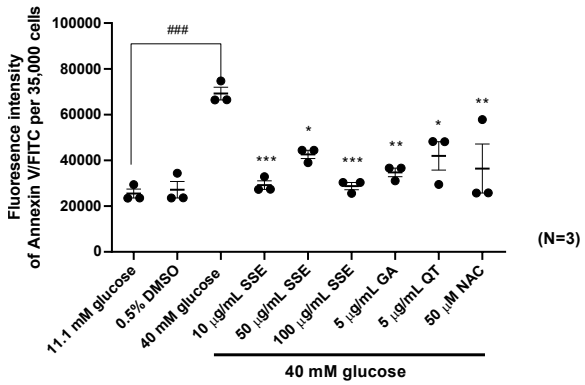

Fig. 5 Antiapoptotic effects of SSE, gallic acid (GA), and quercetin (QT) against glucotoxicity in pancreatic β-cells. Representative percent apoptotic cell of INS-1 after exposure to 40 mM glucose in the presence or absence of 10, 50, and 100 μg/ml SSE for 72 h as well as 5 μg/ml GA or QT. The antioxidant, NAC, was used as a positive control to examine the contribution of antioxidant property in anti-apoptosis. After treatment for 72 h, apoptotic cell death was verified by the annexin V/FITC staining assay. The data are expressed as the mean ± SEM of three independent experiments. $^{\#\#\#}$, $p < 0.001$ vs. control; ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$ vs. 40 mM glucose.

and 10 μg/ml. Notably, gallic acid and quercetin had a strong cytotoxic effect on INS-1 cells at doses of 50, 100, 250, and 500 μg/ml (Fig. S2b-c), reducing cell viability to less than 50% when compared to the DMSO control. Based on these findings, SSE at concentrations of 10, 50, and 100 μg/ml as well as gallic acid and quercetin at concentrations of 1, 5, and 10 μg/ml (Fig. S3) were chosen for assessing antiapoptotic and antioxidant activity against glucotoxicity.

Protective effect of SSE on pancreatic β-cells against glucotoxicity

Both gallic acid and quercetin have been shown to protect against the adverse effects of high glucose [8, 12]. Thus, this study examined the preventive effects of SSE and both phenolic compounds. INS-1 cells were cultivated in the presence of 40 mM glucose alone or in the presence of SSE or phenolic compounds at the same dose (10 μg/ml). MTT was performed 72 h after exposure. The results indicated that 10 μg/ml SSE and phenolic compounds restored the same degree of cell viability (Fig. 4). This finding revealed that SSE and phenolic compounds had a similar beneficial effect on INS-1 cells exposed to high glucose.

Antiapoptotic effect of SSE on pancreatic β-cells against glucotoxicity

To determine the antiapoptotic effects of SSE, INS-1 cells were exposed to high glucose for 72 h in the presence (10, 50, and 100 μg/ml) or absence of SSE with gallic acid and quercetin at 5 μg/ml and 50 μM NAC. Apoptotic cell death was evaluated upon exposure using an annexin V-FITC staining assay. The fluorescence intensity is directly related to the number of annexin V-positive cells. The dot graph in Fig. 5 indicates that the fluorescence intensity increased significantly, indicating a high level of cell death. When exposed to high glucose conditions, apoptotic cells were significantly reduced in the presence of SSE in a dose-independent manner. The reduction in apoptotic cells in the presence of SSE and phenolic compounds was comparable to NAC.

Antioxidant properties of SSE on pancreatic β-cells against glucotoxicity

An additional experiment was performed to investigate whether the antioxidant activity of SSE protects pancreatic β-cells from apoptosis caused by excessive glucose levels. The NBT assay was used to evaluate the intracellular superoxide levels in INS-1 cells. SSE at concentrations of 10, 50, and 100 μg/ml was discovered to drastically reduce intracellular superoxide ion levels in a dose-dependent manner (Fig. 6). These data lend credence to the concept that the phenolics in SSE act as antioxidants, thereby preserving pancreatic β-cells from death caused by glucotoxicity.

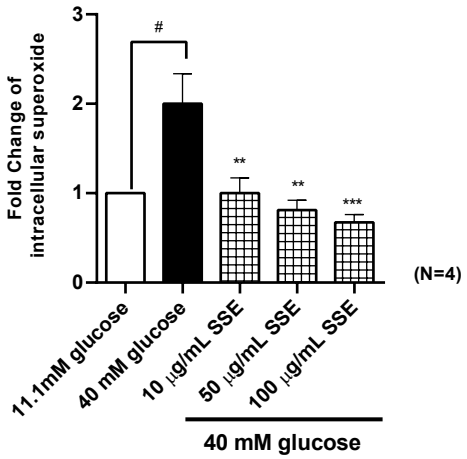

Fig. 6 Effect of SSE on oxidative stress in pancreatic β-cells. INS-1 cells were treated with 11.1 mM glucose RPMI media (control) or 40 mM glucose with or without 10, 50, and 100 μg/ml SSE. After 48 h of treatment, the intracellular superoxide ion level was evaluated by NBT assay. Data are expressed as the mean ± SEM of three independent experiments. $^{\#}$, $p < 0.05$ vs. control; ***, $p < 0.001$ vs. 40 mM glucose; and **, $p < 0.01$ vs. 40 mM glucose.

Overall relations between phenolic compounds and antioxidant and antiapoptotic activities of SSE on protection of pancreatic cells

The present study provides evidence on the potential of phenolics in rose apple extract to reduce oxidative stress and protect against glucotoxicity-induced cell death. We found here that high total phenolic content offered superior antioxidant activity than extract with low phenolic content. This study thus was consistent to our unpublished data which showed that using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, the rose apple extracts obtained from the optimized MAE approach had better antioxidant ability than those obtained from the reflux method as evidenced by lower IC_{50} values. These findings contribute to the existence of knowledge regarding the potential use of the extract in the treatment of diabetes.

This is the first study to examine the antiapoptotic and antioxidant effects of rose apple extract against glucotoxicity in pancreatic β-cells. We hypothesized that rose apple extract exerts its antioxidant properties to protect pancreatic β-cells from apoptosis caused by glucotoxicity. To prove this point, the effects of rose apple extract on cell death induced by high glucose were investigated in cultured INS-1 cells. Additionally, these data demonstrated that high glucose promoted cell death and increased intracellular superoxide generation, both of which are associated with pancreatic β-cell apoptosis. One of the most interesting findings of this study was that rose apple extract treatment protected the tested cells against apoptosis induced by high glucose. These findings corroborate prior research demonstrating that unprocessed rose apple powder treatment can reduce fasting blood glucose and the activity of the apoptotic enzyme cleaved-caspase 3 in pancreatic β-cells in STZ-induced diabetic rats. This activity was associated with elevations in the activities of the antioxidant enzymes CAT and SOD in the pancreas of diabetic rats given unprocessed rose apple powder [19].

Phenolic compounds derived from plants are well established to possess strong antioxidant properties and may be considered a source of holistic treatment in diabetes patients receiving standard therapy [10]. Gallic acid is one of the compounds found in rose apple extract, but quercetin is not. The bioactivity of rose apple extract was thus compared to the activity of the two phenolic compounds previously reported to protect pancreatic β-cells from diabetic conditions [8, 12]. In a recent work conducted by Khamchan et al [19], high-performance liquid chromatography was used to analyze some phytochemicals in crude extract obtained from fruit powder of the rose apple. Gallic acid and quercetin were observed to be present in the samples. This result is inconsistent with our findings. According to the LC-MS/MS method, an aqueous ethanolic extract contained only low levels of gallic acid and no detectable quercetin. Gallic acid and quercetin were employed as reference molecules for comparison since they are well-known hydrophilic and lipophilic antioxidants found in plants, respectively.

Our findings demonstrated that co-treatment of INS-1 cells with 10 μg/ml gallic acid or quercetin decreased apoptosis to a level comparable to the level of 10 μg/ml rose apple extract-treated cells. This finding corresponds to previous studies which demonstrated that gallic acid can protect RINm5F β-cell apoptosis from glucolipotoxicity by enhancing the antiapoptotic Bcl-2 and decreasing caspase-3 activation. Another study showed the antiapoptotic effect of quercetin in diabetic rats. Treatment with quercetin in STZ-induced diabetic rats reduced the levels of nitric oxide, MDA, and blood glucose [36]. Furthermore, quercetin treatment increased antioxidant enzyme activity and enhanced endogenous antioxidant activity, including GSHPx, SOD, and CAT in the pancreas of diabetic rats [36]. With respect to the bioactivity of flavonoids, some molecules such as naringin and kaempferol have previously demonstrated protective capabilities against glucotoxicity [37, 38]. In the present study, naringin glycoside and kaempferol deoxyhexose were found in rose apple extract. Although they share the same structure in aglycone form as naringin and kaempferol, their connection to sugar moieties gives flavonoid glycosides different water solubility properties that could affect their permeability into pancreatic cells. Therefore, we are unable to come to conclusions and suggest that further studies

of the bioactivity of these flavonoid glycosides should be performed. In addition, a recent study demonstrated that amentoflavone and total flavonoids from *Hedyotis diffusa* protected renal tubular epithelial cells (NRK-52E) from high glucose-induced cell damage by inhibiting the PI3K/AKT signaling pathway [39]. Interestingly, our study revealed a major secondary metabolite, matairesinol, a plant-derived lignan in the obtained rose apple extract. Plant-derived lignans have several biological effects, including antioxidant, anti-inflammatory, and anticancer activities [40]. Previously, a clinical study in middle-aged elderly men and postmenopausal women showed a beneficial effect of matairesinol in lowering vascular inflammation and endothelial dysfunction, which may be implicated in cardiovascular prevention [41]. Recently, Wu et al [42] reported that matairesinol can prevent neuronal apoptosis by suppressing the mitogen-activated protein kinase (MAPK) and NF-κB pathways, upregulating adenosine monophosphate-activated protein kinase (AMPK), and increasing the content of antioxidant enzymes in the brain tissue of an animal model of brain sepsis, indicating the anti-inflammatory and antioxidant effects of matairesinol. Taken together, the phytochemical compounds in the obtained rose apple extract could prevent glucotoxicity-induced pancreatic β-cell death primarily through antioxidant activity. These data lend support to the assumption that the phenolics of rose apple extract could function as antioxidants, thereby protecting pancreatic β-cells against glucotoxicity-induced death. Based on our findings, rose apple extract exhibited a dose-independent protective effect against β-cell apoptosis, whereas it showed a dose-dependent antioxidant effect. Thus, we postulated that other mechanisms such as β-cell ER stress underlie β-cell apoptosis. ER is primarily responsible for the synthesis, folding, and quality control of secretory proteins. To maintain ER homeostasis during stressors associated with secretory protein synthesis and folding, cells activate the intracellular signaling system known as the unfolded protein response (UPR) [43]. The UPR reduces the protein load on the ER by decreasing global protein synthesis and enhancing the translation of ER chaperones and foldases involved in correct protein maturation; misfolded proteins are degraded. If the stress cannot be eliminated, the UPR initiates apoptosis [44]. However, other underlying molecular mechanisms of rose apple extract action have multiple plausible pathways that warrant additional investigation.

This study has inherent limitations, as it focused primarily on crude rose apple extract and did not explore the bioactivity of its pure active molecules. Nevertheless, as a result of UHPLC-micrOTOF Q II-MS/MS analysis of the tentative compounds in the aqueous ethanolic extract of rose apple, it is possible to determine which polyphenols are likely to be the active ingredient. Therefore, future research should focus on determining the pancreatic protective capacity of the pure primary constituents presented in the rose apple extract.

CONCLUSION

This is the first report on maximizing the total phenolic content from *S. samarangense* (rose apple) fruits and their antiapoptotic capabilities in the presence of glucotoxicity-induced pancreatic β-cell death. This rose apple extract is protective against glucotoxicity and apoptosis in pancreatic β-cells *in vitro* when prepared under appropriate MAE conditions (Fig. S4). The optimal extraction parameters were investigated. Through HPLC-ESI-MS/MS analysis, 12 compounds could be identified, including cinnamic acid, glucaric acid, maloyl-hexose, citric acid, monocaffeoylquinic acid, gallic acid, 2-methylcitric acid, ellagic acid, naringin-*O*-glucoside, kaempferol deoxyhexose, benzyl-diglycoside, and matairesinol. Pharmacological property studies demonstrated that rose apple extract has an antidiabetic effect by exerting antioxidant properties to protect against glucotoxicity-induced pancreatic β-cell apoptosis, most likely attributable to the composition of the antioxidant polyphenols obtained through appropriate extraction procedures.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.043.

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Appendix A. Supplementary data

Table S1 Analysis of variance data showing the effect of the microwave-assisted extraction conditions on the response of total polyphenol content from *S. samarangense* fruits.

Source	Sum of square	Degree of freedom	Mean square	F value	p value probability $> F$-value	Inference
β_0	13.84	9	1.54	15.81	< 0.0001	***
X_1	3.12	1	3.12	32.07	0.0002	***
X_2	7.22	1	7.22	74.19	< 0.0001	***
X_3	0.05	1	0.05	0.56	0.4726	ns
X_1X_2	0.41	1	0.41	4.26	0.0661	ns
X_1X_3	0.01	1	0.01	0.12	0.7409	ns
X_1^2	0.54	1	0.54	5.53	0.0405	*
X_2^2	1.89	1	1.89	19.39	0.0013	**
X_3^2	0.39	1	0.39	3.97	0.0742	ns
Residual	0.97	10	0.10			
Lack of fit	0.71	5	0.14	2.72	0.1478	ns
Pure error	0.26	5	0.05			
Corrected total sum of squares	14.82	19				

Table S2 Identification of the possible structure of phenolic compounds in *S. samarangense* fruit extract by UHPLC-micrOTOF Q II-MS/MS in negative ion mode.

Peak No.	Retention time (t_R, min)	ESI (−)-micrOTOF-Q II/MS	MS/MS fragment (m/z)	UV spectrum (λ_{max}) (nm)	Tentative identification	Reference
1	0.20	147	103	204, 215, 274	Cinnamic acid	
2	4.11	209	191, 173	210	Glucaric acid	[26]
3	4.51	295	205, 179, 161, 133, 119	210	Malonyl-hexose	[27]
4	5.43	191	173, 161, 133, 111, 97	129, 220	Citric acid	[28, 29]
5	7.24	353	191 [M-H-caffeoyl]$^-$, 173 [M-H-caffeoyl-H_2O]$^-$, 135, 127	201, 244, 296sh, 326	Monocaffeoyl quinic acid	
6	10.39	169	125 [M-COOH]$^-$, 69, 79, 81, 107, 97	213, 228, 271	Gallic acid	[29]
7	11.34	205	205, 191, 173, 143, 111	210	2-Methylcitric acid	
8	21.78	361	341, 179, 121, 147	207, 278, 366	Unknown	
9	32.43	301	301, 284, 257, 255, 229, 201, 185	221,	Ellagic acid	[31]
10	36.57	741	740, 566, 503, 404, 341, 226, 147, 121	290, 310, 335	Naringin-*O*-glucoside	[32]
11	41.55	431	285	265, 365	Kaempferol deoxyhexose	[26]
12	42.13	401	269, 131, 101	258	Benzyl-diglycoside	[33, 34]
13	42.33	357	313, 225, 121	210, 289, 335	Matairesinol	[35]
14	43.60	593	549, 339, 232, 147	214, 236, 294	Unknown	–
15	45.41	489	385, 357, 221, 149, 131	289, 364	Unknown	–

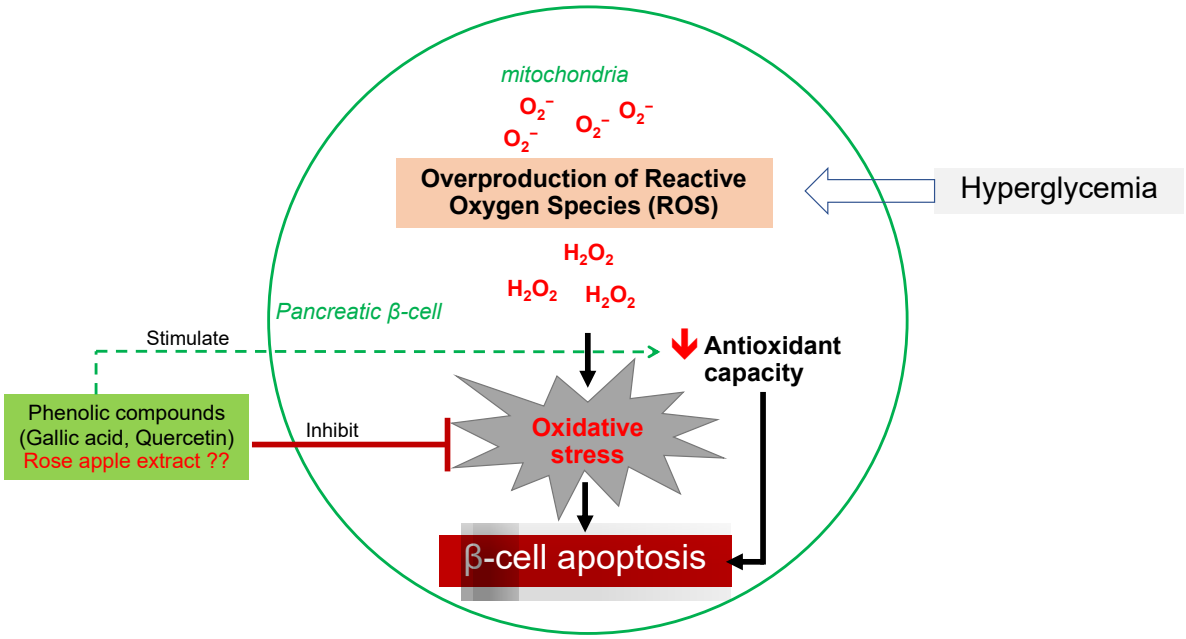

Fig. S1 Oxidative stress mediated β-cell apoptosis and the protective effect of phenolic compounds against glucotoxicity-induced pancreatic β-cell apoptosis.

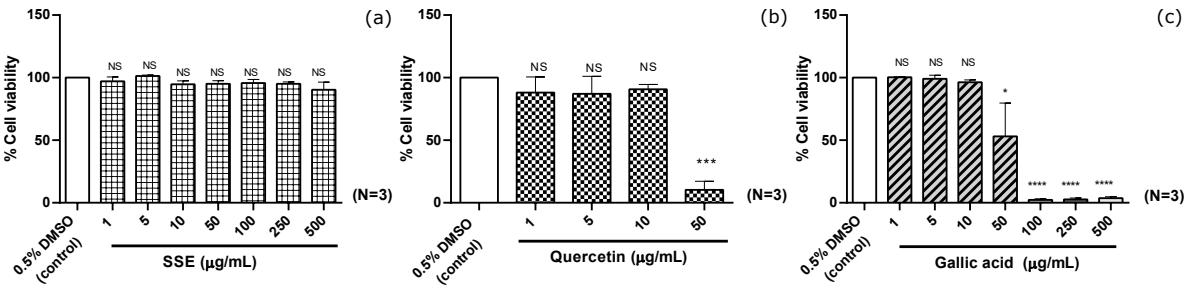

Fig. S2 Effect of SSE, gallic acid (GA), and quercetin (QT) on the viability of pancreatic β-cells. (a) Representative percent cell viability of INS-1 cells after treatment with 1, 5, 10, 50, 100, 250, or 500 μg/ml SSE for 72 h. Representative percent cell viability of INS-1 cells after treatment with 1, 5, 10, and 50 μg/ml QT (b) or with 1, 5, 10, 50, 100, 250, or 500 μg/ml GA (c) for 72 h. Data are expressed as the mean ± SEM of three independent experiments. $p > 0.05$ is not statistically significant (NS).

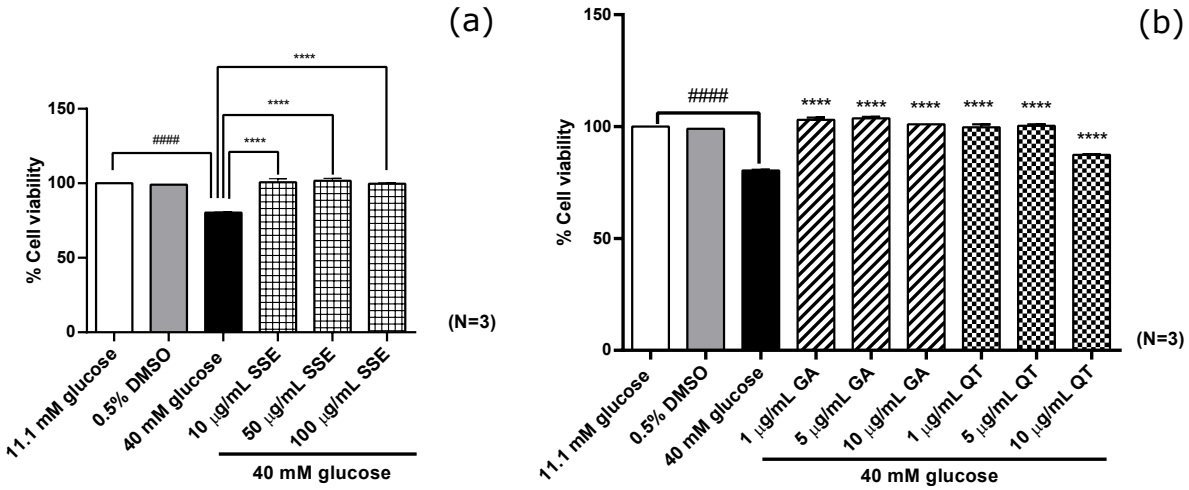

Fig. S3 Cytoprotective effects of SSE, gallic acid (GA), and quercetin (QT) against glucotoxicity in pancreatic β-cells. (a) Representative percent cell viability of INS-1 cells after exposure to 40 mM glucose in the presence or absence of 10, 50, and 100 μg/ml SSE for 72 h. Representative percent cell viability of INS-1 cells after treatment with 1, 5, and 10 μg/ml GA or QT (b). After treatment for 72 h, cell viability was determined by MTT assay. Data are expressed as the mean ± SEM of three independent experiments. $^{\#\#\#\#}$, $p < 0.0001$ vs. control; and ****, $p < 0.0001$ vs. 40 mM glucose.

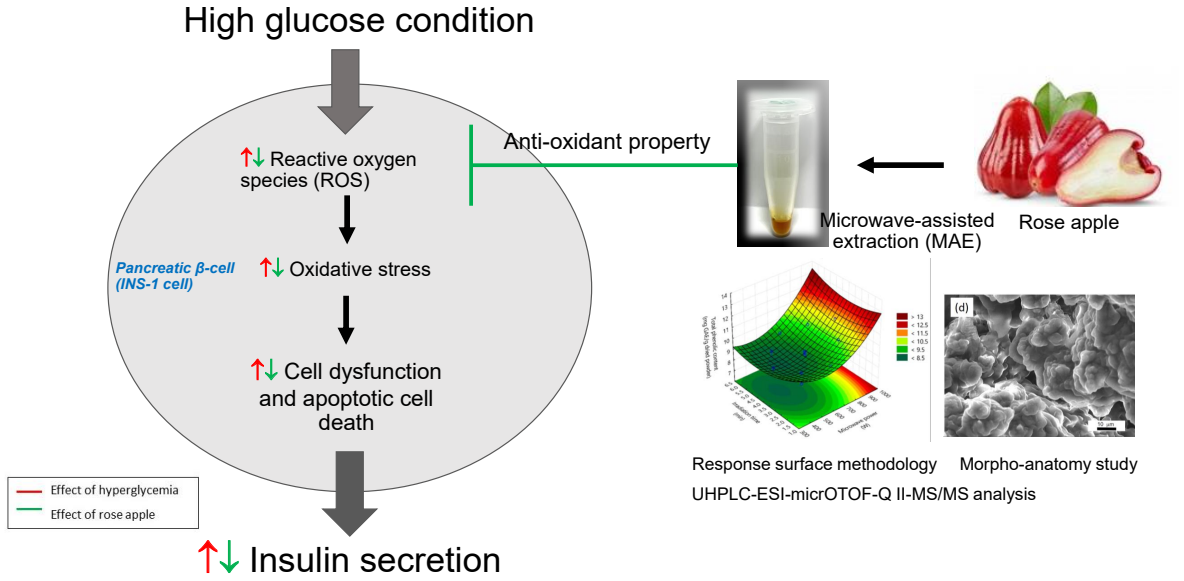

Fig. S4 The protective effect of SSE against glucotoxicity-induced pancreatic β-cell apoptosis.