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In Vitro Studies to Assess the α-Glucosidase Inhibitory Activity and Insulin Secretion Effect of Isorhamnetin 3-*O*-Glucoside and Quercetin 3-*O*-Glucoside Isolated from *Salicornia herbacea*

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Abstract: In this study, we examined the effect of ethanolic extract of *Salicornia herbacea* (ESH), isorhamnetin 3-*O*-glucoside (I3G), quercetin 3-*O*-glucoside (Q3G), quercetin, and isorhamnetin on α-glucosidase activity and glucose-stimulated insulin secretion (GSIS) in insulin-secreting rat insulinoma (INS-1) cells. A portion of the ethyl acetate fraction of ESH was chromatographed on a silica gel by a gradient elution with chloroform and methanol to provide Q3G and I3G. ESH, Q3G, and quercetin inhibited α-glucosidase activity, and quercetin (IC_{50} value was 29.47 ± 3.36 μM) inhibited the activity more effectively than Q3G. We further demonstrated that ESH, Q3G, quercetin, I3G, and isorhamnetin promote GSIS in INS-1 pancreatic β-cells without inducing cytotoxicity. Among them, I3G was the most effective in enhancing GSIS. I3G enhanced the phosphorylation of total extracellular signal-regulated kinase (ERK), insulin receptor substrate-2 (IRS-2), phosphatidylinositol 3-kinase (PI3K), Akt, and activated pancreatic and duodenal homeobox-1 (PDX-1), which are associated with insulin secretion and β-cell function. As components of ESH, Q3G has the potential to regulate blood glucose by inhibiting α-glucosidase activity, and I3G enhances the insulin secretion, but its bioavailability should be considered in determining biological importance.

Keywords: *Salicornia herbacea*; α-glucosidase; insulin; ERK; PI3K; AKT; PDX-1

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1. Introduction

Diabetes mellitus (DM) is one of the most prevalent and costly health conditions impairing patients' quality of life worldwide [1]. It is characterized by hyperglycemia that is the consequence of insufficient secretion of insulin or insulin resistance [2]. Therefore, control of postprandial hyperglycemia is an important target in DM. α-Glucosidase inhibitors such as miglitol, acarbose, and voglibose are antidiabetic drugs that reduce postprandial blood glucose by delaying the process of carbohydrate absorption in the small intestine. However, miglitol, acarbose, and voglibose are known to induce side effects such as stomach pain, diarrhea, and bloating, known as a side effect of any compound that blocks this enzyme [3].

Sulfonylureas such as glibenclamide and gliclazide are widely used for the treatment of type 2 DM. These sulfonylureas potentiate insulin secretion from pancreatic β-cells, but are currently being largely replaced by inhibitors of dipeptidyl peptidase-4 (DPP-4) and glucosurics [4]. At the same time, side effects such as skin reactions, nausea, and dizziness have been reported [5].

Salicornia herbacea is a halophyte that can grow in salt fields or salt marshes along seashores in Korea. It has been used as a culinary vegetable as well as a traditional medicine by people living in coastal areas [6]. Quercetin 3-*O*-glucoside (Q3G) and isorhamnetin 3-*O*-glucoside (I3G). were isolated and identified from this plant and quercetin and isorhamnetin were prepared. It has been reported that Q3G has antidiabetic effects via synthesis and secretion of insulin [7] and inhibition of the sodium glucose co-transporter in rats [8]. It is already well known that quercetin is α-glucosidase inhibitor, and it has been isolated from *Forsythia suspensa* (Thunb) Vahl [9], *Matricaria recutita* L. [10], and *Eucommia ulmoides* [11]. In contrast, little is known about the biological activities of I3G and isorhamnetin. We therefore evaluated the effects of Q3G, I3G, quercetin, and isorhamnetin on α-glucosidase inhibitory activity and insulin secretion in insulin-secreting rat insulinoma (INS-1) cells. Inhibition of α-glucosidase, an intestinal enzyme, is a therapeutic approach in DM patients because α-glucosidase inhibitors can prevent postprandial hyperglycemia by slowing the absorption of glucose [12]. Measuring the activity of glucosidase will provide scientific information on the biochemical potency of Q3G, I3G, quercetin, isorhamnetin and the ethanol extract of *S. herbacea* (ESH) in the development of novel α-glucosidase inhibitors.

Impaired glucose-stimulated insulin secretion (GSIS) is one of the consequences of β-cell dysfunction in diabetes [13]. Quercetin potentiates pancreatic GSIS that enhances the phosphorylation of total extracellular signal-regulated kinase (ERK), playing a key role in β-cell signaling and function [14]. An increase in activated duodenal homeobox-1 (PDX-1) and Akt after treatment with quercetin contributes to increased pancreatic β-cell mass and insulin secretion [15]. Effects of ESH, Q3G, I3G, quercetin, and isorhamnetin on α-glucosidase activity, GSIS from pancreatic β-cells, and the mechanism of action were evaluated as a treatment strategy for DM. This study demonstrates that ESH has the potential to both inhibit α-glucosidase activity and increase insulin secretion. Q3G was found to inhibit α-glucosidase activity and I3G was found to increase GSIS; these effects were associated with overexpression of ERK, PI3K, Akt, insulin receptor substrate-2 (IRS-2), and PDX-1 involved in enhanced insulin secretion and β-cell function.

2. Materials and Methods

2.1. Plant Material

S. herbacea was collected from Suncheonman Byeolryang Yeomjeon, Suncheon, Korea (2019). A voucher specimen was deposited at the Department of Plant Science and Technology, Chung-Ang University (Anseong, Korea).

2.2. Extraction and Isolation of Q3G and I3G

The air-dried powdered samples were extracted with ethanol under reflux. After removal of the solvent, the residue was suspended in water and then fractionated with *n*-hexane, ethyl acetate, chloroform, and *n*-butanol. A portion of the ethyl acetate fraction was eluted on a silica gel chromatography by a gradient elution with chloroform and methanol to afford Q3G and I3G (Figure 1).

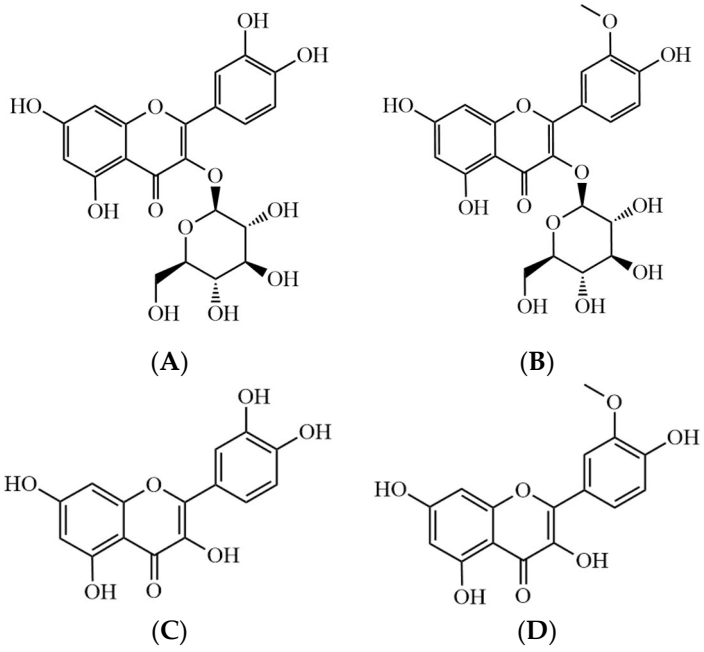

Figure 1. Chemical structures of (**A**) Q3G, (**B**) I3G, (**C**) quercetin, and (**D**) isorhamnetin. Quercetin 3-*O*-glucoside (Q3G), isorhamnetin 3-*O*-glucoside (I3G).

2.3. α-Glucosidase Inhibitory Activity Assay

Previously reported methods with slight modifications were used to determine the α-glucosidase inhibitory activities [16,17]. For the α-glucosidase inhibitory activity assay, the α-glucosidase inhibitor acarbose was used as the positive control. Acarbose, ESH, Q3G, I3G, quercetin, and isorhamnetin were prepared by dissolution in 1% dimethyl sulfoxide (DMSO) in water (the final concentration of DMSO in the reactional mixture was 0.2%). Next, 100 μL of 0.5 U/mL α-glucosidase was mixed with 80 μL of sample in 120 μL of 0.1 M phosphate buffer (pH 6.8) and incubated at 37 °C for 15 min. Then, 100 μL of 5 mM *p*-NPG as the substrate was added and incubated at 37 °C for 15 min, after which the reaction was stopped by adding 100 μL of 0.2 M sodium carbonate solution. The absorbance values of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) were measured at 405 nm using a PowerWave XS microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Inhibitory effects of the samples on α-glucosidase were calculated as follows: α-glucosidase inhibitory activity (%) = $[(A0 - A1)/A0] \times 100$, where A0 is the absorbance without the sample (control) and A1 is the absorbance with the sample.

2.4. Cell Culture and Cell Viability

INS-1 rat insulin-secreting β-cells (Biohermes, Shanghai, China) were maintained in RPMI-1640 medium (Cellgro, Manassas, VA, USA) as described in a published method [18]. Cells were plated on 96-well plates to determine the non-toxic concentration ranges of ESH, Q3G, I3G, quercetin, and isorhamnetin using Ez-Cytox reagent containing water-soluble tetrazolium salts (WSTs) (Daeil Lab Service Co., Seoul, Korea). In viable cells, WSTs produce a highly water-soluble formazan (orange color that absorbs at 450 nm) through mitochondrial electron transport systems. After 24 h of incubation, cells were treated with ESH, Q3G, I3G, quercetin, and isorhamnetin for 24 h. Subsequently, 10 μL of Ez-Cytox reagent was added and incubated for 2 h. A microplate reader (PowerWave XS, Bio-Tek Instruments, Winooski, VT, USA) was used to measure the absorbance values at 450 nm, as described in published methods [19,20].

2.5. GSIS Assay

INS-1 cells were plated on 12-well plates to measure the effects of Q3G, I3G, quercetin, and isorhamnetin isolated from ESH on GSIS in INS-1 cells. After 24 h of incubation, the

cells were washed twice with Krebs–Ringer bicarbonate HEPES buffer (KRBB) and 2.8 mM glucose. After starvation in fresh KRBB for 2 h, the cells were treated with ESH, Q3G, I3G, quercetin, isorhamnetin, and gliclazide as the positive control. After 2 h, cells were simultaneously treated with 2.8 mM (hypoglycemia) or 16.7 mM glucose (hyperglycemia) and incubated for 1 h. GSIS was assessed using a rat insulin ELISA kit (Gentaur, Shibayagi Co. Ltd., Shibukawa, Gunma, Japan), as described previously [18], and then expressed as GSI = insulin concentration in 16.7 mM glucose/insulin concentration in 2.8 mM glucose.

2.6. Western Blot Analysis

INS-1 cells were plated on 6-well plates to measure the effect of I3G isolated from ESH on protein expression changes of P-ERK, ERK, PI3K, Akt, P-IRS-2 (Ser731), IRS-2, P-PI3K, P-Akt (Ser473), and PDX-1 in INS-1 cells. After 24 h of incubation, the cells were treated with I3G for 24 h. Next, the protein extraction and western blot analysis were performed to assess the changes in protein expression, following published methods [21,22].

2.7. Statistical Analysis

All analyses were conducted using SPSS Statistics ver. 19.0 (SPSS Inc., Chicago, IL, USA). Nonparametric comparisons of samples were conducted with the Kruskal–Wallis test to analyze the results. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Preparation of Q3G, I3G, Quercetin, and Isorhamnetin

The structures of Q3G and I3G were elucidated by spectral analysis and comparison with the published literature [23,24]. In brief, Q3G and I3G were obtained as yellow crystals from methanol recrystallization. They responded to the Shinoda and Molisch tests. In the ^{1}H- and ^{13}C-NMR spectra, the typical flavonoid signals were observed. The characteristic fragment ion peaks showed the *retro*-Diels Alder fragmentation of flavonoids. Quercetin and isorhamnetin were obtained from Natural Product Institute of Science and Technology (www.nist.re.kr (accessed on 8 March 2021)), Anseong, Korea. The concentrations of Q3G and I3G as active compounds in the ESH were 0.54 and 1.06 mg/g, respectively.

3.2. α-Glucosidase Inhibitory Activities of ESH, Q3G, I3G, Quercetin, and Isorhamnetin

As shown in Figure 2A, α-glucosidase activity was 44.03 ± 2.45% after incubation with ESH at 500 μg/mL. α-Glucosidase activity was 81.22 ± 3.55%, 75.11 ± 4.84%, 71.83 ± 4.69%, and 61.97 ± 2.16% after incubation with Q3G at 31.25 μM, 62.5 μM, 125 μM, and 250 μM, respectively, compared with that of the control (0 μM) (Figure 2B). However, I3G showed no α-glucosidase inhibitory activity (Figure 2C). α-Glucosidase activity was 46.98 ± 2.86%, 36.03 ± 0.91%, 32.31 ± 1.37%, and 26.47 ± 2.18% after incubation with quercetin at 31.25 μM, 62.5 μM, 125 μM, and 250 μM, respectively, compared with that of the control (0 μM). Its IC_{50} value was 29.47 ± 3.36 μM (Figure 2D), whereas isorhamnetin showed no α-glucosidase inhibitory activity (Figure 2E). Acarbose as the positive control exhibited an IC_{50} value of 29.81 ± 1.31 μM. α-Glucosidase activity was 47.34 ± 2.06% after incubation with acarbose at 31.25 μM compared with that of the control (0 μM) (Figure 2F).

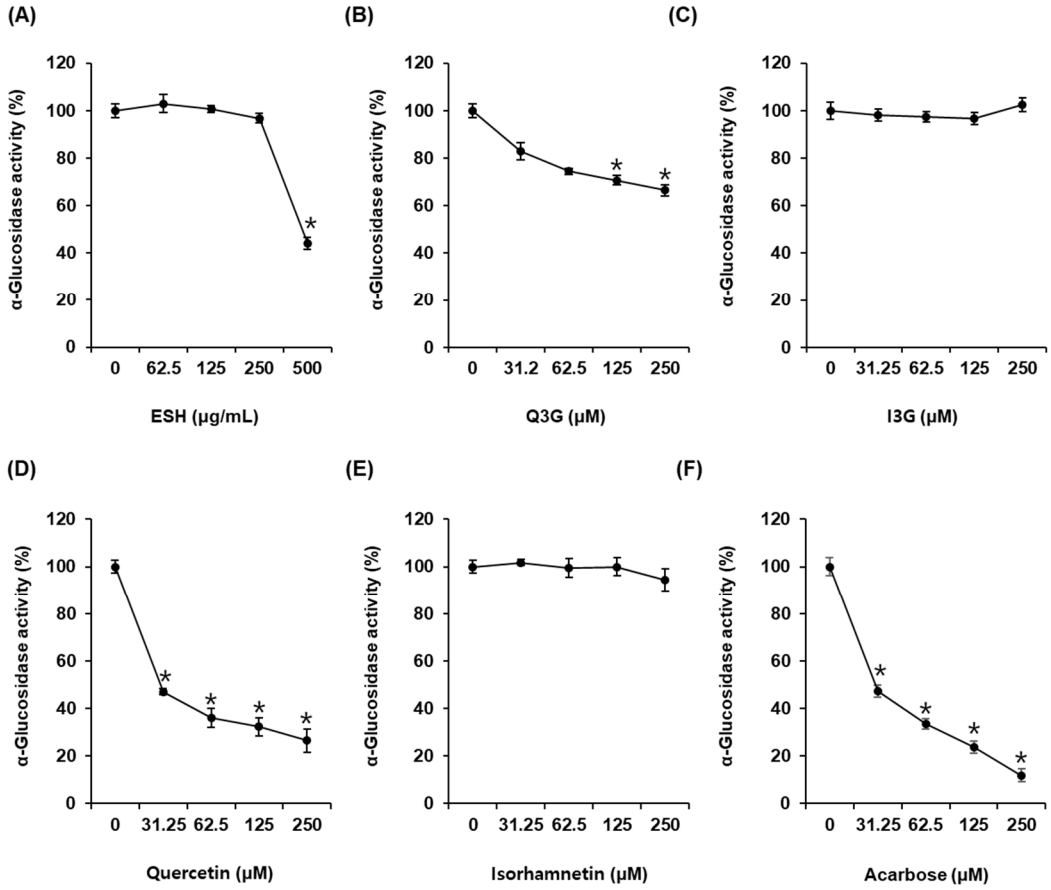

Figure 2. α-Glucosidase inhibitory activities of (**A**) ethanolic extract of *Salicornia herbacea* (ESH), (**B**) quercetin 3-*O*-glucoside (Q3G) (**C**) isorhamnetin 3-*O*-glucoside (I3G), (**D**) quercetin, (**E**) isorhamnetin, and (**F**) acarbose (positive control) compared with that of the control (0 μM) by α-glucosidase inhibitory activity assay (n = 3 independent experiments, * $p < 0.05$, Kruskal–Wallis nonparametric test). Data are expressed as the mean ± SEM.

3.3. Effect of ESH, Q3G, I3G, Quercetin, and Isorhamnetin on GSIS

As shown in Figure 3, at 2.8 mM glucose (hypoglycemia), no significant insulin secretion was observed on treatment with ESH, Q3G, I3G, and gliclazide. However, ESH at 100 μg/mL enhanced GSIS (232.85 ± 3.83 ng/mL) as compared to high glucose concentration at 16.7 mM alone (17.92 ± 8.73 ng/mL) (Figure 3A). In addition, Q3G at 100 μM enhanced insulin secretion (107.01 ± 4.85 ng/mL) as compared to high glucose concentration at 16.7 mM alone (17.92 ± 1.45 ng/mL) (Figure 3B). Interestingly, I3G at 100 μM significantly increased insulin secretion (502.11 ± 12.36 ng/mL) as compared to high glucose concentration at 16.7 mM alone (18.97 ± 11.04 ng/mL) (Figure 3C). Quercetin at 100 μM increased insulin secretion (119.38 ± 10.38 ng/mL) as compared to high glucose concentration at 16.7 mM alone (17.31 ± 10.36 ng/mL) (Figure 3D), whereas isorhamnetin at 100 μM significantly increased insulin secretion (304.37 ± 13.36 ng/mL) as compared to high glucose concentration at 16.7 mM alone (20.23 ± 5.52 ng/mL). In these results, the effect of I3G was similar to that of 100 μM gliclazide (554.26 ± 15.36 ng/mL) in which case also insulin secretion increased as compared to high glucose concentration at 16.7 mM alone (20.07 ± 10.43 ng/mL) (Figure 3D).

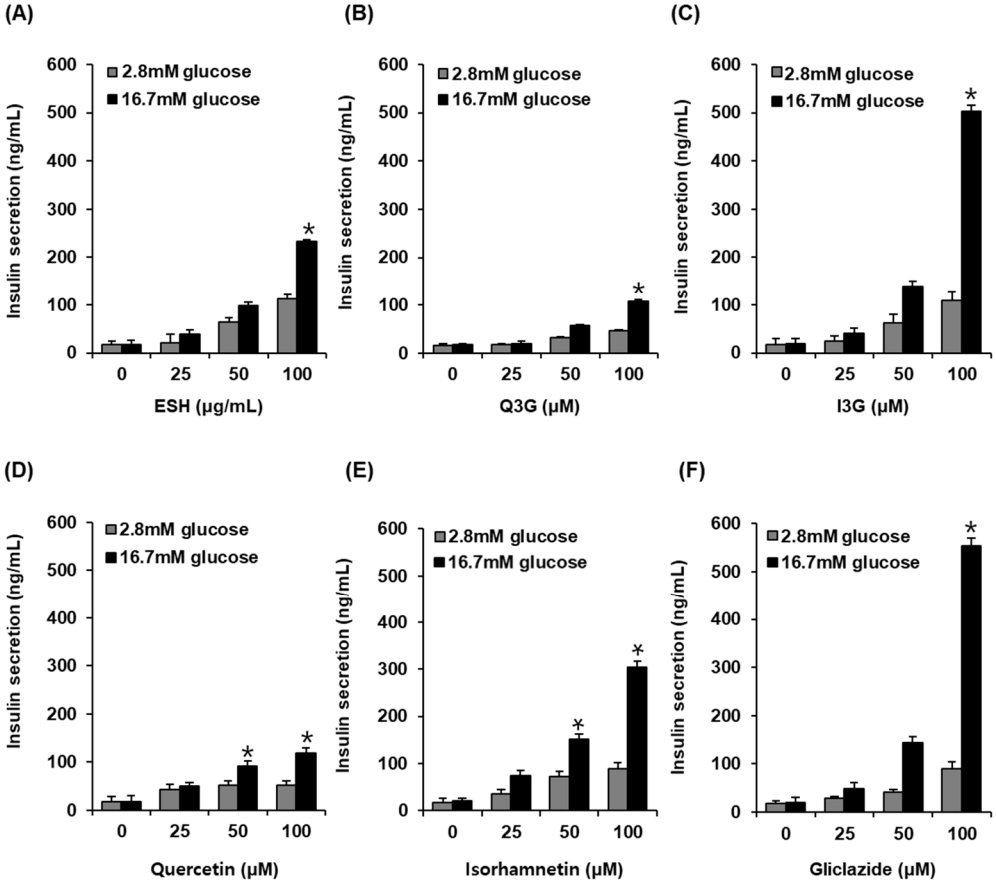

Figure 3. Effect of (**A**) ethanolic extract of *Salicornia herbacea* (ESH), (**B**) quercetin 3-*O*-glucoside (Q3G) (**C**) isorhamnetin 3-*O*-glucoside (I3G), (**D**) quercetin, (**E**) isorhamnetin, and (**F**) positive control (gliclazide) compared with that of the control (0 μM) on glucose-stimulated insulin secretion (GSIS) in INS-1 rat insulin-secreting β-cells for 1 h by insulin secretion assay ($n = 3$ independent experiments, * $p < 0.05$, Kruskal–Wallis nonparametric test). Data are expressed as the mean ± SEM.

3.4. Effect of I3G on the Protein Expression of P-ERK, ERK, P-PI3K, PI3K, P-IRS-2, IRS-2 (Ser731), Akt, P-Akt (Ser473), and PDX-1

To further validate the mechanistic insights on the effect of I3G on GSIS, we measured the expression of proteins including ERK, IRS-2, Akt, PI3K, and PDX-1, which are related to pancreatic β-cell metabolism. As shown in Figure 4A,B, upon treatment with I3G at 50 and 100 μM, protein expression levels of P-ERK, ERK, P-IRS-2 (Ser731), P-Akt (Ser473), P-PI3K, and PDX-1 were increased, compared with the control (0 μM). As shown in Figure 5A,B, increased expression level of Akt upon treatment with I3G at 100 μM was decreased after co-treatment with Akt inhibitor IV (Akt inhibitor), in which case, PDX-1 expression was also decreased. In addition, increased expression level of P-ERK upon treatment with I3G at 100 μM was decreased after co-treatment with U0126 (ERK inhibitor), in which case, PDX-1 expression was also decreased.

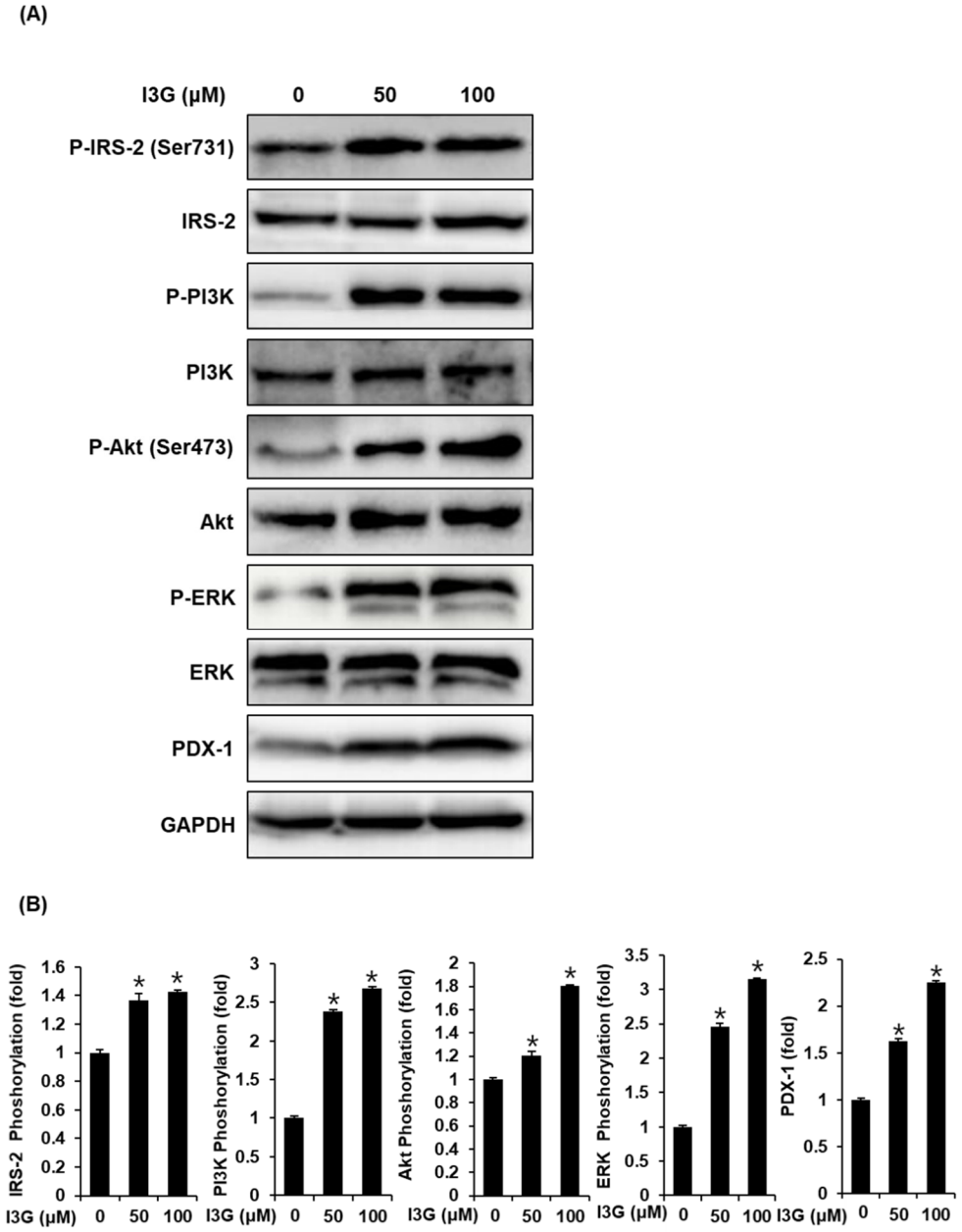

Figure 4. (**A**) Protein expression levels of phospho-insulin receptor substrate-2 (P-IRS-2) (Ser731), IRS-2, phospho-extracellular signal-regulated kinase (P-ERK), ERK, phospho-phosphatidylinositol 3-kinase (P-PI3K), PI3K, phospho-Akt (P-Akt) (Ser473), Akt, pancreatic and duodenal homeobox-1 (PDX-1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in INS-1 cells treated with 50 or 100 μM isorhamnetin 3-*O*-glucoside (I3G), or untreated for 24 h. (**B**) Bar graphs present the densitometric quantification of western blot bands (n = 3 independent experiments, * $p < 0.05$, Kruskal–Wallis nonparametric test). Data are expressed as the mean ± SEM.

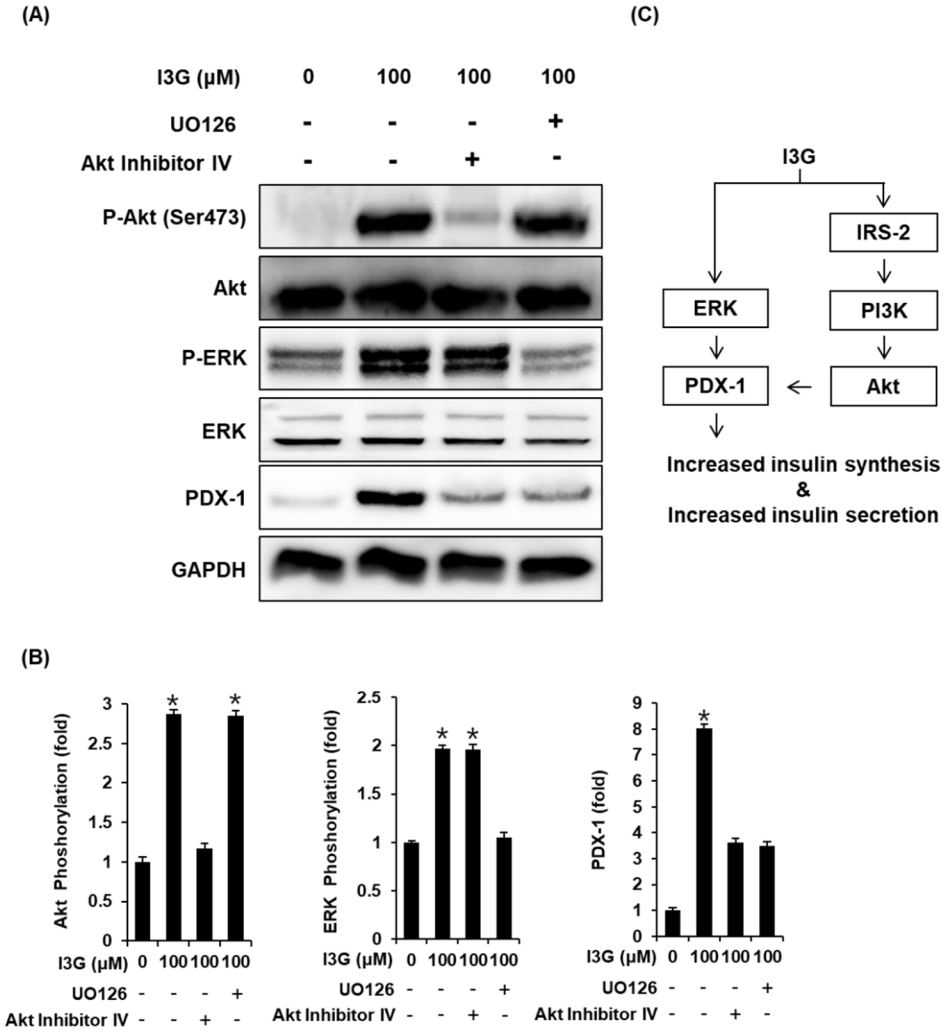

Figure 5. (**A**) Protein expression levels of phospho-Akt (P-Akt) (Ser473), Akt, phospho-extracellular signal-regulated kinase (P-ERK), ERK, pancreatic and duodenal homeobox-1 (PDX-1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in INS-1 cells treated with 100 μM isorhamnetin 3-*O*-glucoside (I3G), and/or (**B**) U0126 (ERK inhibitor, 10 μM) or Akt Inhibitor IV (Akt inhibitor, 500 nM) for 24 h. (**B**) Bar graphs present the densitometric quantification of western blot bands ($n = 3$ independent experiments, * $p < 0.05$, Kruskal–Wallis nonparametric test). Data are expressed as the mean ± SEM. (**C**) Schematic illustration of the effects of I3G isolated from ethanolic extract of *Salicornia herbacea* (ESH) on the protein expression levels related to pancreatic β-cell metabolism.

4. Discussion

This study demonstrates that ESH has the potential to both inhibit α-glucosidase activity and increase GSIS. In addition, Q3G, quercetin, and isorhamnetin were found to inhibit α-glucosidase activity, and I3G, quercetin, and isorhamnetin enhanced GSIS. Dietary carbohydrates are finally hydrolyzed to glucose in the intestine by α-glucosidase located in the mucosal brush-border surface of the membrane of intestinal cells [25]. Effective α-glucosidase inhibitors from natural products, with great structural diversity, have been shown to be a good source for antidiabetic therapies to delay glucose absorption, achieving better glycemic control [26]. To explore biologically active compounds from ESH, we tested the inhibitory activities of Q3G and I3G isolated from ESH against α-glucosidase from *Saccharomyces cerevisiae*. *p*-Nitrophenyl-α-D-glucopyranoside (*p*-NPG) is used as a substrate for the α-glucosidase inhibitory activity assay [16,17].

ESH inhibited the α-glucosidase activity by 44% at a high concentration of 500 μg/mL. It was reported that extract of *S. herbacea* at the same concentration show α-glucosidase

inhibitory activity, where the activity is 10% [27]. I3G and isorhamnetin have no statistically significant α-glucosidase inhibitory activity, whereas Q3G and quercetin inhibited α-glucosidase activity. Quercetin inhibited the activity more effectively than Q3G. These results showed that the sugar moiety of the quercetin glycoside does not enhance the α-glucosidase inhibitory activity. Similarly, it was found that quercetin and Q3G isolated from tartary buckwheat bran have α-glucosidase inhibitory activity, and the inhibitory activity of quercetin is stronger than that of Q3G [28]. The absorption of quercetin glycosides depends on the position and nature of the sugar moiety [29]. In general, after eating quercetin-rich foods, the concentration of quercetin reaching human plasma is in the low range [30]. It can only reach levels of up to 5 μM in plasma [31]. In the present study, the concentrations of quercetin and Q3G required for the α-glucosidase inhibitory activity is too high, so, bioavailability should be considered in determining biological importance of ESH components.

INS-1 pancreatic β-cells have been used to study the effects of GSIS [32]. This method has been used in many previous studies to evaluate insulin secretion capacity. In the previous study, genistein, a soy isoflavone, alone do not affect insulin secretion in INS-1 cells, but significantly enhances in response to glucose stimulation [33]. Importantly, the GSIS assay proceeds after determination of the highest non-cytotoxic concentration. A previous study reported that oleuropein, a phenolic compound, promotes GSIS without inducing cytotoxicity in INS-1 cells [34]. In the present study, ESH enhanced GSIS without inducing cytotoxicity in INS-1 cells. In contrast to the results for α-glucosidase, I3G and isorhamnetin had a better effect than Q3G and quercetin on insulin secretion in response to high-glucose stimulation, without inducing cytotoxicity in INS-1 cells. I3G enhanced the GSIS more effectively than isorhamnetin. These results showed that the sugar moiety of the isorhamnetin glycoside had effect on enhancing GSIS.

Impaired insulin secretion is a feature of type 2 DM which could be due to a reduction in pancreatic β-cell size and number [13]. In an experimental model of type 2 DM, PDX-1, a homeodomain-containing transcription factor for PDX-1 itself, is a primary regulator of insulin secretion and glucose metabolism related to β-cell function [35]. In vitro studies have shown that PDX-1 suppression can lead to a decrease in insulin synthesis [36]. In the present study, I3G resulted in the overexpression of PDX-1. PDX-1 is phosphorylated by phosphorylation of ERK, PI3K, and Akt [37,38]. Enhanced proliferation of INS-1 pancreatic β-cells by ERK and Akt promotes insulin secretion [39]. Oleuropein promotes GSIS and ERK phosphorylation in INS-1 cells in a concentration-dependent manner [34]. In agreement with earlier studies, we found that ERK phosphorylation was increased by I3G. In addition, PI3K-dependent phosphorylation of Akt at Ser473 in the presence of I3G was increased. In both INS-1 cells and high fat diet-fed rats, serine protease inhibitor from visceral adipose improves pancreatic β-cell function though PI3K-dependent Akt phosphorylation at Thr308 and IRS-2 phosphorylation at Ser731, which leads to increases in insulin secretion [40]. Akt phosphorylation can be activated by tyrosine phosphorylation of IRS-2, linking insulin receptors in pancreatic β cells [41]. In the present study, I3G enhanced IRS-2 phosphorylation at Ser731. In addition, treatment with specific Akt and ERK inhibitor attenuated the effects of I3G on expression levels of P-ERK and Akt, respectively, and both Akt and ERK inhibitor attenuated the effects of I3G on the expression level of PDX-1. Together, these data indicated a role of Akt and ERK in the control of PDX-1 expression after treatment with I3G. Consequently, I3G improved insulin secretion by regulating the expression of ERK, PI3K, Akt, IRS-2, and PDX-1. However, bioavailability should be considered in determining clinical importance since I3G is not well absorbed in its parent form [42].

5. Conclusions

Q3G and quercetin inhibited α-glucosidase activity, but in micro molar concentrations. Quercetin inhibited the activity more effectively than Q3G. In addition, I3G was the most effective in enhancing GSIS by regulating the expression of ERK, PI3K, Akt, IRS-2, and

PDX-1. Bioavailability should be considered in determining the clinical importance of these compounds.

Author Contributions: K.S.K., J.Y.P., and S.L. conceived and designed the experiments; D.L. and J.Y.P. performed the experiments; S.L. and J.Y.P. analyzed the data; S.L. and K.S.K. contributed reagents, materials, and analytical tools; and D.L., J.Y.P., and K.S.K. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: There is no conflict of interest.

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