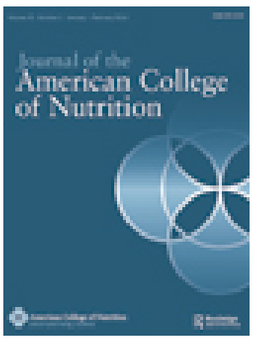

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Antioxidant and Anti-Diabetic Functions of a Polyphenol-Rich Sugarcane Extract

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ABSTRACT

Objectives: Dysfunctional metabolism of carbohydrates is a fundamental component of many dietary-related disorders. It has been hypothesized that plant extracts containing high levels of antioxidants may have the ability to stabilize carbohydrate regulation. The aim of this study was to assess the effects of a polyphenol-rich sugarcane extract on cellular pathways related to carbohydrate metabolism.

Methods: We evaluated the antioxidant activity of a polyphenol-rich sugarcane extract obtained by a patented hydrophobic extract process and its therapeutic potential to regulate carbohydrate metabolism and protect against metabolic disorders such as type 2 diabetes.

Results: Quantitative analytical studies support that the polyphenol-rich sugarcane extract has a high concentration of polyphenols and antioxidant compounds. The follow-up cellular studies via Caco-2 cells and dysfunctional β-cell models suggested that the polyphenol-rich sugarcane extract may help deter glucose and fructose uptake in intestinal cells and restore insulin production in dysfunctional β-cells—key functions in managing diabetic conditions.

Conclusions: These findings suggest that sugarcane polyphenols may modulate cellular mechanism in a manner that is beneficial to health.

Abbreviations: PRSE: Polyphenol-rich sugarcane extract; ROS: reactive oxygen species; RNS: reactive nitrogen species; GLUT: glucose transporter; SGLT: sodium-dependent glucose transporter; GAE: gallic acid equivalency; ORAC: oxygen radical uptake capacity; HepG2: hepatocellular carcinoma cells; PBS: phosphate-buffered saline; DCFH-DA: 2′,7′-Dichlorodihydrofluorescein diacetate; AAPH: 2,2′-Azobis(2-amidinopropane) dihydrochloride; QE: quercetin equivalency; EDTA: ethylene diamine teraacetic acid; DMEM: Dulbecco's Modified Eagle Medium; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HBSS: Hank's Balanced Salt Solution; 2-NBDG: 2-(N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) Amino)-2-Deoxyglucose; KRBB: Krebs-Ringer bicarbonate buffer; NBD-Fructose: 1-(7-nitro-1,2,3-benzadiazole)-Fructose; NAC: N-acetyl-l-cysteine; PCR: Polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay

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KEYWORDS
Antioxidant; anti-diabetic; polyphenol rich sugarcane extract; cell-based assay; glucose uptake; fructose uptake; insulin restoration

Highlights

- Polyphenol rich sugarcane extract (PRSE) produced by a patented hydrophobic extraction process contains rich polyphenol content and exhibits full-spectrum antioxidant activities.
- PRSE deters glucose uptake in Caco-2 human intestinal cells under both active and passive glucose transport channels.
- PRSE inhibits fructose uptake in Caco-2 human intestinal cells, mostly through inhibiting GLUT2 expression.
- PRSE restores insulin production in dysfunctional β-cells—a key indicator for managing diabetic conditions.

Introduction

Sugarcane (*Saccharum officinarum*) is a tall, perennial grass indigenous to tropical South Asia and Southeast Asia. The word *Saccharum* originated from the Sanskrit word "sarkara" or "sakkara" meaning sugar. Sugarcane has long been discovered and cultivated as an important source of sugar and biofuel. Less known to the world is the medicinal value of sugarcane, although sugarcane has long been recognized in traditional Indian medicinal systems as remedies for jaundice, inflammation, constipation, septic shock, and various other diseases (1,2). Modern pharmacological studies discovered a list of phytochemicals in sugarcane, including polyphenolic compounds such as phenolic acids, flavonoids, and different glycosides in juices and its unrefined products (e.g., molasses), as well as fatty acids in sugarcane stems and leaves (1). The finding of these phytochemicals in sugarcane plant substantiated its medicinal values recorded in traditional Indian medicines, since many of these phytochemicals have been identified as bioactives that are responsible for therapeutic effects in a number of disease areas including anti-inflammatory, anti-diabetic, and anti-hyperglycemic. Owing to the discovery of these bioactive compounds in the plant, for the past decade sugarcane has been investigated in numerous *in vivo* and *in vitro*

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studies as an abundant commodity, yet with high therapeutic potential (3).

Diabetes mellitus is a prevalent systemic disease impacting approximately 150 million people worldwide in 2018, potentially doubling by the year 2025 (4). There are two principle forms of diabetes, type 1 and type 2, and type 2 diabetes accounts for around 90% of all diabetes cases worldwide. Type 2 diabetes is characterized by abnormally elevated levels of blood glucose due to insufficiency of insulin secretion, as well as dysfunctions in carbohydrate and sugar metabolism. Increasing evidence revealed that oxidative stress plays a main role in the pathogenesis of diabetes mellitus, and numerous studies report the use of antioxidants to reduce oxidative stress and alleviate diabetic complications (5). Polyphenols (polyphenolic compounds) are known to be a group of naturally occurring compounds that can function as antioxidants mainly via absorbing reactive oxygen or nitrogen species (ROS/RNS) facilitated by the conjugated phenol ring structure they contain in common. Although having not been studied in detail, there is a logical assumption that polyphenolic phytonutrients discovered in sugarcane may function as antioxidants and contribute to diabetic management. In this study, we set out to explore the potential antioxidant effect of phytonutrients found in sugarcane plant and its function in diabetes management.

Sugar uptake and regulation is a functional process that under degenerative conditions may result in various health issues including obesity, diabetes, and cancer (6). Regulating the uptake of sugar into the blood stream and its metabolism after intake have been two major therapeutic approaches to improve diabetic conditions. The major forms of sugar humans consume directly from their diet are sucrose and fructose. Upon intake into human bodies, sucrose, a disaccharide, breaks down into the individual monosaccharides glucose and fructose which are then rapidly absorbed via human intestines into the bloodstream. Glucose uptake takes place via two pathways: (1) facilitated diffusion, a passive process where the movement of ions or molecules going along the concentration gradient, and (2) secondary active transport, an active process with the movement of ions or molecules going against the concentration gradient. The drivers for facilitated diffusion of glucose are glucose transporter (GLUT)1–4, with GLUT2 having been reported to be inhibited by a polyphenol (7). For active glucose transport, a main driver for glucose transport is sodium-dependent glucose transporter 1 (SGLT1). Metabolically, the insulin receptor, a transmembrane receptor that is activated by insulin and insulin-like growth factors, plays a key role in glucose regulation. Under decreased insulin secretion or insulin receptor activity conditions, the cells and tissues are unable to take up glucose, resulting in hyperglycemia (an increase in circulating glucose), leading to type 2 diabetes mellitus.

Fructose uptake mainly takes place passively across membranes by two members of the facilitative glucose transporter (GLUT) family, named GLUT2 and GLUT5. Fructose is absorbed from the lower part of the duodenum and jejunum by the facilitated glucose transporter (GLUT5), then transported to the blood from the intestinal lumen by facilitated sodium-independent transport via the glucose transporters GLUT5 and GLUT2. The GLUT5 transporter plays a major role in fructose uptake, while GLUT2 functions in another major fructose transporter role (8). Studies have shown that overconsumption of fructose leads to a surplus of its metabolites including fatty acids and triglycerides that can lead to increased risk of diabetes, obesity, and metabolic syndrome. Regulating fructose uptake and its transporters therefore is also an effective way to improve diabetic conditions.

In this study, we applied live cells models to assess the antioxidant activities of a polyphenol-rich sugarcane extract (PRSE) produced via a patented hydrophobic extraction process and explored its therapeutic potential in type 2 diabetes mellitus from two perspectives: (1) effect on uptake of glucose and fructose and (2) effect on insulin production in dysfunctional beta-cells.

Materials and methods

Reagents

All chemicals are obtained from Sigma-Aldrich unless otherwise specified.

Study material

A PRSE, a novel proprietary sugarcane plant extract obtained from sugarcane molasses is used in this study. The material is developed and prepared by The Product Makers Pty Ltd based on a patented hydrophobic resin procedure described previously (9). Briefly, deionized water is added to sugarcane mill molasses with constant stirring until the final Brix is 20. Brix defines the sugar content of an aqueous solution. One degree Brix represents 1 gram of sucrose in 100 grams of solution. To a beaker containing 1 liter of 20 Brix feedstock under room temperature (20–25 °C), 500 g of wet weight pretreated ion exchange resin and polymeric adsorbents (Dow Chemical) is added with gentle stirring to ensure effective mixing of the resin particles with the feedstock. The mixture is then filtered under vacuum, and the resin particles are collected and washed by resuspension in 1 liter of deionized water twice. The final washed resins are then suspended in 1 liter of 70% ethanol, stirred for 10 minutes, and the filtrate collected by vacuum filtration. This suspension/filtration process was repeated twice more with 1 liter of 70% ethanol with each filtrate collected. Finally, the three batches of 70% ethanol filtrates were combined, and the ethanol removed under vacuum. The aqueous extract was then lyophilized or spray-dried into a free-flowing brown powder (PRSE) with a final moisture level of 2% to 4% w/w. Preliminary chemical characterization of the extract discovered that the hydrophobic nature of the extraction method resulted in enriched level of hydrophobic compounds including polyphenols, in levels 5- to 10-fold higher than found in the starting material, molasses (data not shown). Specific physical properties of PRSE are described in Table 1.

Quantification of total phenols

Total amount of polyphenols contained in the PRSE is quantified via a total phenolics analysis. Total phenolics analysis is

Table 1. Physical properties of polyphenol-rich sugarcane extract (PRSE).

Appearance:	Dark brown free-flowing powder
Organoleptic:	Bitter-tannic taste
Conductivity:	29 μS/cm (1% in solution @ 20 °C)
pH:	3.74 (1% in solution @ 20 °C)
Total polyphenols:	221 mg/g GAE (as gallic acid equivalency)
Key polyphenols	
Apigenin	1.89 μg per gram
Luteolin	5.30 μg per gram
Tricin	27.40 μg per gram
Total flavonoids:	53.8 mg/g CE (as catechin equivalency)
Total antioxidant activity (ORAC 5.0):	18,837 μmole TE (as trolox equivalency) per gram
ORAC against hydroxyl radicals:	13,785 μmole TE per gram
ORAC against peroxyl radicals:	2,336 μmole TE per gram
ORAC against peroxynitrite:	255 μmole TE per gram
ORAC against singlet oxygen:	2,011 μmole TE per gram
ORAC against super oxide anion:	450 μmole TE per gram
Cellular antioxidant assay:	229.12 μmole QE (as quercetin equivalency) per gram
Color absorbance readings:	
@270 nm:	180 (1% in solution @ 20 °C)
@420 nm:	10 (1% in solution @ 20 °C)
A270/A420:	18 (1% in solution @ 20 °C)
Mineral content (mg/kg):	
Sodium:	170
Potassium:	130
Calcium:	6,600
Magnesium:	3,000
Zinc:	15
Essential trace elements (mg/kg):	
Selenium:	0.3
Chromium:	2.4

a well-established spectrophotometric method that quantifies the total amount of phenolics in a test material. The analysis measures polyphenols based on the Folin-Ciocalteau assay developed by Slinkard and Singleton (10). First, the phenolics constituents in PRSE are reduced by Folin-Ciocalteau reagent (Sigma-Aldrich) to compounds that exhibit maximum absorbance at 765 nm. The intensity of the reaction product is then measured by a spectrophotometer (BioTek Synergy HT) and the amount of total phenols is calculated using the calibration curve generated from a gallic acid standard (Sigma-Aldrich). The total phenolics result is reported as milligram gallic acid equivalency (GAE) per gram of PRSE.

Quantification of polyphenols Liquid Chromatography-Mass Spectrometry with ultraviolet detection (LC-MS/UV)

Quantification of polyphenols in PRSE was completed by Analytical Chemistry Services in Melbourne, Australia. Apigenin, luteolin, and tricin were selected as internal standards for targeted LC-MS with UV detection in series. These compounds have been previously reported in sugarcane juice; however, their concentrations in PRSE was not known (11). In addition, these compounds have been reported to be able to modulate carbohydrate metabolism, increase insulin sensitivity, and have anti-obesity effects (12–14). Therefore, they were useful compounds to target in this trial.

Chemical analysis of antioxidant capacity: ORAC 5.0 analysis

Preliminary antioxidant capacity of the PRSE is assessed via oxygen radical uptake capacity (ORAC) 5.0 analysis, a chemical analysis that quantifies antioxidant scavenging activity of PRSE against oxygen radicals that are known to be involved in the pathogenesis of many chronic diseases. ORAC 5.0 analysis consists of five types of ORAC assays that evaluate the antioxidant capacity of a material against five primary ROSs/RNSs (commonly called "oxygen radicals") found in humans: peroxyl radical, hydroxyl radical, superoxide anion, singlet oxygen, and peroxynitrite. Detailed procedure of the ORAC assays have been reported elsewhere (15,16). In brief, the ORAC assays are based on evaluating the capacity of PRSE to protect a chromagen probe from being damaged by ROSs/RNSs. In each of the ORAC assays, the chromagen probe is exposed to a specific ROS or RNS, which degrades the probe and causes its emission wavelength or intensity to change. When an antioxidant material presents in the environment, the antioxidant absorbs the ROS or RNS and preserves the probe from degradation. The degree of probe preservation is then recorded and calculated against a calibration curve generated from trolox (Sigma-Aldrich), a water-soluble form of vitamin E, which is used as the reference standard. The result of an ORAC assay is expressed as μmole trolox equivalency per gram of PRSE.

In vitro *analysis of antioxidant capacity: cellular antioxidant assay*

As a step forward from the chemical assessment of the antioxidant activity of the PRSE, cellular antioxidant assay is carried out to further understand the potential antioxidant activity of PRSE in a biological system (live human liver cells). The assay protocol has been described previously (17). Briefly, cellular antioxidant assay analyzes the capacity of PRSE to protect fluorescein, a fluorescent molecule served here as a marker, from damage by an ROS in human liver cells. Human hepatocellular carcinoma cells (HepG2) were obtained from American Type Culture Collection and grown in a growth medium and maintained under 37 °C, 5% CO_2/95% O_2 with 95% humidity before use. To start the assay, HepG2 cells are seeded at a density of $3–5 \times 10^5$/well on a 96-well black, clear-bottom microplate in 100 μL of growth medium/well. Sixteen to twenty-four hours after seeding, the growth media was replaced with 100 μL serum free medium/well, and the cells are starved for 3 to 5 hours. Cells are then treated for 1 hour in duplicate with 100 μL of phosphate-buffered saline (PBS) solution containing 25 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and various concentrations of PRSE. The cells are then washed with PBS and treated with 200 μL of an oxygen radical inducer, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) from Sigma-Aldrich. Fluorescent signal of the cells (Excitation 485 nm/Emission 538n) is then recorded via a microplate reader (BioTek, Synergy HT) every 5 minutes for 30 minutes. Quercetin (Sigma-Aldrich), a known antioxidant effective in cellular antioxidant assay, is used as the reference standard. The cellular antioxidant effect of PRSE is quantified by comparing the effect of PRSE on the fluorescent marker with the calibration curve generated from quercetin standard. The result of the cellular antioxidant assay

is expressed as μmole quercetin equivalency (QE) per gram of PRSE.

Glucose and fructose uptake study with Caco-2 cell monolayer

Caco-2 human intestinal cells were obtained from Deutsche Samsung von Microorganismal and Zellkulturen. The cells were maintained under 37 °C in a humidified atmosphere with 5% CO_2 and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 0.05% penicillin/streptomycin/amphotericin (all from Sigma). Cells were removed at 80% to 90% confluence enzymatically using a 0.25% trypsin/0.20% ethylene diamine teraacetic acid (EDTA) solution and subcultured. Media were changed approximately every 2 days.

For glucose uptake studies, Caco-2 cells (passages 26–40) were seeded on 24-well plastic cell culture dishes, and monolayers of Caco-2 cells (100% confluence) were obtained 12 days after the initial seeding. The Caco-2 monolayers were placed in the culture medium (DMEM) free of fetal calf serum for 24 hours and were incubated for 10 minutes at room temperature prior to uptake studies. The Caco-2 cell monolayers were then pretreated with the test materials (six concentrations of PRSE, 100 μM myricetin, or 100 μM phloridzin) or vehicle control for 20 min. Glucose uptake was initiated by the addition of sodium-containing HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)) buffered salt solution (Hank's Balanced Salt Solution [HBSS], pH 7.5; 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES) or sodium free HEPES buffered salt solution (HBSS, pH 7.5; 145 mM KCl, 1 mM K2HPO4, 1 mM CaCl2, 0.5 mM MgCl2, 10 mM HEPES) containing 200 μM of 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose) (Cayman Chemical). 2-NBDG is a fluorescently labeled deoxy glucose analog and was used as a probe for the detection of glucose uptake in Caco-2 cell monolayers as previously reported (18). The glucose uptake process was stopped after 30 minutes by adding a twofold volume of ice-cold PBS into the wells and washed the wells with ice-cold PBS three times. The fluorescent signal was then measured using a microplate reader.

For fructose uptake studies, Caco-2 cell monolayers were obtained and prepared following the same procedure described above. Prior to the study, the Caco-2 monolayers were placed in the culture medium (DMEM) free of fetal calf serum for 24 hours and incubated for 10 minutes at room temperature immediately before the fructose uptake studies. The Caco-2 cell monolayers were then pretreated with the test materials (six concentrations of PRSE or quercetin) or vehicle control for 20 minutes in 0.3 ml glucose-free Krebs-Ringer bicarbonate buffer (KRBB; 129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 0.1% BSA, 10 mM HEPES [pH 7.4]) at 37 °C. Fructose uptake was initiated by the addition of 0.3 ml glucose-free Krebs containing 100 nM 1-(7-nitro-1,2,3-benzadiazole)-fructose (NBD-fructose; Cayman Chemical), a fluorescently labeled fructose analog, which was used as a probe for the detection of fructose uptake in Caco-2 cell monolayers as reported previously (19). Incubation was stopped after 30 minutes by adding a two-fold volume of ice-cold PBS and the Caco-2 monolayers were washed with ice-cold PBS three times. The fluorescent signal of Caco-2 monolayers was then recorded using a microplate reader.

Fructose transporter GLUT2/GLUT5 study in Caco-2 cells

To study the effect of the PRSE on glucose and fructose transporter GLUT2/GLUT5, Caco-2 human intestinal cells were maintained in the same manner as described above, and monolayers of Caco-2 cells (100% confluence) obtained 9 days after the initial seeding are used. Caco-2 cell monolayers were first treated with six concentrations of PRSE or quercetin for 24 hours. Total RNA was then extracted from the treated Caco-2 cells using the MagMAX^{TM}-96 Total RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer's instruction. cDNA was synthesized from total RNA by using High Capacity RNA-to-cDNA Kits (Thermo Fisher Scientific) and a Veriti thermal cycler (Thermo Fisher Scientific). Real-time quantitative Polymerase chain reaction (PCR) was performed in an Applied Biosystems Real-Time Quantitative PCR system (Thermo Fisher Scientific) as described previously (13). Specifically, the primer pair used for GLUT2 was 5′-CAG GAC TAT ATT GTG GGC TAA-3′ (forward) and 5′-CTG ATG AAA AGT GCC AAG T-30 (reverse) and for GLUT5 was 5′-ACC GTG TCC ATG TTT CCA TT-3′ (forward) and 5′-ATT AAG ATC GCA GGC ACG AT-3′ (reverse). Human beta-actin was used as the housekeeping genes. Expression of GLUT2 and GLUT5 mRNA in Caco-2 cells that were treated with six different concentrations of PRSE or quercetin was determined by real-time quantitative PCR and compared with those of Caco-2 cells treated with vehicle.

Insulin secretion maintenance study with dysfunctional β-cells

Syrian hamster pancreatic beta-cells (HIT-T15) were obtained from American Type Culture Collection and cultured in HAMS F12K media with 1% (v/v) penicillin/streptomycin, 10% (w/v) horse serum (Sigma-Aldrich) and 2.5% (w/v) fetal bovine serum (Hyclone), and 5% CO_2/95% O_2 with 95% humidity to an approximate 70% confluency before use.

To study the regulative effect of the PRSE on insulin secretion in dysfunctional pancreatic β-cells (absence of normal insulin secretion caused by high glucose toxicity), the HIT-T15 cells were seeded onto 12-well plates and cultured in HAMS F12K media with 1% (v/v) penicillin/streptomycin, 10% (w/v) horse serum, and 2.5% (w/v) fetal bovine serum containing 16.7 mM glucose, along with different concentrations of PRSE or N-acetyl-l-cysteine (NAC) for 72 h.

Then, the cells were washed two times with KRBB (129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 0.1% BSA, 10 mM HEPES [pH 7.4] and 2.8 mM glucose) and starved for 2 hours in KRBB.

Following starvation, the cells were incubated in fresh KRBB containing 10 mM glucose for 2 hours to induce the release of insulin. The level of insulin secretion from HIT-T15 cells chronically cultured in media containing 16.7 mM glucose with various concentrations of PRSE or NAC was assayed by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions (Crystal Chem. Inc. Briefly, wells were coated with cell media and anti-insulin antibody for 18 hours, rinsed with washing buffer (PBS containing 0.04% (v/v) Tween-20, and incubated with secondary antibody enzyme for 3 hours, and incubated with secondary antibody enzyme conjugate for 3 hours. After thorough rinsing, wells were incubated with enzyme substrate solution and the reaction stopped 30 minutes later by the addition of stop solution. Absorbance (measuring wavelength, 450 nm; subtracting wavelength, 630 nm) was determined 10 minutes thereafter. Insulin concentration was obtained by comparing absorbance with an intra-plate insulin standard curve of concentrations.

Statistical analysis

A statistical analysis was performed for all the study results. First, a correlation analysis was carried out to determine whether there is a relationship between the two variable (x, y) pairs of the study results. Then, a statistical hypothesis testing was performed. Because of the small sample sizes, the Kruskal–Wallis test, a nonparametric test, was used. The Kruskal–Wallis test is the nonparametric alternative to a one-way analysis of variance and does not require normal distributions. The null hypothesis of this test is that all the medians are equal. The alternative hypothesis is that the medians are different. If the Kruskal–Wallis test is significant, it indicates that at least two concentrations have significantly different medians. The statistical analysis was performed using the SAS® software, version 9 (SAS Institute, Inc.).

Results

Polyphenol content and antioxidant capacity of PRSE

Discovery of rich phytochemicals in sugarcane juices, molasses, and other plant parts justified further exploration into this relatively inexpensive plant for therapeutic applications. Among the phytochemicals discovered in sugarcane juices and molasses, polyphenolic compounds are the promising components with high potential of physiological and medicinal benefits (3). The sugarcane extract used in this study is prepared via a patented technology where a hydrophobic extraction process is applied to sugarcane molasses to produce PRSE. The resulting extract is a dark brown free-flowing powder with a bitter-tannic taste. A total polyphenol analysis performed on the extract indicates that one gram of PRSE contains polyphenols that are equivalent to minimum 200 milligrams of gallic acid, i.e., 200 mg GAE per gram of PRSE. A full-spectrum chemical analysis of antioxidant capacity of PRSE against five physiologically relevant radicals, also known as ORAC 5.0 analysis, indicates that one gram of PRSE contains total antioxidant capacity equivalent to 19,457 µmole of trolox, a water-soluble vitamin E analog (Table 1).

Targeted polyphenols analysis with LC-MS also detected three major groups of polyphenols with significant amount of concentrations. Results indicate that the highest concentration of the three polyphenols was tricin, with a detected concentration of 27.40 µg/g, followed by luteolin, 5.30 µg/g, and apigenin, 1.89 µg/g.

As a step further into biological assessment of antioxidant capacity of PRSE in a physiological environment, we carried out a cellular antioxidant assay, an antioxidant analysis performed with human liver cells $HepG_2$. Results indicate that one gram of PRSE contains total antioxidant capacity that was equivalent to 229.12 µmole of quercetin, a known substance that exhibits effective antioxidant activity in a cellular system (17). The physical properties and study findings of polyphenol content and antioxidant capacity of PRSE are summarized in Table 1.

Glucose uptake study with Caco-2 cell monolayers

Glucose uptake study conducted with Caco-2 cell monolayers shows that the treatment of human intestinal Caco-2 cell monolayers with PRSE inhibited the uptake of glucose in a concentration-dependent manner. Figure 1A shows that under sodium-dependent conditions, PRSE caused a significant reduction in glucose uptake at a dose-dependent manner when compared with the control. IC50 values of PRSE on inhibition of glucose uptake under sodium-dependent conditions was 2.465 mg/mL (Figure 1B). Glucose uptake was also decreased with 100 µM phloridzin (78 ± 4%) and 100 µM myricetin (22 ± 4%) under sodium-dependent conditions respectively.

Under sodium-free conditions, PRSE caused a marked and significant reduction in glucose uptake in a dose-dependent manner when compared with the control (Figure 1C). IC50 values of PRSE on inhibition of glucose uptake under sodium-dependent conditions was 1.272 mg/mL (Figure 1D). Myricetin caused a 80% ± 3% reduction in sodium-free glucose uptake while phloridzin exerts no effect on glucose transport under sodium-free conditions.

Fructose uptake and transporter GLUT2/GLUT5 study in Caco-2 cell monolayers

Treatment of human intestinal Caco-2 cells with PRSE inhibited fructose uptake in a concentration-dependent manner, as shown in Figure 2A. IC50 value of PRSE on inhibition of fructose uptake was 4.468 mg/mL. As a comparison, quercetin, a plant polyphenol from the flavonoid group found in many fruits, vegetables, leaves, and grains, also showed inhibition of cellular fructose uptake in a concentration-dependent manner. IC50 value of quercetin on inhibition of fructose uptake was 3.604 µg/mL as shown in Figure 2B.

Treatment of human intestinal Caco-2 cells with PRSE inhibited GLUT2 expression in a concentration-dependent

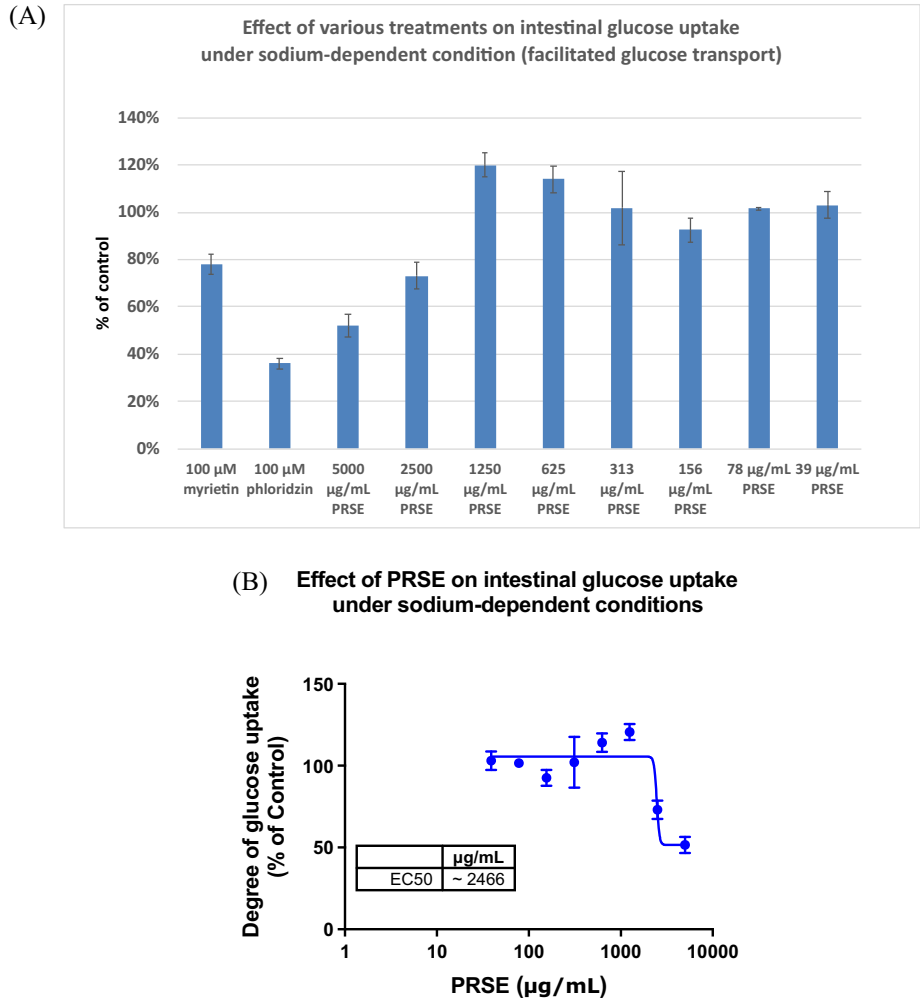

Figure 1. Effect of polyphenol-rich sugarcane extract (PRSE) and other treatments on intestinal glucose uptake as studied under a Caco-2 human intestinal cell model. 1A: Effect of various treatments on intestinal glucose uptake under sodium-dependent condition (facilitated glucose transport); 1B: Inhibition curve of PRSE on intestinal glucose uptake under sodium-dependent condition (facilitated glucose transport); 1C: Effect of various treatments on intestinal glucose uptake under sodium-free condition (active glucose transport); 1D: Inhibition curve of PRSE on intestinal glucose uptake under sodium-free condition (active glucose transport).

manner (Figure 3A), but stimulated GLUT5 expression (Figure 3B). IC50 values of PRSE on inhibition of GLUT2 expression was 3.396 mg/mL. Treatment of Caco-2 cells with quercetin inhibited both GLUT2 and GLUT5 expression in a concentration-dependent manner (Figure 3C, D). IC50 values of quercetin on inhibition of GLUT2 and GLUT 5 expression was 2.645 µg/mL and 1.768 µg/mL, respectively.

Insulin secretion maintenance study with dysfunctional β-cells

PRSE restored insulin production in dysfunctional β-cells that exhibited reduced insulin secretion function. As shown in Figure 4, insulin production in dysfunctional β-cells restored to 39.48% of its normal insulin production level after treating the dysfunctional β-cells with 0.5 mg/mL of PRSE. As a comparison, NAC, a known diabetic treatment agent, showed to restore insulin production up to 77.13% of its normal production level.

Statistical analysis

Correlation analysis

A significant positive or negative nonlinear relationship was found between the two variable (x,y) pairs for each of the studies conducted ($|r| \geqq 0.95$, $r^2 \geqq 0.90$). Higher concentrations show higher effect. All the correlations are significant at $\alpha = 0.05$.

Hypothesis testing

The Kruskal–Wallis test, a nonparametric hypothesis test, was performed following the correlation analysis. The Kruskal–Wallis test was significant at $\alpha = 0.05$ with $H = 10.05$ and p value less than 0.05. This indicates that the medians are different. For all the studies conducted in this work, there is sufficient evidence to support that at least two concentrations in each of the studies have significantly different medians. This suggests that the concentration is a significant factor in the signal changes observed (change in the inhibition %, level of uptake, etc.).

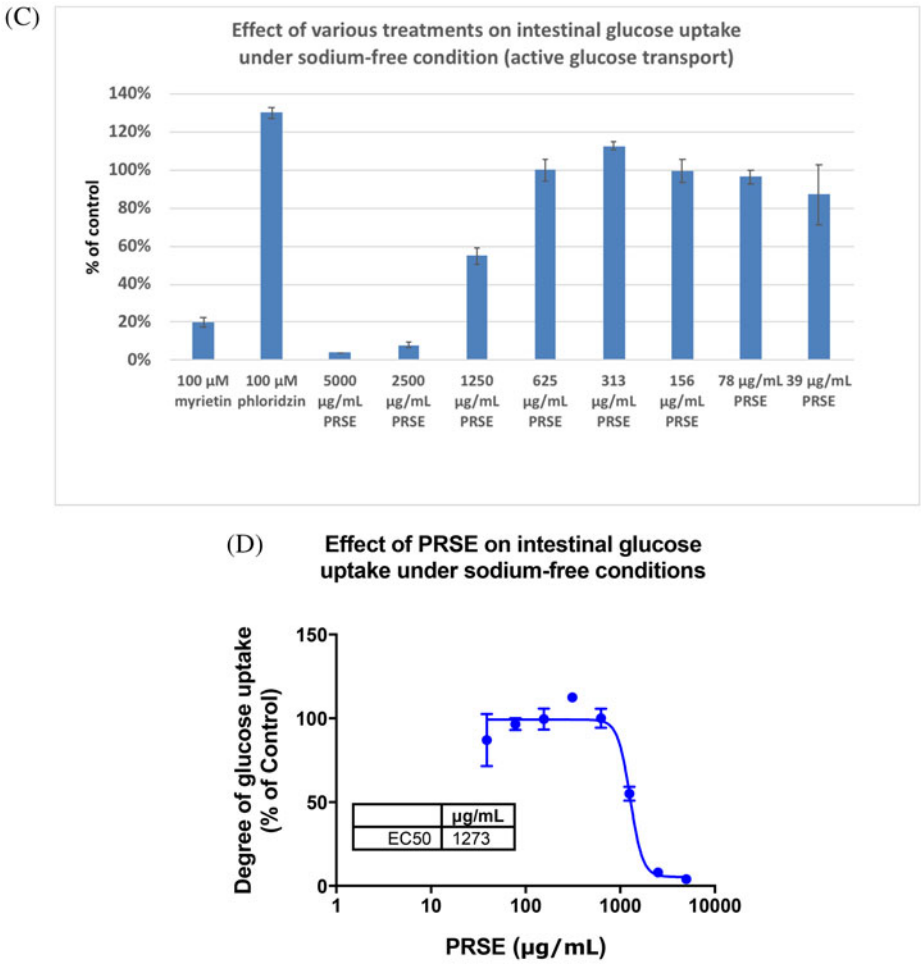

Figure 1. Continued.

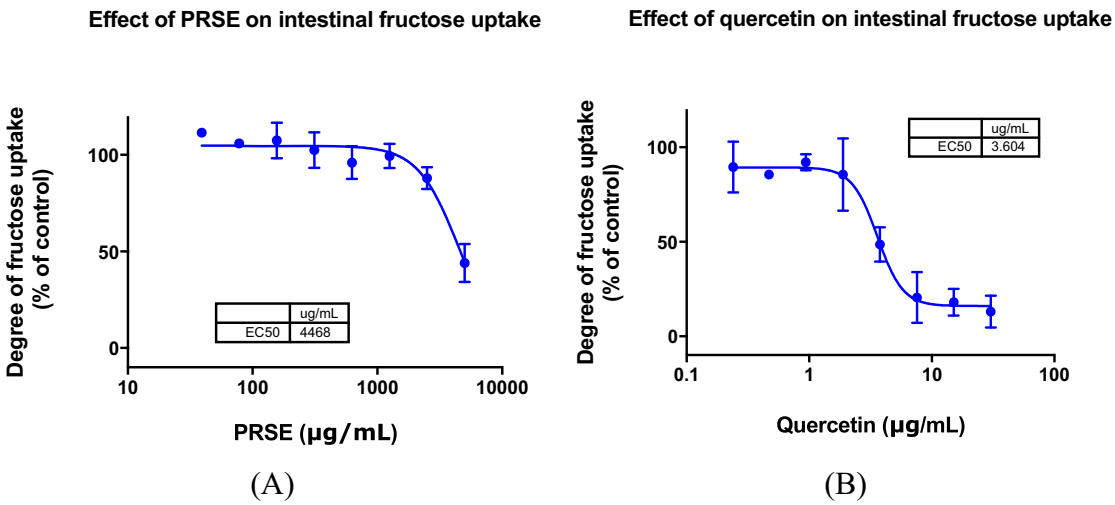

Figure 2. Effect of polyphenol-rich sugarcane extract (PRSE) and quercetin on intestinal fructose uptake as studied using a Caco-2 human intestinal cell model. 2A: Inhibition curve of PRSE on intestinal fructose uptake; 2B: Inhibition curve of quercetin on intestinal fructose uptake.

Discussion

Polyphenol content and antioxidant activity of PRSE

Discovery of phytochemicals in sugarcane plants has generated interests in exploring the use of the plant to help prevent diseases usually thought to be caused by sugar. The availability of these compounds in the waste products from sugar production provides an opportunity to extract the bioactives and the therapeutic material that would otherwise be discarded. In this study, we utilized a patented hydrophobic extraction process to obtain a PRSE. Preliminary characterization work indicates that PRSE contains 221 mg GAE/g of polyphenol, a high level of polyphenols comparing with other natural products known for their polyphenol contents.

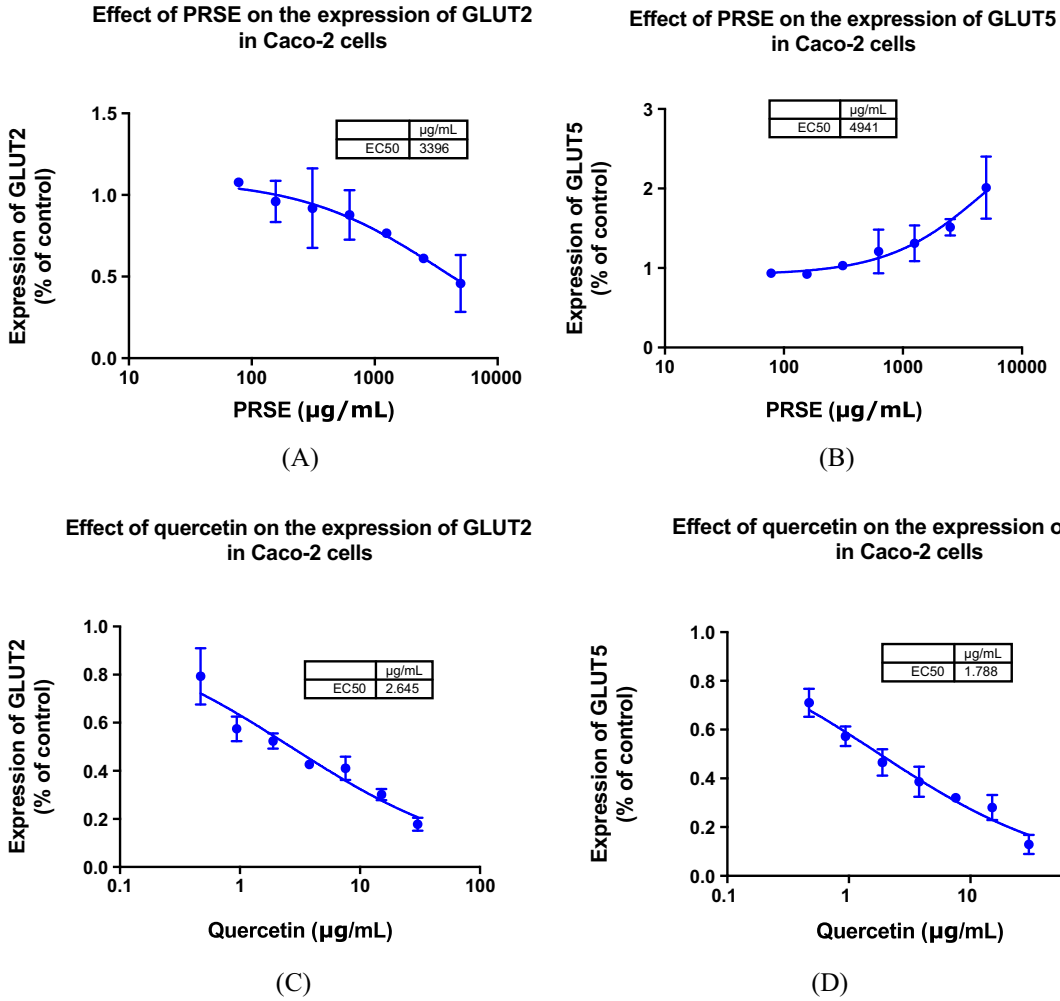

Figure 3. Effect of polyphenol-rich sugarcane extract (PRSE) and quercetin on intestinal GLUT2 and GLUT5 expression as studied using a Caco-2 human intestinal cell model. 3A: Concentration-dependent inhibitive effect of PRSE on intestinal GLUT2 expression; 3B: Enhancing effect of PRSE on intestinal GLUT5 expression; 3C: Concentration-dependent inhibitive effect of quercetin on intestinal GLUT2 expression; 3D: Concentration-dependent inhibitive effect of PRSE on intestinal GLUT5 expression.

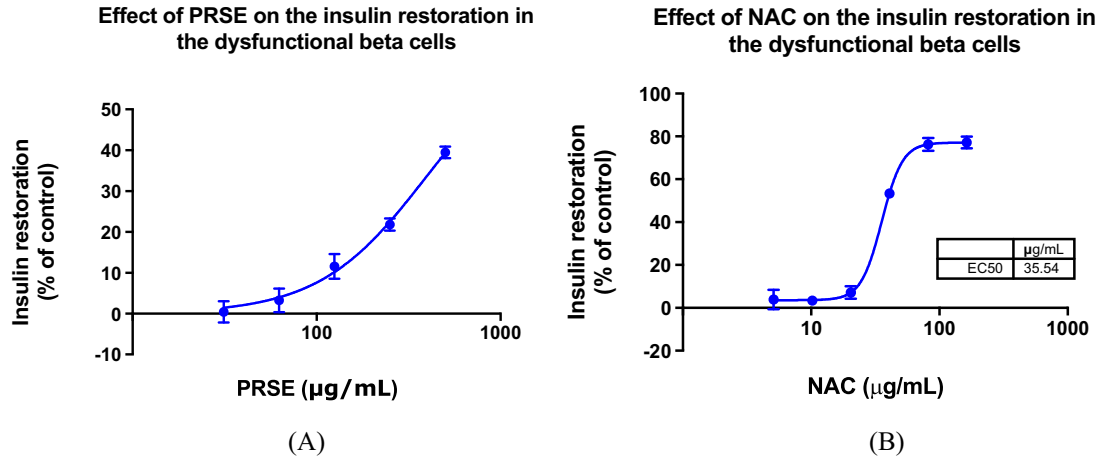

Figure 4. Effect of polyphenol-rich sugarcane extract (PRSE) on insulin production in insulin dysfunctional beta-cells. 4A: Concentration-dependent insulin restoration effect of PRSE on insulin production in insulin dysfunctional beta-cells; 4B: Concentration-dependent insulin restoration effect of NAC on insulin production in insulin dysfunctional beta-cells.

As shown in Table 2, the total phenol content in ground turmeric spice is 27.54 mg GAE/g, 36.63 mg GAE/g for dry cocoa powder, and 66 mg GAE/g for sumac bran. It is remarkable that a hydrophobic resin extraction process enables the production of a polyphenol-rich extract from sugarcane processing waste products that are largely not known as source of polyphenols.

The resulting high level of polyphenols in the extract supported the antioxidant capacity of PRSE, which exhibited a full-spectrum oxidant absorption capacity against five

Table 2. Polyphenol content and antioxidant activity of polyphenol-rich sugarcane extract (PRSE) vs. other natural plant dry matters*.

	Polyphenol (mg GAE/g)	ORAC 5.0 (μmole QE/g)	CAA (μmole QE/g)
Sugarcane extract PRSE	221	18,837	229
Spices, turmeric, ground	27.54**	n/a***	n/a***
Sumac, bran, raw	66.00**		
Cocoa, dry powder, unsweetened	36.63**	1,588	
Green Tea Extract	n/a***	34,819	
Blueberries, freeze dried		3141	222
Aloe, herb, dry powder		n/a***	233
Strawberries, freeze dried			65

*All data in Table 2 are obtained from Brunswick Laboratories, Inc., a U.S. independent bioanalytical testing laboratory, unless otherwise noted.
**Data obtained from USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2.
***n/a: Not available.

primary radicals (peroxyl radical, hydroxyl radical, superoxide anion, singlet oxygen, and peroxynitrite), with a total antioxidant capacity (ORAC 5.0) assessed at 18,837 μmole QE/g. As a comparison, the antioxidant ORAC 5.0 value is only 1,588 μmole QE/g for cocoa powder and 34,819 μmole QE/g for green tea extract. Furthermore, antioxidant assessment with human liver HepG2 cells (cellular antioxidant assay) (17) indicates that PRSE maintained high antioxidant capacity in the context of a living biological system. The cellular antioxidant assay value is 229 μmole QE/g for PRSE. The cellular antioxidant assay of PRSE is of similar scale with other known antioxidants (Table 2) such as blueberries (222 μmole QE/g for freeze-dried blueberries) and herbal powder aloe (233 μmole QE/g). As a comparison, the cellular antioxidant assay value for strawberries (freeze-dried), which is also known as an antioxidant, is 65 μmole QE/g, ∼ 30% of that of PRSE.

Inhibitive effect of PRSE on glucose uptake

As discussed above, previous studies suggest that oxidative stress plays a main role in the pathogenesis of type 2 diabetes mellitus, and antioxidant therapy has been used to reduce oxidative stress and alleviate diabetic complications (5). The rich content of polyphenols and antioxidants in PRSE lends the material therapeutic potentials for type 2 diabetes mellitus. We assessed this potential function of PRSE via Caco-2 cells, a human intestinal cell model widely used in pharmaceutical and nutraceutical industries for *in vitro* prediction of *in vivo* human intestinal permeability and uptake of an orally administered substance. Caco-2 cells have been employed to study the transport kinetics and metabolism of intestinal nutrients (20). In particular, Caco-2 cells are an established cellular model mimicking the human intestinal capacity to absorb sugar (21) and the expression of GLUTs (22). Here, we use a Caco-2 cell model to assess whether PRSE interferes with the intestinal uptake of glucose and, if so, via which transport mechanism(s).

We investigated the effects of PRSE on glucose control in Caco-2 cells under sodium-dependent conditions (favoring facilitated uptake via SGLT1 transporter) and sodium-free conditions (favoring active uptake by GLUT transporters). Results shown in Figure 1 indicate that under sodium-dependent conditions, PRSE induced inhibition of facilitated glucose uptake at IC_{50} 2.465 mg/mL. In comparison, facilitated glucose uptake (sodium-dependent) was inhibited by 78% ± 4% with 100 μM (0.044 mg/mL) phloridzin (an SGLT1 inhibitor) (23) and only by 22% ± 4% with 100 μM (0.32 mg/mL) of myricetin (a GLUT2 inhibitor) (12).

Under sodium-free conditions, PRSE induced significant inhibition of active glucose transport in a dose-dependent manner (IC_{50} 1.272 mg/mL). In comparison, myricetin (a GLUT2 inhibitor) caused an 80% ± 3% reduction in active glucose uptake while phloridzin, as expected for a SGLT1 inhibitor, exerts little inhibitive effect on glucose transport under sodium-free conditions. The results suggest that PRSE inhibits intestinal glucose uptake via interfering with both facilitated and active glucose transport pathways, with inhibiting active glucose uptake playing a larger role.

One consideration in comparing the glucose uptake inhibition effect of above substances is that PRSE is a mixture material, unlike its comparison compounds that are of pure form (>90% purity). Besides potential bioactives such as polyphenols, PRSE also has various other components including minerals, metals, and vitamins that are not known to possess the inhibitive effect being studied. Presumably, the pure compounds by nature are more concentrated and therefore more potent. This consideration is also applicable when evaluating the rest of the study findings of this work.

Effect of PRSE on fructose uptake and fructose transporters

Overall, fructose uptake study results shown in Figure 2 indicate that PRSE induced moderate inhibition of fructose uptake (IC_{50} of 4.468 mg/mL). Fructose uptake mainly takes place through GLUT2 and GLUT5, two facilitative glucose transporters. Previous studies indicated that GLUT5 is responsible for most of luminal fructose uptake, while GLUT2 is particularly important postprandially when a high amount of fructose is ingested (7). The two facilitative fructose transporters, GLUT2 and GLUT5, have been studied actively as therapeutic targets for polyphenols (24). To explore the pathway(s) where PRSE interferes with fructose uptake, we further studied the impact of PRSE on the expression of GLUT2 and GLUT5. Results indicate that PRSE inhibited cellular production of GLUT2, a major fructose transporter, while at the same time, increased the expression of GLUT5, another transporter that is unique to fructose transport and uptake. In the meantime, treatment of Caco-2 cells with quercetin, a plant polyphenol, inhibited

both GLUT2 and GLUT5 expression in a concentration-dependent manner.

The inhibition effects of GLUT2 by PRSE and quercetin we observed are consistent with reports in a recent paper by Andrade et al. that studied the inhibition of pure polyphenols on ^{14}C-labeled fructose in Caco-2 cells (21). Of the range of polyphenols tested, apigenin was found to be one of the strongest inhibitors that induced acute and chronic inhibition of both GLUT2 and GLUT5. Apigenin was detected as a major polyphenol constituent of PRSE and this therefore provides one of the likely mechanisms for the results observed in this study.

Our study shows that PRSE enhanced GLUT5 in Caco-2 cells, which did not disagree with the inhibition of GLUT5 by quercetin that we observed. This GLUT5 activation effect by PRSE was also not consistent with the findings of Andrade et al., which reported that a range of polyphenols inhibited GLUT5 in Caco-2 cells. It is important to acknowledge that PRSE is a complex mixture of compounds including polyphenols, flavonoids, and residual sugar, while quercetin studied in this work and other polyphenols studied by Andrade et al. are pure, isolated compounds. Even though our study and previous studies show that isolated polyphenols induced GLUT5 inhibition, the opposite effects induced by other components in PRSE, for instance, residual glucose and fructose, might have contributed to the overall enhancement of GLUT5 by PRSE. Glucose and fructose have been reported to be potent GLUT5 promoters that enhance the expression of GLUT5 in Caco-2 cells (21,25). This enhancement effect by residual fructose and glucose could overwhelm the inhibition effect by polyphenols in PRSE, resulting an overall GLUT5 enhancing effect by PRSE.

Of interest, although PRSE enhanced the expression of GLUT5, a main fructose transporter, PRSE showed inhibition capability in the overall fructose uptake in Caco-2 cells. This suggests that the inhibition effect of PRSE on GLUT2 overcomes its enhancement effect on GLUT5, resulting an overall inhibition effect on intestinal fructose uptake. Equally important to note is that the GLUT5 activation effect by fructose and glucose observed in cellular studies was not observed in *in vivo* studies (25,26). To understand the function pathways of PRSE further, we are currently designing *in vivo* trials to verify the effect of PRSE on GLUT2, GlUT5, and overall sugar uptake.

PRSE restores insulin level in dysfunctional β-cells

Numerous studies have shown that chronic exposure of pancreatic β-cells (human or rat) to high concentrations of glucose causes cells to be insensitive to glucose and impairs the production of insulin (27). This supports the suggestion that chronic hyperglycemia in type 2 diabetes is detrimental to β-cell function, which causes reduced glucose-stimulated insulin secretion and disproportionately elevated proinsulin. Restoration of insulin production in glucotoxic pancreatic β-cells could help improving glucose regulation, relieving diabetic conditions. In our study, we implemented a glucotoxic-induced insulin dysfunctional rat pancreatic β-cell model to investigate whether PRSE is able to restore insulin production in these insulin dysfunctional β-cells. Results shown in Figure 4 indicate that treatment of PRSE is able to restore insulin production in insulin dysfunctional rat pancreatic β-cells in a concentration-dependent manner. Treating insulin dysfunctional rat pancreatic β-cells with 0.5 mg/mL PRSE (a mixture material) restored the insulin production by 39.48%, equivalent to treating the insulin dysfunctional rat pancreatic β-cells with ∼31 μg/mL (190 μM) of NAC (a pure compound), an amino acid supplement that has been found effective in diabetic treatment (28).

This finding is particularly interesting considering that the restoration of insulin production in dysfunctional beta-cells is a major approach for type 2 diabetic treatment. Current treatments for beta cell dysfunction target enhancement of beta cell structure and/or function or stimulate beta cell proliferation. Most of these treatments are protein-based (29). Previous studies have shown that antioxidants may protect pancreatic beta-cells against glucose toxicity. However, restoration of insulin production in dysfunctional beta-cells by plant extracts have not been reported.

Conclusion

Produced from a patented hydrophobic extraction process, PRSE contains rich polyphenol content and exhibits full-spectrum antioxidant activities. Via Caco-2 cells, a well-established human intestinal cell model for uptake and metabolism study, and insulin dysfunctional β-cells, we explored the effect of PRSE on intestinal glucose and fructose uptake and on insulin secretion in dysfunctional β-cells. PRSE induced significant inhibition of passive glucose uptake (sodium-independent) and moderate inhibition of active transport of glucose (sodium-dependent) uptake. PRSE also exhibited restoration capability of insulin production in insulin dysfunctional β-cells.

In the meantime, PRSE induced moderate inhibition of fructose uptake. The results also suggest that PRSE may inhibit fructose uptake via inhibition of GLUT2, a fructose transporter as well as a glucose transporter.

As mentioned, three polyphenols, apigenin, luteolin, and tricin, identified in the PRSE have been reported to modulate carbohydrate metabolism, increase insulin sensitivity, and reduce obesity in previous studies (12–14). These polyphenols may have contributed to the insulin restoration and other metabolic syndrome modulation effect observed in PRSE. Regardless, this study focuses on the overall health effect of PRSE, a sugarcane extract obtained from sugarcane processing waste products.

The positive findings of the rich polyphenol content of PRSE and its inhibitive effect of glucose and fructose uptake, transporter expression, and insulin restoration ability in cell cultures indicate that PRSE may modulate cellular mechanism in a manner that is beneficial to health. However, further studies using animal models and ultimately in a clinical setting are needed to confirm these therapeutic effects in humans. It is encouraging that a clinical trial using a similar,

but less concentrated extract demonstrated that relatively low dosages of sugarcane extract were able to reduce the glycemic index of foods (30). In our laboratories, a number of follow-up confirmation studies are also under way, including *in vivo* studies on the effect of PRSE on sugar uptake, diabetic conditions, and hyperlipidemia. The success of such studies may result in a new nutritional supplement or therapeutic for diabetes management based on a globally abundant crop.

Ethics statement

This work did not include any human subjects or animal experiments.

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