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Effect of *Cinnamomum Cassia* Stem-Bark Extracts on the Synthesis and Secretion of Insulin in RIN-m5F Cells

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Abstract: *Cinnamomum cassia* (*C. cassia*) crude stem-bark extracts possess some hypoglycemic properties through inhibiting the uptake of glucose by the intestines and/or enhancing insulin sensitivity. To further investigate the mechanism through which the hypoglycemic effect is exerted, this research explored the influence of the extracts of the crude stem-bark of *C. cassia*, with various polarities, on particular aspects of glucose consumption as well as glucose-stimulated insulin synthesis and secretion by RIN-m5F cells. All *C. cassia* extracts did not enhance glucose consumption when compared to the untreated cells ($p<0.001$). However, lower concentrations of the extracts significantly increased mRNA levels of Glucose Transporter 2 (GLUT2) and glucokinase genes. Furthermore, all *C. cassia* extracts significantly stimulated/increased total insulin synthesis (intracellular and secreted) when compared with the control cells, though the effect was not dose-dependent except for water extracts at higher concentration ($p<0.001$). The results of this investigation revealed that generally, the extracts upregulated the expression of Musculoaponeurotic fibrosarcoma homolog A (MafA), the transcription factor involved in insulin gene expression, while that of the Pancreatic Duodenal Homeobox-1 (PDX-1) gene was mostly suppressed. In concluding, the results of this study proposes that *C. cassia* stem-bark extracts may exert hypoglycemic effects through stimulating insulin synthesis and secretion in the RIN-m5F pancreatic beta cells.

Keywords: Diabetes, Pancreatic Beta Cells, *Cinnamomum cassia*, Glucose Uptake, Synthesis and Secretion of Insulin, MafA and PDX-1

Introduction

Diabetes Mellitus (DM) is a sustained condition arising from metabolism. If treatment and the management of this illness is not done appropriately, this may lead to vascular complications that are life-threatening (Klonoff and Schwartz, 2000; Rios *et al*., 2015). The characteristic feature of this illness is a prolonged hyperglycemia, which results from defects in the synthesis and/or release of insulin (Fu *et al*., 2013; Weiss *et al*., 2000). The incidence/occurrence of this disease is gradually increasing and it is projected that 3×10^6 individuals worldwide will be affected by the year 2025 (Rios *et al*., 2015). Medications that are presently in use for the management of DM are not easy to access, have properties that are undesirable or are not affordable (Chaudhury *et al*., 2017). Based on the problems stated above, global research is underway with the purpose of discovering economical, nontoxic, novel hypoglycemic agents from medicinal plants (Katerere and Eloff, 2005; Oh, 2005; Ullah *et al*., 2018).

Cinnamomum cassia, common name Cinnamon, family Lauraceae, is a readily available and inexpensive herb commonly used as a seasoning and flavouring agent for food. This plant has been used historically as herbal medicine for the treatment of diabetes mellitus (Khan *et al*., 2003; Stoecker *et al*., 2010). Ethnobotanical studies claim that cinnamon, in particular, *C. cassia*, has blood glucose lowering effects and this has been explored in animal models of diabetes and in randomized controlled medical trials (Khan *et al*., 2003; Mang *et al*., 2006; Qin, *et al*., 2003; Solomon and Blannin, 2007; Stoecker *et al*., 2010). Some studies have so far successfully demonstrated the capacity of *C. cassia* whole herb powder (1-6 g/day) or

water-soluble extracts (200-500 mg/day) to lower blood glucose, glycosylated haemoglobin A1c and cholesterol levels without any adverse effects in diabetic patients (Dugoua *et al.*, 2007; Khan *et al.*, 2003; Mang *et al.*, 2006; Qin, *et al.*, 2003; Solomon and Blannin, 2007; Stoecker *et al.*, 2010). However, higher doses and long-term use of more than 6 weeks of *C. cassia* products were reported to have toxic effects in hepatic cells of animal simulations and persons due to their elevated coumarin content levels (Iwata *et al*., 2016). The manner in which glucose is lowered *C. cassia* extracts may be due to the existence of a polyphenolic compound known as Methyl Hydroxy Chalcone Polymer (MHCP) (Jarvill-Taylor *et al.*, 2001). MHCP is reported to have insulin-like effects in insulin responsive tissues (Dugoua *et al.*, 2007, Jarvill-Taylor *et al*., 2001). Furthermore, an investigation by Soliman *et al*. (2013) found that H_2O extracts of cinnamon had the capacity to increase/decrease mRNA levels of genes linked with the metabolism of carbohydrates and lipids in rats which were induced with STZ to develop diabetes. With reference to the studies cited above, only *C. cassia* extracts with polar properties were examined for their ability to stimulate insulin secretion. The mechanism in through which *C. cassia* extracts enhance the secretion of insulin in non-insulin dependent secreting cells is not well understood. Therefore, there is need to further examine the effect of *C. cassia* polar and non-polar extracts on glucose stimulated insulin production and secretion.

Hypoglycemic medications stimulating the secretion of insulin from the non-insulin dependent pancreatic cells can improve several steps required in the insulin production and release. These include the activity and the expression of glucokinase and GLUT2, the glucose absorption by cells and the transcriptional and translational activation of the pre-proinsulin (Balekari and Veeresham, 2013). In addition, these agents may enhance the expression levels of the pre-proinsulin gene transcription factors such as MafA and the PDX-1 (Balekari and Veeresham, 2013; Komatsu *et al*., 2013; Subash-Babu *et al*., 2015).

Currently, the mechanism through which stem-bark extracts of *C. cassia* improve/enhance the production and the release of insulin is not well understood or minute or no evidence is available to account for. The purpose of this investigation was to explore the effect of different *C. cassia* crude stem-bark extracts (water, ethanol, ethyl acetate and hexane) on preferred phases of pathways involving the production and release of insulin in non-insulin dependent RIN-m5F beta cells. These aspects comprise of cytotoxicity, glucose consumption, the expression of MafA, PDX-1 and pre-proinsulin gene, glucose sensor genes such as glucokinase and GLUT2 and the quantification of the insulin produced through stimulation of RIN-m5F cells incubated in specified glucose quantities. To our understanding, this is the first study to report on the hypoglycemic effects of *C. cassia* stem-bark extracts on the phases of the production and the release of insulin in non-insulin RIN-m5F cells. Data of this study is important and beneficial when extracts are used along with conventional medication to avoid drug-herb interaction in particular when managing and treating patients with diabetes mellitus.

Materials and Methods

Research Design

The illustration of the investigation approach in the present research is exhibited in the Fig. 1 and the key abbreviations with full terminologies utilized in the research paper is accessible in Table 4.

Stem-Bark Collection, Preservation and Extraction

The *C. cassia* stem-bark (Osmans-Taj Mahal) was purchased from a local store (Shoprite-Checkers, Wonder park Mall, Pretoria North, SA, April 2014) and was exhaustively cleaned in cold water and allowed to dry under dark at room temperature, crushed into acceptable residue by means of Polymix DX-MFC 90D (Kinematica, Italy) and then kept at room temperature in closed brown bottles till required for extraction. The powdered plant material of fifty-thousand micrograms was sequentially extracted with 150 mL of water, ethanol, ethyl acetate and hexane, following method reported by Jeyaseelan *et al*. (2012), with improvements. The ethanol, ethyl acetate and hexane quantities were concentrated at 37°C under *vacuo* by means of Büchi rota vaporator (Lasec, SA), while the water fraction was concentrated by means of the Vacutec freeze-dryer (Lasec, SA). A stock solution of 20 mg/mL of each of the extracts was prepared by dissolving each crude extract in 100% dimethyl sulfoxide (DMSO).

Maintenance of the Liver and Pancreatic Cell Cultures

The hepatic H-4-II-E cells (ATCC® CRL1548™) were incubated in a moistened atmosphere of 5% CO_2 at 37°C in a complete pre-warmed medium (RPMI-1640) improved with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) antibiotic mixture (10 000 units/mL penicillin and 0.1 g/L streptomycin). The pancreatic RIN-m5F insulinoma cells (ATCC® CRL-11065™) were grown in a moistened atmosphere of 5% at 37°C in the same pre-warmed medium complemented with 10% FBS, 1 mm sodium pyruvate and 10 mM HEPES buffer. Cells were tested when they reached eighty-ninety % confluence.

Cytotoxic Effect of C. cassia Extracts Against Liver Cells

Cytotoxic effects of *C. cassia* extracts against H-4-II-E cells was examined by means of 3-(4,5-

dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, following method of Mosmann (1983), with modification. Briefly, hepatic cells grown as previously described, were cultured at 1 x 10^5 cells/well in 96-well plates (Greiner, Lasec, SA) and incubated in a moistened atmosphere of 5% CO_2 at 37°C for 24 h. Thereafter, 100 µL of each extract (3.125-200 µg/mL) was supplemented to the cells and then incubated for 72 h under the same conditions. Dimethyl sulfoxide (1% v/v) and H_2O_2 (1% v/v) were used as negative and positive controls, respectively. Afterwards, 20 µL MTT solution (500 µg/mL) was supplemented to individual wells after the removal of the medium and plates were then incubated at 37°C for 1 h. Formazan crystals produced in each well were then solubilized in $1/10^{th}$ (v/v) of 100% pre-warmed DMSO and the plates were then incubated for 4 h in the shade. The absorbance in each plate was measured at 570 nm using Multiskan Accent ELISA reader (Thermo Fisher Scientific, SA). The survival rate of cells in each plate, measured in percentages (n = 9), was calculated by means of the formula:

$$\%\ Cell\ death\ = 1-\frac{\left(A_{treated\ cells}-A_{blank}\right)}{\left(A_{untreated\ control\ cells}-A_{blank}\right)}\times 100$$

The concentration of plant extracts versus the percentage cell death was used to calculate the LC_{50}, which is the lowest extract concentration causing 50% death of cell population (Sagbo *et al*., 2018).

Glucose Consumption by Pancreatic Cells

The ability of cultured RIN-m5F cells to consume glucose was assessed based on a detailed technique described in Kgopa *et al*. (2020). Concisely, 2 x $l0^5$ cells/well cultured in a pre-warmed media prepared previously for RIN-m5F cells in 96-well plate for 72 h at 37°C in 5% CO_2, were treated with *C. cassia* extracts ranging between 0.16 and 5 µg/mL and 15 mmol/L glucose. Thereafter, glucose absorption by the cells was determined by means of a glucose-oxidase kit (KAT Medicals laboratory, SA) and the absorbance was measured at 540 nm using a Multiskan Accent plate reader (Thermo Fisher Scientific, SA). All the tests were executed in triplicates (n = 9).

Insulin Production and Secretion

Effect of *C. cassia* extracts on the production and the release of insulin by cultured RIN-m5F cells was determined following method described by Kgopa *et al.* (2020). Briefly, $2.5 \times l0^5$ cells/well cultured in pre-warmed RPMI-1640 in 24 well culture plates were grown for 48 h at 37°C in a humidified atmosphere of 5% CO_2.

The cells were incubated with *C. cassia* stem-bark extracts (1.25, 2.5 and 5 µg/mL) or 0.1 µm glibenclamide (positive control) in the occurrence of 15 mmol/L glucose and then grown at 37°C in a 5% CO_2 incubator for 30 min. Insulin content was determined from cells lysed with RIPA lysis buffer and the released insulin was measured from the aliquots of the medium. Analysis of the glucose stimulated insulin synthesis was carried out following the modified method described by Zhang *et al*. (2014) by ELISA (Sigma-Aldrich, SA), subsequent to the manufactures protocol. Concentrations of both the insulin content and secreted insulin were standardized to overall protein content which was determined by means of Lowry protein assay kit (Thermo Fisher Scientific, SA).

Reverse Transcription-PCR

To determine the influence of *C. cassia* stem-bark extracts on pre-proinsulin gene mRNA levels, reverse transcriptase-PCR (RT-PCR) was performed following a procedure detailed by Kgopa *et al*. (2020), with improvements. In a nutshell, pancreatic cells ($3 \times l0^5$ cells/mL) in 6 well plates were cultured in complete media as described previously, then treated for 16 h with varied concentrations of *C. cassia* extracts (1.25, 2.5 and 5 µg/mL) in the presence of 15 mm glucose). DMSO-treated and untreated cells were used as vehicle and negative controls, respectively before RNA isolation.

Gene Jet RNA Purification Kit (Thermo Fisher Scientific, SA) was used for the extraction of the total RNA from both the extract and DMSO treated cells as well as the untreated control cells, according to the manufacturer's instructions. cDNA Revert Aid M-MulVRT kit (Life Technologies, SA) with oligo (dT)12 primers was used for the synthesis of cDNA (0.1 µg) from the RNA template in accordance with the manufacture's protocol. Pre-proinsulin gene, with primers (0.4 µm): forward;5´-TGCCCAGGCTTTTGTCAAAC-3´, reverse; 5´-CTCCAGTGCCAAGGTCTGAA-3´, was amplified by means of KAPA Taq Ready Mix PCR kit (Lasec, SA) using My Cycler (Bio-Rad, SA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primers: forward; 5´-ACTTTGGCATTGTGGAAGG-3´, reverse; 5´-ACACATTGGGGGTAGGAACA-3´) utilized as the housekeeping gene. Amplification parameters of these genes for each of the 32 cycles were: Initial denaturation for 4 min at 95°C, final denaturation for 30 sec at 95°C, annealing for 30 sec at 59°C and extension for 2 min at 72°C and holding time for 4 min at 72°C. All amplicons were visualized on a 1.25% agarose gel and mRNA levels detection and quantification was achieved by Chemiluminescence Imaging System and software version 5.2.1 Program (Image Lab), all from Bio-Rad (SA), respectively.

Table 1: Gene expression primers

	Primer sequences	
	Forward primer	Reverse primer
GAPDH	5´-ACCACACAGTCCATGCCATCAC-3´	5´-TCCACCACCCTGTTGCTGA-3´
Rat glucokinase	5´-TGACAGAGCCAGGATGGAG-3´	5´-TCTTCACGCTCCACTGCC-3´
MafA	5´-AGGCCTTCCGGGGTCAGAG-3´	5´-TGGAGCTGGCACTTCTCGCT-3´
Rat GLUT2	5´-TGGGTTCCTTCCAGTTCG-3´	5´-AGGCGTCTGGTGTCGTATG-3´
PDX-1	5´-AACCCGAGGAAAACAAGAGG-3´	5´-GTTCAACATCACTGCCAGCTC-3´

mRNA Levels Quantification by RT-Real-Time PCR

Amplification of PDX-1, glucokinase, GLUT2 and MafA gene was achieved by RT-qPCR upon treatments of the RIN-m5F cells cultured as described above was incubated with/without 2.5, 5 µg/mL *C. cassia* stem-bark extracts, 15 mmol/L glucose. The RNA extracted in the previous section was also used for this part of the study. Real-Time PCR was performed by means of 2X Sensi FAST SYBR Hi-ROX PCR Mix (Bioline Celtic Diagnostics, SA) based on the producer's procedure using Step One Plus™ Real-Time PCR Detection System (AB Applied Biosystems, Bio-Rad, SA) was used for the amplification of these particular genes. A list with each pair of primer for each gene is presented in Table 1 both showing forward and reverse sequences. mRNA levels of each were normalized to the expression levels of the housekeeping gene by means of a software v2.1 of the same PCR system from Bio-Rad (SA) (AB Applied Biosystems).

Interpretation of Data

The results were presented as mean ± Standard Error of the Mean (SEM) in histograms. The results of treated and untreated controls considered significant when *(p<0.05) and **(p<0.001), assuming equal variance using Student's *t*-test for statistical analysis. Data from this research study was analyzed using Excel software (Microsoft 16).

Results

Yield Percentage of Plant Extracts and the Cell Viability Assay

The percentage yield of plant extract obtained from serial exhaustive extraction and the LC_{50} of the various *C. cassia* extracts attained by the MTT assay (Table 2) is presented. The highest yield in percentage was obtained with ethanol extract while the minimum percentage yield was obtained with the water extraction. The cell viability assay demonstrated that the *C. cassia* hexane extract was the most toxic (LC_{50} = 5.12±0.06 µg/mL), while the ethanol extract was the least toxic (LC_{50} = 247.6±10.4 µg/mL). The 50% lethal concentration presented in Table 2 was used as a guide on the concentration of *C. cassia* extracts to be used in all the assays performed in this study. Based on the table, 5 µg/mL of *C. cassia* extracts was used as the maximum concentrations in all experiments conducted. Therefore, any concentration above 5 µg/mL *C. cassia* extracts was considered toxic.

Effect of C. cassia Stem-Bark Extracts on the Uptake of Glucose

The effect of four *C. cassia* extracts on glucose consumption by RIN-m5F, cells that do not depend on insulin, was explored and the outcomes of this objective are presented in Fig. 2. All *C. cassia* stem-bark extracts at all concentrations tested, did not enhance glucose consumption when compared with the control cells (p<0.001).

Effect of Extracts on the Insulin Content and Secretion by RIN-m5F Cells

The influence of *Cinnamomum cassia* extracts on the insulin content and insulin release by RIN-m5F cells was examined and the results are graphically displayed in Fig. 3. All *C. cassia* extracts under investigation (except the hexane extract at 5 µg/mL) significantly improved the release of insulin in the presence of glucose against cells that were not treated, although the effect was not concentration dependent for most of the extracts (Fig. 3A) (p<0.001). At a dose of 1.25 µg/mL, the ethyl acetate and hexane extract enhanced insulin secretion by 82.7% and 84.4%, respectively compared to 0.1 µm glibenclamide (80.5%), while the ethanol extract enhanced insulin secretion by 79.7%. However, an increase in the dose of 2.5 µg/mL and 5 µg/mL of hexane and ethanol extracts, resulted in low insulin secretion by these cells when compared with the glibenclamide at 0.1 µm (p<0.001). The water extracts at 1.25 and 2.5 µg/mL, enhanced insulin secretion in a manner that is dependent on the extract dose, although the increase was significantly lower than that observed for 0.1 µm glibenclamide (p<0.001).

The intracellular insulin content following treatment of the cells with the extracts ranged from 0.28 µIU/µg protein for the hexane extract to 1.27 µIU/µg protein for the water extract and was therefore lower than the observed insulin secreted for most extracts (Fig. 3A and Fig. 3B). The insulin content of cells treated the water extracts was significantly more than that of the control cells and cells treated with and 0.1 µm glibenclamide (p<0.001) (Fig. 3B). Fig. 3C shows the extracts influence on the total insulin (intracellular and secreted) synthesized

by the cells. Generally, most cinnamon extracts, with the exception of the hexane extracts at 2.5 and 5 μg/mL, showed an substantial increase in total insulin synthesis and secretion when related with the non-treated control cells, although the effect was not dose-dependent except for the water extracts (p<0.001) (Fig. 3C).

The Glucose Sensor mRNA Levels

The effect of four stem-bark extracts of *C. cassia* on the mRNA levels glucokinase and GLUT2 in RIN-m5F cells were examined and the results are graphically demonstrated in Fig. 4A and 4B, respectively while the percent expression levels are presented in Table 3.

A general and a considerable increase in GLUT2 mRNA levels was detected at 2.5 μg/mL of the extracts (p<0.001) (Fig. 4A), except for the ethyl acetate extract which demonstrated an opposite pattern of gene expression. Surprisingly, all *C. cassia* stem-bark extracts, at 5 μg/mL, appeared to significantly increase the GLUT2 mRNA levels, even though the degree of the upregulation decreased with increasing polarity of the extraction solvent (p<0.001). All *C. cassia* stem-bark extracts under investigation increased glucokinase gene mRNA levels when related with the untreated cells (p<0.001) (Fig. 4B). However, this up-regulation was more pronounced at 2.5 μg/mL than at 5 μg/mL of the extracts.

Effect of C. cassia Extracts on MafA and PDX-1 mRNA Levels

In the present investigation, the influence of *C. cassia* extracts on the mRNA levels of MafA and PDX-1 in RIN-5F cells was examined and the results of this study is presented in Fig. 4C and 4D, respectively, while the percent gene expression levels are presented in Table 3.

The findings revealed that the *C. cassia* hexane extracts stimulated a significant amount of mRNA levels of PDX-1 gene by 463.8-fold increase at 2.5 μg/mL relative to the observed expression levels in the negative control cells (p<0.001) (Fig. 4C). All other extracts at both 2.5 and 5 μg/mL except for the hexane extract at 5 μg/mL demostarted to suppress the PDX-1 expression levels. In terms of the influence of the extracts on MafA mRNA levels, ethyl acetate (5 μg/mL) and the hexane extracts (2.5 and 5 μg/mL), demonstrated to increase PDX-1 mRNA levels in a manner that depend on the concentration of the extracts (Fig. 4D). The ethanol extract enhanced the expression of MafA gene only at 2.5 μg/mL concentration while the water extracts down-regulated MafA mRNA levels at all the concentrations tested.

Effect of Extracts of C. cassia on Pre-Proinsulin mRNA Levels

This study investigated the effect of *C. cassia* extraxts on the mRNA levels of the pre-proinsulin gene in the presence of glucose and the results expressed in fold-increase are presented graphically in Fig. 5. For the hexane and water extracts, only the 1.25 and 2.5 μg/mL concentrations led to an increase in pre-proinsulin gene mRNA levels in a manner depending on extract dose (p<0.001). Although ttreatments with water extracts demonstrated opposite patterns, an appreciable expression levels of 0.65-, 1.05- and 0.28-fold increase in 1.25, 2.5 and 5 μg/mL was exhibited when compared to those in cells not treated, respectively. The pre-proinsulin mRNA levels increased for the ethyl acetate and ethanol treatments but with different pattents. Furthermore, treatments using ethanol extracts exhibited a downward pattern where the mRNA levels decreases with increase in treatments concentrations. In general or overall, with the exemption of the hexane extract at 5 μg/mL (Fig. 5A) and the ethyl acetate extract at 2.5 μg/mL (Fig. 5B), all other *C. cassia* stem-bark extracts explored in the current study substantially increased mRNAlevels of the pre-proinsulin gene when compared with cells not treated or DMSO vehicle control (p<0.001).

Table 2: Cytotoxic effects of four *C. cassia* stem-bark extracts on H-4-II-E cells as measured by the MTT assay

Extract	Yield (%)	LC_{50} (μg/mL)
Hexane	1.62	5.12±0.060
Ethyl acetate	2.04	25.4±0.0.74
Ethanol	2.84	247.6±10.400
Water	1.48	20.4±0.3100

LC_{50} = Extracts dose causing 50% cell death presented as Mean ± SEM (n = 9).

Table 3: Percentage mRNA levels of selected genes

	% GLUT2		% Glucokinase		% PDX-1		% MafA	
Extract	25 (μg/ml)	50	25 (μg/ml)	50	25 (μg/ml)	50	25 (μg/ml)	50
Hexane	19.9	87.9	98.9	n.d	99.8	<0.0	99.9	24.6
Ethyl acetate	15.1	90.9	98.6	<0.0	<0.0	<0.0	22.2	47.2
Ethanol	93.5	75.9	98.2	<0.0	<0.0	<0.0	20.6	<0.0
Water	95.4	59.8	95.7	<0.0	<0.0	<0.0	<0.0	<0.0

Note: <0.0% indicate expression levels below control levels. n.d indicates genes not detected

Table 4: Terminologies utilized in the research paper

Name	Description
cDNA	complementary DNA
PBS	Phosphate buffered saline
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
RPMI-1640	Roswell park memorial institute
BSA	Bovine serum albumin
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
RT-PCR	Transcriptase-polymerase chain reaction
HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl]-ethane sulfonic acid
KRBH	Krebs-Ringer-Bicarbonate HEPES
LC_{50}	Lethal concentration at 50%
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
SANBI	South African national biodiversity institute
GSIS	Glucose-stimulated insulin secretion
ELISA	Enzyme-linked immunosorbent assay
SEM	Standard error of the mean
T2DM	Type 2 diabetes mellitus
GAPDH	Glyceraldehyde-3-phosphatehydrogenase
RIN-m5F	Rat insulinoma pancreatic beta cells
NeuroD1	B-2/Neurogenic differentiation 1
MafA	Musculoaponeurotic fibrosarcoma homolog A
PDX-1	Pancreatic duodenal homeobox-1
GLUT2	Glucose transporter 2

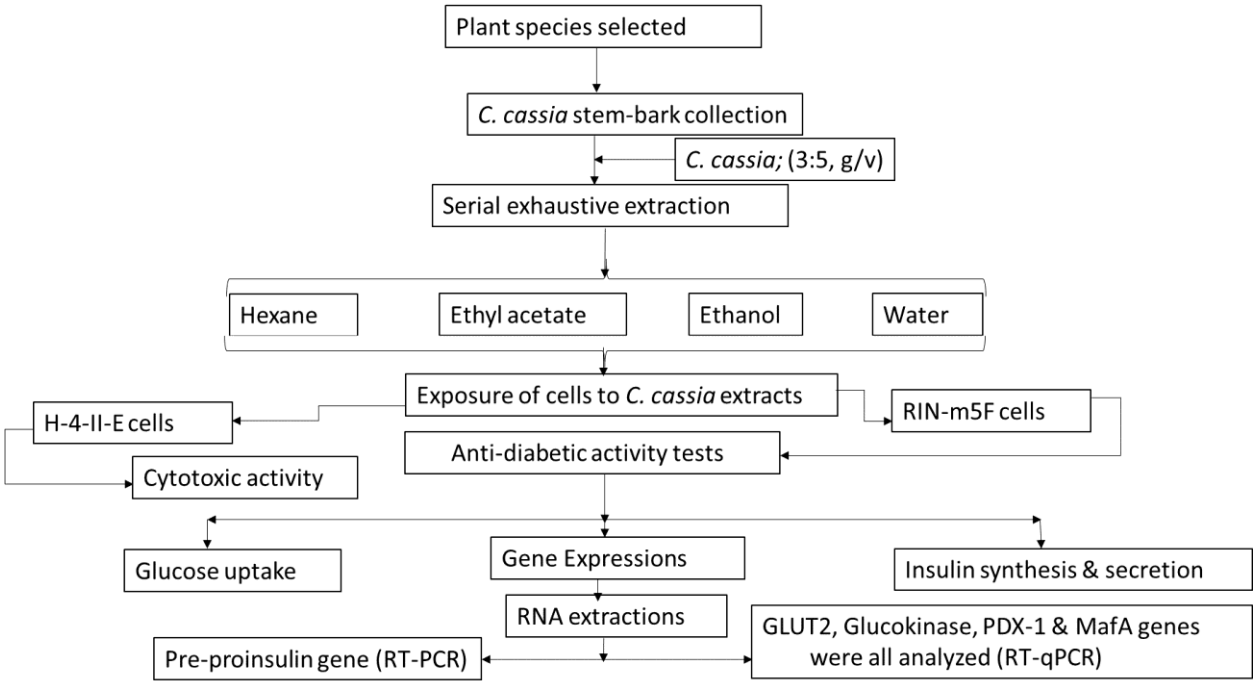


Fig. 1: Flow chart displaying research methodology

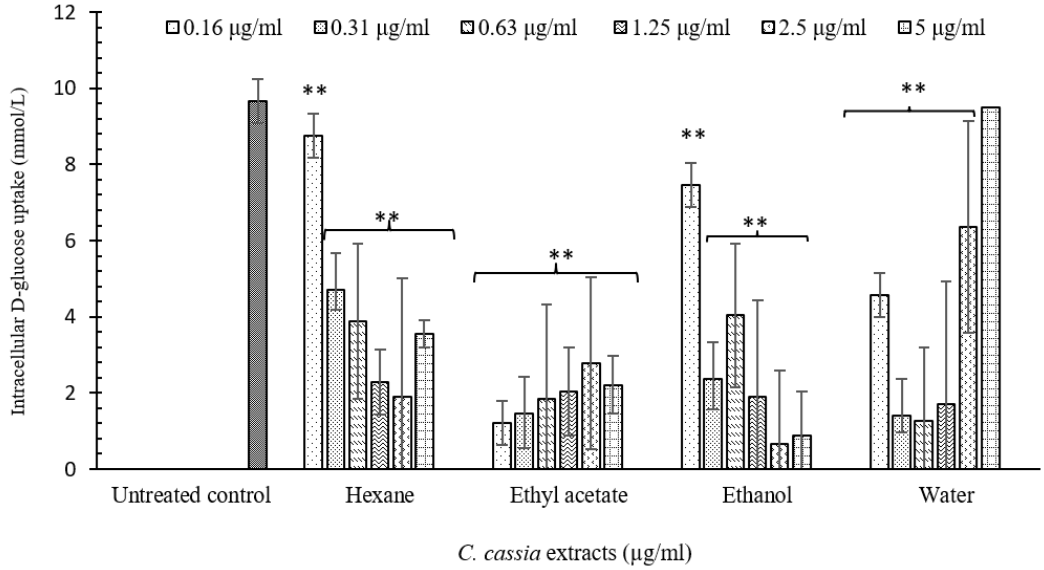
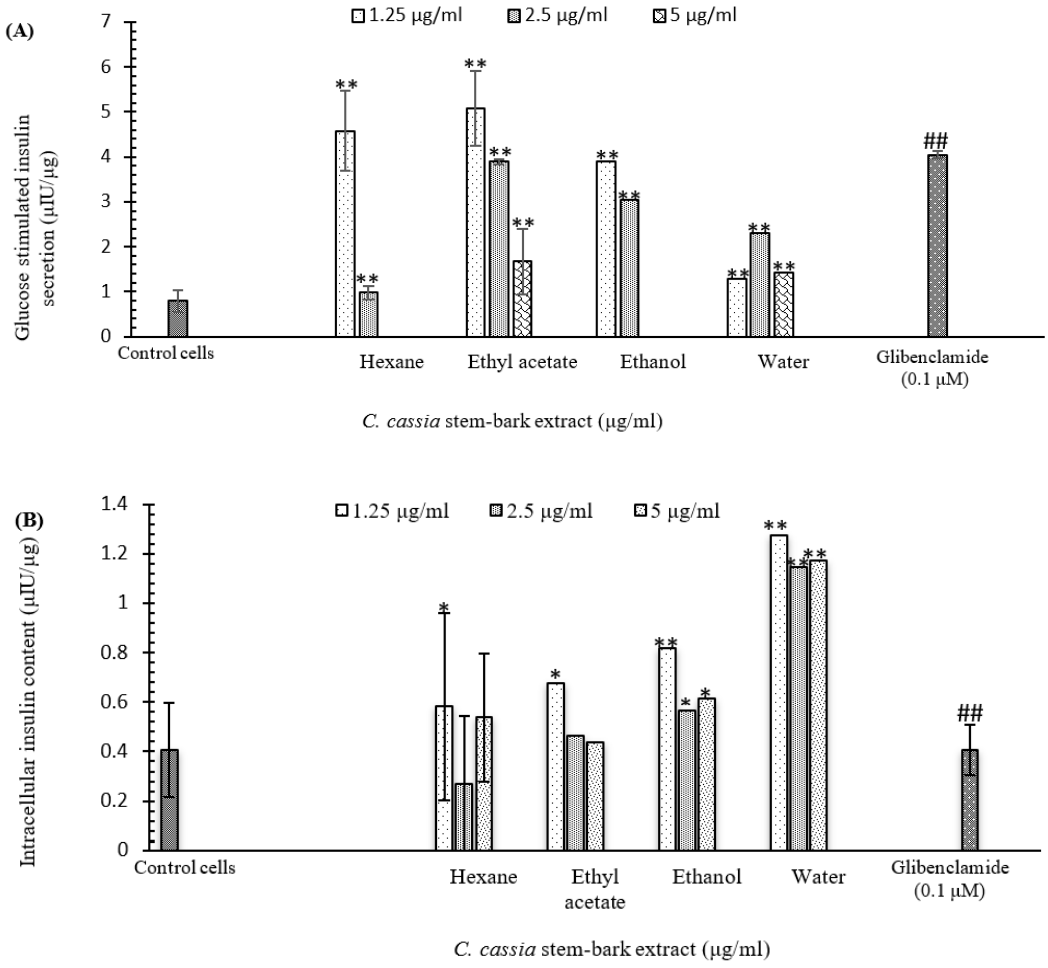


Fig. 2: The glucose consumption by RIN-m5F cells incubated with *C. cassia* stem-bark extracts and 15 mmol/L glucose for 60 min. Each experiment was executed in triplicates (n = 9). The results are expressed as mean glucose uptake ± SEM. Significance differences with * signifying $p<0.05$ and ** signifying $p<0.001$ was considered when compared with cells that were not treated



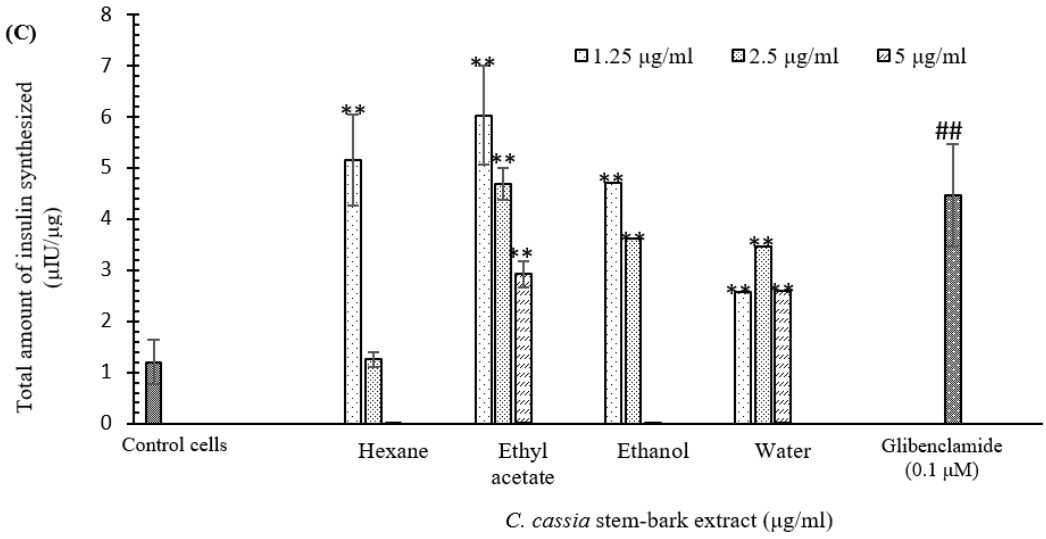


Fig. 3: Influence of *C. cassia* extracts on, (A) glucose stimulated insulin secretion, (B) intracellular insulin concentration and (C) total amount of insulin synthesized by RIN-m5F pancreatic beta cells. Results expressed as mean insulin concentration ± SEM triplicate experiments (n = 9). Significance difference with *signifying $p<0.05$ and ** signifying $p<0.001$ was considered when compared with cells not treated with extracts. ##signifies $p<0.001$ when 0.1 µM glibenclamide were compared to RIN-m5F cells not treated

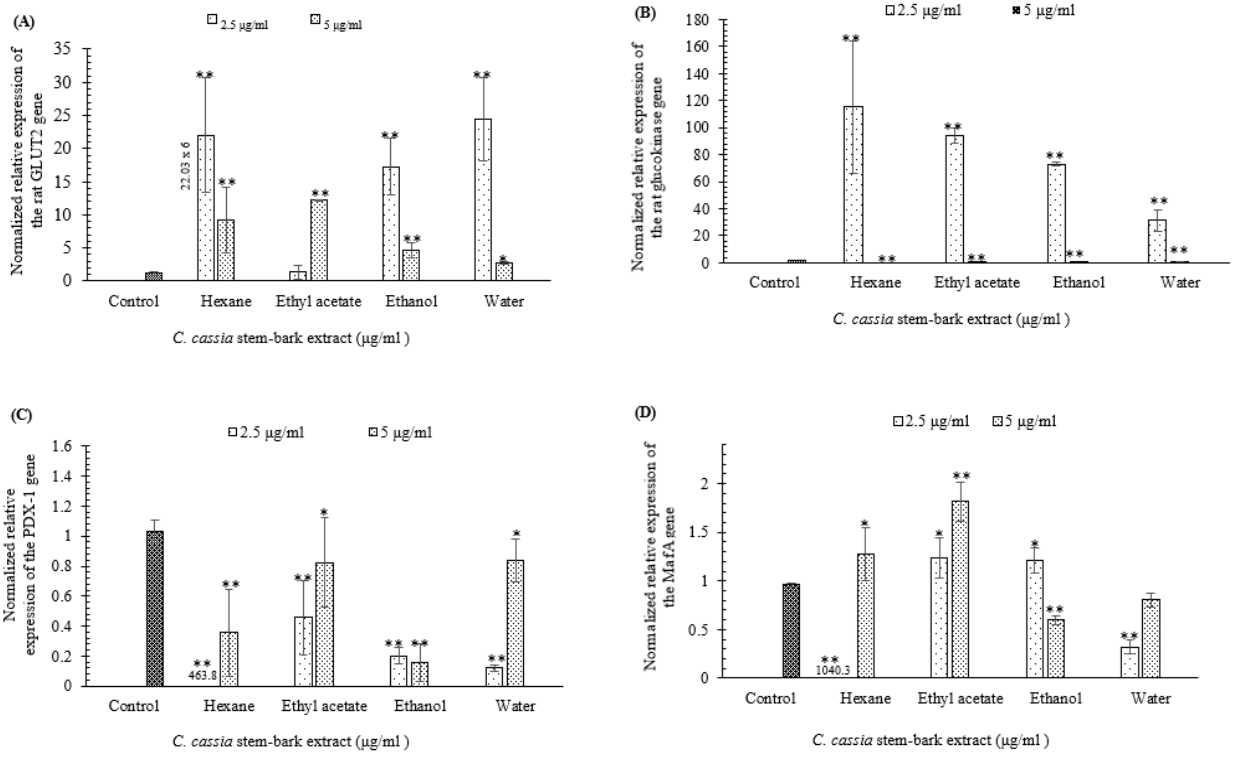


Fig. 4: The mRNA levels of (A) GLUT2, (B) glucokinase (C) PDX-1 and (D) MafA by RIN-m5F cells treated with or without 2.5 and 5 µg/mL *C. cassia* stem-bark extracts for 16 h in the presence of 15 mmol/L glucose. Results presented as mean fold change in mRNA levels ± SEM triplicate experiments (n = 9). Significance difference with * signifying $p<0.05$ and ** signifying $p<0.001$ was considered when compared with cells not treated with the extracts

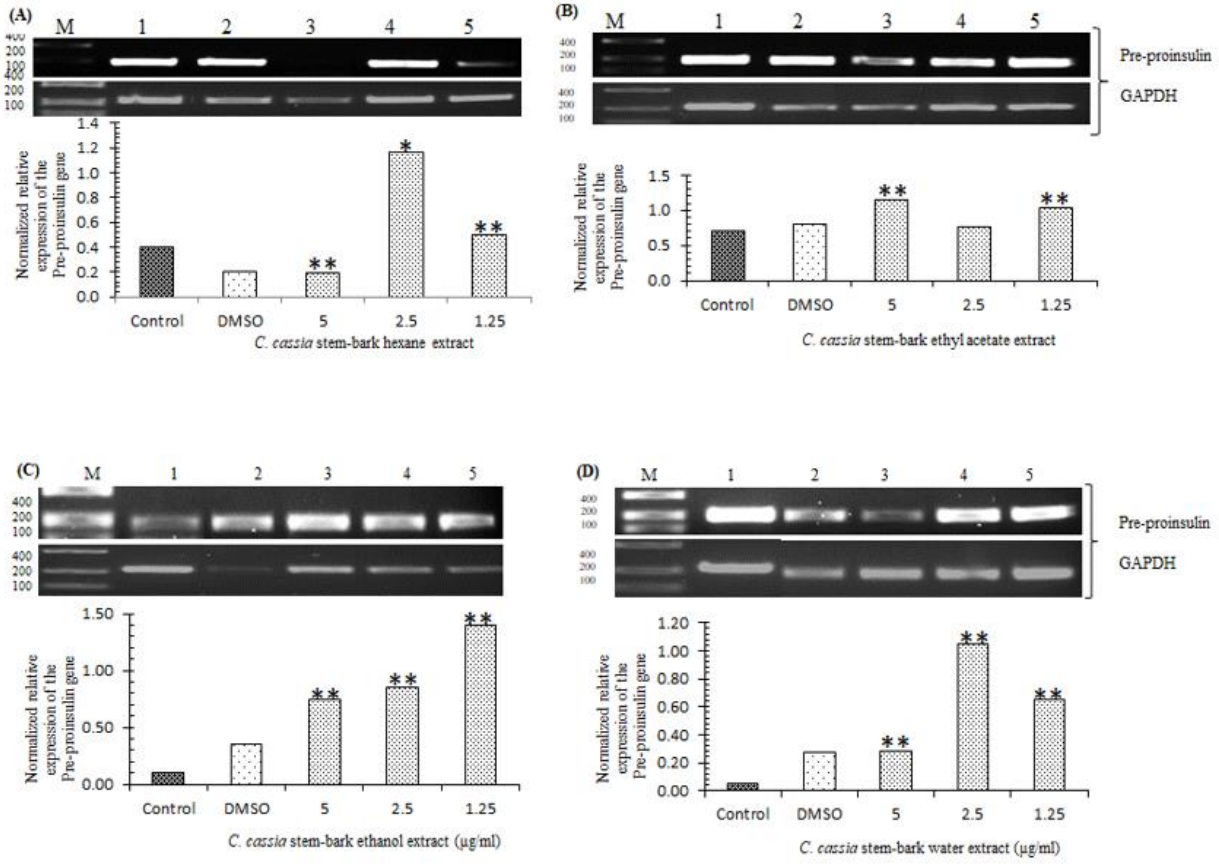


Fig. 5: The Pre-proinsulin gene expression levels by RIN-m5F cells incubated with or without 1.25, 2.5 and 50 µg/mL *C. cassia* stem-bark extracts for 30 min in the presence of 15 mmol/L glucose. (A) Hexane extract, (B) ethyl acetate extract, (C) ethanol extract and (D) water extract. Results were presented as mean fold change expression levels ± SEM triplicate experiments (n = 9). Significance difference with * signifying $p<0.05$ and ** signifying $p<0.001$ was considered when were compared with cells not treated with the extracts

Discussion

Medicinal plants are potential sources of phytochemicals that can be used to develop novel and active therapeutic medications to treat or manage diseases, including Type 2 diabetes mellitus, through varying mechanisms (Beseni *et al*., 2019; Makom *et al*., 2010; Mogale *et al*., 2012; Oh, 2015). Hence, the current research examined the mechanism through which crude stem-bark extracts of *Cinnamomum cassia* exert their hypoglycemic effect. The study focused on the influence of these extracts on the upregulation of genes responsible for glucose uptake, upregulation of the transcription factors for insulin gene and insulin content in RIN-m5F cells incubated together with glucose concentration of 15 mmol/L. Adequate preparation of medicinal plant extracts for anti-diabetic studies is important for ensuring the reliability or the success of the research under study (Beseni *et al*., 2019). In this study, *C. cassia* stem-bark was exhaustively extracted using water, ethanol, ethyl acetate and hexane. *C. cassia* ethanol extract was observed to have the highest percentage yield extract while the non-polar hexane extract had the least. Similar trends were also observed in previous studies where the highest extraction yield was obtained with polar solvents than with the non-polar solvents (Beseni *et al*., 2019; Kgopa *et al*., 2020). This observation is attributed to the influence of sufficient quantities of smaller molecular sized polar phytochemicals present in medicinal plants able to penetrate plant cell wall for advanced extraction (Beseni *et al*., 2019).

In studies that involve medicinal plant extracts for tissue culture and public health programs, it is always necessary to determine and use extracts concentration that is not more-than 50% toxic (Mosmann, 1983; Nemati *et al*., 2013; Sagbo *et al*., 2018). This practice is achieved by performing the cytotoxicity test which allows the determination of LC_{50}. This parameter represents the lowest extract dose at which 50% of the original number of cells remain viable. Any concentration of the plant extract below the LC_{50} value is usually regarded as safe to use in subsequent bioassays involving the cultured cells or related cells (Makom *et al*., 2010; Nemati *et al*., 2013).

In this study, the hexane extract displayed the lowest LC_{50} value of 5 µg/mL, indicating highest toxicity levels, while ethanol had the highest LC_{50} value, when compared with the rest of the extracts tested. This observation could probably be due to the present of plant toxic non-polar phytochemicals in the extract. In order to limit the cytotoxicity of *C. cassia* stem-bark extracts in RIN-m5F cells, the 5 µg/mL extract concentration was used as a marker concentration for all extracts tested in this study. The outcomes of this study propose that the polar *C. cassia* ethanol extracts could be safe to use because of the observed low levels of toxicity. This trend where polar extracts are less toxic to cells has also been observed for other plants (Kgopa *et al*., 2020; Sagbo *et al*., 2018).

The entry of glucose into cells or the consumption of glucose (Fu *et al*., 2013) and the control of the levels of glucose in the bloodstream (Sagbo *et al*., 2018) by insulinoma cells is a binding stage in controlling the insulin production and secretion (Ren *et al*., 2007). In this instance, the glucose transporter proteins play a key role in sensing and taking-up glucose by pancreatic β-cells (Bae *et al*., 2010). While countless deal of study have explored, the influence of extracts from medicinal plants on the absorption of glucose by muscle and adipose tissues (Dimo *et al*., 2007; van de Venter *et al*., 2008), in this investigation, insulin-independent RIN-m5F cells were used to assess the influence of *C. cassia* stem-bark extracts on glucose absorption with/without 15 mmo/l glucose. The amount of glucose was not reduced even overtime when cells incubated with extracts of varied dosages. Failure of some of the medicinal plant extracts to enhance glucose uptake in cell culture study was suggested to be due to the inhibition of the relocation of the glucose transporter protein to the cellular membrane or protein storage after sysnthesis by extracts (Seabe *et al*., 2016). This trend was also observed in these type of extracts when exposed to Type 2 diabetes patients after menopause (Vanschoonbeek *et al*., 2006). Our results are in accordance with previous study which demonstrated the potential of *C. cassia* extracts enhance the activity of glycogen synthase, thus, increasing glycogen storage rather than glucose uptake (Kizilaslana and Erden, 2019). Parallel findings reported by Cao *et al*. (2007) have also showed the reduced levels of insulin receptor-β than the absorption of glucose in the mouse 3T3-L1 adipocytes. This observation is said attributed to specific metabolic or signal transduction pathway feedback inhibition influenced by high concentration of compounds present in the cinnamon extracts.

The present results is different when compared to a study in which *C. zeylanicum* extracts demonstrated an increased activity of glucokinase which may be an indication of increased glucose uptake in STZ-induced diabetic rats (Hassan *et al*., 2012). Our current study contradicts with findings reported in Soliman *et al*. (2013) demonstrating three-fold increase in glucose uptake (108.5±6.2 mg/dL) for diabetic rats treated in crude water-soluble cinnamon extracts compared to non-treated diabetic rats. A study in the literature reported that compounds isolated from cinnamon water extracts enhance glucose uptake in diabetic rats (Qin *et al*., 2003), this was however not the case in the present study. *C. cassia* extract is also regarded unsafe due to high contents of coumarins which may cause health risks if consumed in high amounts (Sartorius *et al*., 2014) and toxic lipid compounds present in the stem-bark (Cao *et al*., 2007). This study is the first to report on *in vitro* investigation of the effects of *C. cassia* stem-bark extracts on glucose absorption by RIN-m5F cells and the results cannot be compared with other results in the literature. Thus, additional studies are necessary to support these results. On the basis of this observation, the current results propose that of *C. cassia* extracts still require thorough investigation when used as anti-diabetic agent to lower glucose levels in diabetic patients.

It has been reported that, the glucokinase enzyme and the glucose transporter 2 protein, which are sensors of glucose, responsible for glucose access into the beta cells of the pancrease and are not influenced by the insulin action (Komatsu *et al*., 2013; Thorens, 2015). Glucose transporter proteins are key molecules playing roles in blood glucose regulation. These proteins are capable of cell surface glucose trasnportation/removal into the cell. Transcriptional down-regulation of GLUT2 gene results in the absence of GLUT2 protein, thus leading to hyperglycaemia. Hence, this study examined the influence of different *C. cassia* extracts on levels of GLUT2 and glucokinase mRNAs in RIN-m5F cells. The purpose of this objective was to confirm whether the observed lack of improvement of glucose consumption by these extracts was associated with the absence or suppression of the glucokinase and/or GLUT2 gene upregulation. It was interesting to note that although all *C. cassia* extracts that did not enhance glucose uptake in this study, all the extracts had the capacity to increase mRNA levels of both the GLUT2 and glucokinase, with only 5 µg/mL ethyl acetate extracts showing dose-dependent effect on GLUT2 mRNA levels. Parallel findings on the expression of glucose transporter protein gene was reported where different plant extracts studied enhanced glucose uptake when cells treated with varying extracts concentrations (Seabi *et al*., 2016). High mRNA levels of the glucokinase gene was detected only at 2.5 µg/mL and not at 5 µg/mL, an effect which was also observed in previous studies with no suitable explanation to account for this observation (Kgopa *et al*., 2020; Zandi *et al*., 2016). These outcome of this objective suggests that the crude *C. cassia* stem-bark extracts through increasing GLUT2 and glucokinase mRNA levels, may improve the glucose uptake though not observed in this case.

The transcription of insulin gene leading to the production and secretion of insulin has been known to be tightly regulated by MafA and PDX-1. Furthermore, the mRNA levels of GLUT2 and glucokinase may be associated with the expression of insulin gene transcription factors. The upregulation of Insulin gene and the matuation of β-cell is regulated by key factor, namley, PDX-1. The deficiency of PDX-1 in mature beta cells is linked with the intolerence of glucose by cells. this suggests how important PDX-1 is, in the maintanance of the funtion of the beta cells (Holland *et al*., 2002). Hence this study, investigated in particular, the effects of *C. cassia* extracts on (i) transcription factors involved in upregulation of insulin gene, namely, MafA and PDX-1 and (ii) upregulation of the pre-proinsulin gene. The manner in which *C. cassia* extracts upregulated the expression of MafA gene also suggest to have activated the glycolytic pathway by initiating glucokinase activity. Like PDX-1, MafA regulates insulin gene expression by synergizing with other transcription factors (Andrali *et al*., 2008). The RIPE3b/C1-A2, is an insulin enhancer, a higly conserved element specifically bound by MafA which triggers the upregulation of insulin gene (Holland *et al*., 2002). On the basis of these observations, it can be concluded that *C. cassia* stem-bark extracts could have a vital influence on the transcription of insulin gene as well as the genes for the insulin transcription factors investigated in the current study. Increased quantities of glucose in the blood lead to the expression of pre-proinsulin gene (Andrali *et al*., 2008), thus, this reserch, explored the influence of extracts of *C. cassia* on the pre-proinsulin mRNA levels in the presence high glucose concentration. Generally, all *C. cassia* stem-bark extracts investigated showed a significant influence on the upregulation of the pre-proinsulin gene relative to untreated control cells or DMSO vehicle, even though this effect did not appear to be dose-dependent in some extracts. Furthermore, different patterns of pre-proinsulin mRNA levels in all extract-treated cells based on increasing concentration were observed, suggesting an observation that cannot be explained but this was not due the effect of high glucose concentration which is reported in the previous study (Andrali *et al*., 2008). Large quantities of glucose in the bloodstream is reported to trigger the upregulation of insulin gene transcription factors (MafA and PDX-1), thus, enhancing mRNA levels of the pre-proinsulin gene resulting in an increase in insulin synthesis and secretion (Vanderford *et al*., 2007), but could not affect the RIN-m5F cells glucose absorption consumption in this study.

Extracts from some medicinal plants known to lower blood glucose levels may do so through stimulating the production of insulinn and release in the presesent of glucose (Ajabnoor, 1990; Balekari and Veeresham, 2013; Patel *et al*., 2012). These effects can be evaluated by quantifying the amount of the extracellular and intracellular insulin after treatment of cells with such anti-diabetic agents (Olaokun *et al*., 2014). In this present research, it was confirmed that the intracellular insulin content obtained was less compared with the secreted insulin. The results obtained from this study, suggest that all extarcts of *C. cassia* stem-bark have the potential to improve the synthesis and secretion of insulin, hence, the insulin-like effect of this plant extract may resemble those of the similar plant studied (Soliman *et al*., 2013). These study results were partially in agreement with those obtained in Soliman *et al*. (2013) and these differences may be due to the study settings, types of cells and extracts used in which *cinnamon* extracts demonstrated insulin like effect in beta cells. The findings of this study suggest that there is a presence of polyphenolic or phytonutrients, most perhaps cinnamaldehyde, eugenol, catechins or terpenes known to be abundant in plant extracts of the same genus but different species (Sartorius *et al*., 2014), which may have contributed in enhancing the observed insulin synthesis.

Limitations and Strengths of the Study

This study investigated designated phases of the synthesis and secretion of insulin and this is considered as one main drawback of the study. In addition, only mRNA levels of the glucose sensors were investigated and thus a comprehensive evidence could have been obtained if the both the activity of glucokinase enzyme and/or glucose transporter protein were also investigated. Another limitation in this study was the use of hepatic cells to measure the toxicity levels of the extracts since RIN-m5F cells were not active to digest MTT reagent. The glucose uptake results obtained with RIN-m5F cells are not conclusive, thus further investigation are required to confirm the results. However, more valuable information was obtained in this study. Firstly, extracts of increasing polarities were investigated to assess the effects of different phytochemicals. Secondly, different concentrations of the extracts were used to determine the presence or shortage of a dosage-dependent effect. Thirdly, a study investigating effect of *C. cassia* extracts on aspects outlined, which includes, glucose uptake, upregulation of transcription factors responsible in insulin gene expression, glucose sensors, pre-proinsulin gene as well as on the synthesized and secreted insulin was conducted.

Conclusion and Recommendation

The stem-bark extracts of *C. cassia* failed to promote the consumption of glucose by beta cells even though all the extracts appeared to increase mRNA levels of the glucokinase and GLUT2 gene. All *C. cassia* stem-bark demonstrated to enhance GSIS when in comparison with negative control cells even though effect was not dose-dependent. The extent to which some of the extracts enhanced insulin content was comparable to that of glibenclamide. This research is first to explore on the effect of *Cinnamomum cassia* extracts on aspects related to glucose uptake by pancreatic beta cells as well as on aspects of insulin synthesis, thus additional studies are recommended to validate these findings. A detailed mechanism regarding how

C. cassia stem-bark extracts regulates the transcription of the insulin gene transcription factors, glucose sensors and the pre-proinsulin genes as well as the identification of active molecules in these extracts may lead to the development of therapeutic medications for the management and treatment of diabetes mellitus specifically in the Southern African countries. Beneficial effects of *C. cassia* stem-bark extracts can be completed when the obtained data is improved with the effects of these extracts on non-diabetic individual. However, *C. cassia* should be prescribed with caution because of its toxicities.

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Authors' Contributions

Ananias Hodi Kgopa: Contributed in the conceptualization of the research project. Conducted the research study, data collection, data analysis and writing of the manuscript.

Motetelo Alfred Mogale: Conceptualization and supervision of the study. planned and designed the research project. Also, involved in the analysis and interpretation of data, edited the manuscript.

Sebolaishi D Makhubela: Contributed to the interpretation of the results, revision and editing of the manuscript.

Leshweni Jerry Shai: Co-supervision of the research study, analysis and interpretation od data. Also, revised and edited the manuscript.

Conflict of Interests

All authors have read and approved the final manuscript. There is no conflict of interests with regard to publishing this research report.

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