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***Costus pictus* D. Don leaf extract stimulates GLP-1 secretion from GLUTag L-cells and has cytoprotective effects in BRIN-BD11 β -cells**

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1 **Abbreviations:** GLP-1, Glucagon-like peptide 1; T2DM, Type 2 diabetes mellitus;
2 CPE, *Costus pictus* extract; CP, *Costus pictus*; ROS, reactive oxygen species; Nrf2,
3 Nuclear Factor Erythroid 2 Like 2; Sod, Superoxide dismutase; Cat, Catalase; Gpx1,
4 Glutathione peroxidase 1; Nfkb, Nuclear Factor Kappa B Subunit; Myc, Proto-
5 Oncogene C-Myc; Erk, Extracellular Signal-Regulated Kinase; Jnk1, C-Jun N-
6 terminal kinase 1; C-Jun, Jun Proto-Oncogene; Ins1, Insulin1; Pdx1, Pancreatic And
7 Duodenal Homeobox 1, MafA, MAF BZIP Transcription Factor A; Gck, Glucokinase;
8 Pc1/Pcsk1, Prohormone convertase 1; Pc2/Pcsk2, Prohormone convertase 2 ; Gcg,
9 Proglucagon; SglT1, Sodium/Glucose Cotransporter 1

Abstract:

Ethnopharmacological relevance: *Costus pictus* D. Don, commonly known as insulin plant, is a traditional Indian antidiabetic herbal medicine with glucose-lowering and insulin secretory effects having been reported in animal models and humans with Type 2 diabetes. However, its effects on GLP-1 secretion from intestinal endocrine L-cells and potential metabolic and protective effects in insulin secreting pancreatic β -cells are not yet fully understood.

Aim of the study: This study is aimed to elucidate the effects of *Costus pictus* D. Don leaf extract (CPE) on L-cell function and GLP-1 secretion using the established murine GLUTag L-cell model and to investigate its potential cytoprotective effects against detrimental effects of palmitate and cytokines in pancreatic β -cells using BRIN-BD11 cells.

Methods: *Costus pictus* D. Don dried leaf powder was extracted by soxhlet method. Cell viability was determined by MTT assay. Changes in gene and protein expression were quantified by qPCR and western blotting, respectively. GLP-1 and insulin secretion were measured by ELISA.

Results: CPE significantly enhanced cell viability in both BRIN-BD11 and GLUTag cells and protected BRIN-BD11 cells against palmitate- and proinflammatory cytokine-induced toxicity. CPE enhanced acute GLP-1 secretion 6.4 -16.3-fold from GLUTag cells at both low (1.1 mM) and high (16.7 mM) glucose ($P < 0.01$) concentrations. Antioxidant (Nrf2, Cat & Gpx1) and pro-proliferative (Erk1 and Jnk1) gene expression were upregulated by 24 h culture with CPE while proinflammatory transcription factor NF- κ B was downregulated.

Conclusion: Diminished postprandial GLP-1 secretion and loss of insulin secreting β -cells are known contributors of T2DM. Our data suggests that CPE acutely

1 stimulates GLP-1 secretion from L-cells. Long term exposure of the BRIN-BD11 cells
2 to CPE enhances cell number and protects against palmitate and proinflammatory
3 cytokines by activating multiple pathways. Thus, the current study suggests that the
4 possible antidiabetic properties of CPE may be linked to enhanced GLP-1 secretion
5 and β -cell protection which could be beneficial in management of T2DM.
6 **Keywords:** GLP-1, *Costus pictus*, β -cell, L-cell, cytoprotection

1. Introduction:

Obesity-associated lipotoxicity is one of the drivers of cellular demise in Type 2 Diabetes Mellitus (T2DM). Elevated circulating free fatty acids (FFA) and cytokines in obesity are associated with pancreatic β -cell reactive oxygen species (ROS) accumulation and β -cell dysfunction in diabetes (Yang and Li, 2012). Under physiological conditions, ROS such as superoxide and hydroxyl radicals are produced in β -cells as a by-product of mitochondrial metabolism. These are then converted to less toxic hydrogen peroxide by superoxide dismutase (SOD) and further broken down to oxygen and water molecules by catalase (Cat) and glutathione peroxidase (Gpx) (Wang and Wang, 2017). Due to very low expression of antioxidant enzymes in β -cells compared to liver (Tiedge et al., 1997), coupled with high demand for insulin to compensate peripheral tissue insulin resistance and exposure to high FFA, cytokines and glucose, ROS accumulate in β -cells which drives cellular dysfunction and apoptosis (Drewe et al., 2010). Expression of these antioxidants are regulated by the transcription factor Nrf2 (Nuclear Factor, Erythroid 2 Like 2), with previous studies in rodent and murine diabetic models reporting that induction of Nrf2 could alleviate high fat diet-mediated oxidative stress by promoting β -cell self-repair (Abebe et al., 2017; Yagishita et al., 2014).

Endogenous gut L-cell derived glucagon-like peptide-1 (7-36 amide) (GLP-1) is known to protect β -cells against apoptosis by promoting β -cell proliferation and survival (Yusta et al., 2006), however its effects are short-lived due to rapid N-terminal cleavage to GLP-1 (9-36) by the ubiquitous enzyme dipeptidyl peptidase-4 (DPP-4) (Hansen et al., 1999). GLP-1 acts through its specific G protein-coupled receptor (the GLP-1R), to potentiate postprandial glucose-stimulated insulin secretion (GSIS) along with gut K-cell derived glucose-dependent insulintropic

polypeptide (GIP) which is known as the incretin effect. GLP-1R activation enhances insulin gene transcription and promotes β -cell proliferation and cytoprotection (Lee and Jun, 2014). It is known that there is a diminished incretin effect in Type 2 diabetes (Baggio and Drucker, 2007), and although the causes for the loss of incretin effect are unclear, it is believed that defects in nutrient-induced GLP-1 secretion may be one possible factor (Jorsal et al., 2018).

The proglucagon (*Gcg*) gene is expressed in both pancreatic islet α -cells and gut L-cells, but post-translational changes by prohormone convertase 1/3 (*Pcsk1*) in L-cells produces GLP-1, whereas prohormone convertase 2 (*Pcsk2*) produces glucagon. Previous studies in GLUTag L-cells reported that chronic exposure to palmitate (a saturated free fatty acid) elevated endoplasmic reticulum stress and caused L-cell apoptosis indicating that L-cells may be susceptible to lipotoxicity similar to observations in β -cells (Vasu et al., 2015). Thus, any cytoprotective agents that offer β -cells protection by promoting antioxidant defence or agents that stimulate endogenous GLP-1 production may be of therapeutic benefit in the treatment of T2DM.

Costus pictus D. Don (CP), also known as 'spiral flag', is indigenous to South and Central America, but was introduced to India and has been used in Indian folk medicine as a herbal remedy for diabetes (Elavarasi and Saravanan, 2012; Gireesh et al., 2009; Hegde et al., 2014; Shetty et al., 2010). Due to its insulin secretory effects, it is known in south India as the insulin plant. In streptozotocin-induced diabetic rats, *Costus pictus* extract (CPE) improved glycaemic control and elevated circulating insulin levels (Gireesh et al., 2009). Furthermore, methanolic CP extract enhanced insulin secretion from isolated murine and human islets, and in MIN6

1 insulin-secreting cells it increases intracellular calcium levels through L-type calcium
2 channels (Al-Romaiyan et al., 2010). Methanolic leaf extract of CP is reported to be
3 rich in flavonoids such as isoquercetin, astragalin, kaempferol and quercetin along
4 with some phenolic compounds which might be associated with its antioxidant
5 effects in diabetic rat models (Ashwini et al., 2015). Previous studies focused on
6 CPE effect on insulin secretion, but its acute and longer-term effects on expression
7 of insulin and cytoprotective effects in pancreatic β -cell, including antioxidant gene
8 transcription and translation are unknown. Furthermore, CPE effects on gut L-cell
9 function and GLP-1 secretion have not previously been investigated. Thus, the
10 current study aimed to investigate the effects of CPE on GLP-1 secretion and
11 function of L-cell using the murine L-cell GLUTag model along with β -cell protective
12 effects using the rat insulinoma BRIN-BD11 cell line.

2. Materials and Methods

2.1 *Costus pictus* D. Don Extract:

Commercially available 100% CP dried leaf powder was purchased from Aditi Herbals, Chennai, India (Batch number: 0316). Using 100ml of absolute ethanol, 40g of CP leaf powder was extracted by Soxhlet method over 1hour (Irobekhan et al., 2016). The resultant extract was concentrated by rotary drum evaporator, weighed and reconstituted at 30mg/ml in ethanol. Reconstituted stock was stored at -20°C.

2.2 Cell Culture and viability:

Rat clonal BRIN-BD11 cells (generated by the PR. Flatt laboratory at Ulster University, U.K) were established by electrofusion of RINm5F cells with New England Deaconess Hospital (NEDH) rat pancreatic islet cells (McClenaghan et al., 1996). They are glucose responsive and their insulin secretion is 5-10 times greater than the parental RINm5F cells. They are stable up to >50 passages in the cell culture and grow in monolayers with epithelioid characteristics.

BRIN-BD11 cells (gifted by Prof Peter Flatt, Ulster University) were cultured in RPMI 1640 medium with 2mM L-Glutamine (Lonza, Belgium), supplemented with 10%FBS (v/v), 50U/ml Penicillin/streptomycin and maintained at 37°C with 5% CO₂ and 95% air. Cells were trypsinised and sub-cultured at 1:5 dilutions when 80-90% confluence was reached. Passages from 20 to 40 were used for experiments. To determine the effects of CPE on cell viability, BRIN-BD11 cells were seeded in 96 well tissue culture plates at 10,000 cells/well. After overnight culture, cells were incubated with CPE (0.39 to 50µg/ml) for 24h-48h and cell viability assessed using MTT assay.

The GLUTag cell line (generated by the Drucker laboratory at University of Toronto, Ontario, Canada) was isolated from a glucagon-producing enteroendocrine cell tumor that occurred in glucagon gene-SV40 T antigen transgenic mice (Lee et al., 1992). Compared to other murine L-cell model STC-1, these cells are well differentiated and respond to physiological and pharmacological stimulants similar to the primary L-cells. They are stable in cell culture up to 100 passages/ 18 months and maintain the L-cell phenotype (Drucker et al., 1994).

GLUTag cells (a kind gift from Prof. D Drucker) were routinely cultured in Dulbecco's modified eagle medium (DMEM) (5.5 mmol/L D-glucose) (Lonza, UK), supplemented with 10% (v/v) foetal bovine serum (v/v), 50U/ml penicillin/streptomycin and 2mM L-glutamine, and were maintained at 37°C with 5% CO₂ and 95% air (Drucker et al., 1992; Lee et al., 1992). Cells were trypsinised and sub-cultured at 1:5 dilutions when 80-90% confluence was reached. Passage numbers from 25 to 35 were used for experiments. To determine the effects of CPE on cell viability over 24h, GLUTag cells were seeded in Geltrex™ (Gibco, UK) coated 96 well plates at 10,000 cells/well. After overnight culture, cells were incubated with CPE (0.78 to 200µg/ml) for 24h and cell viability assessed using MTT assay.

2.3 GLP-1 and insulin secretion:

For GLP-1 and insulin secretion tests, GLUTag and BRIN-BD11 cells were seeded into 12 well plates (2.5X10⁵ cells/well) (precoated with Geltrex™ for GLUTag cells) and incubated for 24h prior to use. On the day of experiment, cells were washed and incubated in KRB buffer (both basal (1.1 mmol/L) and high (16.7 mmol/L) glucose concentrations) with test reagents at 37°C for 1h. The KRB buffer was composed of (mmol/L): 115 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.28 CaCl₂, 25 NaHCO₃, 20

1 HEPES and 0.1% (w/v) bovine serum albumin (BSA) (adjusted to pH 7.4 with 1M
2 NaOH). After test incubations, supernatant was collected, centrifuged at 900rpm for
3 5min and GLP-1 & insulin levels measured using the total GLP-1 ELISA kit (Millipore,
4 UK) and Rat Insulin ELISA (Mercodia, Sweden) according to manufacturer's
5 specifications.

6 **2.4 Immunofluorescent determination of cellular GLP-1:**

7 To analyse GLP-1 content, GLUTag cells (1×10^5 cells/ coverslip) were seeded
8 on Geltrex™ coated glass coverslips and cultured overnight until confluent. After
9 treatment with 6.25µg/ml CPE for 24h in standard culture media, cells
10 were permeabilised and fixed by addition of 500µl of ice-cold methanol for 5mins at -
11 20°C. Non-specific binding was blocked with normal horse serum for 1h at room
12 temperature and cells were then incubated with primary anti-GLP-1 antibody (1:200
13 dilution in PBS containing 2%BSA) (Goat pAb to GLP-1, SC-26637, Santa Cruz
14 Biotechnology), overnight at 4°C. Alexa Fluor 594 conjugated donkey anti-goat IgG
15 secondary antibody (1:500 dilution in PBS) (abcam, ab150132) was added to cells
16 and incubated at 37°C for 1h. Mounting media containing DAPI (Abcam,
17 ab104139) was used to stain the nuclei. Cells were visualised and images captured
18 using an EVOS® FL fluorescence microscope. Random images were taken from
19 each coverslip and average intensity of 60 cells/coverslip was measured using
20 ZEN lite software. Data was plotted as mean pixel intensity per 1000 pixel ².

21 **2.5 RNA extraction And Quantitative real-time PCR:**

22 Total RNA was extracted from CPE pre-treated BRIN-BD11 and GLUTag cells
23 using NucleoSpin® RNA kit (Macherey-Nagel, UK) according to manufacturer's

1 protocol. From total RNA, cDNA was synthesised using High-Capacity cDNA reverse
2 transcription kit (Applied Biosystems, UK), according to manufacturer's protocol.

3 Quantitative real-time PCR was performed on a CFX96™ Real-Time PCR detection
4 system using iQ™ SYBR® Green Supermix (BIO-RAD, UK). Rat and mouse primer
5 sequences used are listed in appendix Tables 1 and 2, respectively. Cycling
6 conditions used were: one cycle at 95°C for 3mins followed by 40 cycles at 95°C for
7 15sec and 60°C for 1min. At the end of each experiment, melting curve analysis was
8 done to analyse primer specificity.

9 **2.6 Western blot:**

10 Cells were lysed with ice cold radioimmunoassay precipitation buffer
11 (RIPA) (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5%
12 sodium deoxycholate, 0.1% sodium dodecyl sulphate) and centrifuged at 10,000 rpm
13 for 15mins (4°C) to separate any cell debris. Protein concentration in the supernatant
14 was measured using DC™ Protein assay kit (BIO-RAD, UK) according
15 to manufacturer's specifications using bovine serum albumin (BSA) as standard.
16 Proteins were separated by SDS-PAGE and transferred to nitrocellulose
17 membrane (20V for 10mins) using iBlot® blotting system (Thermo scientific,
18 UK). Membranes were blocked with 5% (w/v) BSA for 1h at room temperature
19 and incubated with primary antibodies overnight at 4°C. Primary antibodies used
20 were: Anti-Pcsk1 (1:1000) (GTX113797S, Genetex), Anti-Pcsk2 (2µg/ml) (MAB6018-
21 SP, Novus Biologicals) , Anti-Nfkb p65 (1:1000) (8242, Cell Signalling Technology) ,
22 Anti-Pdx1 (1µg/ml) (AF2517, R&D Systems) and Anti-Actin-beta (1:1000) (ab-
23 1801, Abcam UK). Blots were incubated with IRDye® conjugated specific secondary
24 antibodies (1:5000) for 1h at room temperature. Signals were detected using the

- 1 Odyssey® Fc imaging system (LI-COR, UK) and analysed using Image Studio™
- 2 software.

1 **2.7 Data Analysis and Statistics:**

2 Results are presented as mean $\pm$ S.E.M. Data was analysed
3 using GraphPad PRISM® software (ver 6.01) with unpaired student's t-
4 test (parametric, two-tailed) for comparing two groups or one-way ANOVA for
5 comparing more than 2 groups (Dunnett's post hoc test) with a significance threshold
6 of $p < 0.05$.

3. Results:

3.1 Effects of CPE on cell viability:

To investigate the effects of CPE on BRIN-BD11 cell viability, cells were incubated with various concentrations of CPE between 0.39 – 50 µg/ml for 24-48h. High concentrations of CPE (25 & 50µg/ml) caused significant reductions in cell viability ($P<0.0001$) both at 24 and 48h (Fig. 1 A&B). However, lower concentrations of CPE (12.5 and 6.25 µg/ml) enhanced BRIN-BD11 cell viability ($P<0.01$ & $P<0.05$, respectively) following 24h treatment whereas other concentrations had no significant effects (Fig. 1A). Treatment with CPE for 48h caused significant ($P<0.0001$) increases in BRIN-BD11 cell viability at all concentrations between 0.39 and 12.5 µg/ml (Fig. 1B).

In GLUTag cells, CPE at 50µg/ml and above reduced cell viability significantly ($P<0.0001$) over 24h treatment and did not show any improvement over 48h. We observed a significant increase in cell viability ($P<0.01$) at concentrations of 3.125 and 6.25 µg/ml at 24 h (Fig. 1C). CPE treatment for 48h at 12.5µg/ml slightly enhanced viability ($P<0.05$) and concentrations between 1.56 and 6.25 µg/ml also increased cell viability ($P<0.001$) (Fig. 1D).

2 **Figure 1: Effects of CPE on BRIN-BD11 and GLUTag cell viability.** (A) and (B), Effects of CPE at
3 concentrations between 50- 0.39 $\mu\text{g/ml}$ on BRIN-BD11 cell viability over 24 h (A) and 48h
4 (B) treatment. (C) and (D), Effects of CPE at concentrations between 200- 0.78 $\mu\text{g/ml}$ on GLUTag cell
5 viability over 24 h (C) and 48h (D) treatment. Plotted as % change in cell viability compared to control
6 (vehicle control). Values represent mean $\pm$ S.E.M. from four different experiments conducted in
7 duplicates (n=4). Data analysed by one-way ANOVA with Dunnett's posthoc test. *P<0.05; **P<0.01;
8 ***P<0.001; ****P<0.0001, compared to control (no CPE addition).

3.2 Cytoprotective effects of CPE against palmitate and cytokine induced toxicity in BRIN-BD11 cells: As low concentrations of CPE enhanced cell viability over 24h, the potential protective effects of CPE on BRIN-BD11 cell viability against detrimental concentrations of palmitate (125& 250 μ M) and a cytokine cocktail containing IL-1 β (50U), TNF- α (1000U) and IFN- γ (1000U) were tested. Exposure of BRIN-BD11 cells for 24 h to palmitate (125 and 250 μ M) reduced cell viability significantly by 28.8% and 16.7% respectively. Concentrations of CPE between 3.125 and 12.5 μ g/ml showed significant ($P<0.0001$) protective effects against palmitate (Fig. 2A). CPE treatment (3.125 - 12.5 μ g/ml) over 24h also significantly protected against the cytotoxic effects of the pro-inflammatory cytokine cocktail in BRIN-BD11 cells (Fig. 2B).

Figure 2: Cytoprotective effects of CPE on palmitate and cytokines induced toxicity in BRIN-BDE11 cells. Protective effects of CPE against detrimental effects of palmitate (A) and cytokine cocktail (B) on BRIN-BD11 cell viability. Cells were cultured with palmitate or cytokine cocktail containing IL-1 β (50U), TNF- α (1000U) and IFN- γ (1000U) either alone or in combination with CPE for 24 h prior to cell viability measurement by MTT assay. ** $P<0.01$; **** $P<0.0001$ compared to 125 μ M palmitate or cytokine cocktail treatment alone; $\Delta P<0.05$, $\Delta\Delta\Delta\Delta P<0.0001$ compared to 250 μ M palmitate alone, $\Psi\Psi\Psi\Psi P<0.0001$, compared to untreated vehicle control. Values represent mean $\pm$ S.E.M. from four different experiments conducted in duplicates ($n=4$). Data analysed by one-way ANOVA with Dunnett's posthoc test.

3.3 Effects of CPE on antioxidant gene expression levels: As CPE treatment protected the BRIN-BD11 cells against palmitate and proinflammatory cytokine-induced toxicity; we decided to examine the antioxidant gene expression levels following 24-48h treatment of the cells with 6.25µg/ml CPE. First, Nrf2 expression along with ROS scavenging enzymes Sod1, Sod2, Cat and Gpx1 was investigated. Nrf2 (Fig. 3A) was upregulated significantly at both 24h and 48h along with Cat expression (Fig. 3B). Gpx1 expression was not altered at 24h but was upregulated at 48h (Fig. 3B), while Sod1 and Sod2 levels were unchanged (Fig. 3B). Proinflammatory cytokine-induced apoptosis is mediated through Nfkb activation, so expression of Nfkb1 and Nfkb2 was examined. At 24h, both Nfkb1 and Nfkb2 were downregulated (Fig. 3A), whereas no significant changes were observed following 48h (Fig. 3A). Activation of mitogen-activated protein kinases Erk1/2 and Jnk1 are associated with β -cell proliferation. As, 24 and 48h CPE treatment enhanced BRIN-BD11 cell viability, the expression of these genes along with their associated proto-oncogenes cJun and Myc were examined. Erk1 was upregulated at both 24h and 48h whereas Erk2 expression was unchanged (Fig. 3C). Myc and cJun were downregulated by 24 h CPE treatment (Fig. 3A and 3C), whereas Jnk1 and cJun were upregulated at 48h (Fig. 3C). To determine the effects of CPE on insulin, we checked expression of Ins1 and insulin gene transcriptional activators Pdx1 and Mafa along with β -cell glucose sensor, Gck. At 24h, CPE did not alter expression of these genes, however, interestingly at 48h all four were downregulated (Fig. 3D).

Figure 3: Effect of 6.25µg/ml CPE over 24-48h on BRIN-BD11 (A, B, C and D) and GLUTag (E) cell mRNA levels. Data represent fold change in mRNA levels compared to vehicle control/untreated BRIN-BD11/GLUTag cells and normalised to β -actin expression. Values represent mean $\pm$ S.E.M. from three different experiments performed in duplicates (n=3). Student's t test was used for statistical analysis. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared to their respective 24h/48h control/untreated expression.

As proglucagon (Gcg) and Pcsk1 are necessary to produce GLP-1 in L-cells, we examined effects of CPE treatment on expression of these genes. GLUTag cells treated with 6.25µg/ml CPE showed reduced expression levels of Gcg at both 24 and 48h, whereas Pcsk1 expression was downregulated at 24h but unchanged at 48h compared to controls (Fig. 3E). Contrary to pancreatic β -cells, Sglt1 plays a major role in glucose transport in gut L-cells. As shown in Fig. 3E, CPE treatment did not alter the expression of Sglt1 nor the glucose sensor Gck. Nfkb1 which was downregulated by CPE in BRIN-BD11 cells was unchanged in GLUTag cells following both 24h and 48h CPE treatment (Fig. 3E).

3.4 Effects of CPE on protein levels: As CPE treatment in BRIN-BD11 cells downregulated Ins1 mRNA along with its associated Pdx1 and Mafa expression, we decided to check Pdx1 protein levels. Furthermore, expression of Pcsk1 and Pcsk2, which are necessary for insulin post translational processing, were also studied. CPE (6.25µg/ml) treatment for 24h significantly downregulated protein levels of Pcsk1, Pcsk2 and Pdx1 levels in BRIN-BD11 cells (Fig. 4 A & B).

In L-cells, posttranslational modifications of proglucagon by Pcsk1 produce GLP-1 whereas Pcsk2 promotes glucagon production. In GLUTag cells, both Pcsk1 and Pcsk2 protein levels were unchanged following 24h treatment with 6.25µg/ml CPE (Fig. 4 C & D). Expression of Nfkb p65 (a Nfkb1 associated protein) was significantly reduced in CPE treated GLUTag cells (Fig. 4 C& D).

Figure 4: Effects of 6.25µg/ml CPE over 24h treatment on BRIN-BD11 (A and B) and GLUTag (C and D) translational changes; Data plotted as % change in relative density of CPE treated compared to untreated/vehicle control and normalised to actin expression. Values represent mean $\pm$ S.E.M. (n=4 for BRIN-BD11 or n=3 for GLUTag). Student's t test was used for statistical analysis. *P<0.05, **P<0.01, ****P<0.0001.

3.5 Effects of CPE on GLP-1 Content: As gene expression studies showed a significant reduction in Gcg and Pcsk1 expression, we checked staining intensity to investigate GLP-1 cellular content after 24h (6.25µg/ml) CPE treatment in GLUTag cells. Quantification of immunofluorescence images showed no significant changes in GLP-1 staining intensity between control and CPE treated cells (Fig. 5).

Figure 5: Effects of CPE treatment on GLP-1 content in GLUTag cells. GLUTag cells treated with 6.25µg/ml CPE for 24h followed by staining with anti-GLP-1 (red) and nuclear stain DAPI (blue). Fluorescence from vehicle control and CPE treated cells was measured (60 cells per coverslip from 3 separate experiments) and data was plotted as mean ±S.E.M. fluorescence intensity (n=3). Student's t test was used for statistical analysis (ns, no significance).

3.6 Effects of CPE on insulin and GLP-1 secretion: To determine potential acute effects of CPE on insulin and GLP-1 secretion from BRIN-BD11 and GLUTag cells, respectively, the cells were incubated with various concentrations of CPE (6.25, 12.5 & 25 µg/ml for BRIN-BD11 and 25 & 50 µg/ml for GLUTag cells) in KRB buffer over 1h at both low (1.1mM) and high (16.7mM) glucose concentrations. In BRIN-BD11 cells, CPE stimulated insulin secretion significantly by 1.6-1.7-fold at low and 1.3-1.6-fold at high glucose concentrations, although high glucose failed to increase insulin secretion from these cells. CPE stimulated GLP-1 secretion by 2.9 - 6.4-fold at low (1.1 mM) glucose and by 3.8 – 16.3-fold at high (16.7 mM) glucose as shown in Figure 6. Unexpectedly, high glucose had no effect on GLP-1 secretion compared to low glucose. However, secretory effects of 50 µg/ml CPE at high glucose was significantly greater than the respective effects at low glucose.

Figure 6: Effects of CPE on insulin/GLP-1 secretion from BRIN-BD11/GLUTag cells. Insulin and GLP-1 secretions were measured in the presence of various concentrations of CPE at low (1.1mM) (Black) or high (16.7mM) (dotted) glucose concentration. Data plotted as concentration of Insulin/GLP-1 secreted from 2.5×10^5 cells per hour. Values plotted as $\pm$ S.E.M. from 3 independent experiments conducted in duplicates ($n=3$). Student's t test was used for statistical analysis. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$ compared to their respective 1.1/16.7 mM glucose control. $\Delta\Delta P<0.01$, compared to respective 1.1 mM glucose.

4. Discussion:

Inflammatory cytokines and ROS are known contributors of β -cells dysfunction and apoptosis, leading to a decline in insulin output and promoting hyperglycaemia (Cnop et al., 2005). Under physiological conditions, elevated circulating glucose levels result in glucose uptake into β -cells via GLUT transporters, glucose phosphorylation by glucokinase (Gck) and generation of pyruvate which undergoes mitochondrial metabolism to produce ATP. The change in the ATP/ADP ratio closes ATP sensitive K^+ channels to depolarise the plasma membrane thereby enabling an increase in cytoplasmic Ca^{2+} $[Ca^{2+}]_c$ through the opening of voltage-gated calcium channels (Klemen et al., 2017). Increased $[Ca^{2+}]_c$ is essential to drive insulin granule docking and exocytosis. However, mitochondrial metabolism of glucose and $[Ca^{2+}]_c$, also produce reactive oxygen species such as hydroxyl and superoxide radicals (Starkov et al., 2002). Superoxide dismutase (Sod) isoforms convert these free radicals into hydrogen peroxide which is converted to oxygen and water molecule by enzymes catalase (Cat) and glutathione peroxidase (Gpx). In rat islets and insulinoma cell lines RINm5F, it is previously reported that expression of Sod1 is less than 50% compared to expression in the liver, whereas Cat and Gpx expression are even lower at less than 10% compared to the liver (Tiedge et al., 1997). A more recent study in human islets demonstrated that β -cells express significantly low levels of GPX and Catalase compared to α -cells indicating β -cells are more susceptible to oxidative stress (Miki et al., 2018). In previous studies, CP methanolic extract treatment in fructose-fed Wistar rats (to mimic the inflammation) caused significant increases in circulating Gpx levels, although, β -cell specific effects of CPE are unclear (Ashwini et al., 2015). Similar to these results, the current study supports the potential for CPE to significantly elevate Gpx mRNA levels as well as catalase

1 levels in BRIN-BD11 cells. Thus, CPE may be beneficial for the protection of β -cells,
2 but further studies in primary β -cells and islets are required.

3 Nrf2 is a master regulator of antioxidant gene transcription in response to oxidative
4 stress, and when associated with its regulatory protein, Keap1, is steadily degraded
5 in a keap-1-dependent manner through the ubiquitin-proteasome pathway (Taguchi
6 et al., 2011). Upon activation by stress signals, Nrf2 is released from Keap-1 and
7 translocate to the nucleus to enable upscaling of cellular defence by promoting
8 antioxidant gene transcription, including Sod1, Cat, Gpx1 and Ho1. This requires
9 Nrf2 heterodimerisation with small Maf proteins, which allow binding to the promoter
10 region of the antioxidant response element (ARE) (Itoh et al., 2004; Zhu et al., 2005).
11 Nrf2 activation protects β -cells against fatty acid-induced oxidative stress and
12 promotes self-repair (Abebe et al., 2017; Yagishita et al., 2014). In line with our
13 observation of increased Cat and Gpx1, longer-term (24-48h) CPE treatment of the
14 BRIN-BD11 cells also caused increases in Nrf2 expression. Interestingly, we noticed
15 an increase in Sod1 expression, but it was not significant. Perhaps, changes in Sod1
16 expression may have occurred earlier than 24h, which could possibly be the reason
17 for lack of significant change observed in the current study. Investigation into
18 changes throughout the initial 24h treatment may shed further light on changes in
19 antioxidant profiles within these cells.

20 Pancreatic islet inflammation is a trademark of Type 1 Diabetes Mellitus, and it is
21 also observed in T2DM. Inflammation caused by increased cytokine levels and
22 immune cells infiltration of islets is observed in both Type 1 and Type 2 diabetes and
23 in animal models of diabetes (Ehses et al., 2007). Proinflammatory cytokines IL-1 β ,
24 TNF- α and IFN- γ are linked to β -cell apoptosis in T2DM. IL-1 β is produced by

1 activated macrophages and also by β -cells themselves under high glucose
2 conditions (Maedler et al., 2002). TNF- α and IFN- γ are secreted by macrophages
3 and T-helper cells, respectively. *In vitro* studies have shown that IL-1 β alone and in
4 combination with TNF- α and IFN- γ , reduces insulin secretion and induces β -cell
5 apoptosis (Keane et al., 2015). These cytokines, by signalling through their specific
6 receptors, promote proinflammatory transcription factor NF κ B activation by
7 phosphorylation of inhibitor of κ B (I κ B) (Karin, 1999). Cytokine activated NF κ B
8 translocate to the nucleus, binds to DNA and promote proapoptotic gene
9 transcriptions (Heimberg et al., 2001). Previous studies in isolated rat β -cells
10 reported that inhibiting the NF κ B activation showed a protective effect against
11 cytokines induced apoptosis (Heimberg et al., 2001). In our studies, CPE treatment
12 in BRIN-BD11 cells protected against cytokines induced reduction in cell viability,
13 with notable downregulation of NF κ B1 & 2 expression levels which may contribute to
14 protective effects of CPE against cytokines. Elevated levels of FFA are observed in
15 obesity which is associated with an increased risk for the development of T2DM.
16 Chronic exposure to saturated FFAs, like palmitate, lead to reduced GSIS, a
17 reduction in insulin biosynthesis and increased β -cell apoptosis (Hagman et al.,
18 2005). It is known that palmitate-induced apoptosis is linked to ER stress and
19 mitochondrial dysfunction due to increased ROS production and inflammation
20 (Karaskov et al., 2006; Yang and Li, 2012). Consistent with these results, in the
21 current study, chronic exposure of BRIN-BD11 cells to palmitate (24h), reduced cell
22 viability. Since CPE offered some degree of protection against the actions of
23 palmitate, this could be attributed to its ability to enhance Nrf2-mediated antioxidant
24 and anti-inflammatory properties and also through reducing NF κ B expression.

1 Pdx1, NeuroD1 and Mafa transcription factors are necessary for defining β -cell
2 phenotype and enabling insulin gene transcription and secretion in response to
3 elevated glucose levels (Andrali et al., 2008). In response to high glucose, Pdx1 and
4 NeuroD1 are translocated to the nucleus along with Mafa where they bind to the
5 insulin promoter and driving insulin gene transcription (Andrali et al., 2008).
6 Posttranslational changes by prohormone convertases 1 & 2 (Pc1/Pcsk1 &
7 Pc2/Pcsk2) convert proinsulin into insulin and c-peptide (Itoh et al., 1996).
8 Unexpectedly, CPE treatment of BRIN-BD11 cells for 48h reduced Ins1, Pdx1 and
9 Mafa gene expression, while Pdx1, Pcsk1 and Pcsk2 protein levels were also
10 reduced at 24h. Numerous studies have confirmed the important role of Erk1/2 and
11 Jnk1/c-Jun pathways in enhancing cell proliferation (Ma et al., 2012; Mebratu and
12 Tesfaigzi, 2009; Prause et al., 2014). Interestingly, proliferating β -cells are prone to
13 immaturity and often reduced expression of Pdx1 along with insulin gene expression
14 which is mediated by increased expression of cMyc and cJun (Henderson and Stein,
15 1994; Puri et al., 2018). Since 48h CPE treatment enhances Erk1, cJun and Jnk1
16 expression, along with enhanced cell viability, this might be related to the reduction
17 in Pdx1, Ins1 and Mafa expression, in compromise for enhancing cell proliferation.
18 Further studies into the proliferative effects of CPE in β -cells using specific markers
19 of proliferation such as Ki67 are warranted. Our data suggests that CPE stimulates
20 insulin secretion acutely at both low and high glucose concentrations. Unexpectedly,
21 we did not observe any changes in glucose stimulated insulin secretion from these
22 cells, a limitation of *in vitro* culture, however the actions of CPE in primary cells
23 would provide more clarity in regards to the actions of CPE at low and high glucose
24 concentrations. In previous studies, methanolic extract of CP (100 μ g/ml) stimulated
25 insulin secretion from MIN-6 cell lines and human islets (Al-Romaiyan et al., 2010).

1 In our study, lower concentrations of ethanolic extract (6.25, 12.5 and 25µg/ml)
2 stimulated insulin secretion from BRIN-BD11 cells, although further studies are
3 needed to investigate these effects in pancreatic islets.

4 The incretin hormone GLP-1 is secreted by enteroendocrine L-cells in response to
5 nutrients after meal ingestion. Although GLP-1 secreting L-cells are mostly
6 concentrated in the ileum, they are distributed throughout the small intestine
7 (Mortensen et al., 2003) and large intestine. The proglucagon (Gcg) gene is
8 expressed in both gut L-cells and within the pancreatic α -cells and yields a 180
9 amino acid precursor protein. Although both Pcsk1 and Pcsk2 are expressed in gut
10 L-cells, posttranslational changes in the L-cells by Pcsk1 produces GLP-1, whereas
11 in the α -cells Pcsk2 predominates and yields glucagon (Rouillé et al., 1995). Our
12 data suggest that CPE treatment in GLUTag cells acutely enhances GLP-1 secretion
13 at high concentrations (25 & 50µg/ml). Although, 25µg/ml has no effect on cell
14 viability and 50µg/ml is cytotoxic over long-term 24-48h treatment, acute secretion
15 studies were conducted over 1h treatment, thus high concentration-induced
16 cytotoxicity could have minimal or no effect on secretion. Further pharmacological
17 investigation into the acute CPE versus chronic effects of CPE treatment on L-cell
18 function are required. Although, current results suggest that CPE stimulate GLP-1
19 secretion from GLUTag cells, further studies into the in vivo effects of CPE on
20 incretin biology are warranted. Over 24h CPE treatment significantly reduced
21 proglucagon and Pcsk1 gene expression levels whereas both Pcsk1 and Pcsk2
22 protein expression were unchanged along with cellular GLP-1 staining intensity.
23 Although it is unexpected, long term exposure to GLP-1 secretagogue reducing
24 proglucagon expression is not entirely new, with previous reports that 24h exposure
25 of GLUTag cells to ghrelin reduced proglucagon expression whereas acutely ghrelin

1 stimulated GLP-1 secretion (Lindqvist et al., 2017). Furthermore, perhaps it may be
2 possible that similar to pancreatic β -cells, L-cell proliferation causes a reduction in
3 secretory function.

4 It has previously been reported in *in vitro* studies that, the GLUTag L-cell model is
5 prone to gluco- and lipotoxicity in a similar manner to pancreatic β -cells, although
6 whether such effects occur *in vivo* has yet to be established (Vasu et al., 2015). A
7 study by Filipello et al (2018) reported that palmitate treatment in GLUTag cells
8 caused a significant increase in proglucagon and Pcsk2 expression, with a switch in
9 protein processing result in glucagon secretion instead of GLP-1. In the present
10 study, we noted that the treatment of GLUTag cells with low concentrations of CPE
11 enhanced cell viability and reduced proinflammatory transcription factor NF κ B
12 associated NF κ Bp65 protein levels. Thus, CPE may have a protective effect against
13 inflammation in L-cells similar to our observations in BRIN-BD11 cells; however, this
14 requires further investigation as the current study did not investigate cytoprotective
15 effects of CPE against palmitate and cytokines in GLUTag cells.

16 Although BRIN-BD11 and GLUTag cell lines are established and widely used *in vitro*
17 models, like any other cell line, these cells have some limitations such as lack of
18 glucose responses. Thus, current results need to be further validated in primary cells
19 and also it would be necessary to evaluate role of CPE metabolism on its anti-
20 diabetic effects *in vivo*. In a previous study by Ashwini et. al, (2015), methanolic
21 extract of CP was used to investigate its chemical composition and anti-diabetic
22 effects. While in the current study we used ethanolic extract, although it showed
23 significant beneficial effects in β -cells and L-cells, further studies are needed to

1 clarify the bioactive constituents within the ethanolic extract and to compare the
2 bioactive composition in relation to other chemical extraction methods.

3 **5. Conclusion:**

4 In conclusion, we have confirmed that acutely CPE stimulates GLP-1 secretion from
5 GLUTag L-cells, and this might be partly responsible for its anti-diabetic properties.
6 However, studies into the *in vivo* effects of CPE on GLP-1 and insulin secretion are
7 warranted. CPE also enables insulin secretion and cytoprotection of BRIN-BD11 β -
8 cells by stimulating multiple antioxidant pathways, which may protect them against
9 metabolic stress. Further studies are needed to confirm the long-term effects of CPE
10 both *in vitro* and *in vivo*, on both β - and L-cell secretory gene expression and
11 function.

1 **Author Contributions:**

2 Chinmai Patibandla and Steven Patterson designed experiments. Chinmai
3 Patibandla performed and analysed experiments. MD Zahidul Islam Khan, Louise
4 MacGregor and Mark James Campbell repeated experiments. Chinmai Patibandla
5 and Steven Patterson prepared and revised the manuscript.

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9 **Conflict of interest:**

10 The authors declare no conflict of interest.

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