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# *Costus Pictus* Extracts Stimulate Insulin Secretion from Mouse and Human Islets of Langerhans *In Vitro*

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**Key Words**

Diabetes • Insulin stimulation • Plant-derived secretagogues

**Abstract**

Plant-derived extracts have been used as folk remedies for Type 2 diabetes mellitus (T2DM) for many centuries, and offer the potential of cheap and readily available alternatives to conventional pharmaceuticals in developing countries. Extracts of *Costus pictus* (CP), a plant belonging to the Costaceae family, are reported to have antidiabetic activity *in vivo*. The exact molecular mode of action(s) of CP is unclear but the antihyperglycemic effect seen in animal studies was associated with dramatic increases in insulin secretion so in our study we have measured the effect of aqueous CP extract on insulin secretion *in vitro* from the MIN6 β-cell line and isolated mouse and human islets. Our data demonstrate that CP has a direct stimulatory effect on insulin secretion at basal but not stimulatory glucose concentrations which was not associated with compromised membrane integrity or decrease β-cell viability. Single cell calcium microfluorimetry measurements showed that CP caused elevations in β-cell intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$), an effect which was completely abolished by the removal of extracellular $Ca^{2+}$ or blockade of voltage-gated $Ca^{2+}$ channels (VGCC). These *in vitro* observations suggest that one mode of action of CP is through stimulating insulin secretion which may be mediated, in part, by the ability of CP to increase $[Ca^{2+}]_i$ levels through VGCC. CP extracts may provide an affordable and inexpensive alternative for treating patients with T2DM.



## Introduction

Diabetes mellitus is a global health problem affecting both developed and developing countries. It is defined as a metabolic disorder characterized by an elevation of blood glucose concentration leading to chronic hyperglycemia

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and is associated with abnormal metabolism of carbohydrates, fat and protein. The prevalence of the disease is increasing and the estimated number of people with diabetes will be 440 million by the year 2030, more than double the current estimate [1]. Ninety-five percent of those individuals will have Type 2 diabetes mellitus (T2DM) which is characterized by β-cell dysfunction and insulin resistance. Untreated or poorly controlled T2DM greatly increases the risk of developing macrovascular and microvascular complications, involving many different organs of the body [2]. Macrovascular complications, which involve large vessels, can lead to coronary heart disease, stroke and peripheral vascular disease and it has been shown that such complications are responsible for about 70-80% of the mortality among patients with T2DM. Microvascular complications, which affect small arteries and arterioles, develop as a result of hyperglycemia and may lead to neuropathy, retinopathy and nephropathy, which are the leading causes of lower extremity amputations, blindness and end-stage renal disease, respectively [3-4].

In the past few years the pharmacotherapy of T2DM has changed dramatically with the addition of new classes of drug to achieve tighter glycemic control to reduce the risk of developing microvascular and macrovascular complications and to improve the quality of patients' lives. Despite the introduction of new chemical classes of drugs and the effort spent on drug development, the appropriate glycemic control in most patients with T2DM is still under target and may require intensive multiple drug therapy, increasing the risk of developing adverse drug effects and drug-drug interactions [5].

The use of herbal medicines for the treatment of diabetes mellitus has been known for centuries [6]. Many plants have reported efficacy in the treatment of T2DM [7], and recently extracts of *Costus pictus* (CP) have been reported to show antidiabetic properties. The plant, commonly known as spiral ginger, belongs to the Costaceae family and grows in gardens as an ornamental climbing plant [8-9]. The antidiabetic activity of a CP extract has been tested chronically in animals *in vivo*, where it was reported to reduce blood glucose levels in rats in which hyperglycemia had been induced by administering the β-cell toxins alloxan or streptozotocin (STZ) [10-12]. The precise mechanism of the glucose-lowering effect of CP is not completely clear but it may be due to 1) an inhibition of α-amylase and α-glucosidase enzymes resulting in reduced carbohydrate absorption [13]; 2) an increase in GLUT4 translocation and glucose uptake in insulin-responsive target tissues [14]; or 3) a direct stimulation of insulin secretion from pancreatic β-cells [12].

The aim of this study was, therefore, to determine whether an aqueous extract of CP acutely and directly stimulates insulin secretion from the mouse MIN6 β-cell line and from primary mouse and human islets. Many insulin secretagogues initiate insulin secretion by increasing cytosolic $Ca^{2+}$ ($[Ca^{2+}]_i$) through facilitating extracellular $Ca^{2+}$ entry into β-cells via voltage-gated $Ca^{2+}$ channels (VGCC) in the plasma membrane, so we have also examined the dependence of CP-induced insulin secretion on $Ca^{2+}$.

## Materials and Methods

*Materials*

Insulin-secreting MIN6 cells were kindly provided by Prof J I Miyazaki (University of Tokyo, Japan). GS4 (*Gymnema sylvestre* 4) was prepared as described previously [15]. $Na^{125}I$ for insulin iodination was from Perkin Elmer (Bucks, UK). All consumables were obtained from Sigma Aldrich (Dorset, UK).

*Methods*

*Plant material and preparation.* CP leaves were collected from Kerala Agricultural University Mannuthy and a voucher specimen is deposited in the VIT University herbal garden, Vellore, Tamil Nadu (VIT/CP/G1). The leaves were processed as described previously [16]. Briefly, shade dried plant material was ground into powder and an extract was prepared by successive maceration of the powder (10 g) at room temperature with methanol for 48h in a shaker. The final extract was filtered and the filtrate was lyophilized to obtain a powdered extract. The extract was stored as a 100 mg/ml stock and diluted as appropriate in a physiological salt solution containing in mM: 111 NaCl, 27 $NaHCO_3$, 5.0 KCl, 1.0 $MgCl_2.6H_2O$, 0.28 $MgSO_4.7H_2O$ and 0.22 $KH_2PO_4$ [17] for use in experiments. The yield of dry extract as a percentage weight of the starting fresh leaves of CP was 1.42%.

*Maintenance of MIN6 cells.* MIN6 cells (passage 32-39) were maintained as monolayers at 37°C (95% air/ 5% $CO_2$) in DMEM supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine and 100 U/ml penicillin with 0.1 mg/ml streptomycin. MIN6 cells grow as adherent monolayers on negatively-charged tissue culture plastic. The medium was changed every 3-4 days and when the cell confluency reached ~70-80% the MIN6 cells were detached for use in experiments by incubation with 0.1% trypsin/0.02% EDTA.

*Formation of MIN6 Pseudoislets (PIs).* MIN6 cells were cultured in bacterial Petri dishes to prevent the adhesion of the cells to the substrate, resulting in the formation of three dimensional islet-like cell clusters known as pseudoislets (PIs) which show greatly enhanced insulin secretory responses over MIN6 cells maintained as monolayers [18]. PIs were maintained in DMEM supplemented with 10% FCS, 2mM L-glutamine and 100 U/ml penicillin with 0.1 mg/ml streptomycin at 37°C (95%

air/ 5% $CO_2$). The medium was changed every 3-4 days and PIs were used in the experiments after 7-10 days.

*Isolation of mouse and human islets.* The pancreata of outbred albino ICR mice were digested by collagenase as described previously [19]. The islets were purified on Histopaque density gradients and cultured in RPMI medium supplemented with 10% FCS and 100 U/ml penicillin/0.1 mg/ml streptomycin. Human islets were aseptically isolated from pancreata of non-diabetic heart-beating organ donors at the Human Islet Transplantation Unit at King's College Hospital, with appropriate ethical approval [20] and maintained in CMRL medium supplemented with 10% FCS, 2mM glutamate and 100 U/ml penicillin/0.1 mg/ml streptomycin.

*Insulin secretion*

*Insulin secretion from MIN6 monolayer cells.* MIN6 cells were seeded into 96-well plates at a density of 30,000 cells/well and maintained as monolayers in DMEM under standard tissue culture conditions for 1-2 days. For experimental use, MIN6 cells were pre-incubated in physiological salt solution supplemented with 2mM glucose, 2mM $CaCl_2$ and 0.5 mg/ml BSA at 37°C for 120 min. The cells were then incubated with buffer containing 2mM glucose in the presence or absence of CP extract (0.06-1.0 mg/ml) for 30 min at 37°C. At the end of the incubation period, 100µl of incubation medium was removed and stored at -20°C until insulin was measured by RIA [21].

*Insulin secretion from MIN6 pseudoislets, isolated mouse and human islets.* To measure the time-course of the effects of CP extract on insulin secretion, perifusion experiments using MIN6 PIs, isolated mouse and human islets were performed [22]. PIs or islets were transferred into chambers lined with 1 µm pore-size nylon filter and perifused with physiological buffer supplemented with 2mM glucose, 2mM $CaCl_2$ and 0.5 mg/ml BSA, in a temperature-controlled (37°C) environment. At the start of each experiment the PIs or islets were perifused for 60 min at a flow rate of 0.5 ml/min, during which time the perifusate was discarded. At t=60 min, the tissues were perifused with buffer supplemented with either 2mM or 20mM glucose in the presence or absence of 0.1 mg/ml of CP extract. Perifusate samples were collected every 2 min and stored at -20°C until insulin content was determined by RIA.

*Insulin content of mouse islets.* To investigate the chronic effect of CP on insulin content of mouse islets, 5 mouse islets were transferred into 1.5 ml Eppendorf tubes and incubated for 24hrs, under normal tissue culture conditions, with DMEM supplemented with 2mM glucose in the presence or absence of 0.1 mg/ml CP. Following 24hrs, the islets were washed with PBS, sonicated in acidified alcohol and stored at -20°C until assayed for insulin content by RIA.

*Cell viability.* The effects of CP on membrane integrity were assessed using a Trypan Blue exclusion test [15]. MIN6 monolayers that had been exposed to CP extract (1.0 mg/ml) for 30 min were incubated with 0.1% (w/v) Trypan Blue dye for 15 min at 37°C. The cells were then washed with phosphate buffered saline (PBS) and visualized under a light microscope. Images were captured using a Nikon Coolpix 4500 digital camera (Surrey, UK).

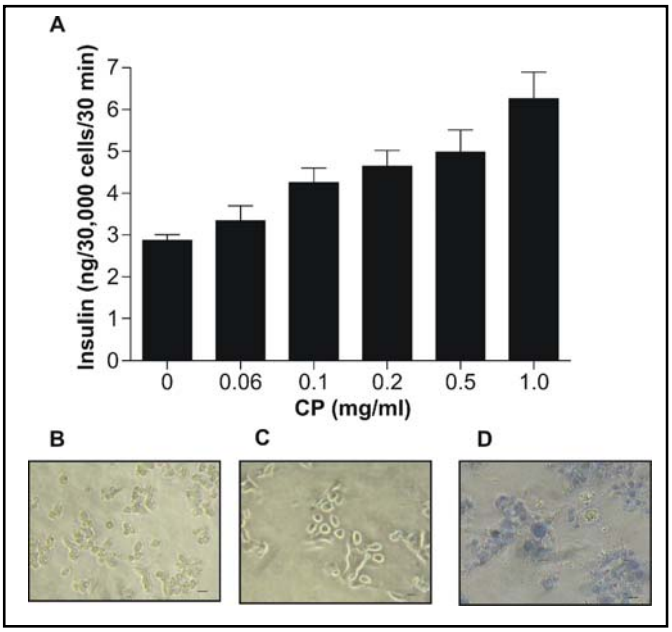
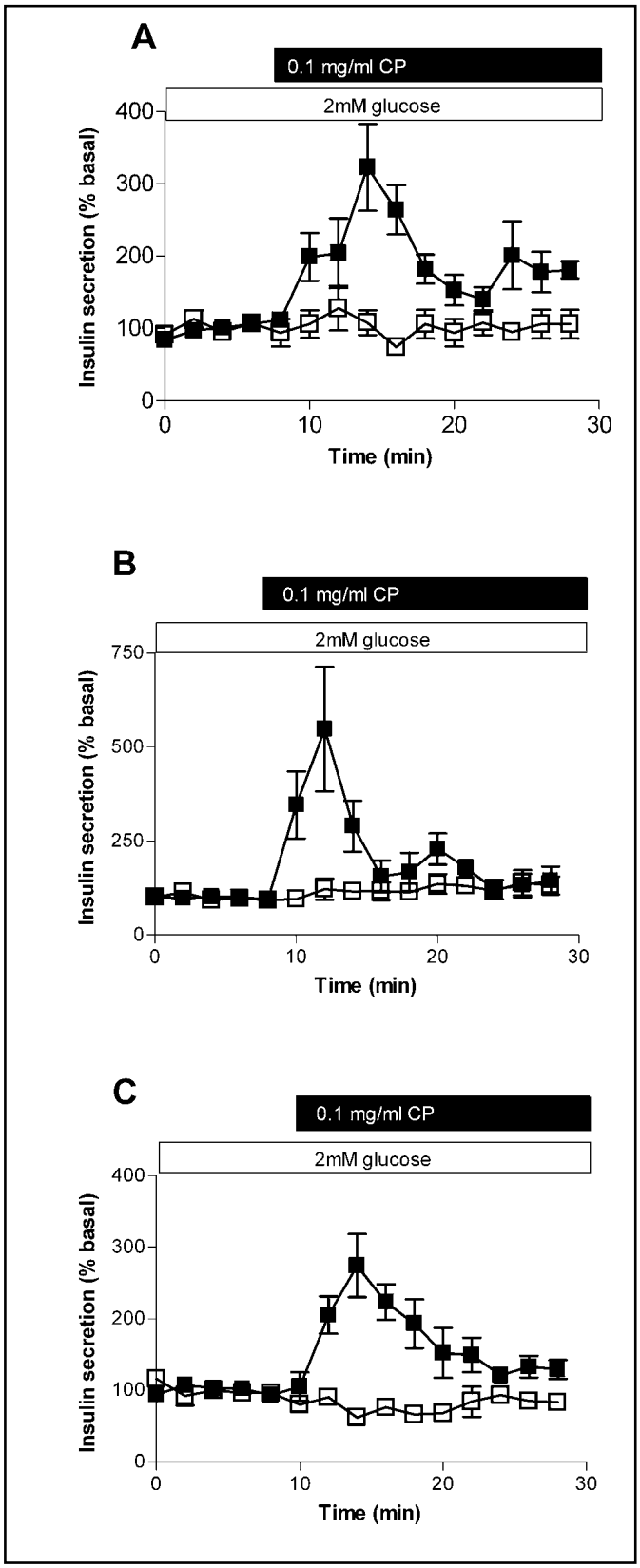



**Fig. 1.** Effect of CP extract on insulin secretion from MIN6 cells and MIN6 cell viability. (A) MIN6 cells were incubated for 30 min at 37 °C with CP extract (0.06-1.0 mg/ml) and insulin content of the supernatant was measured by RIA. CP extract induced a concentration-dependent increase ($p<0.01$) in insulin secretion. Data are means ± SEM, n=12 for each experimental treatment. In parallel, membrane integrity was measured by the Trypan Blue exclusion method. MIN6 cells were exposed to 1.0 mg/ml CP for 30 min after which they were incubated with 0.1% Trypan Blue dye in PBS at 37 °C for 15 min. The micrographs in panels B and C show MIN6 cells that had been incubated with 2mM glucose in the absence (B) or presence (C) of 1.0 mg/ml CP after staining with 0.1% (w/v) Trypan Blue dye. Panel D shows blue-stained MIN6 cells following 30 min incubation with 2mM glucose in the presence of GS4, a high saponin containing-extract. Bar shows 10µm.

*Calcium microfluorimetry.* The effects of CP extract on $[Ca^{2+}]_i$ were assessed using single cell calcium microfluorimetry as described previously [22]. Briefly, MIN6 cells were seeded on ethanol-washed glass coverslips at a density of 50,000 cells/ coverslip and allowed to adhere overnight in DMEM under standard tissue culture conditions. The cells were loaded with 5µM of the $Ca^{2+}$-fluorophore Fura-2/AM for 30 min at 37°C. The coverslips were placed in a steel chamber which was mounted into a heating platform (37°C) on the stage of an Axiovert 135 Research Inverted Microscope. The cells were perifused with 0.1 mg/ml CP in the presence or absence of ethylene glycol tetraacetic acid (EGTA: 0.1mM) or nifedipine (10µM), a voltage-gated $Ca^{2+}$ channel (VGCC) blocker, at a flow rate of 1 ml/min. Cells were illuminated alternately at 340 and 380nm, and emitted light at 510nm was detected using a CCD camera.

**Fig. 2.** Effect of CP extract (0.1 mg/ml) on insulin secretion from MIN6 PIs, primary mouse and human islets at a substimulatory glucose concentration. MIN6 PIs (A), mouse (B) or human (C) islets were perifused with physiological salt solution supplemented with either 0.1 mg/ml CP (■) or vehicle (□) in the presence of 2mM glucose. Samples were collected every 2 min and insulin content was measured by RIA. Insulin secretion data are expressed as % of basal (2mM glucose). Insulin secretion was significantly stimulated from all cell types in the presence of 0.1 mg/ml CP at 2mM glucose ($P<0.05$). Data show mean ± SEM, n=4 per each treatment.




*Data Analysis*

Data are represented as mean ± SEM. Differences between treatment groups were assessed using one way analysis of variance (ANOVA), Student's t-test, or Bonferroni's multiple comparison test as appropriate, and differences between treatments were considered significant at $P< 0.05$.

## Results

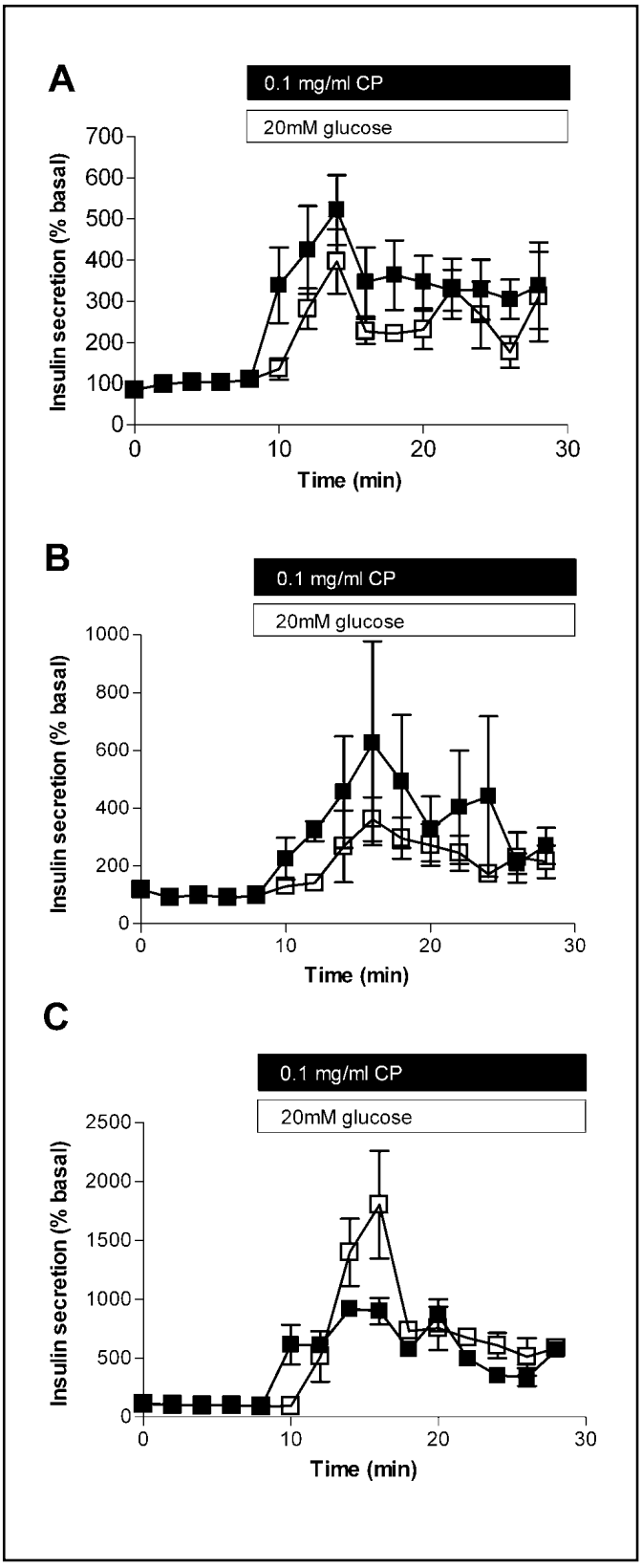
*Effects of CP extract on insulin secretion from MIN6 monolayers and MIN6 membrane integrity*

The effect of CP on insulin secretion from MIN6 cell monolayers at a basal concentration of glucose (2mM) is shown in Fig. 1A. Thirty min exposure of MIN6 cells to the CP extract (0.06-1.0 mg/ml) caused a concentration-dependent increase in insulin secretion ($P<0.01$) which could not be attributed to the extract causing non-specific leakage of insulin from the cells by damaging their plasma membranes. Thus, the effect of CP extract on membrane integrity was assessed using the Trypan Blue exclusion test. Incubating MIN6 cells with 0.1% (w/v) Trypan Blue dye for 15 min in the presence of CP extract (1.0mg/ml) was not accompanied by increased Trypan Blue uptake (Fig. 1 B, C). Fig. 1 D shows the effect of inducing membrane damage in MIN6 cells by incubating them in the presence of GS4, a plant extract with a high saponin content.

*Effects of CP extract on insulin secretion from MIN6 PIs, isolated mouse and human islets*

A multichannel, temperature-controlled perifusion system was used to examine the effects of CP extract on the pattern and rate of insulin secretion. MIN6 PIs, mouse and human islets were perifused with buffers supplemented with 0.1 mg/ml CP extract at glucose concentrations selected to mimic conditions of fasting blood glucose (2mM) and uncontrolled hyperglycemia (20mM). In the presence of 2mM glucose, the CP extract induced a rapid but transient increase in insulin secretion from MIN6 PIs, reaching a peak of 323 ± 60% basal ($p<0.01$) after 4-6 min (Fig. 2A). The rate of insulin secretion subsequently subsided to almost basal levels

**Fig. 3.** Effect of CP extract (0.1 mg/ml) on insulin secretion from MIN6 PIs, primary mouse and human islets at a stimulatory glucose concentration. MIN6 PIs (A), mouse (B) or human (C) islets were perifused with physiological salt solution supplemented with 20mM glucose in the presence ( ■ ) or absence ( □ ) of 0.1 mg/ml CP, as shown by the bars. Samples were collected every 2 min and insulin content was measured by RIA. Data are expressed as % of basal (2mM glucose). Insulin secretion was not significantly potentiated in the presence of 0.1 mg/ml CP extract at 20mM glucose (P>0.2). Data show mean ± SEM, n=4 per each treatment.




despite the continued presence of the CP extract. Similarly, CP-induced insulin secretion from perifused mouse (Fig. 2B) and human (Fig. 2C) islets was also rapid in onset but transient during the 20 min exposure to CP. The CP extract (0.1 mg/ml) did not potentiate glucose-induced (20mM) insulin release from MIN6 PIs or from mouse or human islets, as shown in Fig. 3. In these experiments exposing MIN6 PIs, mouse or human islets to 20mM glucose evoked a bi-phasic and maintained increase in insulin secretion. Addition of CP extract slightly elevated insulin release from MIN6 PIs and mouse islets over the first phase of glucose-induced insulin secretion but this effect was not statistically significant neither when assessing the rate of insulin secretion at individual time points, nor when comparing area under the curve estimates of the total mass of insulin secreted during the experiment.

*Effect of CP extract on insulin content of mouse islets*

Prolonged exposure of mouse islets to CP was not associated with any changes in islet insulin content. The total insulin content of islets incubated in the presence of 2mM glucose alone was 29.5 ± 5.5 ng/islet. Addition of CP did not cause any changes in islet insulin content (28.7 ± 2.1 ng/islet, p>0.2).

*Effects of CP extract on MIN6 cell $[Ca^{2+}]_i$*

Exposure to CP extract (0.1 mg/ml) at 2mM glucose was associated with an increase in $[Ca^{2+}]_i$ in Fura-2-loaded MIN6 cells (Fig. 4). The elevations in $[Ca^{2+}]_i$ levels were delayed in onset, with a lag time of approximately 3 min, consistent with the time-course of the effects of the extract on insulin secretion (Fig. 2). The CP-induced increase in $[Ca^{2+}]_i$ was completely abolished in the absence of extracellular $Ca^{2+}$ or in the presence of nifedipine. MIN6 cells that had been exposed to CP showed further elevation of $[Ca^{2+}]_i$ levels upon exposure to 100μM ATP (basal to peak amplitude: 0.3±0.01, p<0.01, 30/30 cells, data not shown).

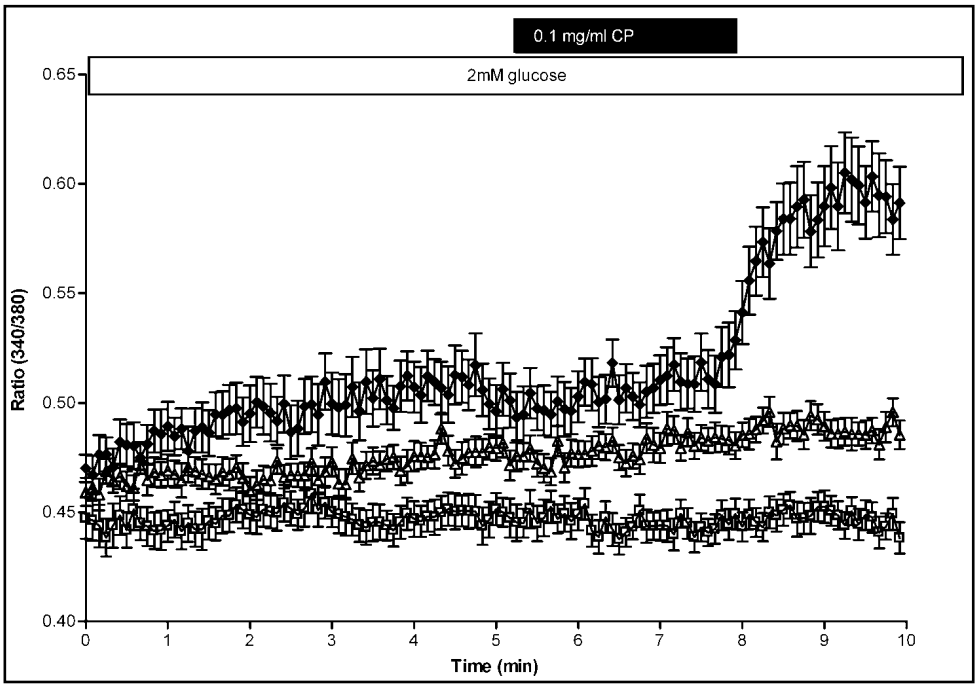



**Fig. 4.** Effect of CP extract on intracellular $Ca^{2+}$ levels in MIN6 β-cells. Fura-2-loaded MIN6 cells were perifused throughout with a buffer containing 2mM glucose and supplemented with 0.1 mg/ml CP extract for 3 min, as shown by the black bar, in the presence (◆) or absence (□) of extracellular $Ca^{2+}$ and in the presence of nifedipine (△). Changes in $[Ca^{2+}]_i$ were determined by single cell microfluorimetry and expressed as 340/380nm ratiometric data. CP significantly ($P<0.01$) elevated $[Ca^{2+}]_i$ which was completely abolished in the absence of extracellular $Ca^{2+}$ or in the presence of nifedipine ($P<0.01$). Data are mean ± SEM, n=30-34 cells for each experimental treatment. All observations are representative of at least two experiments.

## Discussion

Plant extracts have been used medicinally since antiquity for treating the symptoms of diabetes. One promising candidate is *Costus pictus* (CP), which has glucose-lowering activity in diabetic rats. The beneficial antidiabetic effect of CP seen in hyperglycemic rats was reported following chronic and prolonged treatment with the plant extract. Thus, 28 d oral administration of the same aqueous CP extract as used in the current study to hyperglycemic rats at 2 gm/kg body weight induced a significant ($p<0.001$) reduction in fasting blood glucose levels, associated with increased serum insulin levels [11]. Other studies have also reported similar results [10-12]. It is difficult to investigate cellular mechanisms of action using *in vivo* models, so in our current study we investigated the acute effect of a CP extract *in vitro* using MIN6 cells and primary mouse and human islets. MIN6 cells, which are a transformed insulin-secreting β-cell line derived from a mouse insulinoma, are a useful experimental model for studies of insulin secretion because, unlike other β-cell lines, they contain a relatively high insulin content [23-24].

Our preliminary data using MIN6 cells grown as adherent monolayers demonstrated that CP extract caused a concentration-dependent increase in insulin secretion, and that the extract was capable of initiating an insulin secretory response in the absence of a stimulatory concentration of glucose. In all our experiments, the basal glucose concentration was maintained at 2mM, a sub-stimulatory concentration of glucose, to eliminate any possible contribution of glucose to the secretory response. Plant extracts often contain high concentrations of chemicals such as triterpine saponins that can damage plasma membranes and thus increase their permeability, resulting in a non-specific and uncontrolled release of insulin from β-cells into the incubation medium [15]. However, the CP extract did not compromise plasma membrane viability even at a concentration as high as 1.0 mg/ml, suggesting that its effects on insulin secretion in our experiments were due to an activation of a regulated exocytotic response.

MIN6 cells grown as monolayers offer a model for rapid screening of potential insulin-releasing compounds but they are less responsive to secretagogues than cells which are aggregated into three-dimensional islet-like clusters (pseudoislets), probably because of enhanced β-cell to β-cell communication within the pseudoislet structures [18]. Therefore, for our subsequent perifusion measurements of the effects of CP extract on insulin secretion, the MIN6 cells were configured as pseudoislets to ensure maximum insulin secretory responses. Perifusion experiments have the additional advantages of measuring the rate and duration of insulin output, which facilitate the detection of relatively transient responses

of the extract. These experiments confirmed the observations obtained using monolayer cells that CP extract acutely initiated insulin secretion in the presence of a substimulatory concentration of glucose, and further revealed that the effect was transient. These observations are in accordance with the lack of effect of CP extract on plasma membrane permeability because such damage is associated with irreversible leakage of insulin from compromised cells. In addition, CP extract induced an insulin secretory response from primary mouse and human islets confirming that the extract had a direct stimulatory effect in primary β-cells. Furthermore, the direct stimulatory effect of CP extract on mouse islets was not associated with increases in insulin expression at either mRNA (data not shown) or protein levels, further supporting the results of our perifusion experiments which reported a transient effect of CP on insulin secretion.

The initiation of insulin secretion by CP could not be attributed to the presence of high $K^+$ levels in the plant extract since our trace element analysis of CP leaves using proton-induced X-ray emission indicated that the $K^+$ concentration was only 0.4±0.12μg/ml. Thus, CP leaf extracts contain, at most, micromolar concentrations of $K^+$ while millimolar concentrations are required to stimulate insulin secretion [25].

Although CP slightly potentiated the first, but not the second, phase of glucose-induced insulin secretion from MIN6 PIs and mouse islets, the increment in insulin secretion did not reach statistical significance. On the other hand, CP did not enhance insulin output over the bi-phasic glucose-induced insulin secretion from human islets. This may be a consequence of the human islets showing a robust glucose-induced secretory response (maximally 18 fold basal) and any small changes in insulin secretion that may have been caused by CP are likely to have been masked. A supra-physiological glucose concentration (20mM) was used in these experiments to preclude an action of the plant extracts through metabolism-induced generation of ATP, similar to the action of glucose. Our observations are in contrast to a recent report that aqueous CP extract stimulated insulin secretion above that induced by 20mM glucose [12]. The differences in reported effects of CP extracts most likely reflect differences in experimental protocols between studies. Our perifusion experiments were designed to look at rapid changes in the rate of insulin secretion over a minute-to-minute time scale, whereas the previous study [12] used a static incubation protocol which found stimulatory effects on glucose-induced insulin secretion after prolonged (24h) exposure to the extract.

Many physiological insulin secretagogues act by facilitating the influx of extracellular $Ca^{2+}$, or the release of $Ca^{2+}$ from intracellular stores leading to increased $[Ca^{2+}]_i$ and insulin exocytosis. Our measurements of $[Ca^{2+}]_i$ in Fura-2-loaded MIN6 cells demonstrated that the CP extract induced elevations in $[Ca^{2+}]_i$, consistent with its effects to initiate insulin secretion. The removal of extracellular $Ca^{2+}$ or blockade of VGCC by nifedipine completely inhibited the CP-induced increases in $[Ca^{2+}]_i$, suggesting that CP raised cytosolic $Ca^{2+}$ levels in β-cells by increasing $Ca^{2+}$ influx through VGCC. The ability of the cells which had been exposed to CP to further increase $[Ca^{2+}]_i$ in response to ATP further demonstrated that the extract did not impair the ability of the cells to maintain a membrane potential and $Ca^{2+}$ gradient across the plasma membrane, consistent with the Trypan Blue measurements of membrane integrity.

In summary, our *in vitro* studies suggest that an important mechanism of action of *Costus pictus* extract is through a direct effect on islet β-cells to stimulate insulin secretion. Our measurements of $[Ca^{2+}]_i$ suggest that CP initiates an insulin secretory response by increasing $Ca^{2+}$ influx through VGCC, consistent with a mode of action similar to that of other depolarizing agents such as sulphonylurea drugs. This ability to by-pass glucose metabolism and induce insulin secretion by depolarization-induced $Ca^{2+}$ influx offers a mechanism for increasing insulin secretion from glucose-unresponsive β-cells in T2DM. This is the first demonstration that CP induces insulin secretion from human islets of Langerhans, suggesting that CP may provide an affordable and inexpensive alternative for treating patients with T2DM in developing countries. Characterization of the active ingredient(s) in CP extracts offers an interesting area for further investigations.

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