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ANTI-DIABETIC AND ANTI- INFLAMMATORY EFFECTS OF MEDICINAL PLANTS IN A TYPE 2 DIABETIC ANIMAL MODEL

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Anti-diabetic and anti-inflammatory effects of medicinal plants in a type 2 diabetic animal model

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Dedicated to my family with love

ABSTRACT

Medicinal plants have been suggested since ancient times to be a good source of drugs for treating diabetes. The aims of this thesis were to identify the anti-diabetic effects and to understand further the mechanisms involved for two traditional plants, borapetol B (C1) isolated from *Tinospora crispa*, and *Gynostemma pentaphyllum* (*GP*), in diabetic rats. The effects of *GP* extract on innate immune mechanisms in the urinary tract were also investigated.

In **Paper I,** an oral administration of C1 significantly decreased blood glucose levels and increased plasma insulin levels in treated rats, compared to placebo groups, in both Wistar (W) and diabetic Goto Kakizaki (GK) rats. C1 dose-dependently increased insulin secretion from W and GK rat islets. In perifusions of W and GK rat islets, insulin secretion was increased by C1 in low and high glucose, and returned to basal levels when C1 was omitted, indicating that the compound did not cause leakage of insulin by damaging islet beta cells. This study provides evidence that C1 possesses anti-diabetic properties mainly due to its stimulation of insulin release.

In **Paper II,** when exploring the mechanisms of insulin release in W and GK pancreatic islets, we showed that the C1 effect was partly via K-ATP channels, since the potassium channel blocker diazoxide partly but not totally suppressed C1 stimulation at 16.7 mM glucose. The C1 effect was also dependent on L-type Ca^{2+} channels, since the calcium blocker nifedipine suppressed the insulin response to C1 at 16.7 mM glucose. There was no modulation by PKA and PKC inhibitors. Furthermore, the C1 effect was partly dependent on pertussis toxin sensitive G_e-protein. Therefore, the major stimulatory effect of C1 might be on the process of exocytosis.

In **Paper III,** two weeks' treatment with *GP extract* in GK rats significantly improved blood glucose, plasma insulin levels and insulin secretion from islets isolated from the treated rats. Futhermore, when tested *in vitro, GP* extract dose-dependently stimulated insulin release from the isolated rat islets at high glucose concentrations. *GP*-induced insulin release is partly mediated via K-ATP and L-type Ca^{2+} channels. The effects of *GP* were also mediated via the PKA system, and partly dependent on pertussis toxin sensitive G_e-protein at high glucose concentrations.

In **Paper IV**, *ex vivo* infection experiments demonstrated that the pro-inflammatory response to *E. coli* was attenuated in bladder tissue from diabetic GK rats receiving *GP* extract, compared to untreated rats. *In vitro* assays using uroepithelial cells challenged with *E. coli* corroborated these results. Moreover, *GP* treatment modulated the expression of antimicrobial peptides. With these properties, *GP* might be a beneficial supplement for diabetic patients with a history of urinary tract infection.

Keywords: Type 2 diabetes, Goto Kakizaki rat, insulin secretion, anti-inflammatory, antimicrobial peptides

LIST OF PUBLICATIONS

I. **Ezarul Faradianna Lokman**, Harvest F. Gu, Wan Nazaimoon Wan Mohamud, Mashitah M. Yusoff, Keh Leong Chia, Claes-Göran Östenson (2013). Anti-diabetic effect of oral borapetol B compound, isolated from the plant *Tinospora crispa*, by stimulating insulin release. *Evidence Based Complementary and Alternative Medicine* (http://dx.doi.org/10.1155/2013/727602)

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III. **Ezarul Faradianna Lokman**, Harvest F. Gu, Wan Nazaimoon Wan Mohamud, Claes-Göran Östenson. Evaluation of anti-diabetic effects of *Gynostemma Pentaphyllum* on insulin release. (*Submitted manuscript*)

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* These authors contributed equally to the study.

LIST OF PUBLICATIONS NOT LISTED IN THESIS

I.   Gu HF, Zheng X, Abu Seman N, Gu T, Botusan IR, Sunkari VG, **Lokman EF**, Brismar K and Catrina SB (2013). Impact of the hypoxia-inducible factor-1 alpha (HIF-1 alpha) Pro582Ser polymorphism on diabetes nephropathy. *Diabetes Care* 36(2):415-21

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Anti-microbial peptides
ATP	Adenosine triphosphate
AUCs	Area under the glucose curves
cAMP	Cyclic adenosine monophosphate
DAG	Cathelicidin antimicrobial peptide LL-37/hCAP18
GK	Goto Kakizaki
CO_2	Carbon diazoxide
IL	Interleukin
KRB	Kreb's Ringer Bicarbonate buffer
PTX	Pertussis toxin
PKA	Protein kinase A
PKC	Protein kinase C
OGTT	Oral glucose tolerance test
RIA	Radioimmunoassay
RPMI	Roswell Park Memorial Institute medium
T2D	Type 2 diabetes
W	Wistar
T.crispa	*Tinospora crispa*
GP	*Gynostemma pentaphyllum*
UTI	Urinary tract infection

1 INTRODUCTION

1.1 TYPE 2 DIABETES

Type 2 diabetes (T2D) is an endocrine metabolic disorder characterized by hyperglycaemia due to impaired insulin secretion and insulin resistance [1-3] **(Figure 1)**. Impaired insulin secretion is associated with beta-cell secretory dysfunction and/or diminished beta-cell mass, which results in the failure of islets to secrete adequate amounts of insulin in order to achieve normoglycaemia [4]. The failure of pancreatic islets to secrete sufficient levels of insulin in response to elevated blood glucose concentrations reduces insulin signaling in its target tissues, which include the liver, muscle and adipocytes. This leads to decreased glucose uptake by the muscles, increased glucose output by the liver and increased lipolysis in the fat tissues. The increased levels of glucose and fatty acids in the bloodstream lead to both reduced insulin secretion and insulin resistance [5,6].
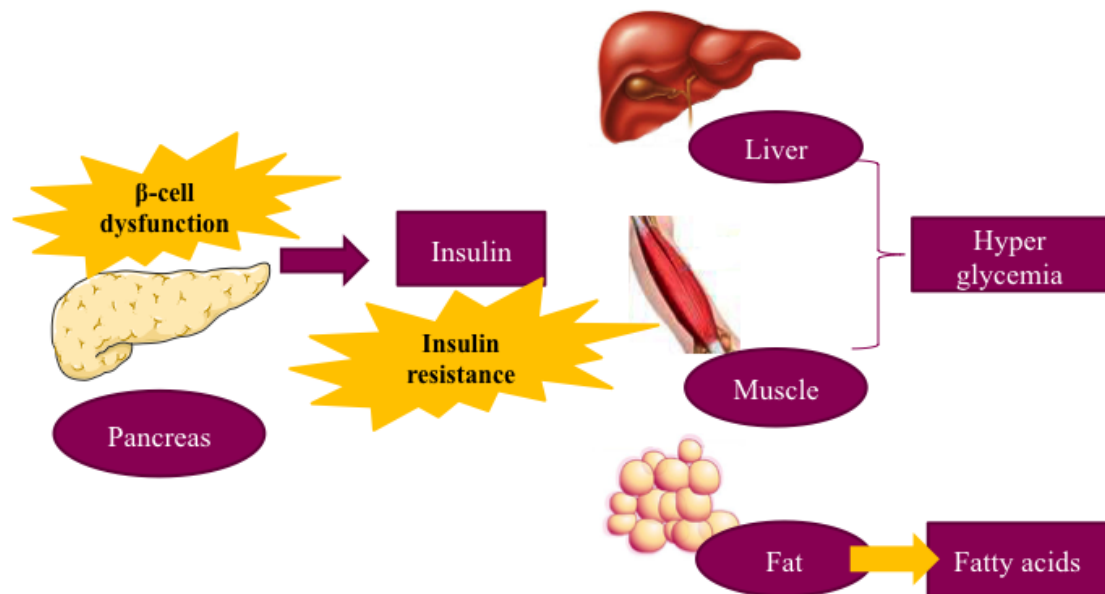
Figure 1: The schematic model of T2D development.

The number of people with diabetes is increasing worldwide and is expected to rise from 366 million in 2011 to 552 million in 2030 [7]. Several factors lead to the development of diabetes, including an aging population, genetic factors, sedentary life style, obesity and an unhealthy diet [8,9]. Complications in diabetes are both macrovascular (heart attack, stroke and peripheral vascular disease) and microvascular

(neuropathy, retinopathy and nephropathy), which lead to increased morbidity and mortality [10].

1.1.1 Insulin secretion pathway in pancreatic beta islets

The K-ATP channel -dependent and -independent pathways are two major signaling processes involved in controlling insulin secretion and maintaining the regulation of glucose homeostasis in pancreatic islets [4,11]. In the K-ATP channel-dependent pathway, glucose enters the beta cells via glucose transporter 2 (GLUT-2). Glucose is phosphorylated to glucose-6-phosphate (G6P) by glucokinase; subsequent glycolysis and glucose oxidation steps generate ATP, which leads to an increase in the cellular adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio **(Figure 2)**. This results in the closure of K-ATP channel-dependent channels, causing membrane depolarization and the opening of voltage-dependent Ca^{2+} channels, which facilitates the entry of free extracellular Ca^{2+} into the beta cells, causing increased cytosolic Ca^{2+} levels and stimulated insulin release by exocytosis [12-14]. The K-ATP channel-independent pathway, in which glucose exerts direct stimulatory effects on the exocytosis of insulin, works in synergy with the K-ATP channel-dependent pathway [11,15].
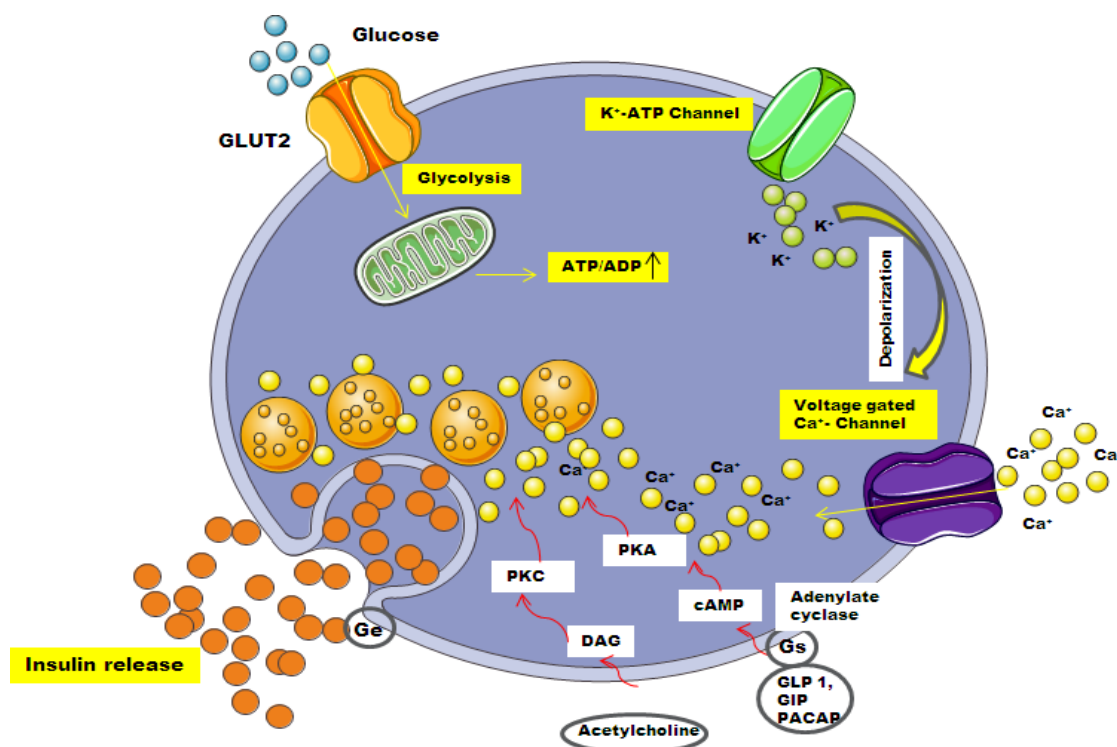
Figure 2: Schematic model of glucose induced insulin secretion in pancreatic β-cells

Second messengers such as cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG) increase insulin release via protein kinase A (PKA) and protein kinase C (PKC) pathways [16]. Guanyl nucleotide-binding (GTP-coupled) proteins, also known as G proteins, are involved in the process of exocytosis [17,18]. Several inhibitors were used to block insulin release in order to understand the mechanism by which herbal plant extracts regulate insulin secretion (**Figure 3**). Diazoxide acts on the K-ATP site by opening K-ATP channels, which results in an increase of intracellular K^+ and thus inhibits insulin release [12]. Nifedipine plays an important role in the Ca^{2+} channel closure by blocking Ca^{2+} influx, thereby inhibiting insulin release [19]. While an increase in the intracellular Ca^{2+} concentration is the principal signal in insulin secretion, other intracellular signals such as cAMP and DAG also are important in the regulation of exocytosis through protein phosphorylation mediated by PKA and PKC, respectively [11,15]. H89 and calphostin C are used to block the PKA and PKC systems, respectively. G-proteins are involved in signal transduction in many tissues, and act as mediators of hormonal inhibition of insulin release [20]. G_i protein is known to be involved in the inhibition of adenylyl cyclase, whereas G_e protein acts during insulin exocytosis [17]. Both G_e and G_i proteins are inhibited by pertussis toxin (expressed by *Bordetella pertussis*) via ADP-ribosylation [20,21].
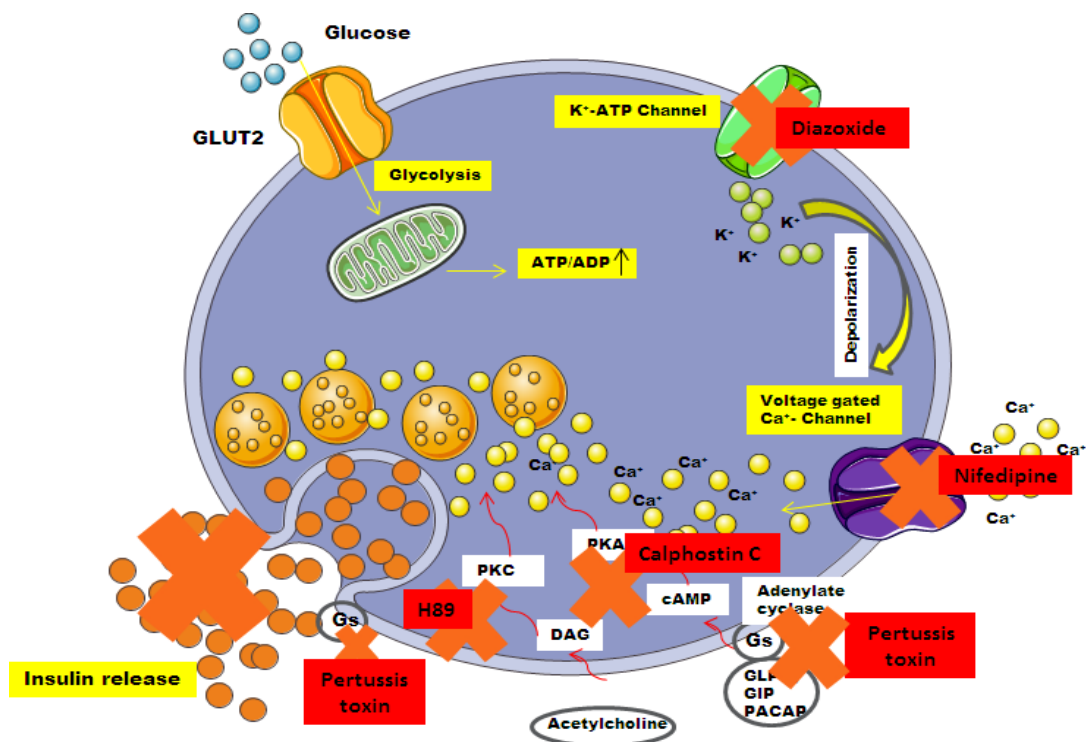
Figure 3: Schematic model of the inhibitors used to understand the effects of *Tinospora crispa* and *Gynostemma pentaphyllum* on the insulin secretion in pancreatic β-cells

1.1.2 Urinary tract infection (UTI) in type 2 diabetes mellitus

Urinary tract infection (UTI) is a common infection detected in type 2 diabetes patients [22-24]. UTIs can be divided into cystitis, pyelonephritis and urosepsis, depending on which part of the urinary tract is involved. In addition, catheter-related bacteriuria and infections frequently occur in association to urinary catheters [25]. The clinical symptoms of UTI include dysuria, frequent micturations, abdominal distention and fever [26]. Women are more often affected by UTI than men, and diabetes is an additional risk factor [26,27]. Due to increasing antibiotic resistance among bacteria, there is a search for more effective drugs to replace currently available conventional antibiotics, which have become less effective over time.

Susceptibility to bacterial infection increases in parallel with the duration and severity of diabetes. It has previously been shown that the presence of *E. coli* in urine was significantly higher in diabetic patients, and that an increased level of glycosylated hemoglobin (HbA1c) predisposes diabetic patients to UTI [24,28]. Increased adherence of bacteria to uroepithelial cells, the presence of glycosuria, and neutrophil dysfunction predispose diabetic individuals to genital infections and UTI [22,23,29]. The overall elevated levels of pro-inflammatory cytokines found in diabetic patients have also been suggested to contribute to the development of UTI [30]. Interleukin (IL)-6 and IL-8 are important inflammatory cytokines in bacterial infections which are produced in response to bacterial pathogens [31].

Apparently, a high glucose content in the urine, which facilitates bacterial growth and a defective host immune response, predisposes diabetes patients to infection due to impaired phagocytic functions such as chemotaxis and phagocytosis. Hyperglycaemia leads to neutrophil dysfunction by increasing intracellular calcium levels and interfering with actin, diapedesis and phagocytosis [32]. Other factors, including age, diabetic nephropathy and vascular complications, all increase the risk of UTIs in diabetic patients [23].

1.2 TREATMENT OF TYPE 2 DIABETES WITH TRADITIONAL PLANTS

The current treatment of T2D includes anti-diabetic agents such as sulphonylureas, biguanides and incretins. When these drugs fail to correct hyperglycaemia, insulin theraphy is initiated. However, the presence of a number of adverse effects with

conventional drugs has stimulated the search for more effective anti-diabetic drugs from medicinal plant sources which exhibit comparatively less side effects [33-36].

Medicinal plants have been suggested since ancient times to be a good source of medicine for the treatment of diabetes. The anti-diabetic properties of medicinal plants are attributed to the presence of several substances such as glycosides, alkaloids, terpenoids, flavonoids and others. Currently, there are about 800 plants which are reported to possess anti-diabetic properties both in *in vivo* and in *in vitro* studies. Several medicinal plant parts have demonstrated promising results in terms of achieving normoglycemia by improving insulin secretion from pancreatic beta cells, while others have been shown to increase glucose uptake in adipose and skeletal muscle, to improve hepatic insulin sensitivity and to suppress glucose absorption in the intestinal tract. However, further investigations are needed, since not all medicinal plants have been properly evaluated and characterized in terms of the mechanism of action, long term side effects and toxicity [1,9].

1.2.1   *Tinospora crispa*

Tinospora crispa (*T. crispa*) belongs to the family of *Menispermaceae*, and is known to the Malays by various vernacular names such as 'akar patawali' or 'akar seruntum'. It is an indigenous climber plant that commonly grows wild in Asian countries from the southwestern part of China to Southeast Asia, including Malaysia [37,38]. It has been used in traditional medicine for various healing puposes including septicaemia, fever, malaria and others [37,39]. Several studies have confirmed that *T. crispa* possesses antioxidant properties and also anti-proliferative effects on selected human cancer cell lines [40-42]. Other studies have shown that *T. crispa* exhibits anti-inflammatory [43], immunomodulatory [44] and anti-malarial properties [45]. It has been shown as well that *T. crispa* helps to maintain blood pressure and heart rate in anesthetized rats [46]. The anti-diabetic effects of *T. crispa* extract have been clearly demonstrated both *in vivo* and *in vitro*, and include improving insulin secretion and insulin sensitivity [38,47-50].

1.2.2   *Gynostemma pentaphyllum*

Gynostemma Pentaphyllum (GP) Makino, also known as Jiao Gu Lan, is a climbing perennial vine plant which grows in several parts of Asian countries including China, Vietnam, Japan and Malaysia [51,52]. Consumed as tea or food, the traditional benefits

of *GP* as claimed by practitioners have been pharmacologically and clinically proven in several studies. *GP* has been reported previously to have a high radical scavenging capacity, as well as anti-proliferative, anti-inflammatory [51,53,54], hypoglycemic [55,56], anti-cancer [57,58] and anti-microbial effects [59].

Gypenosides are saponins isolated from *GP*, and currently there are about 90 different gypenosides that have been isolated and characterized. Randomized clinical trials and experiments in an animal model of T2D have demonstrated that *GP* exerts a significant anti-diabetic effect by enhancing insulin sensitivity [60-62] and insulin secretion [63-65]. The anti-diabetic effects and the insulin release mechanisms of a gypenoside (phanoside) compound isolated from *GP* have also been identified [63]. Furthermore, using diabetic rat models, *GP* saponins were found to be associated with the regulation of glycemia, dyslipidemia and immunocompetence with antioxidant activities [66,67].

Previous studies have also shown the anti-inflammatory effects of extracts using different types of cell cultures. Components of *GP* have previously been observed to exhibit anti-inflammatory properties in cell lines by inhibiting the nuclear factor (NF) κB [68] and the inflammatory mediators IL-1β, IL-6, TNF-α and COX-2 mRNA [51,53,69].

1.3   GOTO-KAKIZAKI (GK) RAT

The GK rat strain is an inbred non-obese model of T2D that displays mild fasting hyperglycemia mainly due to impaired insulin secretion and a degree of insulin resistance. In the GK rat, glucose intolerance is most likely primarily due to reduced beta cell mass and impaired beta cell function. The morphological feature of GK rat pancreatic islets is starfish-shaped islets due to fibrosis [70-72].

Established by Goto and collaborators in Sendai, Japan in 1970s, this rat substrain was derived from normoglycemic Wistar (W) rats by selective repeated inbreeding in each successive generation of the siblings with the highest blood glucose levels, as identified by an oral glucose tolerance test. The glucose intolerance and impairment of glucose-induced insulin release have been constant features of the GK rat [70,72]. Impaired glucose-stimulated insulin secretion has been shown *in vivo* [73], in perifused isolated

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pancreatic islets [72,73] and in isolated pancreatic islets [72,74]. GK rats also exhibit impaired insulin sensitivity in the liver, skeletal muscle and adipose tissues [75,76].

Although there are differences in islet cell morphology and islet metabolism in some GK rat colonies, common features between the colonies include reduced beta cell mass, mild hyperglycemia, insulin resistance and a lean phenotype [77]. At the embryonic stage of 16 weeks, the beta cell mass is only half of that of normal W rats, and the fetuses show both decreased insulin levels [78]. Basal hyperglycemia and decreased insulin levels are detectable at 3 weeks after birth due to defects in the neonatal beta cell mass [79].

The GK rat model has been used not only for studies of complications in diabetes (retinopathy, neuropathy and nephropathy) [80-82], but also in investigations of defective of insulin release [72] and hypertension. These reports have shown that both acute and chronic hyperglycemia increase islet capillary pressure [83]. In conclusion, the GK rat has been widely used in experimental diabetes research, and can be regarded as one of the best available rodent strains for the study of inherited T2D.

2 AIMS

The aim of this thesis was to investigate the anti-diabetic effects of two plants used in traditional medicine, *Tinospora crispa* and *Gynostemma pentaphyllum (GP)* in an animal of type 2 diabetes, the Goto Kakizaki rat. The effects of *GP* extract on innate immune mechanisms in the urinary tract were also identified.

The specific aims of the individual papers were:

I.   To evaluate the anti-diabetic effects of borapetol B (C1) isolated from *Tinospora crispa in vivo* and in isolated rat pancreatic islets.

II. To identify the mechanisms by which borapetol B (C1) isolated from *Tinospora crispa* stimulates insulin secretion in rat pancreatic islets.

III. To elucidate the anti-diabetic effects of *GP* extract, and to understand the mechanisms behind its stimulation of insulin release.

IV. To investigate if oral administration of *GP* extract exhibits immune-modulatory properties in the urinary bladder of rats, and to identify the resulting changes in the expression of pro-inflammatory cytokines and antimicrobial peptides.

3   MATERIALS AND METHODS

3.1   RAT MODELS

Male normoglycemic control Wistar (W) and spontaneously type 2 diabetic Goto-Kakizaki (GK) rats (200-350 g) were used in this study. GK rats, originating from W rats, were bred in our department [72]. W rats were purchased from a commercial breeder (Charles River). The animals were kept at 22°C with an alternating 12-hour light-dark cycle (6 am to 6 pm) and were allowed access to food and water before being anesthetized for isolation of pancreatic islets. The study was approved by the Laboratory Animal Ethics Committee of the Karolinska Institutet.

3.2   PLANT MATERIALS

3.2.1   *Tinospora crispa*

Tinospora crispa stems were collected in Kota Belud (Sabah, Malaysia) in May 2005, identified by Berhaman Ahmad (Universiti Malaysia Sabah) and voucher specimen (FRI54832) deposited at the Forest Research Institute Malaysia. Isolation and purification of borapetol B (C1) from *T*. *crispa* were modified from a previous study [44,84,85].

During the isolation procedure, fractions stimulating insulin secretion in a bioassay with isolated pancreatic islets from W rats were selected for subsequent purification. The stem powder (5 kg) was extracted by sonicating with solvents at room temperature (25°C) for 15 minutes. It was first defatted with hexane followed by methanol-water (4:1) solvent extraction. Extracts were consolidated and reduced to one-third volume by vacuum evaporation yielding a brown syrup. The syrup was acidified to pH 2 with sulphuric acid (50% v/v) and partitioned four times with chloroform. The chloroform layer was evaporated to dryness to obtain brownish mass which showed prominent insulin stimulatory effect. The brown mass was chromatographed over normal phase silica gel eluted with 100% chloroform followed by chloroform methanol (9.5: 0.5) and subsequently increasing eluent polarity with methanol.

Chromatographic fractions were monitored by thin layer chromatography (TLC) visualized at 365 nm. Fractions containing spots possessing Rf value within the range of 0.20 to 0.75 (chloroform methanol 9.5:0.5) were examined further as these fractions were also inducing insulin secretion. These fractions were consolidated and re-chromatographed to yield 10 subfractions. When cooled (4°C), subfractions seven and eight yielded colourless crystals. Upon recrystallization with chloroform-methanol, crystals (450 mg) were recovered by vacuum filtration and washed with cold chloroform. TLC revealed a single compound known as C1 which stimulated insulin secretion. The identity of C1 as borapetol B was further confirmed using 1H-NMR [86]. The compound was suspended to a stock solution of 320 µg of C1 diluted in 640 µl of 3.3 mM KRB buffer with 5% of DMSO for *in vitro* experiments **(Paper I)**; and to 240 µg of C1 diluted in 21 ml of water with 5% of DMSO **(Paper II)** for the treatment of animals.

3.2.2   Extraction procedure of *Gynostemma pentaphyllum*

GP water extracts were prepared according to standardized procedures from the whole herb (Legosan Co., Kumla, Sweden) and consists of 98% gypenosides (German LEFO-Institut für Lebensmittel und Umwelt GmbH, Ahrensburg, Germany). The dried extract was suspended to a stock solution of 10 mg/ml PBS for *in vitro* experiments **(Paper IV)**; and to 0.85 mg/20 ml **(Paper III)** water for the treatment of animals.

3.3   ORAL GLUCOSE TOLERANCE TEST

An oral glucose tolerance test was performed to identify the effect of C1 **(Paper I)** and *GP* **(Paper III)** on the blood glucose levels. C1 (10 µg/100 g of body weight) was given orally 30 min prior to the OGTT **(Paper I)** to W and GK rats whereas *GP* (0.03 g/100 g of body weight) was administrated orally to GK for 2 weeks **(Paper III)**. The rats were fasted overnight (14 to 15 hours), allowing access only to plain drinking water. Blood for glucose determination was obtained by tail-prick method at different time points: 0 minute (before glucose load at 2 mg/g body weight), then at 30, 60, and 120 minutes **(Paper I)** post glucose administration or at 30, 60, 90, 120 and 150 minutes **(Paper III)**. Blood glucose level was measured using a glucometer, Accu-check Aviva (Roche Diagnostic GmbH, USA). Blood samples were collected for the measurement of plasma insulin level (about 20 µl/blood sample) at 0 and 30 min **(Paper I)** and 0, 30 and 120 minutes **(Paper III)**.

3.4 TISSUE COLLECTION

After two weeks' treatment with *GP*, the animals were euthanized using CO_2. Islets **(Paper III)** and bladder tissues **(Paper IV)** were collected immediately for further analysis and kept in -80ºC.

3.5 ISOLATION OF PANCREATIC ISLETS

The isolation of islets was performed using collagenase digestion method **(Paper I, II, III)** [63]. The islets were washed several times with Hank's Balanced Salt Solution (HBBS), and then hand-picked under a stereomicroscope and cultured for 24 hours at 37°C, with an atmosphere of 5% CO_2-95% air in RPMI 1640 culture medium (SVA, Sweden) supplemented with 30 mg *L*-glutamine (Sigma-Aldrich, USA), 11 mM glucose (Sigma-Aldrich, USA), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin) (Invitrogen, USA). Inactivated fetal calf serum (10 %) was added to the culture medium.

3.6 BATCH INCUBATIONS FOR INSULIN SECRETION

Following overnight culture, islets were pre-incubated at 3.3 mM glucose at 37°C with an atmosphere of 5% CO_2 – 95% air for 30 to 45 min. The islets were further incubated with different concentrations of compound in a slowly shaking 37°C waterbath for 60 min. C1 was tested at 10, 1, 0.1 μg/ml **(Paper I).** *GP* was tested at 15, 10, 5, 1 mg/ml **(Paper III)**. The medium used was Krebs-Ringer bicarbonate (KRB) buffer solution containing 10 mmol/l HEPES (Sigma-Aldrich, USA) and 0.2% bovine serum albumin. After incubation, 200 μl of the medium were transferred to new tubes for RIA and kept in freezer -20°C until assayed for insulin secretion.

3.7 PERIFUSIONS OF ISLETS

Perifusions of islets were done to investigate how the C1 substance affects the kinetics of insulin release [63] **(Paper I)**. Islets were perifused with medium containing 10 μg/ml of C1 at 3.3 mM and 16.7 mM glucose using a peristaltic pump (Ismatec SA, Zurich, Switzerland) [87]. Fractions (200 μl) of the perifusion medium were collected for radioimmunoassay of insulin [88].

3.8 EFFECTS OF TRADITIONAL PLANTS ON THE INSULIN SECRETION FROM ISLETS

To investigate mechanisms behind effects of C1 and *GP* extract on insulin secretion, rat islets were incubated in both KRB containing 3.3 mM and 16.7 mM glucose **(Paper II, III)**. The medium was added with different incubation mixtures and inhibitors; 0.25 mM diazoxide (Sigma-Aldrich, USA) only (to open the K-ATP channels); 50 mM of KCl (for depolarization of beta cells). To investigate the effects of compounds on L-type Ca^{2+} channels on insulin secretion, rat islets were incubated in both KRB containing 3.3 mM and 16.7 mM glucose with addition of L-type Ca^{2+} channel inhibitor, nifedipine (Sigma-Aldrich, USA). To investigate the effect of C1 and *GP* in the presence of protein PKC and PKA on insulin release, rat islets were incubated with or without compounds and the PKA-inhibitor, H89 (10 uM) (Sigma-Aldrich, USA) or the PKC inhibitor, calphostin-C (1.5 uM) (Sigma-Aldrich, USA) for 60 min in KRB containing 3.3 mM and 16.7 mM glucose.

To assess the possible involvement of exocytotic G-protein induced by C1 and *GP* extract in insulin release, rat islets were pretreated at 37°C overnight with 100 ng/ml pertussis toxin, RPMI 1640 culture medium containing 11 mM glucose. The islets exposed overnight with or without pertussis toxin were incubated with C1 and *GP* extract at 3.3 mM and 16.7 mM glucose to study insulin secretion.

3.9 MEASUREMENT OF INSULIN SECRETION FROM ISLETS

Aliquots obtained from batch incubations **(Paper I, III)**, perifusions **(Paper I)** and the insulin release experiments **(Paper II, III)** were analyzed for insulin content using RIA [88].

3.10 BACTERIA

The uropathogenic *E. coli* strain CFT073 was used for all infection experiments. Bacteria from frozen stock were cultured on blood agar plates overnight at 37ºC and then inoculated into 3 ml of LB broth for another overnight culture. This overnight culture was diluted 1:100 into fresh LB broth and grown until bacteria reached the logarithmic growth phase. Bacteria were collected by centrifugation and washed once with PBS. Then, the bacterial density was adjusted to an OD=0.125 at 600 nm, corresponding to approximately 10^8 CFU/ml.

3.11 AGAR DIFFUSSION ASSAY

The sensitivity assay was carried out to test the antimicrobial activity of *GP* against *E. coli* strain CFT073 in an agar diffusion assay **(Paper IV)**. Briefly, bacteria from logarithmic culture at approximately 5×10^4 CFU/ml was mixed with 1% low-electrolyte agarose, vortexed and quickly poured intro Petri dish which was distributed evenly on the whole plate. After 30 min, several 3- mm holes were made in the agar layer and 3 µl of 10 mg/ml of *GP* was added into the holes. The agar was left for an hour at room temperature and then incubated overnight at 37°C. The antimicrobial potential of *GP* was determined by measuring the diameter of the zone of inhibition around the holes.

3.12 *EX VIVO* INFECTION MODEL

The GK rats which were administrated orally with either *GP* or water were sacrificed after 2 weeks and bladder tissues were collected **(Paper IV)**. Immediately after collection, bladder tissue pieces were placed in DMEM medium (Gibco) with 1 µg/ml gentamicin with or without 10^8 CFU/ml *E. coli* CFT073 and incubated in a water bath at 37°C. This concentration of gentamicin was previously found to inhibit bacterial growth during the 2-hour incubation period without significantly affecting bacterial viability [89]. After 2 hours, pieces were transferred into RNAlater reagent (Qiagen) and kept at 4°C until processing for gene expression analyses.

3.13 CELLS CULTURE, *GP* TREATMENT AND BACTERIAL INFECTION

Bladder epithelial cells T24 and 5637 were cultured in McCoy's 5A modified medium or RPMI 1640, respectively, containing 10% fetal bovine serum at 37°C and 5% CO_2 in a humidified incubator **(Paper IV)**. For experiments, 10^5 cells/well were seeded in 24-well tissue culture plates (Sarstedt Inc, Newton, NC, USA and Costar, Corning, NY, USA, respectively) and incubated for 24 hours. The cells were then treated with 0.5, 0.1 and 0.05 mg/ml of *GP*. After another 24 hours, the cells were infected with 10^6 CFU/ml *E. coli* CFT073 in medium with 0.5, 0.1 and 0.05 mg/ml of *GP*, respectively and gentamicin (final concentration of 40 µg/ ml). The infected cells were incubated at 37º C in a humidified incubator with 5% CO_2 for 24 hours. Thereafter, the medium was collected and centrifuged at 300 g for 10 min. The supernatant was collected and stored at -80˚C for further analysis. The cells on the plate were collected for RNA

extraction. Viability of the cells was not significantly affected by *GP* at the used concentrations as determined by XTT assay.

3.14 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

The secreted IL-8 in cell culture supernatants was determined by ELISA using the Human CXCL8/IL-8 DuoSet (R&D systems) according to the manufacturer's recommendations **(Paper IV)**. The ELISA microplate reader at 560 nm was used and concentrations in the samples were calculated with help of the standard curve.

3.15 TOTAL RNA EXTRACTION AND CDNA SYNTHESIS (PAPER IV)

Total RNA isolation from bladder pieces was processed according to the manufacturers' protocol using RNeasy Mini Kit (Qiagen) **(Paper IV)**. Tissue pieces were homogenized in RLT buffer containing *β*-mercaptoethanol using a mortar and pestle and subsequent passing through a QIAshredder; cells were collected by addition of the same lysis buffer. Tissue and cell lysates were then processed following the recommendations of the manufacturer. Total RNA at 1 μg of was transcribed into cDNA High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The concentration and purity of RNA was determined with a Nanodrop**.**

3.16 GENE EXPRESSION ANALYSIS

Changes in gene expression were assessed by RT real-time PCR using TaqMan gene expression system (Applied Biosystems) **(Paper IV)**. Primer/probe mixtures specific for human and rat IL6; human IL8 and rat CXCL1, the rat orthologue to IL8; and human cathelicidin antimicrobial peptides LL-37/hCAP18 (CAMP), RNase7, psoriasin (S100A7) and DEFB4 encoding human beta defensin 2 (HBD2) were used; human or rat GAPDH served as reference genes. Expression levels are presented as $2^{-\Delta CT}$, and gene induction as $2^{-\Delta\Delta CT}$ compared to non-infected, non-treated samples.

3.17 FLUORESCENCE IMMUNOCYTOCHEMISTRY

Cells were grown on glass coverslips and treated as described above **(Paper IV)**. Cells were fixed by incubation with 4% paraformaldehyde in PBS for 15 minutes at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes and then blocked with 5% BSA in permeabilization buffer for 30 minutes. Incubation with

primary antibodies diluted in buffer against psoriasin (1:800; Abcam), RNase7 (1:500; Icosagen), CAMP (1:50; Santa Cruz Biotechnologies) and HBD2 (1:50; Santa Cruz Biotechnologies) was performed at 4°C overnight, followed by incubation with an Alexa488-labeled secondary antibody (1:600; Invitrogen) for 1 hour at room temperature. Images were acquired on a Leica confocal microscope SP5 with a 40x or 63x objective. In each experiment, 5 images were recorded per condition and images taken with 40x objective were used to quantify peptide expression with the microscope software (LAS AF Lite).

3.18 STATISTICAL ANALYSIS

All data were analyzed using Prism Graph Pad Software (CA, USA). The results were presented as mean ± SEM. For OGTT experiments, differences between experimental groups were analyzed using paired *t*-test **(Paper I)** and unpaired *t*-test **(Paper III)**. The differences between means in the batch incubations and the mechanisms of insulin release were analyzed for significance using paired *t*-test **(Paper II)** and for **Paper III,** one-way ANOVA was performed, if significant followed by Bonferroni's Multiple Comparison Post Hoc Test. Data obtained from *ex vivo* analyses of rat tissue were overall analyzed by Mann Whitney test; data from cell culture experiments were analyzed by ANOVA with Dunnett's multiple comparison tests **(Paper IV)**. Differences with *P*-values below 0.5 were considered statistically significant.

4 RESULTS AND DISCUSSIONS

4.1 ANTIDIABETIC EFFECT OF ORAL BORAPETOL B COMPOUND, ISOLATED FROM THE PLANT TINOSPORA CRISPA, BY STIMULATING INSULIN RELEASE (PAPER I)

In **Paper I**, we evaluated the antidiabetic properties of borapetol B (C1) isolated from *Tinospora crispa* in normoglycemic control Wistar (W) and spontaneously type 2 diabetic Goto-Kakizaki (GK) rats. An acute oral administration of C1 reduced blood glucose levels in treated versus placebo groups in W ($P<0.01$) **(Figure 4)** and with decreased areas under the glucose curves (AUCs) (0 min to 120 min; $P<0.001$) and in GK ($P<0.05$) **(Figure 5)** with AUCs (0-120 min; $P<0.01$). Plasma insulin levels were increased by 2-fold in treated W and GK rats versus placebo group at 30 min ($P<0.05$). The incubation of islets at different C1 concentrations at low and high glucose showed that C1 dose-dependently increased insulin secretion from W and GK isolated islets **(Table 1)**. The perifusion of isolated islets indicated that C1 did not cause leakage of insulin by damaging islet beta cells both in W and GK rats since insulin release was stimulated only in the presence of C1.
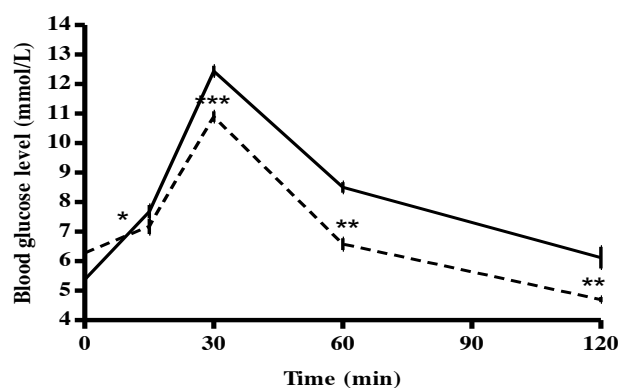
Figure 4: Blood glucose level in the oral glucose tolerance test in W rats. 10 µg/100 g of b.w of C1 (---) or Placebo (—) was given orally 30 minutes prior to the glucose challenge (0.2 g/100 g of b.w.). Data are presented as means ± SEM (n=5). **$P<0.01$ vs. Placebo; ***$P<0.001$ vs. Placebo
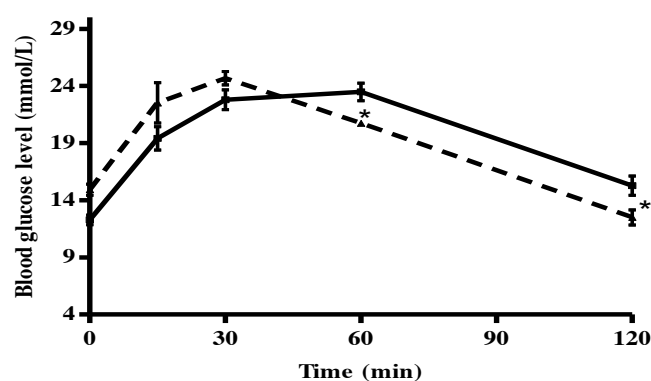
Figure 5: Blood glucose level in the oral glucose tolerance test in GK rats. 10 µg/100 g of b.w of C1 (---) or Placebo (—) was given orally 30 minutes prior to the glucose challenge (0.2 g/100 g of b.w.). Data are presented as means ± SEM (n=5). *$P<0.05$ vs. Placebo

Table 1: The effect of different concentrations of C1 at low (3.3 mM) and high (16.7 mM) glucose on insulin secretion from W (n=5) and GK (n=3) rat islets.

Addition to incubation medium		Insulin release (μU/islet/h)	
		W islets	GK islets
Glucose (mM)	C1 (μg/ml)		
3.3	None	2.4 ± 0.1	1.2 ± 0.2
	0.1	$15.1 \pm 2.2^{**}$	$4.7 \pm 0.3^{*}$
	1	$19.5 \pm 4^{*}$	$7.5 \pm 0.9^{*}$
	10	$21.9 \pm 2^{***}$	$10.5 \pm 1.8^{*}$
16.7	None	32.5 ± 1.8	13.6 ± 0.6
	0.1	$48.8 \pm 6^{\#}$	$20.6 \pm 1.1^{\#}$
	1	$63.1 \pm 8.9^{\#}$	$30.9 \pm 3.8^{\#}$
	10	$164.5 \pm 11.3^{\#\#\#}$	$57.3 \pm 6.3^{\#}$

$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ when compared with the control group

Previous findings have shown the effectiveness of extract and isolated compounds from *Tinospora crispa* in the stimulation of insulin release and insulin sensitivity in normal and diabetic animal model [38,47-50]. We show that oral administration of C1 30 minute before an oral glucose challenge significantly decreased blood glucose levels in W rats. This was most likely mediated through enhanced insulin secretion, since the plasma insulin level in treated W rats increased by 2-fold compared to the placebo group. This is further supported by our findings that C1 stimulates insulin secretion from isolated pancreatic islets, both in batch incubations and in perifusions. To further assess the insulinotropic properties of C1 in T2D, studies were performed in spontaneously diabetic GK rats.

We now demonstrated that oral treatment with C1 decreased blood glucose levels in parallel with an increase in plasma insulin levels during the OGTT, not only in W rats but also in GK rats. In addition, C1 at different concentrations increased insulin release from GK rat islets in low and high glucose and the stimulatory effect was observed in a dose dependent manner. In the perifusions of islets experiment, C1 stimulated insulin secretion in both W and GK rat islets. The insulin secretion gradually returned to basal level on the removal of C1, supporting that C1 did not cause nonspecific insulin leakage by damaging islets beta cells.

In conclusion, the results of this study show that C1 improves the diabetic condition in GK rats by stimulating insulin secretion. Further studies are needed to understand the mechanisms involved by which C1 induces insulin release from pancreatic islets.

4.2 THE POSSIBLE MECHANISMS BY WHICH BORAPETOL B (C1) STIMULATES INSULIN RELEASE FROM RAT ISLETS (PAPER II)

In **Paper II**, we explored the mechanisms by which C1 stimulates insulin release in the isolated pancreatic islets of normoglycemic control W and GK rats via K-ATP dependent pathway. C1 significantly stimulated insulin release at both low and high glucose in W and GK rat islets. The opening of K-ATP channels by adding diazoxide inhibited insulin release at 16.7 mM glucose in W ($P<0.01$) and GK ($P<0.05$) rat islets compared to control **(Table 2)**. Diazoxide decreased insulin response to C1 in W and in GK (both $P<0.01$) only at 16.7 mM glucose. The insulin release of both W and GK rat islets incubated with C1 + diazoxide + KCl was significantly higher (when compared with islets incubated either with C1 only or diazoxide + KCl) at both 3.3 mM and 16.7 mM glucose.

Table 2: Effect of diazoxide on the insulin response to C1 in the isolated W and GK rat islets. Results of insulin release (µU/islet) are the mean ± S.E.M of three batch incubations at each condition.

	W rat islets		GK rat islets	
Glucose (mM)	3.3 mM	16.7 mM	3.3 mM	16.7 mM
Addition to the medium				
None	2.5 ± 0.3	30.9 ± 1.4	1.2 ± 0.1	13 ± 0.2
T.crispa C1 (10 µg/ml)	$22.1 \pm 0.4^{***}$	$140.8 \pm 5.1^{**}$	$10 \pm 0.3^{**}$	$56.2 \pm 2.7^{**}$
Diazoxide (0.25 mM)	2.6 ± 0.1	$3.6 \pm 0.2^{**}$	1.2 ± 0.2	$10.1 \pm 0.5^{*}$
Diazoxide (0.25 mM) + KCl (50 mM)	$22.8 \pm 2.3^{**}$	$131.2 \pm 7.7^{*}$	$10.2 \pm 1^{**}$	$54 \pm 5.5^{**}$
T.crispa C1 (10 µg/ml) + Diazoxide (0.25 mM)	$23.6 \pm 0.7^{\#\#}$	$85.4 \pm 5.3^{\#\#}$	9.9 ± 0.6	$37.4 \pm 3.4^{\#\#}$
T.crispa C1 (10 µg/ml) + KCl (50 mM) + Diazoxide (0.25 mM)	$38.5 \pm 1.2^{\#\#\dagger}$	$155.7 \pm 7.4^{\#\dagger}$	$14.8 \pm 0.3^{\#\#\dagger}$	$84.5 \pm 5.8^{\#\dagger}$

$^{*}P<0.05;^{**}P<0.01;^{***}P<0.001$ when compared with control group with no addition
$^{\#}P<0.05;^{\#\#}P<0.01$ when compared with group with only T.crispa C1
$^{\dagger}P<0.05$ when compared with diazoxide + KCl

Nifedipine decreased insulin release in W ($P<0.05$) and in GK ($P<0.01$) rat islets at 16.7 glucose only **(Table 3)**. In the presence of nifedipine, C1-induced insulin secretion of islets was decreased in W ($P<0.01$) and GK rat islets at 16.7 mM glucose. H89 and calphostin C inhibitors of PKA and PKC, respectively, did not affect the insulin response to C1 respectively in W and GK islets at both 3.3 mM and 16.7 mM glucose **(Table 4)**. At 16.7 mM glucose, pertussis toxin decreased the insulin response to C1 in the W ($P<0.01$) and GK rat islets ($P<0.05$). When exploring the mechanisms of insulin release in the W and GK pancreatic islets, we showed that the C1 effect was exerted partly via K-ATP channels since diazoxide partly, but not totally suppressed C1 stimulation at 16.7 mM glucose. C1 effect was also dependent on L-type Ca^{2+} channels

since nifedipine suppressed the insulin response to C1 at 16.7 mM. There was no modulation by PKA and PKC inhibitors. Furthermore, C1 effect was partly dependent on pertussis toxin sensitive Ge-protein.

Table 3: Effect of nifedipine on the insulin response to C1 in the isolated W and GK rat islets. Results of insulin release (µU/islet) are the mean ± S.E.M of three batch incubations at each condition.

	W rat islets		GK rat islets	
Glucose	**3.3 mM**	**16.7 mM**	**3.3 mM**	**16.7 mM**
Addition to the medium				
None	2.6 ± 0.2	30.3 ± 3.1	1.3 ± 0.2	13.4 ± 0.6
T.crispa C1 (10 µg/ml)	$24 \pm 3.3^*$	$157.5 \pm 7.7^{**}$	$10.5 \pm 0.6^{**}$	$59.3 \pm 5.3^*$
Nifedipine (10 µM)	2.3 ± 0.2	$5.3 \pm 0.7^*$	1 ± 0.1	$4.4 \pm 0.1^{**}$
T.crispa C1 (10 µg/ml) + Nifedipine (10 µM)	$22.2 \pm 1.6^{**}$	$132 \pm 10^{**\#\#}$	$10.3 \pm 0.5^{**}$	$30.6 \pm 3.2^{*\#}$

$^*P<0.05;^{**}P<0.01$ *when compared with control group with no addition*
$^{\#}P<0.05;^{\#\#}P<0.01$*when compared with group with only C1*

Table 4: Effect of H89 and calphostin C on the insulin response to C1 in the isolated Wistar and GK rat islets. Results of insulin release (µU/islet) are the mean ± S.E.M of three batch incubations at each condition.

	W rat islets		Gk rat islets	
Glucose	**3.3 mM**	**16.7 mM**	**3.3 mM**	**16.7 mM**
Addition to the medium				
None	3.1 ± 0.2	31 ± 3.6	2.4 ± 0.7	15.3 ± 1.2
H89 (10 µM)	4.4 ± 0.3	25.6 ± 1.2	2.1 ± 0.2	15.6 ± 0.9
Calphostin C (1.5 µM)	5.0 ± 0.3	24.1 ± 1.5	1.8 ± 0.4	14.3 ± 1.1
T.crispa C1 (10 µg/ml)	$21.4 \pm 3.2^*$	$152.4 \pm 8.3^{**}$	$11.2 \pm 1.2^*$	$56 \pm 2.4^{**}$
T.crispa C1 (10 µg/ml) + H89 (10 µM)	$20.1 \pm 1.8^*$	$159.7 \pm 4.9^{**}$	$8.0 \pm 1.0^{**}$	$45.7 \pm 2.8^*$
T.crispa C1 (10 µg/ml) + Calphostin C (1.5 µM)	$21 \pm 0.6^{**}$	$150.5 \pm 6.7^{**}$	$6.6 \pm 1.0^*$	$53 \pm 2.8^*$

$^*P<0.05;^{**}P<0.01$ *when compared with control group with no addition*

We showed that C1 significantly stimulated insulin release at both low and high glucose. The mechanism by which C1 stimulates insulin release in W and GK was partly mediated via K-ATP and L-type Ca^{2+} channels rat islets. The effect of C1 was also partly dependent on pertussis toxin sensitive Ge-protein at low and high glucose. C1 effect was not mediated via the PKA and PKC system. The effect of C1 is partly in contrast to the sulfonylureas drug which stimulates insulin by closing the K-ATP channel [90,91].

4.3 EVALUATION OF ANTIDIABETIC EFFECTS OF *GYNOSTEMMA PENTAPHYLLUM* ON INSULIN RELEASE (PAPER III)

In **Paper III**, the effects of *GP* water extract on GK rat blood glucose and serum insulin levels were determined. In addition, *GP* extract effects on mechanisms behind insulin secretion from GK rat islets were investigated. The oral glucose tolerance test was performed to identify the effects of *GP* on the blood glucose and insulin levels. After two-weeks' treatment with *GP* extract, glucose tolerance was significantly improved. The mean blood glucose levels at 120 were decreased in the treated group as compared to the placebo group (11.0 ± 1.1 *vs* 13.9 ± 1.1 mM, respectively **(Figure 6)** with AUCs (0-120 min) for glucose being 639.6 ± 38.5 *vs* 842.4 ± 43.8) mM ($P<0.01$). Plasma insulin levels after *GP* treatment were significantly increased both at 0 min and after glucose stimulation at 30 and 120 min when compared with the control group ($P<0.01$) .
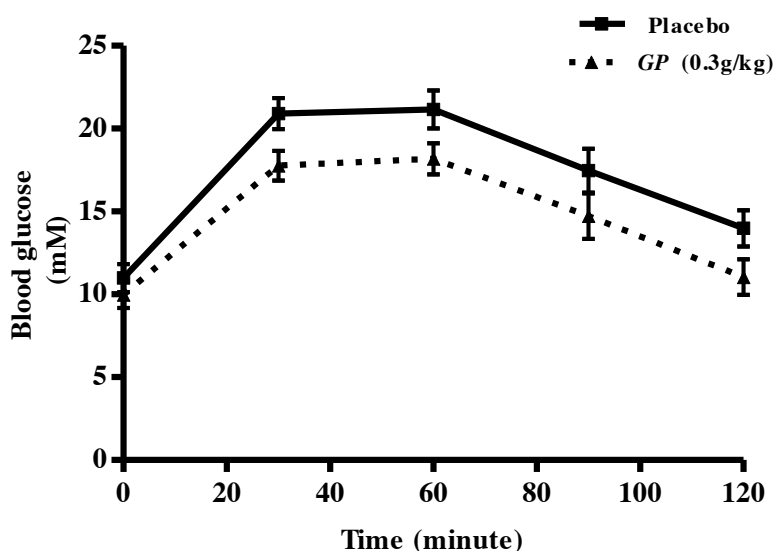
Figure 6: Blood glucose level in the oral glucose tolerance test in GK rats after two-week treatment. *GP* (0.3 g/kg of body weight) (---) or placebo (—) was given orally daily for two weeks. Data are presented as means ± SEM (n=5).

To identify if the oral administration of *GP* has an effect on insulin release, the pancreas of rat treated with *GP* for two-week were collected, and islets isolated for studies of insulin secretion. At 3.3 mM glucose, insulin release was not significantly different between islets of GK rats treated with *GP* as compared to islets from control GK rats (14.6 ± 1 *vs* 14.3 ± 1.9 μU/islet). However, insulin release at 16.7 mM glucose was significantly higher in islets from *GP*-treated rats as compared to islets from control rats (61.8 ± 4.9 *vs* 32.7 ± 2.8 μU/islet; $P<0.001$).

In separate *in vitro* experiments, isolated GK rat islets were incubated with different concentrations of *GP* to identify the insulin-stimulatory effect. At 3.3 mM glucose, *GP* at any concentration did not stimulate insulin release when compared to the control incubations **(Figure 7)**. At 16.7 mM glucose, the addition of 5, 10 and 15 mg/ml *GP* dose-dependently stimulated insulin release 1.6-fold, 2.2-fold (*P<0.01*) and 3.6-fold (*P<0.001*), respectively, when compared to the control group.
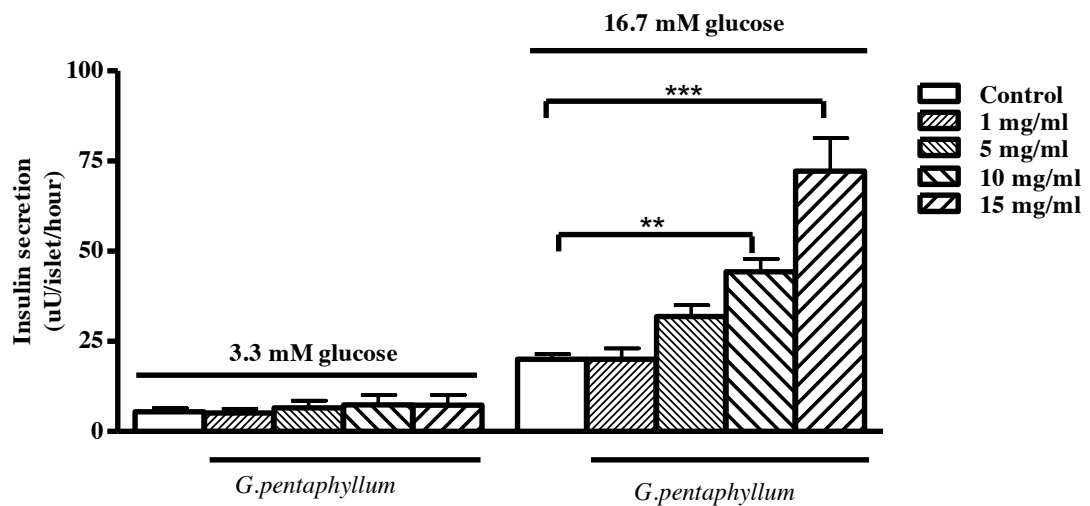
Figure 7: Effect of different concentrations of *GP* (1,5,10,15 mg/ml) on the insulin secretion in GK rat islets (n=5). **P<0.01, ***P<0.001 (when compared with the control group at 16.7 mM glucose only). Results of insulin release (μU/islet/hour) are the means ± SEM of five independent experiments with three replicates for each experiment. Differences between means were analyzed by one-way ANOVA, if significant followed by Bonferroni's Multiple Comparison Post Hoc Test.

Our findings have shown that *GP* has a potent insulin-stimulating activity in the GK rat islets. Therefore, to further understand the mechanisms behind *GP*-stimulated insulin release, we have explored the different sequences of pancreatic beta cell stimulus-secretion coupling for glucose [63]. We first elucidated the role of the ATP-sensitive potassium (K-ATP) channels.

To understand if *GP* stimulates insulin release via the K-ATP channels, we used diazoxide. The opening of K-ATP channels by adding diazoxide (0.25 mM) inhibited insulin release by 63% at 16.7 mM glucose **(Figure 8A)**. In addition, at 16.7 mM glucose, diazoxide decreased insulin response to *GP* from 78.1 ± 15.6 to 26 ± 17.0 μU/islet (*P<0.001*). The addition of potassium chloride (KCl) to islets incubated with diazoxide to depolarize the *β*-cells increased insulin release at both 3.3 mM and 16.7 mM glucose compared to the control group. At 16.7 mM, the insulin response to *GP* +

diazoxide and KCl was significantly higher compared to islets incubated with diazoxide + KCl (at $P<0.05$). Diazoxide however did not affect the insulin response to *GP* at low glucose.

To further understand if *GP* exerts its effect via L-type Ca^{2+} channel, nifedipine was used as an inhibitor of these channels [92]. At 16.7 mM glucose, *GP* stimulated insulin release by 2.6 fold ($P<0.001$) compared to the control **(Figure 8B)**. The addition of nifedipine (10 µM), decreased insulin release from 12.6 ± 1.7 to 6.5 ± 1.3 (µU/islet). Incubation with nifedipine and *GP* at 16.7 mM glucose decreased insulin secretion significantly compared to secretion induced by *GP* only, from 32.8 ± 4.5 to 19.4 ± 1.3 µU/islet ($P<0.01$).

PKA or PKC pathways potentiate the insulin response to a metabolic stimulus [93]. To understand if *GP* exerts its effect via the PKA and PKC pathways, H89 (PKA inhibitor) and calphostin C (PKC inhibitor) were used. Incubation of islets at 16.7 mM glucose with *GP* stimulated insulin release 2.2-fold ($P<0.01$) **(Figure 8C)**. H89 (10 µM) and calphostin C (10 µM) decreased insulin release from 22.1 ± 2.8 to 7.7 ± 3.9 and to 5.4 ± 0.2 µU/islet, respectively at 16.7 mM glucose compared to the control group. H89 decreased the insulin response to *GP* from 48.8 ± 0.9 to 26.07 ± 3.2 µU/islet ($P<0.05$). Calphostin C did not affect the insulin response to *GP* at 16.7 mM glucose.

To understand if *GP* stimulates insulin release via exocytotic G_e proteins, pertussis toxin (PTX) was used as an inhibitor. PTX prevents the G proteins from interacting with their associated G protein-coupled receptors [21]. The pre-incubation of GK rat islets with pertussis toxin in the presence of *GP* at 11.1 mM glucose decreased the insulin secretion compared to the control group, from 29.6 ± 2.2 to 13.8 ± 0.7 µU/islet at 16.7 mM glucose ($P<0.05$) **(Figure 8D)**.

Our present findings suggest that *GP*-induced insulin release is partly mediated via K-ATP and L-type Ca^{2+} channels. *GP*-induced insulin secretion is also modified by the PKA, but not the PKC, pathway. It seems plausible that *GP*, at least partly, exerts its stimulatory effects on insulin exocytosis, since preincubation of islets with the G-protein inhibitor pertussis toxin also decreased *GP*-induced insulin release.

In conclusion, our findings have shown that *GP* acts via K-ATP and L-type Ca^{2+} channels. In addition, *GP* seems to interact with the PKA system and partially via

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exocytotic G_e-proteins. Thus, it seems possible that these effects, at least to some extent, are exerted by phanoside, a gypenoside that has been purified from *GP* [63,64]. Phanoside was also shown to exert its insulin-stimulatory effect distal to K-ATP channels and L-type Ca^{2+} channels, i.e. mainly on the exocytotic machinery of the beta cells [63,65].
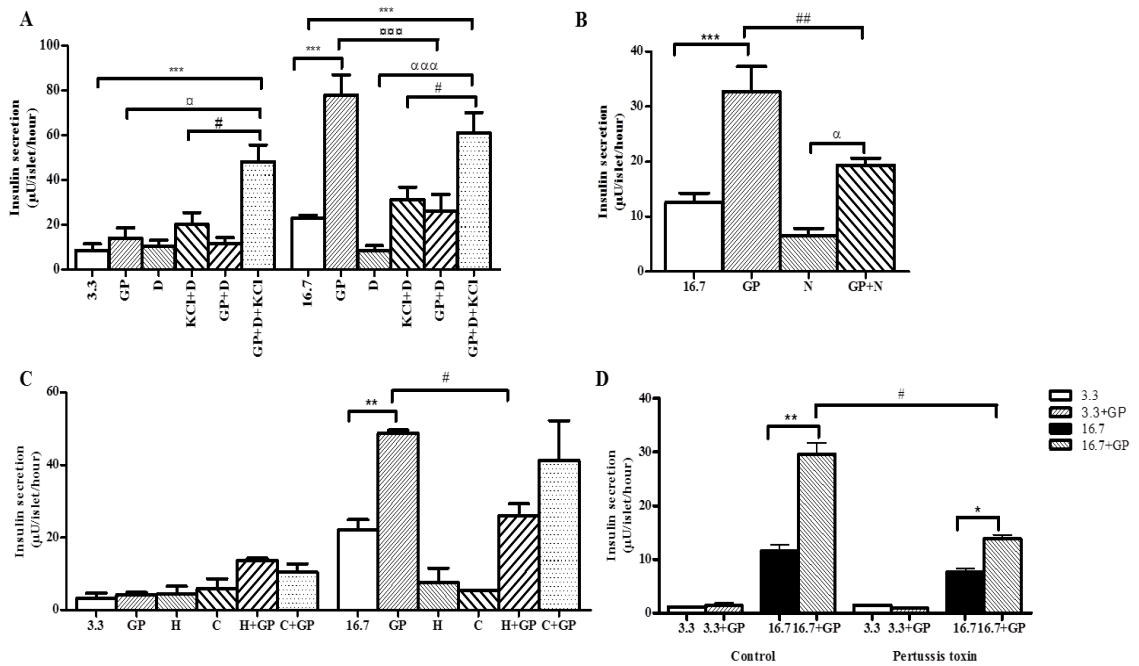

Figure 8: The mechanisms of insulin release of *GP* in the isolated GK rat islets via

(A) K-ATP channel, opened by diazoxide (D) and depolarized by kalium chloride (KCl) at 3.3 mM and 16.7 mM glucose (n=5). [***]$P<0.001$ (when compared with control group with no addition); [#]$P<0.05$ (when compared with group with only D+KCl); [¤]$P<0.05$, [¤¤¤]$P<0.001$ (when compared with group with only *GP*); [ααα]$P<0.001$ (when compared with D) [ab]

(B) Ca^{2+} channel, inhibited by nifedipine (N) at 16.7 mM glucose (n=6). [***]$P<0.001$ (when compared with control group with no addition); [##]$P<0.01$ (when compared with group with only *GP*); [α]$P<0.05$ (when compared with N)[ab]

(C) PKA and PKC mediators, inhibited by H89 and calphostin C (n=3) at 3.3 mM and 16.7 mM glucose. [**]$P<0.01$ (when compared with control group with no addition); [#]$P<0.01$ (when compared with group with only *GP*) [ab]

(D) G protein, inhibited by pertussis toxin (n=3). [*]$P<0.05$, [**]$P<0.01$ (when compared with control group with no addition at 16.7 mM glucose); [#]$P<0.05$ (when compared with islets incubated with *GP* at 16.7 mM glucose without exposure to pertussis toxin) using paired t-test[a]

[a]Results of insulin release (µU/islet) are the mean ± S.E.M. [b] Differences between means were analyzed by one-way ANOVA, if significant followed by Bonferroni's Multiple Comparison Post Hoc Test.

4.4 *GYNOSTEMMA PENTAPHYLLUM* EXHIBITS ANTI-INFLAMMATORY PROPERTIES AND MODULATES ANTIMICROBIAL PEPTIDE EXPRESSION IN THE URINARY BLADDER (PAPER IV)

In **Paper IV**, we investigated if oral administration of *GP* impacts the inflammatory response to *E. coli* in the urinary bladder of diabetic GK rats and further the influence *in vitro* in epithelial bladder cell lines. We also investigated if *GP* treatment modulates the expression of antimicrobial peptides in response to *E. coli*.

At baseline, no significant difference in the expression of pro-inflammatory IL-6 and CXCL-1, the rat homolog for human IL-8, could be established **(Figure 9A)**. In response to bacteria, our results showed that *GP* reduced the IL-6 levels in the rat bladder tissue compared to the control group on mRNA level (P<0.05). The CXCL1 induction was also attenuated **(Figure 9B)**.
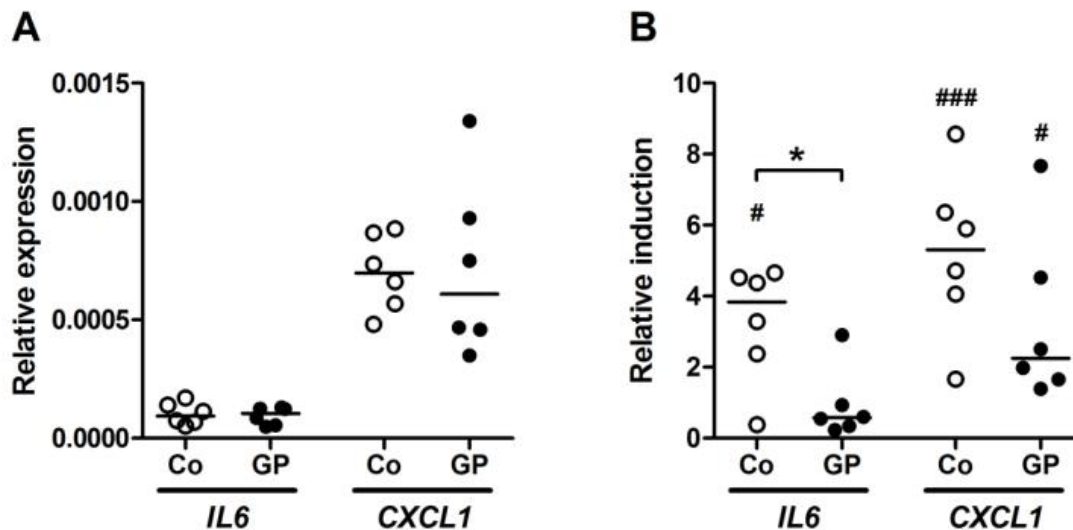
Figure 9: The pro-inflammatory response to *E. coli* is reduced in bladder tissue from *GP*-treated rats. Bladders were removed from GK rats (Co) or GK rats receiving *GP* for two weeks (*GP*). Total RNA was extracted directly (A) or after a 2-hour *ex vivo* infection with uropathogenic *E. coli* in the presence of sublethal concentrations of gentamicin (1 µg/ml) (B). Relative expression levels are expressed as $2^{-\Delta C_T}$ in relation to GAPDH levels (A) and as $2^{-\Delta\Delta C_T}$ in relation to non-infected control tissue incubated alongside (B). Differences between control and *GP*-treated rats were analyzed by two-tailed *t*-test, *P<0.05; gene induction by *E. coli* was evaluated by one-tailed, paired *t*-test between non-infected and infected tissue pieces from the same animal, [#]P<0.05, [###]P<0.001.

We then confirmed these initial findings with *in vitro* experiments using the epithelial bladder cell lines, T24 and 5637. Our results in T24 cells showed that IL-6 induction was reduced after *GP* treatment at 0.1 and 0.5 mg/ml in response to bacteria, however no significant difference was observed when compared to untreated cells **(Figure 10A)**. A similar pattern was seen in the IL-8 induction which was reduced in the cells treated

with *GP* at 0.5 mg/ml (P<0.05) and 0.1 mg/ml on mRNA level when compared to the untreated cells infected with *E.coli* **(Figure 10B)**. On protein level, the IL-8 was significantly downregulated at 0.5 mg/ml **(Figure 10C)**. Using 5637 cell line, no induction of IL-6 was observed in response to *E.coli* **(Figure 10D)**. *GP* significantly attenuated the IL-8 level at 0.05 (P<0.05), 0.1 (P<0.05) and 0.5 mg/ml (P<0.001) on mRNA level when compared with the cells infected with only *E.coli* **(Figure 10E)**.
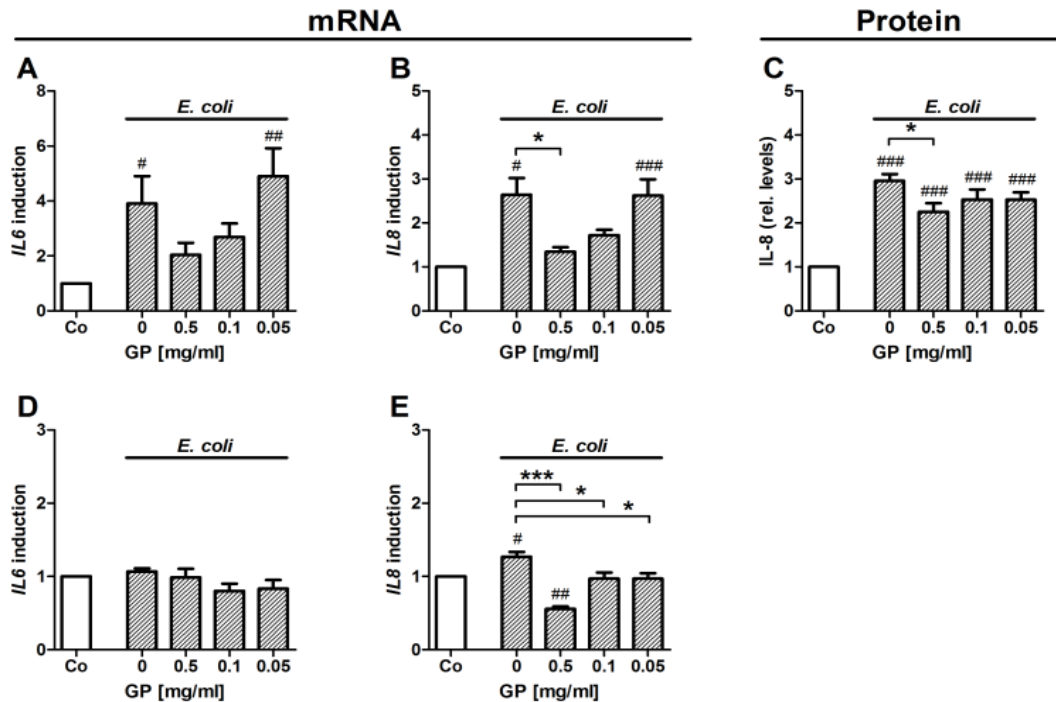

**Figure 10: *GP* reduces bacteria-mediated induction of IL-6 and IL-8 in bladder epithelial cells.**
Bladder epithelial cells T24 (A-C) and 5637 (D+E) were treated with *GP* at indicated concentration for 24 hours and infected with *E. coli* in the presence of 40 µg/ml gentamicin for another 24 hours. Gene expression (A+B, D+E) was determined by RT real-time PCR and the influence of *GP* on bacteria-mediated gene induction is expressed in relation to non-infected, non-treated control cells (Co). Secretion of IL-8 into the cell culture medium was quantified by ELISA (C). Differences to control cells are indicated by $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$; differences to infected, non-treated cells are indicated by *P<0.05 and ***P<0.001; analysis was performed with ANOVA and Dunnett's multiple comparison test. Results from 5-6 (A-C) or 3 (D+E) experiments are shown with mean and SEM.

Our results have confirmed that *GP* exerts anti-inflammatory effects by suppressing the pro-inflammatory response to *E.coli* both *ex vivo* and *in vitro* which could protect the tissue from destruction. However, the reduction of anti-inflammatory levels by *GP* might decrease the immune response against invading pathogens to a critical limit. Therefore, we sought to investigate whether *GP* activates alternative pathways in the host defense against bacteria. Our results demonstrated that *GP* modulates expression of antimicrobial peptides.

In the bladder epithelial cell line 5637, psoriasin (S100A7) expression was significantly induced at 0.05, 0.1 and 0.5 mg/ml (P<0.001) **(Figure 11A)** whereas the expression level of human beta defensin 2 (DEFB4) was downregulated by *GP* **(Figure 11B)**. Furthermore, cathelicidin antimicrobial peptide LL-37/hCAP18 (CAMP) levels was also increased **(Figure 12A)**. The expression of RNase 7 level was not significantly affected **(Figure 12B)**. Microscopic analyses of cells treated with *GP* corroborated the result on transcript level **(Figures 11C and 12C)**. In further experiments, the antimicrobial peptide levels found in the animals treated with *GP* supported the results obtained from cell culture. Transcription of S100A7A was also higher in bladder tissue after *ex vivo* infection in *GP*-treated GK rats, as compared to control rats, and a similar trend was seen for CAMP (data not shown). This study has confirmed that *GP* has anti-inflammatory effects by suppressing the IL-6 and IL-8 inductions and compensates with an increased AMP expression.
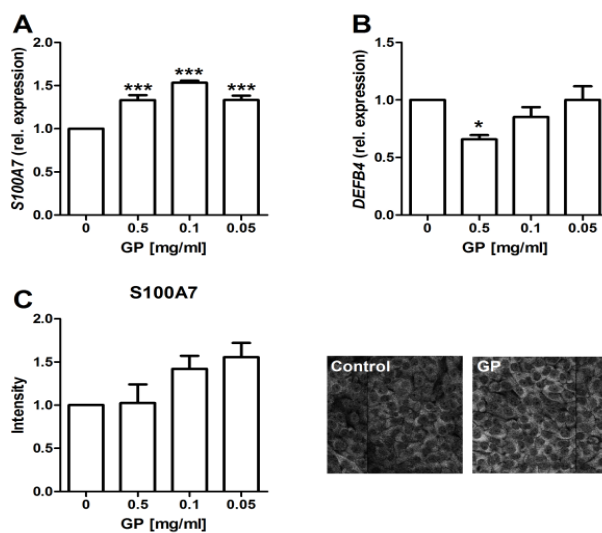
Figure 11: *GP* modulates the expression of antimicrobial peptides in bladder epithelial cells. Bladder epithelial cells 5637 were treated with *GP* at indicated concentrations for 24 hours. The expression of antimicrobial peptides psoriasin (S100A7) (A+C) and human beta defensin 2 (DEFB4) (B) was evaluated by RT real-time PCR (A+B) and psoriasin by immunofluorescence microscopy (C). Analysis was performed with ANOVA and Dunnett's multiple comparison test. Results from 4 (A+B) or 2 (B) independent experiments are shown as mean and SD.
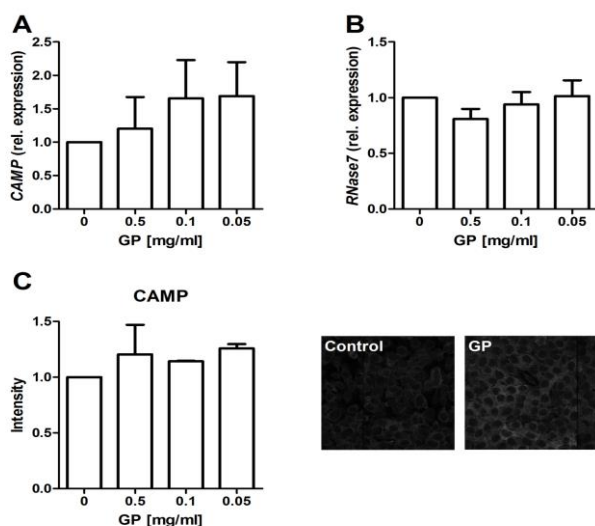
Figure 12: *GP* modulates the expression of antimicrobial peptides in bladder epithelial cells. Bladder epithelial cells 5637 were treated with *GP* at indicated concentrations for 24 hours. The expression of CAMP (A+C) and RNase 7 (B) was evaluated by RT real-time PCR (A+B) and CAMP by immunofluorescence microscopy (C). Analysis was performed with ANOVA and Dunnett's multiple comparison test. Results from 4 (A+B) or 2 (B) independent experiments are shown as mean and SD.

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Both NF-κB and mitogen-activated protein kinases (MAP kinases) are activated by tumor necrosis factor α (TNFα) [30]. *GP* has been shown previously to exhibit anti-inflammatory action by inhibition of nuclear factor (NF) κB signaling [68]. Gypenoside isolated from *GP* has been shown to inhibit NF-κB activation via a PPAR-α-dependent pathway in murine macrophages [94]. In contrast, some gypenosides have been demonstrated to promote phosphorylation of the MAP kinase ERK1/2 [95]. In another study, *GP* compounds have been reported to suppress expression of IL-1β, IL-6 and COX-2 mRNA in LPS-induced RAW 264.7 macrophage cells which may be used to promote the potential application of GP in functional foods [56].

Our results suggested that *GP* exerts its anti-inflammatory effects by suppressing IL-8 and IL-6 via the NF-κB signaling pathway and that it activates psoriasin and CAMP peptides via the MAP kinase. Apart from NF-κB, mMAP kinases have also been shown to be involved in the regulation of AMP expression [96,97].

In conclusion, our study has confirmed that an oral consumption of *GP* leads to a reduction of the pro-inflammatory response in the bladder epithelium of type 2 diabetic rat in response to *E. coli* infection. The upregulation of neutrophil-attractant AMP psoriasin might compensate the suppression of IL-8 level but at the same time also inhibit *E. coli* directly. Thus, the administration of *GP* might be useful to support innate defence mechanisms from long term complications in T2D patients.

5 THESIS SUMMARY

We have investigated the antidiabetic effects of two traditional plants, borapetol B (C1) isolated from *Tinospora crispa* and *Gynostemma pentaphyllum (GP)* using an animal of type 2 diabetes, the Goto Kakizaki rat. The anti-inflammatory and anti-microbial effects of *GP* were also analyzed. Therefore, the following conclusions can be made from our results;

Paper I – The study confirms that an oral administration of C1 improves the diabetic condition in GK rats which was most likely mediated through enhanced insulin secretion. This is further supported by the *in vitro* study showing stimulated insulin secretion when C1 was added in batch incubation and perifusion experiments.

Paper II – This study provides evidence that the C1 has an effect partly via K-ATP and L-type Ca^{2+} channels and the major stimulatory effect might be on the exocytosis of insulin. The findings may be important for drug development in the future.

Paper III – This study indicates that the anti-diabetic effects of *GP* are associated with increased level of insulin release from the islets. The mechanisms of insulin release of *GP* involved might be partly mediated via K-ATP, L-type Ca^{2+} channels, PKA system and partly dependent on pertussis toxin sensitive G_e-protein at high glucose. These findings may be used to promote the potential use of *GP* as an anti-diabetic drug.

Paper IV – This study demonstrates that the oral administration of *GP* exhibits both anti-inflammatory properties and modulates antimicrobial peptide expression in the urinary bladder. Further *GP* may be a promising candidate to support the innate immune system and to protect from long-term complications in diabetic patients.

In conclusion, both C1 and *GP* provide a potential source of anti-diabetic drugs which exert effects via stimulating insulin secretion. Furthermore, *GP* has the potential to support innate immunity defence mechanisms in diabetes.

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