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ANTI-DIABETIC EFFECT OF GYNOSTEMMA PENTAPHYLLUM TEA IN TYPE 2 DIABETES

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To my family with love

ABSTRACT

Type 2 diabetes mellitus (T2D) is a major non-communicable disease and an important health burden worldwide, especially in the developing countries including Vietnam. *Gynostemma pentaphyllum* has been widely used in Vietnam as a herbal medicine for the prevention and treatment of T2D but the mechanism related to its effects is unknown.

This thesis aims to evaluate the anti-diabetic effect and safety of GP extract (tea) in T2D patients and to investigate the major mechanisms of action of GP in T2D patients and in Goto-Kakizaki (GK) rats.

The medication was provided as GP tea (dose 6g/day). The studies were implemented on newly diagnosed T2D patients to investigate the anti-diabetic effect of GP tea (**Paper I**), the effect of GP tea as add-on therapy with sulfonylurea (SU) (**Paper II**), and the effect of GP tea on insulin sensitivity (**Paper III**). Effects of GP tea on glucose tolerance and hepatic glucose output (HGO) were studied in GK rats (**Paper IV**). There was a randomized, placebo-controlled, double blind design for **Papers I, II and IV**, and a cross-over design for **Paper III**.

Significant anti-diabetic effects and improved insulin sensitivity of GP tea were clearly demonstrated in **Paper I**. After 12 weeks of treatment, fasting plasma glucose (FPG) levels decreased 3.0 ± 1.8 mmol/l in the GP group, compared to a decrease of 0.6 ± 2.2 mmol/l in the control group ($p < 0.01$). HbA_{1C} levels decreased approximately 2%-units in the GP group, compared to 0.2%-unit in the controls ($p < 0.001$). Changes in Homeostasis Model Assessment-Insulin Resistance between baseline and the 12th week indicated that insulin resistance decreased significantly in the GP group (-2.1 ± 3.0) compared with that (+1.1 ± 3.3) in the control group ($p < 0.05$). As add-on therapy to SU, GP tea further improved glycemic control and this improvement was sustained over 12 weeks (**Paper II**). After 4 weeks of SU treatment, FPG and HbA_{1C} decreased significantly ($p < 0.001$). FPG was further reduced after add-on therapy by 2.9 ± 1.7 and 0.9 ± 0.6 mmol/l in the GP and control groups, respectively ($p < 0.001$). HbA_{1C} levels decreased approximately 2%-units in the add-on GP group compared to 0.7%-units in the controls ($p < 0.001$). Furthermore, there were potential benefits of maintaining low-dose SU without symptoms of hypoglycemia. The biosecurity of GP tea was suggested clinically, since no hepatotoxicity, nephrotoxicity or other adverse effects were observed in the trials. The GP tea exerted anti-diabetic effects by improving insulin sensitivity, as demonstrated in the placebo-controlled cross-over study using the somatostatin-insulin-glucose infusion test (SIGIT) (**Paper III**). FPG and steady-state plasma glucose (SIGIT mean) were lower after GP treatment, compared to placebo treatment ($p < 0.001$). These glycometabolic improvements were achieved without any major change of circulating insulin levels. Finally, oral administration of GP tea for three weeks to GK rats exerted anti-diabetic effects by reducing plasma glucose (PG) levels and suppressing HGO levels significantly (**Paper IV**). The PG levels decreased from 9.8 ± 0.6 to 6.8 ± 0.4 mmol/L ($p = 0.027$) in GP-treated rats, whereas PG levels were not significantly decreased in the placebo rats. Glucose tolerance, assessed by an intra-peritoneal glucose tolerance test, was significantly improved in GP-treated rats, compared to placebo-treated group (areas under the glucose curves, AUCs, from 0 to 120 min were 1150 ± 200 vs. 1761 ± 87 mmol/L; $p = 0.013$). The glucose response in an intra-peritoneal pyruvate tolerance test was significantly lower in the GP group, indicating suppression of gluconeogenesis by GP treatment. In liver perfusions, the AUCs for HGO during 18 min (0-18 min) were significantly decreased in GP-treated rats, compared with control rats (302.8 ± 36.5 vs. 423.5 ± 44.7 μmol, $p < 0.05$). Three-week GP treatment significantly reduced hepatic glycogen content, but not glycogen synthase activity ($p < 0.007$), compared to the placebo group.

Our studies indicate that GP tea improves glucose tolerance and FPG, most likely by increasing insulin sensitivity and suppressing HGO. GP tea could offer an alternative to the addition of other oral medications in the treatment of T2D patients in Vietnam.

Keywords: Herbal medicine; Type 2 diabetes; *Gynostemma pentaphyllum* tea; Insulin sensitivity; Sulfonylureas; Glucose tolerance.

LIST OF PUBLICATIONS

I. ***Huyen VT,*** *Phan DV, Thang P, Hoa NK, Ostenson CG*. Antidiabetic effect of Gynostemma pentaphyllum tea in randomly assigned type 2 diabetic patients. Horm Metab Res 2010; 42: 353-357.

II. ***Huyen VT,*** *Phan DV, Thang P, Ky PT, Hoa NK, Ostenson CG*. Antidiabetic effect of add-on Gynostemma pentaphyllum tea therapy with sulfonylureas in randomly assigned type 2 diabetic patients. Submitted manuscript.

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IV. *Kamal Y,* ***Huyen VT,*** *Hoa NK, Ostenson CG.* Herbal extract of *Gynostemma pentaphyllum* decreases hepatic glucose output in type 2 diabetic Goto-Kakizaki rats. Int J Biomed Sci Vol.7 No.2 June 2011.

CONTENTS

LIST OF ABBREVIATIONS

AUCs	Areas under the glucose curves
BMI	Body Mass Index
CAM	Complementary and alternative medicine
DBP	Diastolic blood pressure
DPP4	Dipeptidyl peptidase-4
EHC	Euglycemic-hyperinsulinemic clamp
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
GAD	Glutamic Acid Decarboxylase
GK	Goto-Kakizaki
GLP-1	Glucagon-like-peptide 1
GLUT 2	Glucose transporter 2
GP	*Gynostemma pentaphyllum*
GS	Glycogen synthase
HbA_{1C}	Glycosylated hemoglobin
HGO	Hepatic glucose output
HOMA	Homeostasis model assessment
IA-2	Islet antigen 2
i.p	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IPPTT	Intraperitoneal pyruvate tolerance test
KRB	Krebs-Henseleit bicarbonate buffer
OGTT	Oral glucose tolerance test
PG	Plasma glucose
PTP-1B	Protein tyrosine phosphatase 1B
RIA	Insulin radioimmunoassay
SBP	Systolic blood pressure
s.c	Subcutaneous
SCITT	Subcutaneous insulin tolerance test
SIGIT	Somatostatin-insulin-glucose infusion test
SSPG	Steady-state plasma glucose
SSPI	Steady-state plasma insulin
SU	Sulfonylurea
T2D	Type 2 diabetes mellitus
TZD	Thiazolidinedione

1 INTRODUCTION

Type 2 diabetes mellitus (T2D) is one of the major non-communicable diseases and recognized as an important health burden worldwide, because it is highly prevalent in the general population and causes substantial morbidity, mortality, and long-term complications [1,2]. It now represents one of the most challenging public health problems of the 21st century. The global prevalence of type 2 diabetes mellitus is projected to increase from 2.8% in 2000 to 4.4% in 2030, with the actual number of people with the disease doubling during the same period [3]. A large increase in this global prevalence will take place in the developing world, with approximately 80% of all new cases expected to appear in these countries by 2025 [4,5]. The Asia-Pacific region, including Vietnam, has been identified as of prime importance to the epidemiology of diabetes [6,7] because the prevalence of T2D in this part of the world is higher than in other developing countries [1]. In Vietnam, in the early of 1990s, the reported prevalence of diabetes in the two largest cities, Hanoi and Ho Chi Minh City, was 1.2% and 2.5% respectively [8,9]. In 2002, however, the prevalence increased to 2.7% in Hanoi [10] and 4.8% and 6.9% in rural and urban areas, respectively, of Ho Chi Minh City [9]. This is possibly reflecting the result of lifestyle changes known as the "Nutrition Transition," characterized by over-consumption of food, increased consumption of total fat, animal fat, and protein, as well as decreased physical activity [11].

Knowledge of the pathogenesis of T2D is important in understanding the appropriate modes of treatment. T2D is a heterogeneous syndrome due to the interaction of various environmental factors with multiple diabetogenic genes, which cause various combinations of beta cell failure and insulin resistance. Both defects are partly

genetically and environmentally determined, and both are exacerbated by hyperglycemia ("glucose toxicity") [12,13]. Genetic predisposition accounts for 40-80% of susceptibility to T2D and is polygenic. So far, most of T2D-associated genes relate to beta cell function and insulin secretion, or unknown functions [14,15] Environmental risk factors for T2D include obesity (which accounts for 90% of acquired risk) and physical inactivity, which decreases insulin sensitivity. About 55 percent of T2D patients are obese at diagnosis [16]; chronic obesity leads to the increased insulin resistance that can develop into T2D, most likely because adipose tissue (especially that in the abdomen around internal organs) is a source of several types of molecules, including hormones and cytokines, which signal to other tissues. Inflammatory cytokines, for example, may activate the NF-κB pathway which has been linked to the development of insulin resistance [17]. Other research shows that T2D causes obesity due to changes in metabolism and a gradual failure of cells, leading to insulin resistance [18]. However, environmental factors (almost probably diet and weight) play a large part in the development of T2D, in addition to any genetic component. This can be seen from the adoption of the T2D epidemiological pattern in those who have moved to a different environment as compared to the same genetic pool who have not, e.g. in immigrants to Western developed countries, as compared to their relatives in lower incidence countries of origin [19]. Such developments can also be found in environments which have had a recent increase in social wealth, increasingly common throughout Asia.

Insulin resistance is first demonstrated in skeletal muscle, in which higher concentrations of insulin are necessary to allow glucose to enter cells. Extra-hepatic insulin resistance predicts the development of T2D [13,20] and is detected in normoglycemic first-degree relatives of patients with T2D [21-23]. It is influenced by both genetic and environmental (for example, obesity) factors. Insulin-resistant

individuals frequently exhibit a constellation of other characteristics, including visceral obesity, dyslipidemia, hypertension, hyperinsulinemia, impaired fibrinolysis, endothelial dysfunction, hyperuricemia, vascular inflammation, and premature atherosclerosis [24]. Such individuals are said to have the metabolic syndrome [25], or insulin resistance syndrome, and emphasize the pathogenic role of insulin resistance. Initially, in the face of insulin resistance, compensatory increases in pancreatic insulin secretion may be able to maintain normal glucose concentrations. However, as the disease progresses, due to inherited beta cell impairments, insulin production gradually diminishes, leading to progressive stages of hyperglycemia. Hyperglycemia is first exhibited in the postprandial state, since uptake by skeletal muscle is the metabolic fate of the majority of ingested carbohydrate energy, and then during fasting. As insulin secretion decreases, hepatic glucose production, normally attenuated by insulin, increases. This increase is primarily responsible for the elevation of fasting glucose levels in patients with T2D. Superimposed upon these mechanisms is the well-recognized deleterious effect of hyperglycemia itself – glucotoxicity - upon both insulin sensitivity and insulin secretion [26]. Adipose tissue plays an important but often overlooked role in the pathogenesis of T2D. Insulin resistance is also demonstrated at the adipocyte level, leading to unrestrained lipolysis and elevation of circulating free fatty acids. Increased free fatty acids, in turn, further dampen the insulin response in skeletal muscle [27] while further impairing pancreatic insulin secretion as well as augmenting hepatic glucose production ("lipotoxicity") [28]. Therefore, T2D results from coexisting defects at multiple organ sites: resistance to insulin action in muscle, defective pancreatic insulin secretion, and unrestrained hepatic glucose production, all of which are worsened by defective insulin action in fat. These pathophysiological lesions are to blame for the

development and progression of hyperglycemia. They are also the primary targets for pharmacological therapy.

1.1 TREATMENT OF TYPE 2 DIABETES

The goal in treating T2D is to maintain blood glucose concentrations within normal limits and to prevent the development of long-term complications of the disease. Weight reduction, exercise and dietary modification decrease insulin resistance and correct the hyperglycemia of T2D in some patients. Oral hypoglycemic agents and insulin therapy may be required to achieve satisfactory serum glucose levels. In addition, treatment of dyslipidemia, hemostatic disturbances and hypertension may be indicated.

Non-pharmacological therapy

Diet, exercise, and weight loss are at the center of any therapeutic diabetes program. Not only do these lifestyle modifications lower blood glucose concentrations, they also ameliorate many of the frequently coexisting risk factors for cardiovascular disease. Unfortunately, most patients are unable to achieve adequate control with lifestyle interventions alone, which should not detract from their critical role, since they enhance the effectiveness of medical regimens. An energy-controlled diet and regular aerobic exercise are therefore recommended for the majority of patients with T2D, who are usually overweight [29,30].

Pharmacological Therapy

A number of oral anti-hyperglycemic agents have been introduced in recent years, each with its own mode of action. For example, reversal of the above mentioned defects, either individually or in concert, improves glycemic control: sulfonylureas, metformin, α-glucosidase inhibitors, thiazolidinediones, nonsulfonylurea insulin secretagogues, glucagon-like peptide 1 (GLP-1) analogues and dipeptidyl peptidase-4

(DPP4) inhibitors. An understanding of their mechanisms of action is important for their proper use, especially when they are administered in combination. Sulfonylureas (SU) bind to the sulfonylurea receptor, found on the surface of pancreatic beta cells. This interaction leads to a closure of ATP-dependent potassium (KATP) channels, facilitating cell membrane depolarization, calcium entry into the cell through voltage-dependent calcium channels, and insulin secretion [31]. Thus, SU allow for insulin release at lower glucose thresholds than normal. They partially reverse the attenuated insulin secretion that characterizes T2D. Understandably, in the face of SU therapy, circulating insulin concentrations are increased. As a result, and despite the presence of insulin resistance, glucose concentrations fall. SU increase insulin secretion and they often lead to weight gain and may cause hypoglycemia [31].

Metformin induces suppression of hepatic glucose output [32] and may also increase glucose uptake in muscle via stimulation of AMP kinase [33]. In patients with diabetes, metformin lowers plasma glucose concentrations both alone [34,35] and in combination with SU [35-37] while simultaneously decreasing plasma insulin concentrations. A third category of drugs, *a*-glucosidase inhibitors, decreases postprandial plasma glucose concentrations by delaying the absorption of carbohydrates [38]. Thiazolidinedione (TZD) acts by increasing overall insulin sensitivity with evidence of effects in both the liver, the primary glucose-producing organ, and skeletal muscle, the main site of glucose disposal [31]. TZD is effective both when given alone and when given in combination with either SU or metformin [31,39]. Meglitinides (repaglinidin and nataglinide) help the pancreas produce insulin and are often called "short-acting secretagogues". They act on the same potassium channels as SU, but at a different binding site [40]. By closing the potassium channels of the pancreatic beta cells, they open the calcium channels, hence enhancing insulin secretion [40]. The most recent additions to this armamentarium of anti-diabetic

drugs are the DPP4 inhibitors and GLP-1 analogues, which work by increasing blood concentrations of the incretin GLP-1, which in turn increases insulin production from pancreatic beta cells. They also lower glucagon release from pancreatic alpha cells, which causes reduced hepatic glucose production and may decrease body weight by reducing food intake. Although a growing number of oral pharmacologic options are now available for management of T2D, acting through a variety of mechanisms that confer additive glucose lowering effects, the effectiveness of such therapies still remains inadequate [31]. In addition, all oral anti-diabetic agents have side effects. SU and the shorter-acting insulin secretagogues are associated with weight gain and hypoglycemia [31]. The major side effects of metformin and the GLP-1 analogues are seen in the gastrointestinal tract, with nausea, cramps, and diarrhea or constipation, respectively [31]. The major side effect of α-glucosidase inhibitors is flatulence, which occurs when undigested carbohydrate enters the large bowel, where it is digested by colonic bacteria resulting in gas formation [31]. At high doses or in the presence of renal decompensation, hepatic necrosis may occur due to high serum a-glucosidase levels. The main side effect of TZD is water retention, leading to edema, generally a problem in less than 5% of individuals, but a big problem for some and in those with with significant water retention, potentially leading to decompensation of previously unrecognized heart failure. Therefore, TZD should be prescribed with both caution and patient warnings about the potential for water retention/weight gain, especially in patients with decreased ventricular function (NYHA grade III or IV heart failure). Recent studies have shown that there may be an increased risk of coronary heart disease and heart attacks with rosiglitazone [41] and a possible link between pioglitazone and bladder cancer [42] and osteoporotic fractures [43]. With increasing reliance on multiple patented pharmacological agents to meet glucose targets, the cost of treatment has also become a real concern. The cost of metformin

and sulfonylureas is much lower than that of the meglitinides, TZD, GLP-1 analogues and DPP4 inhibitors [44]. The ability of developing countries, such as Vietnam, to afford this level of treatment is dubious. These concerns point to the need for more effective, safer and less expensive management. Complementary and alternative medicine approaches that include herbs may hold promise in this regard; this research has focused on novel approaches to the treatment of T2D.

1.2 TREATMENT OF T2D IN TRADITIONAL MEDICINE

Many modern pharmaceuticals used in conventional medicine today also have natural plant origins. Among them, metformin was derived from the flowering plant, *Galega officinalis* (Goat's Rue or French Lilac), which was a common traditional remedy for diabetes [45,46]. Plant derivatives with purported hypoglycemic properties have been used in folk medicine and traditional healing systems around the world (e.g., Native American Indian, Jewish, Chinese, East Indian, Mexican, Vietnamese [47]). Traditional herbal medicines have played a major role in the management of diabetes in Vietnam and many Asian countries for centuries [47]. Several hundred plants are known to have anti-diabetic properties, and a large number of compounds from plant extracts have been reported to have beneficial effects for the relief of diabetes [48,49]. In addition, currently available pharmacological agents for T2D exhibit a number of limitations, such as side effects and high rates of secondary failure. Thus, diabetic patients and healthcare professionals are considering complementary and alternative approaches [50-52]. Physicians now indicate that more than 75% of their patients use complementary and alternative medicine (CAM) therapies including herbs [53-55]. A survey conducted in 1997–1998 reported that about one-third of subjects with diabetes use CAM to treat their condition [56]. In other surveys of specific diabetic populations, 39% of Navajo, two-thirds of Vietnamese, and 49% of a largely Hispanic population in South Texas used CAM [57-59]. While herbology is growing in popularity, some

surveys found that only 21% of people with diabetes who used any form of CAM talked to a healthcare professional about it [60]. In general, the scientific literature on the efficacy of herbal medicine in the treatment of diabetes is relatively sparse and heterogeneous. As growing numbers of patients are turning to alternative medicines, diabetes educators need to ask about the use of herbs and to be aware of their benefits and risks.

1.3 GYNOSTEMMA PENTAPHYLLUM

Gynostemma pentaphyllum Makino (Family Cucurbitaceae) (Giảo cổ lam – Vietnamese name or Jiaogulan – Chinese name) is a perennial creeping herb growing wild in the mountainous regions of Vietnam, Japan, China, and many other Asian countries. It is widely used in Vietnam and Southeast Asian countries as a herbal medicine, being beneficial for the prevention and treatment of diabetes. In addition to an anti-diabetic effect, GP extracts reportedly have various effects, such as cholesterol-lowering and immunopotentiation, as well as antitumor and antioxidant effects [61-63]. Saponins, also known as gypenosides, are a large group of substances in GP extract. Currently, there are about 90 different gypenosides that have been isolated and characterized. Crude saponin fractions of GP extracts have shown to exert hypoglycemic and hypolipidemic effects in rats [62,63]. However, the actions of the functional component that is responsible for the anti-diabetic effect of GP extracts need to be elucidated.

Our previous works on GP have revealed the anti-diabetic effect of GP extracts in normal rats, associated with a novel insulin-releasing gypenoside - phanoside [64,65]. Extracts of GP have been shown to reduce both hyperglycemia and hyperlipidemia in diabetic Zucker Fatty rats [66]. A recent experimental study demonstrated that ethanol extract of GP, produced in Vietnam, inhibited protein tyrosine phosphatase 1B (PTP-1B) activity, which may lead to enhanced insulin sensitivity and thereby improved

glucose tolerance [67]. Therefore, it is highly relevant to use GP extracts to do research in subjects with diabetes.

1.4 MODELS FOR ASSESSING ANTI-DIABETIC ACTIVITY

There are some research techniques that are relevant to the study of diabetes, namely methods to assess insulin secretion and action, energy balance and body composition, and vascular structure. These techniques are applied to clinical investigations in humans; most approaches, with appropriate modifications, have also been used to study diabetes in animal models

1.5 CLINICAL RESEARCH METHODS IN DIABETES

Measurements of insulin action

Insulin resistance refers to impairment to the physiological action of insulin. This is generally measured in terms of insulin's glucose-lowering effect, which is convenient and easy to quantify. However, insulin has numerous other metabolic actions (antilipolytic, protein-anabolic) and also affects vascular and other tissues. Sensitivity to these different actions of insulin may vary considerably among individuals and also in different pathophysiological states. For example, subjects with T2D are relatively resistant to insulin's hypoglycemic effect, but remain sensitive to its sodium-retaining action in the kidney. Thus identification of insulin resistance using any of the glucose-based methods does not necessarily predict insensitivity to other actions of insulin. The modern methods that measure insulin sensitivity can be conveniently divided into three groups:

- Steady-state techniques involve the continuously intravenous infusion of insulin with or without glucose, until a nearly constant plasma glucose concentration is reached.

- Dynamic methods use a bolus challenge of intravenous insulin allowing tissue glucose uptake to be derived from the changes in plasma glucose concentrations.

- Basal-state methods calculate insulin resistance from simultaneous fasting plasma glucose and insulin concentrations.

Steady-state methods

All steady-state techniques exploit the principle that maximal insulin-stimulated glucose disposal, which occurs primarily in skeletal muscle, equates with the rate of glucose infusion required to maintain euglycemia under conditions of supraphysiological hyperinsulinemia

- Euglycemic-hyperinsulinaemic clamp (EHC): this technique, developed to study kinetics and pioneered by DeFronzo and colleagues [68], is regarded as the gold standard method for determining insulin sensitivity.

- Modification of the clamp technique: the EHC is a controlled and versatile technique that can be adapted to examine other aspects of insulin action. Using stepped increases in insulin infusion rates, together with somatostatin to suppress endogenous insulin secretion, the EHC has been used to construct a dose-response curve for insulin's suppression of lipolysis and free fatty acid release *in vivo*.

- Somatostatin-insulin-glucose infusion test (SIGIT): Patients initially are infused continuously with an intravenous infusion of somatostatin (270 μg/h) to suppress endogenous insulin and glucose secretion. Insulin and 20% glucose is then infused at doses of 0.4 mU/kg/min and 6 mg/kg/min, respectively. Plasma glucose and insulin are checked at zero, 30, 60, 90, 120 and 150 minutes. These

last 3 values are averaged to determine the steady-state plasma glucose level [69-71].

- Measurement of hepatic glucose output (HGO): HGO can be estimated during steady-state conditions, in which it primarily provides an index of gluconeogenesis. HGO can be measured in the basal state and under conditions of hyperinsulinemia - giving an index of hepatic insulin action.

- Insulin sensitivity tests: these methods involve the coinfusion of insulin and glucose at fixed doses until a steady-state is reached.

Dynamic methods

- Oral glucose tolerance test (OGTT): modification of the diagnostic OGTT has been employed to assess insulin sensitivity, as inferred from changes in insulin concentrations caused by the glucose challenge; however, the approach is invalid in diabetic patients, because of their impaired insulin secretion.

- Insulin tolerance test: this test can be used to determine insulin sensitivity from the rate at which the blood glucose concentration falls following an intravenous injection of insulin.

Basal-state methods

- Homeostasis model assessment (HOMA): the widely used HOMA model, developed by Matthews and colleagues, uses fasting measurements of blood glucose and insulin concentrations to calculate indices of both insulin sensitivity and beta cell function [72]. The principle of HOMA is that blood glucose and insulin concentrations are related by feedback of glucose on beta cells to increase insulin secretion. For a given level of blood glucose, prevailing insulin levels therefore reflect both insulin sensitivity and beta cell function.

Evaluation of beta cell function

Insulin secretory reserve can be assessed by several methods. To distinguish type 1 (and other severe insulin-deficiency states) from type 2 diabetes, C-peptide concentrations can be measured after challenge with insulin secretagogues such as intravenous glucagon or a large carbohydrate load given orally. C-peptide is used in preference to insulin, because it is cleared more slowly and so provides a more reliable index of beta cell secretion.

- Hyperglycemic clamp: the hyperglycemic clamp [68] can be used to measure insulin secretion under steady-state conditions of controlled hyperglycemia (≈10 mmol/L). The insulin response is biphasic, with an early peak, followed by a sustained rise. Indices of glucose-stimulated insulin secretion include the amplitude of the early peak, the final plasma insulin concentration achieved, or the absolute rise from baseline. These data can be expressed as percentages of levels achieved in normal subjects. Alternatively, systemic insulin delivery has been calculated as the product of the metabolic clearance rate (from EHC) and the total insulin response during the hyperglycemic clamp [68].

- Glutamic Acid Decarboxylase (GAD) and islet antigen 2 (IA-2) antibodies are commonly found in diabetes mellitus type 1. In making an early diagnosis for type 1 diabetes mellitus, GAD and IA-2 antibodies tests are used for differential diagnosis between latent autoimmune diabetes of adults (LADA) and type 2 diabetes and may also be used for differential diagnosis of gestational diabetes, risk prediction in immediate family members for type 1 diabetes, as well as a tool to monitor prognosis of the clinical progression of type 1 diabetes [73].

1.6 ANIMAL MODELS IN T2D RESEARCH

Due to complex interactions among multiple susceptibility genes and between genetic and environmental factors, genetic analyses of diabetes are difficult to perform and the genetics of T2D are poorly understood in humans. Moreover, diabetes research in humans is impeded by obvious ethical considerations, because provocation of disease is strictly impermissible in man. Animal models of diabetes are therefore of great use in biomedical studies because they offer promise of new insights into human diabetes. Inbred animal models, in which the genetic background is homogeneous and environmental factors can be controlled, are therefore valuable in the genetic dissection of such a multi-factorial disease. Most of the available models are based on rodents because of their small size, short generation interval, easy availability and economic considerations. Spontaneously diabetic animals of T2D may be obtained from these animals which exhibit one or several genetic mutations transmitted from generation to generation (*e.g.*, *ob/*ob mice, *db/db* mice), or by selection from non-diabetic outbred animals by repeated breeding over several generations *e.g.*, Goto-Kakizaki (GK) rats and Tsumara Suzuki Obese Diabetes (TSOD) mice [74,75].

Goto-Kakizaki (GK) rat

The GK rat is a non-obese Wistar sub-strain which develops T2D early in life. The model was developed by Goto and Kakizaki at Tohoku University, Sendai, Japan in 1975 [76]. The selections of breeding were based on selecting male and female Wistar rats with the highest glucose levels within the normal range, during a glucose tolerance test [76,77]. Repetition of the selective breeding over numerous generations resulted in the production of non-obese diabetic rats from Wistar rats. Colonies were then initiated with breeding pairs from Japan, including the Paris, Stockholm, Seattle and other colonies [78-81]. The Stockholm rat colony was initiated from progenitors delivered from the F40 generation of the original colony. Impaired insulin release appears in

early life and seems to be the primary defect [82]. Insulin resistance in skeletal muscle and liver is moderate and most likely secondary to hyperglycemia, at least in the muscle [82-84]. In GK rats, impaired glucose-stimulated insulin secretion has been demonstrated *in vivo* [78,85,86], in isolated pancreatic islets [79,87], and in the perfused isolated pancreas [78,79,82]. In GK rats from our colony, the pancreatic insulin content and density of beta cells are normal [82,88]. The defective insulin response to glucose in GK rats is accompanied by a number of abnormalities in islet glucose metabolism e.g. increases in glucose cycling and glucose utilization [79,87], and decreases in the activities of FAD-linked glycerol phosphate dehydrogenase, pyruvate carboxylase [79,89] and pyruvate dehydrogenase [90].

Glucose transporter 2 (GLUT 2) is found to be under-expressed, but not to the degree that could lead to the accounted impairment for the insulin release [91]. This hypothesis could be supported by the fact that glucokinase/hexokinase activities were found to be normal [79,92]. Also, glycolysis rates in GK rat islets are unchanged or increased compared with control islets [79,93,94]. Moreover, oxidation of glucose has been reported to be unchanged [79,87].

In GK rats, hepatic insulin resistance is accompanied by an increase in HGO in association with dysregulation of hepatic fructose-2-6-bisphosphate. Hepatic insulin resistance was also characterized by decreased insulin receptor number but normal tyrosine kinase activity [83].

In the skeletal muscle, defective activation of glucose accumulation into glycogen, possibly due to the chronic activation of protein kinase C [95,96], has been suggested to contribute to insulin resistance and hyperglycemia in GK rats. Additionally, defective post-receptor signaling was characterized by alterations in insulin-stimulated glucose transport and PI3K-activated Akt kinase [84].

2 AIMS

The overall aim of this thesis is to investigate the anti-diabetic effect and safety of the traditional Vietnamese herb *Gynostemma pentaphyllum* (GP) extract (tea) in type 2 diabetic patients (T2D) and identify the major mechanisms of action of GP in patients with T2D, as well as in Goto-Kakizaki (GK) rats.

Specific aims for the four papers are as follows:

I. To investigate the anti-diabetic effect of GP tea in drug-naïve T2D patients.

II. To study the anti-diabetic effect of GP tea as add-on therapy with sulfonylureas in drug-naïve T2D patients.

III. To evaluate the effect of GP tea on insulin sensitivity in drug-naïve T2D patients.

IV. To explore the effect of GP tea on hepatic glucose output in spontaneously type 2 diabetic GK rats.

3 MATERIALS AND METHODS

3.1 MEDICATION

The medication was provided in the form of GP tea at a dose of 6g/day (3g/packet, twice a day). The whole plants of *Gynostemma pentaphyllum* Makino-Cucurbitaceae were collected from the Hoa Binh province, in the north of Vietnam, and identified by Professor Pham Thanh Ky, Department of Material Medica, Hanoi College of Pharmacy. The production of GP tea included two stages. The first stage was to confirm authenticated GP plants which were compared with the voucher specimen (HN-0152) deposited in the herbarium at the Department of Material Medica, Hanoi College of Pharmacy. The second stage was to produce the GP tea as specified. Briefly, the process included extraction of the authenticated GP plants for 2h in boiling water, and with a following precipitation of impurities by adding concentrated 70% ethanol. The 70% ethanol was then removed by distillation at low pressure, and impurities were removed by filtration. Thereafter, the tea was inspected as a semi-finished brown powder with the typical odour of GP tea. This powder had a humidity of approximately 6.7%, and could be dissolved in water to produce a brown liquid with a sweet-bitter flavour. The tea contained flavonoids, as shown by a positive cyanidin reaction with the base $FeCl_3$ (5%) [97,98], and furthermore about 18% saponins, as indicated by a positive foaming test [97,98]. Thus, the standardization of the GP tea included confirmation of its typical odour, state and sweet-bitter flavour, approximately 7% in humidity, and positive reactions in the flavonoid (cyanidin reaction) and saponin (foaming) tests. The placebo extract was green tea extract (tea) (*Camellia sinensis*), which was supplied at the same dose and was similar to the GP tea in shape and packaging. After grinding of the resulting GP and placebo material into a powder to form soluble particles, the powder was packed in tin foil packets (6x5 cm) by an

automatic packing machine. Each packet contained 3 g of powder, and 10 packets were packed in one box to be distributed to the patients. Both GP and placebo tea were easily dissolved in 60 mls water (room temperature) and taken 30 minutes prior to breakfast and dinner.

3.2 SUBJECTS

Patients for Paper I, II and III

The studies were implemented on newly-diagnosed T2D patients. All subjects were recruited from outpatients at the National Institute of Gerontology and some district hospitals in Hanoi. The treatment options and possibility of beneficial effects and risks of the treatment were explained to newly-diagnosed patients with T2D. The treatment option was left to the subject's discretion. Consecutive patients were enrolled into the study. Written informed consent was obtained from all subjects. The study protocol was approved by the research ethics board at Hanoi Medical University, Hanoi, Vietnam and the Regional Ethics Committee at the Karolinska Institute, Stockholm, Sweden.

Inclusion criteria were: 1) newly-diagnosed patients with T2D according to WHO criteria [99] were recruited from outpatients at the National Institute of Gerontology and some district hospitals in Hanoi, Vietnam; 2) age from 40 to 70 years old, 3) anti-diabetic drug naïve; 4) mean (of two) fasting plasma glucose (FPG) measurements from 9 to 14 mmol/L; and 5) glycosylated hemoglobin (HbA_{1C}) from 9 to 13%.

Exclusion criteria were: 1) previous pharmacological treatment for diabetes, 2) chronic complications related to type 2 diabetes, 3) smoking subjects, 4) pregnancy or lactation and 5) increased titres of GAD and IA-2 antibodies.

Evaluation at baseline included detailed medical history and examination, fasting and oral glucose tolerance test, HbA_{1C}, liver and renal function tests, fasting lipid profile, and plasma insulin and C-peptide levels.

Animals for Paper IV

Diabetic Goto-Kakizaki (GK) rats (200-300g), originating from Wistar rats, were bred in our department. The animals were kept at 22˚C on a 12-hour light-dark cycle (6 a.m and 6 p.m) with free access to food and water during GP treatment (see below) and before being anesthetized for liver perfusion. The study was approved by the Laboratory Animal Ethics Committee of the Karolinska Institute (N367/08, N72/08).

3.3 METHODS FOR PATIENTS WITH T2D

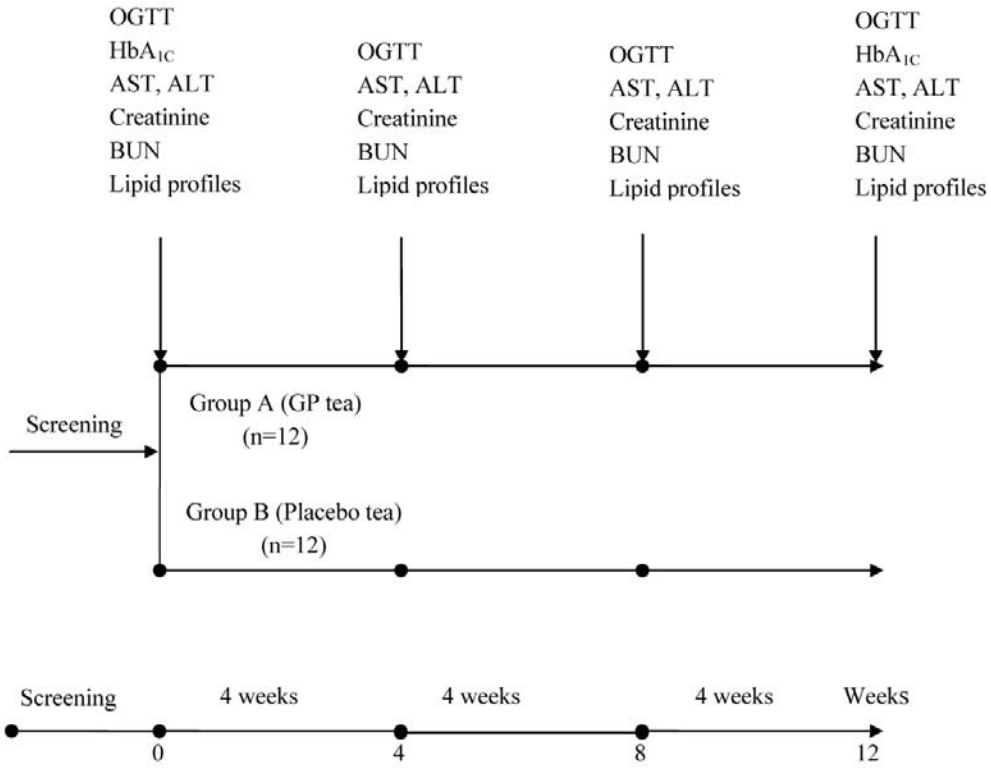
The study used a randomized, placebo-controlled, double-blind design for Papers I and II, and a cross-over design for Paper III. Group assignment of patients was blinded for both the main investigator and other investigators performing outcome analyses by use of a coding system, where the codes were kept by the independent allocator and revealed only after completing treatment periods and analyses. The patients were instructed to follow the diet as always recommended for newly diagnosed T2D patients. The patients were also told to walk 30 minutes a day and at least three days a week during the trial. This was reinforced at each follow-up visit every week. All patients were treated on an out-patient basis with individual medical records and were provided tea for a week; all tea bags were counted out at each visit. The subjects were encouraged to call the physicians at any time if they had problems.

Paper I

The design of the study is shown in Figure 1a. After screening by the main investigator, the selected patients were randomly assigned by another, independent allocator by use of numbered containers into 2 groups, matched by sex: group A and group B received

GP tea and placebo tea, respectively, in a divided dose (twice a day, 30 minutes before breakfast and dinner).

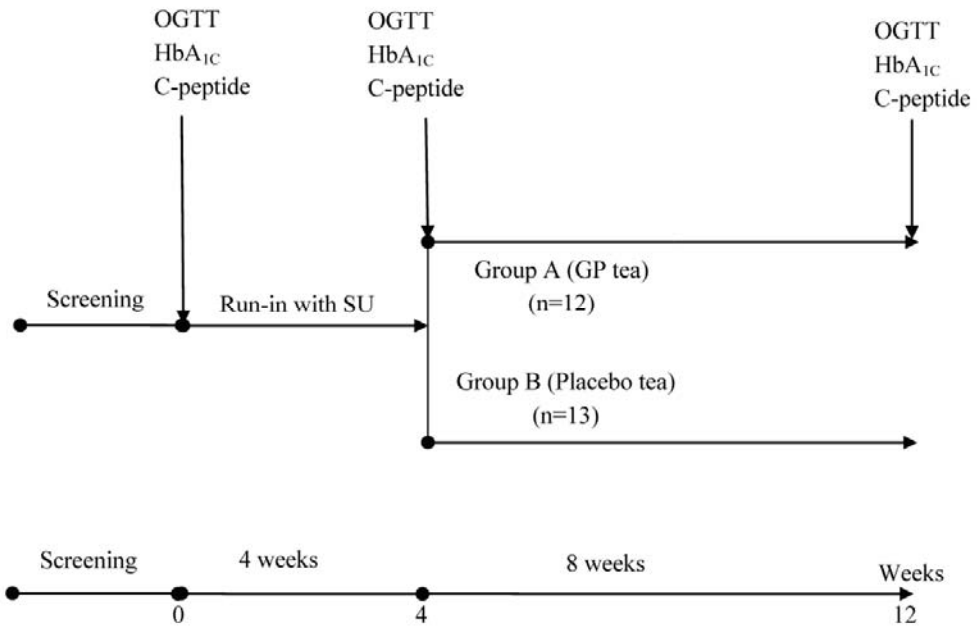
Figure 1a.



Paper II

The protocol of this clinical trial is presented in Figure 1b. After screening, the selected subjects received gliclazide modified-release preparation 30 mg daily as a single dose, and entered a 4-week run-in period of reinforcement of lifestyle education, followed by concealed randomization. After the four week-run in, all selected patients were randomly assigned by another, independent allocator by use of numbered containers into 2 groups, matched by age, sex and HbA_{1C}: group A and group B received GP tea and placebo tea, respectively, in a divided dose (twice a day, 30 minutes before breakfast and dinner).

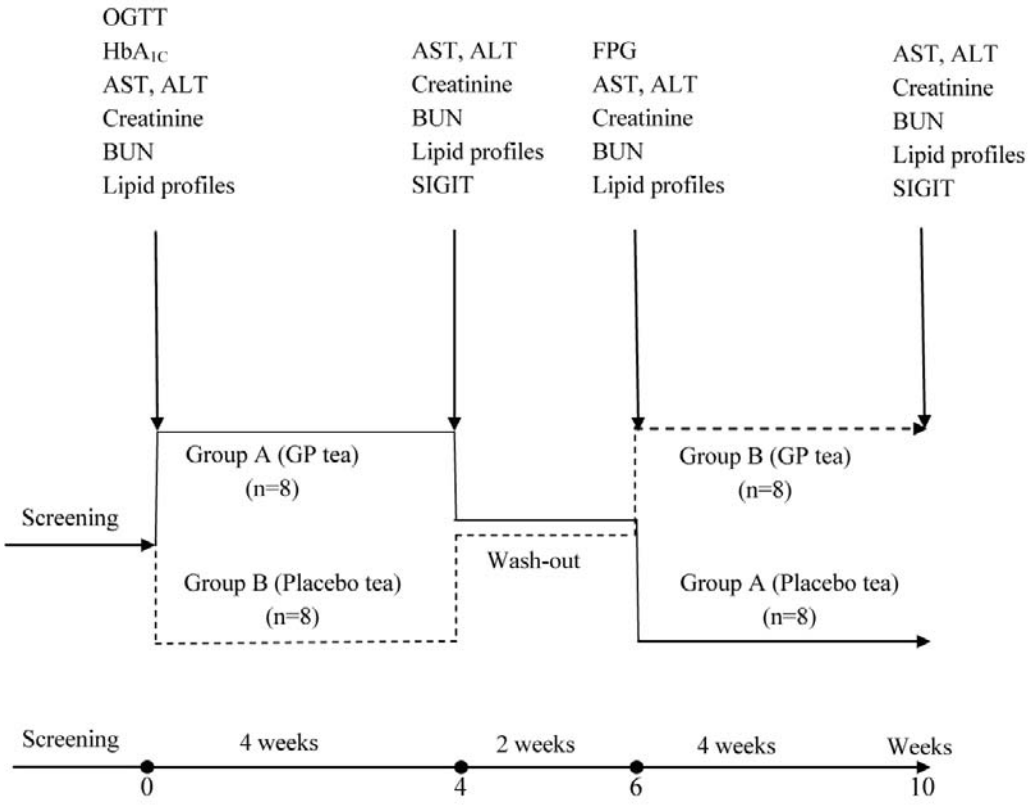
Figure 1b.



Paper III

The protocol of this clinical trial is presented in Figure 1c. After screening by the main investigator, the selected patients were randomly assigned by another, independent allocator by use of numbered containers into two groups, matched by age, sex, fasting plasma glucose and HbA_{1C}: group A and group B received GP tea and placebo tea, respectively, 6 g daily in a divided dose (twice a day, 30 minutes before breakfast and dinner). After a two-week wash-out period on non-tea therapy, the patients were switched to another four weeks of 6 g GP tea/day (group B) and placebo-tea (group A). At the end of both four-week periods of treatment with GP or placebo tea, all patients underwent a somatostatin-insulin-glucose infusion test.

Figure 1c.



Somatostatin-Insulin-Glucose Infusion Test (SIGIT) (Paper III)

All subjects participated in the SIGIT, performed at 8am after an 8-10h overnight fast with only tap water allowed *ad libitum*. The SIGIT, lasting 150 minutes, was conducted as described earlier [70,71]. Briefly, an i.v cannula (Exeflon, Exelint, Los Angeles, California, USA) was inserted into an antecubital vein for infusion of all test substances. A second cannula was inserted into a wrist vein contra-laterally to the infusion site for blood sampling. Each subject was given a 150 minute intravenous infusion of somatostatin (270 µg/h; Somatosan, Wasserburger Arzneimittelwerk GmbH, Wasserburg, Germany), insulin (0.4 mU/kg/min; Actrapid HM, Novo Nordisk, Denmark) and glucose (6 mg/kg/min; in a 20% solution). Ten ml blood of each patient was added to the mixture to prevent absorption of insulin and somatostatin onto the

glass surface. Plasma glucose and insulin were measured at 0, 30, 60, 90, 120 and 150 minutes. Somatostatin was used to suppress endogenous insulin release, thereby allowing an estimation of sensitivity to exogenously administered insulin by measuring blood glucose values at 90, 120 and 150 minutes of the test (SIGIT mean). Since similar steady-state plasma insulin levels are achieved in all subjects, this test allows us to compare the ability of the recruited subjects to dispose of identical glucose loads under the same insulin stimulus. Therefore, the mean of several plasma glucose concentrations measured during the steady-state period from 90 to 150 minutes is a measure of efficiency of insulin-mediated glucose utilization, i.e., insulin sensitivity.

Biochemical and anthropometric analyses

Blood samples of fasting subjects were taken before, during (every week or second week for 10-12 weeks), and after the treatment for measurement of plasma glucose, HbA_{1C}, liver enzymes (ALT, AST), creatinine, lipid profiles, glucagon, cortisol, insulin and C-peptide levels. Serum samples were obtained by centrifugation and stored at minus 20ºC pending for assay. Analysis of glucose concentration of each sample was done by enzymatic colorimetric test, GOD-PAP in a glucose analyzer (Autolab Instrument, Boehringer Mannheim, Germany, wave-length Hg 546 nm). HbA_{1C} was measured with a BIO-RAD D-10^{TM} (Bio-Rad, Strasbourg, Schiltigheim, France). The insulin concentration was measured by insulin radioimmunoassay (RIA), using our own antibodies, human insulin as a standard, and charcoal addition to separate antibody-bound and free insulin [100], glucagon was determined by RIA (Euro-Diagnostica AB, Medeon, Malmö, Sweden), and cortisol was analyzed by Cortisol RIA DSL-2000 (Cherwell Innovation Centre, Upper Heyford, Oxon, UK). C-peptide was measured by human C-peptide RIA kit (HCP-20K, Millipore, 6 Research Park Drive, St. Charles, Missouri 63304 USA). Oral glucose tolerance tests (OGTT) (75g glucose)

were performed four times for Paper I, three times at baseline, after four and twelve weeks for Paper II, and at baseline to confirm the diabetic diagnosis for Paper III. Venous blood samples were drawn before, 30 and 120 minutes after glucose intake. The Homeostasis Model Assessment (HOMA) was used to assess insulin sensitivity and beta cell function, based on fasting insulin and glucose levels and according to published algorithms: HOMA insulin resistance (HOMA-IR) = (insulin x glucose)/22.5, and HOMA beta cell function (HOMA-β) = 20 x insulin/(glucose -3.5) [72]. Body weight, body mass index (BMI), waist and hip circumference, blood pressure, and registered adverse effects were noted in medical records during the visits.

3.4 METHODS FOR GK RATS

Oral administration of GP and placebo tea

GP and placebo tea were given to unanesthetized male GK rats by gavage through an enteral feeding tube (polyvinyl chloride, sterile VYCON, Lab. Pharmaceutiques Vycon, Ecouen, France) connected to a syringe with the solutions. The GK rats were divided into two groups in each experiment; 800 mg/kg of GP tea or placebo tea were given twice a day at 9:00 and 15:00 for three days or three weeks.

Plasma glucose (PG) measurement

Blood samples for determination of glucose (about 20µl/sample) were taken after a small tail incision. PG levels were monitored by the glucose dehydrogenase method (Accu-Check, Aviva**)** every 2 days before the oral administration of GP or placebo tea.

Intraperitoneal Glucose Tolerance Test (IPGTT) and Intraperitoneal Pyruvate Tolerance Test (IPPTT)

IPGTT and IPPTT were carried out in overnight fasted GK rats. PG concentrations were obtained at 0, 15, 30, 60, and 120 minutes after an intraperitoneal (i.p.) injection

of glucose (2 mg/g body weight; Glukos APL 500 mg/ml) or pyruvate (2 mg/g body weight; sodium pyruvate, SIGMA), respectively.

Subcutaneous (s.c) insulin tolerance test (SCITT)

For the SCITT, insulin was injected s.c. at a dose of 0.5 U/kg and PG levels were measured in fasted GK rats before the injection of insulin (0 min) and every 15 minutes for 2 hours and then every 30 minutes for another 2 hours.

Isolation and perfusion of GK rat liver for determination of hepatic glucose output (HGO)

Liver perfusions were started between 10 a.m. and noon. The rats were anesthetized with an i.p. injection of ketamine (60-70 µg/g body weight; Pfizer AB, Täby, Sweden). Livers were perfused *in situ* without recirculation in a 37°C cabinet via the portal vein using Krebs-Henseleit bicarbonate buffer (KRB), pH 7.4, which was equilibrated with 95% O_2 and 5% CO_2. The perfusion pressure was kept constant with a flow rate of 3.0-4.0 ml/min/g liver.

Adrenaline (Merck AB NM, Stockholm, Sweden) was diluted into the perfusion medium (KRB) to a final concentration of 50 nmol/L. Livers were perfused for 8-18 minutes. In rats treated for three days, livers were perfused with KRB for eight minutes. In rats treated for three weeks, the first eight minutes of perfusion with KRB only was followed by addition of adrenalin (50 nmol/L) in KRB for 10 minutes. Samples of the perfusate were taken at 2-minute intervals from the inferior caval vein during perfusion, and their glucose levels were measured by the glucose oxidase method using a glucose analyzer (Yellow Springs Instruments). Hepatic glucose output was calculated using the mean glucose concentration in relation to flow rate and hepatic dry weight. These livers were not used for any other measurements.

Hepatic glycogen content

Liver homogenates were extracted in 80% ethanol to remove glucose. An aliquot of each homogenate was mixed with amyloglucosidase (Roche Applied Science) and incubated at 60°C for 15 minutes to degrade glycogen into glucose residuals. The samples were diluted and incubated with 1 ml Glucose Assay Reagent (o-Dianisidine Reagent + Glucose Oxidase /Peroxidase Reagent, Sigma-Aldrich) at 37°C for 30 minutes, followed by the addition of 1 ml 12N H_2SO_4 to stop the reaction. The absorbance of glucose was read at 540 nm. In parallel, different concentrations of rabbit liver glycogen type III (Sigma-Aldrich) were treated as the samples and used as a standard curve.

Hepatic glycogen synthase (GS) activity

GS was determined by a method based on the incorporation of ^{14}C-labelled uridine diphosphate-glucose into glycogen. The active form of GS (the form which is activated by insulin) was measured at a low concentration of glucose-6-phosphate (0.3 mm) and the total GS at a high concentration (6.0 mm).

3.5 STATISTICAL ANALYSIS:

Results are expressed as means ± SD in Papers I, II and III, and means ± SEM in Paper IV. Paired t-tests and ANOVA were used to analyze data in the same group before and after treatment, with Bonferroni correction for multiple testing. The unpaired sample t-test was used for normally distributed variables to compare differences in mean change between the treatment group and the control group (SPSS version 12.0 in Papers I, SPSS version 16.0 in Papers II and III, Sigmaplot 2001 in Paper IV). Differences were considered significant if the p-value was below 0.05.

4 RESULTS

4.1 BASELINE CHARACTERISTICS OF THE PATIENTS IN 3 TRIALS (PAPERS I, II, III)

In Paper I, the baseline evaluations of all selected T2D patients revealed that there were no statistically significant differences between the treatment group and the control group on age, gender, systolic and diastolic blood pressure (SBP and DBP), body weight, BMI, waist, hip circumference, FPG, and HbA_{1C} (Table 1a). All patients were compliant with the treatment protocol and completed the study. There were no adverse effects, such as gastrointestinal (nausea or vomiting) and hypoglycemic symptoms, from either GP tea or placebo tea during the research period. Body weights and BMI values at the end of the study were not significantly different from those at the baseline value.

In Paper II, after a 4-week treatment with SU, FPG was significantly decreased from 11.4 ± 1.3 to 9.3 ± 1.3 mmol/L ($n = 25$, $p < 0.001$). A similar decrement was observed with HbA_{1C} from 9.9 ± 1.0 to 8.9 ± 0.7% ($p < 0.001$). Increases in C-peptide and insulin levels, and improvements of lipid profile were also found. At the fourth week, evaluations of all selected T2D patients revealed no statistically significant differences between groups allotted to treatment with GP and placebo tea regarding age, gender, SBP and DBP, body weight, BMI, waist, hip circumference, FPG, and HbA_{1C} (Table 1b). All patients were compliant with the treatment protocol.

In Paper III, the baseline characteristics of the patients receiving GP treatment initially did not differ significantly from those starting with placebo and *vice versa* at week 6, after two weeks of wash-out (Table 1c). There were no statistically significant differences between the groups regarding age, gender, SBP and DBP, body weight, BMI, waist, hip circumference, FPG, and HbA_{1C}.

Table 1a. Baseline clinical characteristics and laboratory findings of the patients

	GP group	Control group
N	12	12
Age (years)	63.5 ± 6.5	57.2 ± 8.2
Sex (Male:Female)	8:4	9:3
Body weight (kg)	61.1 ± 10.0	57.6 ± 10.6
BMI (kg/m^2)	24.0 ± 2.3	23.0 ± 2.6
Waist	90.3 ± 6.7	88.3± 8.9
Hip	99.3 ± 6.4	98.2 ± 6.0
SBP (mm Hg)	120 ± 10.0	117 ± 10.0
DBP (mm Hg)	78 ± 4.5	75 ± 6.8
FPG (mmol/L)	10.0 ± 2.2	9.3 ± 2.3
FPI (pmol/L)	94.4 ± 31.2	79.5 ± 27.8
HbA_{1C} (%)	9.4 ± 2.0	8.2 ± 1.6
Cholesterol (mmol/L)	4.9 ± 1.3	4.8 ± 1.2[a]
Triglyceride (mmol/L)	2.8 ± 1.5	2.6 ± 1.8[a]
HDL-cholesterol (mmol/L)	1.0 ± 0.2	1.0 ± 0.3[a]
LDL-cholesterol (mmol/L)	3.1 ± 0.9	2.7 ± 0.9[a]

Results are means ± SD. [a] n=11, after removal of one patient with an extreme triglyceride value (22 mmol/l). SBP = Systolic Blood Pressure, DBS = Diastolic Blood Pressure, FPG = Fasting Plasma Glucose, FPI = Fasting Plasma Insulin

Table 1b. Clinical characteristics and laboratory findings of the patients at baseline and after 4 weeks

	GP group		Control group	
	Baseline	**After 4 weeks**	**Baseline**	**After 4 week**
N	12	12	13	13
Age (years)	55.6 ± 9.9	55.6 ± 9.9	54.5 ± 8.4	54.5 ± 8.4
Sex (Male:Female)	7:5	7:5	8:5	8:5
Body weight (kg)	54.8 ± 6.8	54.9 ± 6.9	55.3 ± 11.9	55.3 ± 11.4
BMI (kg/m^2)	21.7 ± 2.7	21.8 ± 2.7	22.5 ± 4.4	22.5 ± 4.2
Waist	86.0 ± 5.8	85.8 ± 5.9	87.1 ± 11.4	87.3 ± 11.5
Hip	95.8 ± 3.8	95.3 ± 4.3	97.9 ± 8.5	97.3 ± 8.5
SBP (mm Hg)	122.5 ± 8.7	123.5 ± 9.1	119.2 ± 6.4	119.2 ± 6.4
DBP (mm Hg)	79.6 ± 5.4	79.6 ± 5.3	77.7 ± 4.2	76.2 ± 5.1
FPG (mmol/L)	11.6 ± 1.1	9.6 ± 1.6	11.1± 1.5	9.0 ± 1.0
FPI (pmol/L)	96.1 ± 33.0	120.0 ± 42.0	101.4 ± 34.2	131.4 ± 67.2
HbA_{1C} (%)	9.9 ± 1.0	9.0 ± 0.7	9.9 ± 1.1	8.8 ± 0.6
C-peptide (ng/mL)	0.9 ± 0.5	1.7 ± 0.7	0.8 ± 0.6	1.5 ± 0.8
30-minute C-peptide (ng/mL)	1.4 ± 1.0	2.2 ± 1.3	1.5 ± 1.4	2.9 ± 1.3
Cholesterol (mmol/L)	5.5 ± 1.3	4.8 ± 1.1	4.8 ± 1.1	4.3 ± 0.8
Triglyceride (mmol/L)	3.2 ± 2.5	2.4 ± 1.5	2.7 ± 2.0	2.0 ± 1.2
HDL-cholesterol (mmol/L)	1.3 ± 0.3	1.3 ± 0.4	1.2 ± 0.2	1.1 ± 0.2
LDL-cholesterol (mmol/L)	3.0 ± 0.8	2.8 ± 0.9	2.9 ± 0.9	2.3 ± 0.6

Results are means ± SD, SBP = Systolic Blood Pressure, DBS = Diastolic Blood Pressure, FPG = Fasting Plasma Glucose, FPI = Fasting Plasma Insulin

Table 1c. Clinical characteristics and laboratory findings of the patients

	Group A (GP-Placebo tea)		Group B (Placebo tea - GP)	
	Baseline	**Week 6**	**Baseline**	**Week 6**
N	8	8	8	8
Age (years)	58.8 ± 5.9	58.8 ± 5.9	58.1 ± 6.6	58.1 ± 6.6
Sex (Male:Female)	5:3	5:3	5:3	5:3
Body weight (kg)	58.6 ± 5.3	58.8 ± 5.4	56.8 ± 4.9	57.1 ± 5.0
BMI (kg/m^2)	23.3 ± 1.9	23.4 ± 2.0	22.6 ± 1.8	22.7 ± 1.9
Waist	85.8 ± 6.2	85.4 ± 5.8	81.0 ± 6.1	81.9 ± 6.0
Hip	94.4 ± 3.5	94.1 ± 3.3	91.8 ± 4.2	91.6 ± 4.0
SBP (mm Hg)	119.4 ± 10.2	119.4 ± 11.5	118.2 ± 8.3	118.5 ± 7.9
DBS (mm Hg)	74.4 ± 5.0	74.4 ± 4.9	76.3 ± 4.2	76.3 ± 5.1
FPG (mmol/L)	8.3 ± 0.8	7.4 ± 0.6	8.4 ± 0.7	8.0 ± 1.0
FPI (pmol/L)	141.0 ± 38.2	132.0 ± 109.2	113.2 ± 65.3	122.9 ± 78.5
HbA_{1C} (%)	8.1 ± 0.9		8.1 ± 0.7	
Cholesterol (mmol/L)	5.4 ± 0.8	5.4 ± 1.0	5.0 ± 0.9	5.4 ± 1.2 [a]
Triglyceride (mmol/L)	2.2 ± 1.5	2.1 ± 0.9	2.1 ± 1.1	2.7 ± 1.3[a]
HDL-cholesterol (mmol/L)	1.5 ± 1.0	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.2 [a]
LDL-cholesterol (mmol/L)	3.3 ± 1.2	3.3 ± 1.0	3.0 ± 0.7	3.0 ± 0.8 [a]

Results are means ± SD of sixteen patients. [a] n=7, after removal of one patient with an extreme triglyceride value (12 mmol/l). SBP = Systolic Blood Pressure, DBS = Diastolic Blood Pressure, FPG = Fasting Plasma Glucose, FPI = Fasting Plasma Insulin

4.2 EFFECTS OF GP TEA ON GLUCOSE REGULATION

In Paper I, after 12-weeks treatment, the FPG in the GP group decreased significantly (from 10.0 ± 2.2 to 7.0 ± 1.4 mmol/L, $p < 0.001$), while the levels of FPG in the control group did not change significantly (Figure 2a). Thus, at the end of treatment period, FPG had decreased 3.0 ± 1.8 mmol/L in the GP tea group, as compared to 0.6 ± 2.2 mmol/L in the control group ($p < 0.01$). There was no change in fasting plasma insulin levels between the groups. Therapy with GP tea did not decrease 30 and 120 minute OGTT post-load glucose values significantly. The HbA_{1C} values decreased from 9.4 ± 2.0 to 7.4 ± 1.0% in the GP tea group and from 8.2 ± 1.6 to 8.1 ± 1.3 in the control group. Thus, the decrement was significantly larger in the intervention group than in the control group (2.0 ± 1.3 versus 0.2 ± 0.5 %, $p < 0.001$).

Change in HOMA-IR between baseline and the twelfth week indicated that insulin resistance decreased significantly in the GP group (-2.1 ± 3.0) compared with that (+1.1 ± 3.3) in the control group ($p < 0.05$), but there were no significant changes in HOMA-β during 12 weeks (Table 2).

In Paper II, FPG was not different between groups after four weeks treatment with SU, but was significantly decreased after six weeks of add-on GP tea with SU ($p < 0.001$) (Figure 2b). At the end of the treatment course, the value of FPG decreased by 2.9 ± 1.7 mmol/L to a level of 6.8 ± 0.4 mmol/L ($p < 0.001$) in the GP tea group. The levels of FPG in the control group were slightly reduced (0.9 ± 0.6 mmol/L, $p<0.001$ vs. reduction in GP group) at the end of the treatment period to a level of 8.1 ± 1 mmol/L. Therapy with GP tea also significantly decreased 30 and 120 minute OGTT post-load glucose values. The HbA_{1C} values decreased from 9.0 ± 0.7 to 7.0 ± 0.7% in the GP group, and from 8.8 ± 0.6 to 8.1 ± 0.6% in the placebo group. Thus, the decrement was significantly larger in the GP group than that in the control group (2.0 ± 0.9 versus

0.7 ± 0.5 % units, $p < 0.001$) (Table 3). The glycometabolic improvements were achieved without any major change of circulating insulin and C-peptide levels.

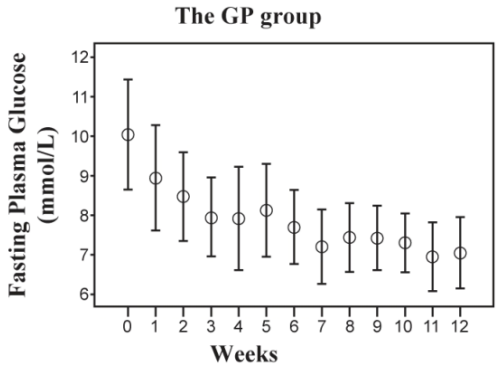
Table 2- Change in HOMA-IR and HOMA-β

	HOMA-IR			HOMA-β		
	GP group (n=11)	Control group (n=12)	p	GP group (n=11)	Control group (n=12)	p
Baseline	6.96 ± 2.32	5.32 ± 1.91	0.077	54.79 ± 32.51	54.79 ± 32.28	1
After 12-week	4.82 ± 2.64	6.89 ± 3.8	0.236	50.36 ± 30.65	69.6 ± 53.03	0.305
Change (0-12 weeks)	-2.14 ± 3.05	1.1 ± 3.27	0.023	- 4.43 ± 26.17	14.82 ± 37.2	0.17

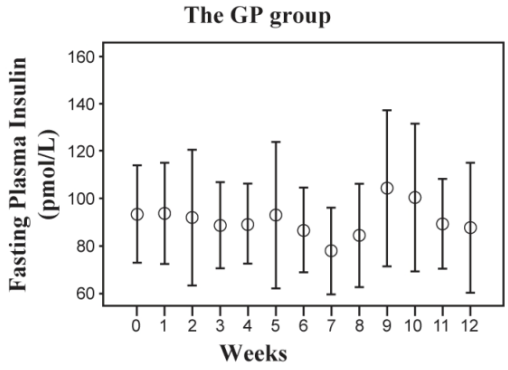
Results are means ± SD. Insulin value missing in one patients of GP group

In Paper III, treatment with GP tea compared to placebo induced no differences in fasting plasma insulin (FPI) but resulted in lower FPG ($p < 0.001$; Table 4). Following treatment with GP tea, the FPG decreased by 1.9 ± 1.0 mmol/L, while the levels of FPG in the placebo treatment were not significantly reduced (-0.2 ± 1.5 mmol/L, $p < 0.001$). These effects were reversed after exchanging treatment (Figure 2c) and the glycometabolic improvements were achieved without any major change of circulating insulin levels (Table 4, Figure 2c).

Figure 2a. Fasting plasma glucose (mmol/L) and insulin (pmol/L) levels in GP (A and C) and control (B and D, respectively) groups. Means ± SD (n=12 in each group except n=11 for insulin in GP group).



A



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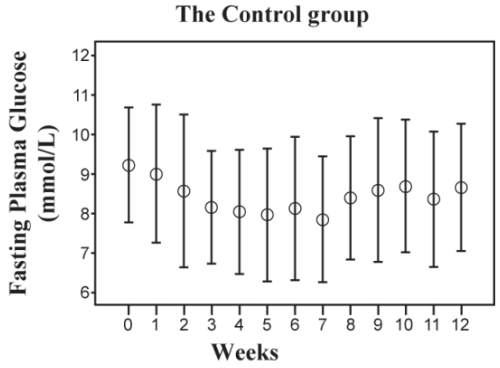

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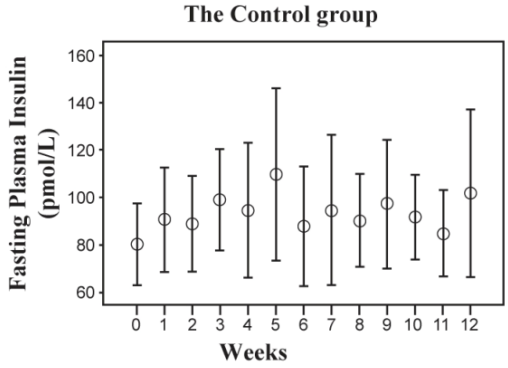


D

Figure 2b. Fasting plasma glucose (mmol/L) and insulin (pmol/L) levels in GP (A and C) and control (B and D, respectively) groups after adding GP or placebo tea. Means ± SD (n=12 in GP group and n=13 in control group).

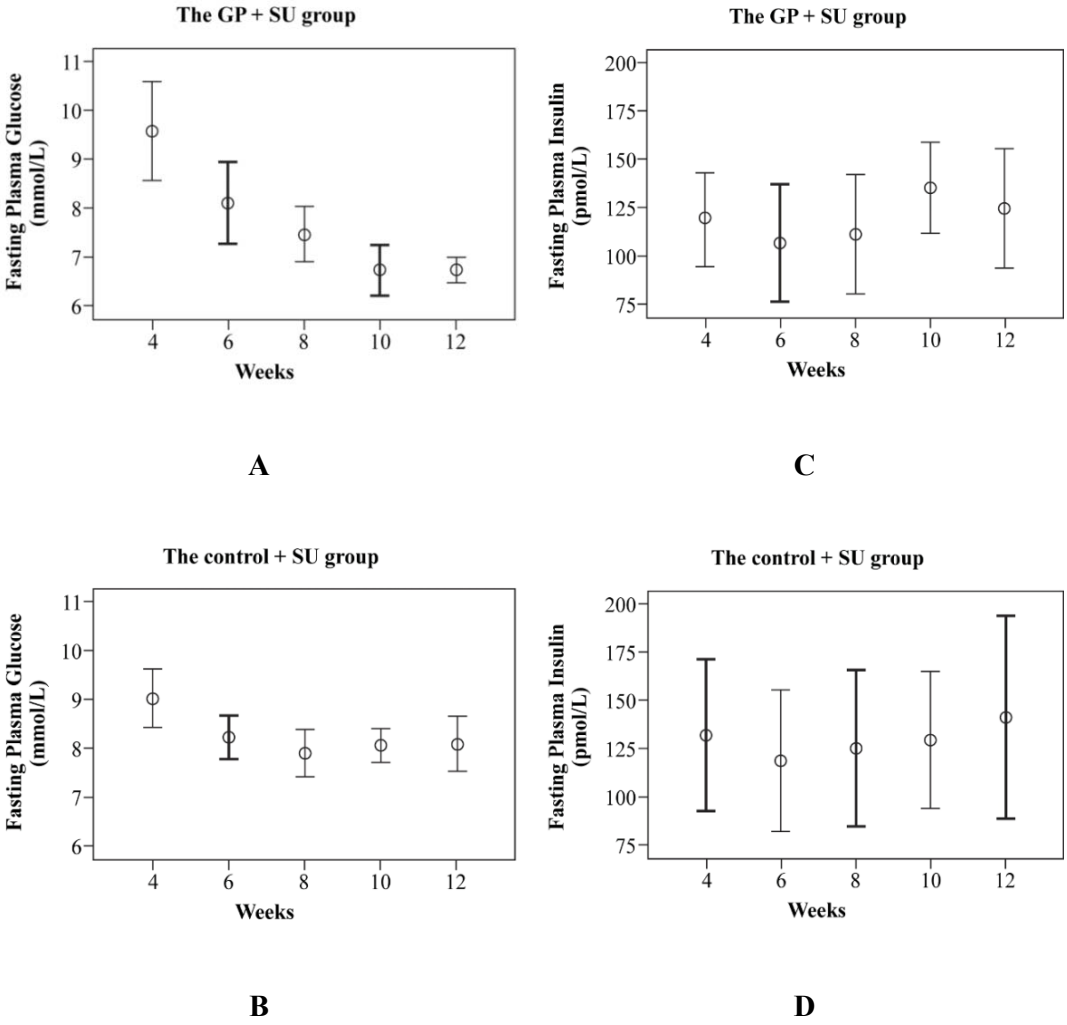


Table 3. Changes in HbA_{1C} from week 4 to week 12

HbA_{1C} (%)	GP tea group n=12	Placebo group n=13	p
Week 4	9.0 ± 0.7	8.8 ± 0.6	0.516
Week 12	7.0 ± 0.7	8.1 ± 0.6	< 0.001
Change (4-12 weeks)	-2.0 ± 0.9	-0.7 ± 0.5	< 0.001

Results are mean ± SD

The mean steady-state plasma insulin levels and plasma glucose responses of the two groups during the SIGIT study are given in Table 4. The steady-state plasma insulin (SSPI) response clearly indicates that similar plasma levels of exogenous insulin were attained as a result of the infusion in all subjects. In contrast, the mean steady-state plasma glucose (SSPG) responses were markedly decreased after GP extract treatment ($p < 0.01$), indicating improved insulin sensitivity.

Changes in body weight and other parameters

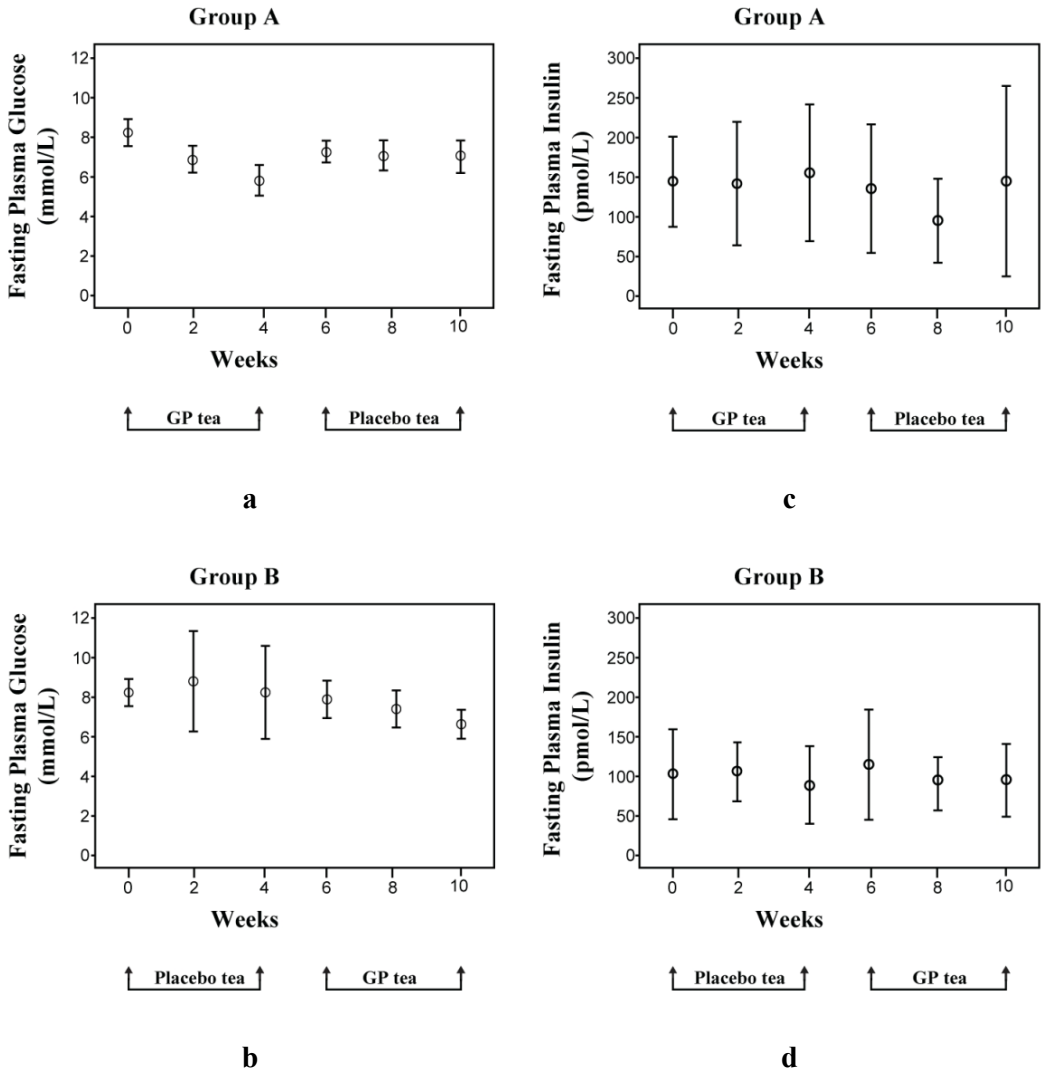
There were no significant differences within or between groups regarding changes in serum triglyceride, total cholesterol, HDL and LDL cholesterol levels. Similarly, no significant changes in the plasma levels of AST, ALT, creatinine, BUN were detected during the study. Neither the GP tea -treated nor placebo groups experienced any acute adverse effect such as gastrointestinal, diarrhea and hypoglycemic symptoms during the research period, and all patients were compliant with the treatment protocol and completed the study.

Table 4. Plasma glucose, insulin levels and SIGIT mean in two kinds of treatment

	GP treatment	Placebo treatment	p
FPG before treatment (mmol/L)	8.2 ± 0.9	7.9 ± 0.8	0.385
FPG after treatment (mmol/L)	6.3 ± 0.8	7.7 ± 1.9	0.039
Change in FPG (mmol/L)	-1.9 ± 1.0	-0.2 ± 1.5	< 0.001
SSPG (SIGIT mean) (mmol/L)	12.5 ± 3.2	16.2 ± 4.1	< 0.01
FPI before treatment (pmol/L)	123.6 ± 59.7	122.2 ± 79.9	0.971
FPI after treatment (pmol/L)	130.6 ± 81.3	120.1 ± 106.3	0.773
Change in FPI (pmol/L)	-0.2 ± 7.8	-0.3 ± 9.8	0.984
SSPI (pmol/L)	223.6 ± 91.0	218.1 ± 104.9	0.696

Results are means ± SD of sixteen patients in each treatment. FPG = Fasting Plasma Glucose, FPI = Fasting Plasma Insulin, SSPG: steady-state plasma glucose; SSPI: steady-state plasma insulin.

Figure 2c. Fasting plasma glucose (mmol/L) and insulin (pmol/L) levels in Group A (a and c) and Group B (b and d). Means ± SD (n = 8 in each group).



4.3 EFFECT OF GP EXTRACT ON PG IN GK RATS

The three-day treatment of GK rats with 800 mg/kg GP tea, or placebo tea, given orally twice a day had no significant effect on PG levels (from 9.9 ± 1.2 to 8.9 ± 0.6 mmol/L in GP treated, and from 8.5 ± 0.4 to 8.6 ± 0.3mmol/L in placebo treated rats). In the three-week treatment, the PG concentrations were reduced significantly in GP treated rats from 9.8 ± 0.6 to 6.8 ± 0.4 mmol/L ($p = 0.027$), while in the placebo rats, PG levels were slightly but not significantly decreased from 8.8 ± 0.8 to 7.5 ± 0.3 mmol/L.

Effect of GP tea on IPGTT, SCITT and IPPTT in GK rats

In the IPGTT, the baseline glucose tolerance test (day 0) was similar in both groups, the areas under the glucose curves (AUCs) during 120 min (0-120 min) being 1995.1 ± 102.2 vs. 2029.5 ± 135.1 mmol/L, ($p = 0.843$). However, after the three-week treatment with GP tea, the glucose tolerance was significantly improved as compared to that in the placebo group, with AUCs 1149.6 ± 200.0 vs. 1727.9 ± 95.5 mmol/L, respectively ($p < 0.05$; Figure 3). After the three-week treatment, the PG concentrations in rats administered with s.c. insulin (0.5 U/kg, SCITT) were reduced similarly in both groups, AUCs (0-240 min) (-149.7 ± 71.8 vs. -110.4 ± 65.1 mmol/L, $p = 0.696$; Figure 4). In the IPPTT, there were no significant differences in the total glucose responses between the two groups, AUCs (0-120 min) (1137.7 ± 67.8 vs. 1240.1 ± 103.6 mmol/L; $p = 0.432$; Figure 5). However, when analysing the glucose response from minute 15 to minute 120, the AUC (15-120 min) was significantly lower in the GP group (415.5 ± 68.0 vs. 641.5 ± 41.8 mmol/L; $p < 0.05$).

Figure 3. Mean of plasma glucose levels in the intraperitoneal glucose tolerance test after the three-week treatment.

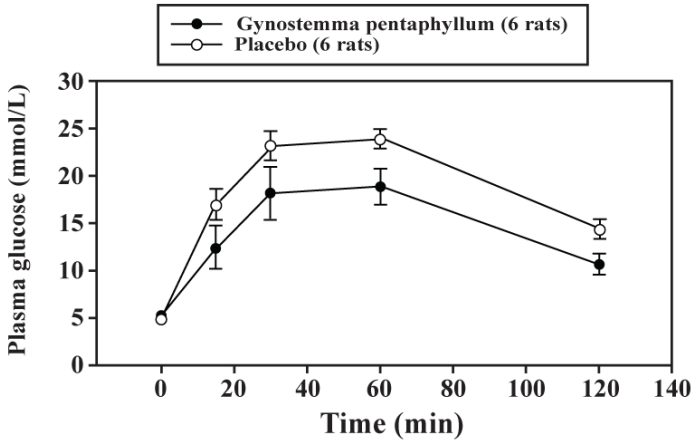


Figure 4. Mean of plasma glucose levels in the subcutaneous insulin tolerance test after the three-week treatment.

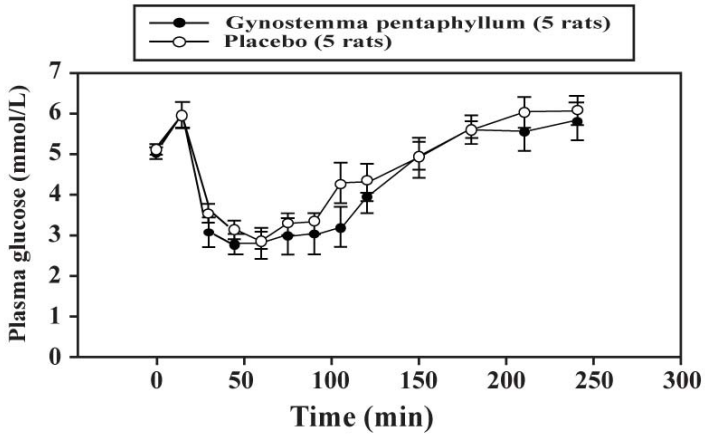
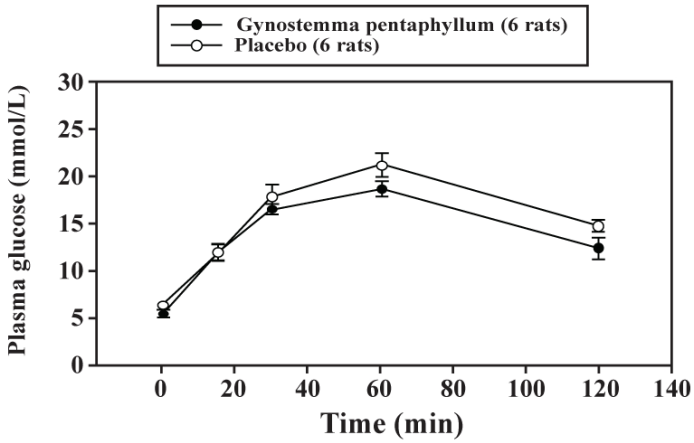


Figure 5. Mean of plasma glucose levels in intraperitoneal pyruvate tolerance test after the three-week treatment.

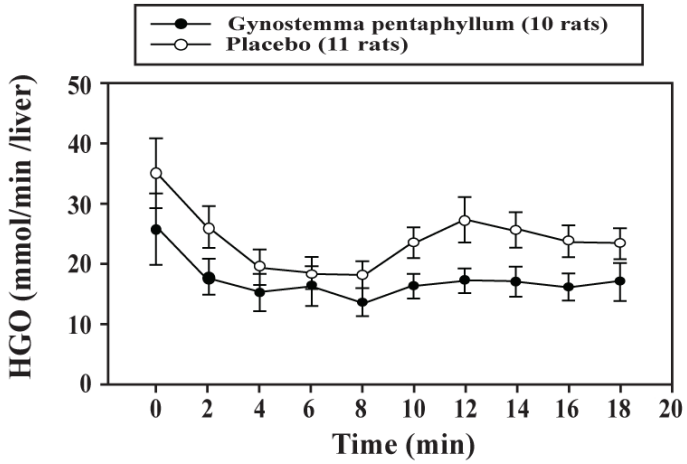


Effect of GP tea on HGO levels in GK rats

After the three-day treatment with GP tea, basal HGO was 20% lower in comparison with that in the placebo group (28.8 ± 6.5 vs. 36.1 ± 11.6 μmol/min). After three weeks, the basal HGO in GP treated rats was 27% lower (25.7 ± 5.9 vs. 35.1 ± 5.8 μmol/min in treated and placebo groups, respectively), but these differences were not statistically significant. However, in the three-week treated GK rats, AUCs for HGO during 18

minutes (0-18 min) was significantly decreased, as compared with the placebo rats (302.8 ± 36.5 vs. 423.5 ± 44.7 μmol/min, p = 0.05; Figure 6). In addition, infusion of 50 nmol/L adrenaline beginning after 8 min of perfusion (8-18 min, adjusted to the 8 min of perfusion) did increase the HGO in all rats but the response to adrenaline tended to be lower in the treated rats compared to the placebo rats (27.5 ± 8.9 vs. 61.0 ± 17.5 μmol/min, p = 0.114).

Figure 6. Mean of hepatic glucose output after the three-week treatment, (basal perfusion 0-8 min, and addition of 50 nM adrenaline 8-18 min).



Effect of GP tea on hepatic glycogen content and GS activity in liver of GK rats

The three-week treatment with GP tea significantly reduced the hepatic glycogen content, as compared to the placebo group (18.3 ± 4.6 vs. 35.6 ± 2.9 mg/g liver, respectively, p < 0.007). The hepatic glycogen synthase activity did not differ in the group treated with GP tea (40.5 ± 6.3 percent), compared to that in the placebo group (35.9 ± 6.2 percent).

5 DISCUSSION

The main findings of our studies were that GP tea exerted a significant anti-diabetic effect, and that this was accounted for by enhanced insulin sensitivity.

The placebo group and the GP group did not differ in baseline characteristics and diabetic parameters (drug naïve T2D, HbA_{1C}, FPG) in Papers I, II, and III. The GP tea was mainly responsible for the reduction in glucose levels because all patients received similar diet and exercise therapies, with only a minor effect on FPG in the control group. Green tea (*Camellia sinensis*) was reported to induce anti-hyperglycemic effects in mice and stretozotocin-diabetic rats [101,102], but there is little evidence that it improves glycemic control substantially in human T2D [102,103].

In Paper I, during 12 weeks of the therapy with GP tea, HbA_{1C} levels decreased significantly by approximately 2%-units, while in control subjects using placebo HbA_{1C} levels did not change. In Paper II, these results were reaffirmed by improving glycemic control in patients treated with SU who received the addition of GP tea, as compared to patients on SU therapy alone. Add-on GP tea therapy with SU lead to decreases in HbA_{1C} of approximately 2%-units, and in FPG of nearly 3 mmol/L over 8 weeks. This HbA_{1C} lowering effect was clearly comparable with other oral anti-diabetic agents used as monotherapy, e.g. metformin with a 1.4% to 2% reduction of HbA_{1C} [104], and the effect of the combination therapy of SU and GP tea was comparable with that of SU combined with metformin [105]. In Paper III, the major anti-diabetic role of GP tea was also proven by the reduction of FPG, and by the glucose responses to insulin during SIGIT after GP treatment, with the reverse effect after switching to placebo treatment.

Regarding the possible mechanism by which GP tea decreases plasma glucose levels, we have previously isolated an insulin-releasing compound, phanoside, from GP extract [64]. However, our present data do not support an increased insulin response to

a glucose challenge in GP tea-treated patients, since the 30 minute plasma insulin levels in OGTT were not enhanced. The improvement of diabetic control with the combined therapy in Paper II occurred without any significant changes in insulin and C-peptide levels after adding GP tea to the insulin secretagogue SU. In addition, HOMA-β did not indicate that GP tea enhanced beta cell secretion. The anti-diabetic effect of GP tea treatment could rather be explained by improved insulin sensitivity, as demonstrated by HOMA-IR. With respect to these observations, it appears that GP tea may provide improved glycemic control via a mechanism that is independent of the stimulation of insulin release, and that does not place any additional burden on defective beta cells. This is agreement with another study showing a significant reduction of HOMA-IR in subjects treated by GP extract [106]. In Paper III, the GP tea decreased FPG and the glucose response to insulin in the SIGIT steady-state, whereas plasma insulin levels during the SIGIT did not change after GP tea treatment in our study population. In a previous study, endogenous C-peptide concentrations, reflecting insulin secretion during SIGIT, were almost entirely abolished [71]. Thus, these results in Paper III are in agreement with our previous findings in Papers I and II [107,108], and indicate that the decrease in blood glucose levels is explained by an improvement in insulin sensitivity. Interestingly, another experimental study demonstrated that an ethanol extract of GP, produced in Vietnam, inhibited protein tyrosine phosphatase 1B activity, which may lead to enhanced insulin sensitivity and thereby improved glucose tolerance [67].

In Paper IV, our results in GK rats were consistent with those in humans. Oral administration of GP tea for three days did not change the PG levels, whereas long-term three-week treatment revealed significant decreases in PG. In addition, the three-week treatment with GP tea significantly improved glucose tolerance and reduced HGO compared to the placebo rats. Since the results of an insulin tolerance test reflecting

insulin sensitivity mainly in muscle and other extra-hepatic tissues did not differ between the two groups, it seems unlikely that the primary effect of GP tea is exerted on the extra-hepatic tissues. Thus, these findings suggest that GP-induced improvements in glucose tolerance in GK rats is accounted for, at least partly, by decreased HGO. Moreover, although addition of adrenaline did increase the HGO in all rats, the adrenaline effect tended to be suppressed in GP tea -treated rats.

HGO plays a prominent role in glucose homeostasis. Insulin decreases HGO by activating glycogen synthesis and glycolysis, and by suppressing gluconeogenesis [109]. Glycogen is the intracellular stored form of glucose, and its levels in various tissues, particularly in liver and skeletal muscle, reflect insulin's action in stimulating glycogen synthase and inhibiting glycogen phosphorylase [110]. We have shown that hepatic glycogen content after three weeks of GP tea treatment was significantly lower than that after placebo treatment. In parallel, hepatic glycogen synthase activity did not differ between GP-treated and placebo rats. In the liver, insulin-dependent glucose regulatory functions are controlled by a number of different mechanisms. Among these, phosphotyrosine phosphatase 1B (PTP1B) is known to negatively modulate insulin's action on hepatic glucose metabolism through tyrosine dephosphorylation of the insulin receptor and/or insulin receptor substrates [111]. GP tea has been demonstrated to inhibit PTP1B, and this action has been linked to enhanced insulin sensitivity and improved glucose tolerance [67,112]. Interestingly, a more recent study has shown that hepatic glycogen content was significantly reduced in PTP1B -/- transgenic mice as compared to wild-type controls [113]. Therefore, it can be speculated that GP tea improves hepatic insulin sensitivity to some extent through the inhibition of PTP1B. In addition, it seems likely that the improvement in hepatic insulin sensitivity is partly accounted for by a reduction of gluconeogenesis, as shown by the decreased glucose response during the pyruvate tolerance test in the GP tea -treated GK rats.

The anti-hyperlipidemic effect was not observed after treatment with GP tea. These results differ from those observed in a study of obese Zucker fatty rats, in which triglyceride and cholesterol levels were reduced after 4 days of treatment with GP extract [66]. The difference between doses and species may be an explanation for these divergent outcomes. In addition, GP extract has been reported to exert many other effects, such as anti-tumor [114,115], antioxidant and hepatoprotective [106,116], anti-inflammatory [117], and anti-gastric ulcer effects [118,119]. However, an evaluation of these effects of GP was beyond the scope of our study.

In our studies, there was ideal compliance (100%), and GP tea was well-tolerated. In the GP tea groups in Papers I and III, no patient developed abnormalities of the liver or renal function tests throughout the study period. In Paper II, no adverse effects were documented in the combination treatment of SU and GP tea, compared with SU as monotherapy or SU and placebo tea. Compared with the most common combination, SU plus metformin, no patients experienced adverse gastrointestinal effects such as diarrhea, nausea, epigastric discomfort, and anorexia, which might be the causes of treatment discontinuation in some studies [120,121]. In addition, neither hepatoxicity nor nephrotoxicity developed, in support of the biosecurity of GP tea used as add-on therapy with SU. We used a low dose of the SU, gliclazide, and no patient experienced symptoms of hypoglycemia, even when SU was used in combination therapy with GP tea. The risk of hypoglycemia often increases in parallel with the dose increment of SU [122]; however, with the addition of GP tea, the observed results proved that this tea can be efficacious for the maintenance of the effects of low dose SU treatment. During our studies, no patients experienced any symptoms of hypoglycemia, and no acute hepatoxic or nephrotoxic effects were observed. This notion is supported by a study in rats, in which no signs of chronic toxicity were found after a 6-month administration of rather high GP tea extract doses (up to 0.75 g/kg per day) [123]. However, larger and

longer trials are needed to assess, with higher accuracy, the prevalence of possible adverse effects. In addition, further research is needed to determine the durability of GP extract's anti-diabetic effect, as well as effects on patient-reported outcomes, morbidity and mortality.

6 CONCLUSIONS

I. The significant anti-diabetic effects and improved insulin sensitivity of GP tea were clearly demonstrated (Paper I), although this randomized, double-blind, placebo-controlled trial only enrolled a modest number of patients. The biosecurity of the extract was indicated clinically, since no hepatotoxicity and nephrotoxicity or other acute adverse effects were observed in the trial. Therefore, this trial (Paper I) provided a basis for a novel, easy access, effective and safe approach to treat type 2 diabetic patients using traditional Vietnamese medicine.

II. As add-on therapy to sulfonylurea, GP extract improved glycemic control, and this improvement was sustained over 12 weeks (Paper II). Furthermore, there were potential benefits in terms of the maintenance of low-dose SU without any symptoms of hypoglycemia. The biosecurity of GP extract was suggested clinically, since no hepatotoxicity and nephrotoxicity or other acute adverse effects were observed in the trial. Therefore, our results using Vietnamese herbal medicine could offer an alternative to the addition of other oral medications to treat type 2 diabetic patients.

III. The GP extract exerted anti-diabetic effects by improving insulin sensitivity as the major mechanism, in a placebo-controlled crossover study (Paper III).

IV. The oral administration of GP extract for three weeks to GK rats exerted anti-diabetic effects by reducing plasma glucose levels and suppressing HGO levels significantly (Paper IV). The mechanism behind the improved hepatic insulin sensitivity may relate to suppression of gluconeogenesis and inhibition of PTP1B.

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