Assessment of anti-diabetic effect of Vietnamese herbal drugs

av

Nguyen Khanh Hoa

AKADEMISK AVHANDLING

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Abstract

The prevalence of type 2 diabetes mellitus is increasing in Vietnam as well as in other developing countries (China, Indian subcontinent, and Africa). Searching for hypoglycemic agents with origin from domestic herbals was considered as a useful way to find novel therapy of the disease.

After treatment i.p. or orally of normal mice with extract of *Anemarrhena asphodeloides* (A.a.), *Angiopteris evecta* (A.e.) and *Gynostemma pentaphyllum* (G.p.), blood glucose levels of the mice were decreased. All of those 3 extracts also suppressed the rise in blood glucose in normal mice during a glucose tolerance test.

At both 3.3 and 16.7 mM glucose, 2, 4 and 8 mg/ml *Anemarrhena asphodeloides* (A.a. or TH2) increased the insulin release of Wistar (W) and Goto-Kakizaki (GK) rat islets. In perifusions of islets, A.a. also increased insulin secretion that returned to basal levels when A.a. was omitted from the perifusate. Thus, ethanol extract of the roots of A.a. contains a substance, TH2, that stimulates insulin secretion from islets of normal W and GK rats. The mechanism behind TH2-stimulated insulin secretion involves an effect on the exocytotic machinery of the B-cell, mediated via pertussis toxin-sensitive G\_e-proteins.

*G.p.* extract had a hypoglycemic effect in rats and mice. We have isolated the active compound, phanoside, a gypenoside with molecular mass of 914.5 Da. When given orally to rats, phanoside (40 and 80 mg/kg) improved glucose tolerance and enhanced plasma insulin levels. Phanoside stimulated insulin release at 3.3 and 16.7 mM glucose from isolated rat pancreatic islets of both W and GK rats. Interestingly, B-cell sensitivity to phanoside is higher at 16.7 mM than at 3.3 mM glucose, since significant insulin responses were observed with phanoside between 31.25 and 125 μM only at the high glucose levels. When W rat islets were incubated at 3.3 mM glucose with 150 μM phanoside and 0.25 mM diazoxide to keep K-ATP channels open, insulin secretion was similar to that in islets incubated in 150 μM phanoside alone. At 16.7 mM glucose, phanoside-stimulated insulin secretion was reduced in the presence of 0.25 mM diazoxide. In W islets depolarized by 50 mM KCl and with diazoxide, phanoside stimulated insulin release 2-fold at 3.3 mM glucose but did not further increase the release at 16.7 mM glucose. When using nimodipine to block L-type Ca\(^{2+}\) channels in B-cells, phanoside-induced insulin secretion was unaffected at 3.3 mM glucose but decreased at 16.7 mM glucose. In perifusion of islets, phanoside (75 and 150 μM) dose-dependently increased insulin secretion that returned to basal levels when phanoside was omitted. Thus, the effect of phanoside seems to be exerted distal to K-ATP channels and L-type Ca\(^{2+}\) channels that is on the exocytotic machinery of the B-cells.

In conclusion, from 8 Vietnamese herbal drugs, we have found 3 extracts which decreased blood glucose of the mice. Two of them (A.a. and G.p. extract) stimulated insulin secretion from rat islets. Ethanol extract of A.a. (TH2) stimulated insulin secretion by an effect on the exocytotic machinery of the B cell mediated via pertussis toxin sensitive G\_e-protein. From G.p. we isolated a novel substance, phanoside, that directly stimulates the exocytosis of insulin.

**Keywords:** G-protein, type 2 diabetes, phanoside, antidiabetic plant, hypoglycemic agent, insulin secretion, pancreatic islets, *Gynostemma pentaphyllum, Anemarrhena asphodeloides, Angiopteris evecta,*
## Abstract


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## Acknowledgements


## Financial supports


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## Paper I-IV


Abstract

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After treatment i.p. or orally of normal mice with extract of *Anemarrhena asphodeloides* (*A.a.*), *Angiopteris evecta* (*A.e.*) and *Gynostemma pentaphyllum* (*G.p.*), blood glucose levels of the mice were decreased. All of those 3 extracts also suppressed the rise in blood glucose in normal mice during a glucose tolerance test.

At both 3.3 and 16.7 mM glucose, 2, 4 and 8 mg/ml *Anemarrhena asphodeloides* (*A.a.* or TH2) increased the insulin release of Wistar (W) and Goto-Kakizaki (GK) rat islets. In perifusions of islets, *A.a.* also increased insulin secretion that returned to basal levels when *A.a.* was omitted from the perifusate. Thus, ethanol extract of the roots of *A.a.* contains a substance, TH2, that stimulates insulin secretion from islets of normal W and GK rats. The mechanism behind TH2-stimulated insulin secretion involves an effect on the exocytotic machinery of the B-cell, mediated via pertussis toxin-sensitive G\(_e\)-proteins.

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release at 16.7 mM glucose. When using nimodipine to block L-type Ca\(^{2+}\) channels in B-cells, phanoside-induced insulin secretion was unaffected at 3.3 mM glucose but decreased at 16.7 mM glucose. In perfusion of islets, phanoside (75 and 150 µM) dose-dependently increased insulin secretion that returned to basal levels when phanoside was omitted. Thus, the effect of phanoside seems to be exerted distal to K-ATP channels and L-type Ca\(^{2+}\) channels that is on the exocytotic machinery of the B-cells.

In conclusion, from 8 Vietnamese herbal drugs, we have found 3 extracts which decreased blood glucose of the mice. Two of them (A.a and G.p. extract) stimulated insulin secretion from rat islets. Ethanol extract of A.a. (TH2) stimulated insulin secretion by an effect on the exocytotic machinery of the B cell mediated via pertussis toxin sensitive G\(_{\alpha}\)-protein. From G.p. we isolated a novel substance, phanoside, that directly stimulates the exocytosis of insulin.

**Keywords:** G-protein, type 2 diabetes, phanoside, antidiabetic plant, hypoglycemic agent, insulin secretion, pancreatic islets, *Gynostemma pentaphyllum*, *Anemarrhena asphodeloides*, *Angiopteris evecta*, *
List of original papers

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. Nguyen KH, Dao Van Phan, Nguyen Duy Thuan, Claes-Göran Östenson. Screening of the hypoglycemic effect of eight Vietnamese herbal drugs. (Manuscript)


IV. Nguyen Khanh Hoa, Åke Norberg, Rannar Sillard, Dao Van Phan, Dang Thi Ngoc Dzung, Hans Jörnvall, Claes-Göran Östenson. The mechanism by which phanoside stimulates insulin secretion from rat islets. (Manuscript)

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Abbreviations

A.a  Anemarrhena asphodeloides
ACN  Acetonitrile
ADP  Adenosine diphosphate
A.e.  Angiopteris evecta.
Ambe.  Amberlite exchange
ANOVA  Analysis of variance
ATP  Adenosine triphosphate
BuOH  Butanol
b.w.  Body weight
cAMP  cyclic adenosine monophosphate
CV  Column volumes
DAG  Diacylglycerol
DMSO  Dimethyl sulfoxide
DQF  Double quantum-filtered
EtOH  Ethanol
E.u.  Eucommia ulmoides
GIP  Gastric inhibitory polypeptide
GK  Goto-Kakizaki
GLP-1  Glucagon like peptide-1
G.p.  Gynostemma pentaphyllum
G-proteins  GTP-binding proteins
HEPES  N-[2-Hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid]
H.m.  Hydrangea macrophylla
HPLC  High performance liquid chromatography
HSQC  Heteronuclear single quantum correlation
iNOS  inducible isoform of nitric oxide synthase.
i.p.  intraperitoneal
IP$_3$  Inositoltriphosphate
K-ATP channels  ATP-dependent potassium channels
KRB  Krebs-Ringer bicarbonate
<table>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear overhauser effect</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>P</td>
<td>Phanoside</td>
</tr>
<tr>
<td>P.Ginseng</td>
<td><em>Panax ginseng</em></td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>R.g.</td>
<td><em>Rehmannia glutinosa</em></td>
</tr>
<tr>
<td>ROESY</td>
<td>Rotating overhauser effect spectroscopy</td>
</tr>
<tr>
<td>RIA</td>
<td>Radio immunoassay</td>
</tr>
<tr>
<td>S.b.</td>
<td><em>Scrophularia buergeriana</em></td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total correlation spectroscopy</td>
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<tr>
<td>TH2</td>
<td><em>Anemarrhena asphodeloides</em> extract</td>
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Background

1. General

Type 2 diabetes mellitus is a common endocrinological disease. The number of adults with diabetes in the world will rise from 170 millions in the year 2000 to more than 360 millions in the year 2025 (King et al. 1998). In Vietnam as well as in other developing countries (China, Indian subcontinent, and Africa) the prevalence of diabetes is also increasing (Green et al. 2003; Zimet 2003). In Vietnam the crude prevalence of diabetes in 1992 was 1.2% in Hanoi, and 2.5 % in Hochiminh city (Quoc et al. 1994), and increased in 2001 to 6.6% in Hochiminh city (Duc Son et al. 2004).

The pathogenesis of type 2 diabetes is complex, involving progressive development of insulin resistance and relative deficiency in insulin secretion, leading to overt hyperglycemia (Ostenson 2001). Currently available therapies for type 2 diabetes include insulin and various oral agents: sulfonylureas; metformin; α-glucosidase inhibitors, such as acarbose; thiazolidinedions, e.g. pioglitazone and rosiglitazone (DeFronzo 1999). In addition, repaglinide and nateglinide are recently launched non-sulfonylurea insulin secretagogues that act via a similar mechanism as sulfonylurea drugs. Moreover, glucagon-like peptide-1 (GLP-1) analogues will soon be introduced worldwide. The latter exert several effects including glucose-dependent stimulation of insulin release, suppression of glucagon release, prolongation of gastric emptying and reduced food intake (Nauck 1998; Perfetti and Merkel 2000). These agents are used as monotherapy in newly diagnosed patients. In patients where the disease is more advanced, such drugs are frequently used in combination to achieve better glycemic control (DeFronzo 1999). Most of the above oral agents suffer from inadequate long-term efficacy (as monotherapy) and treatment failure after long time of treatment (DeFronzo 1999) and also a number of adverse effects. Moreover, with increasing reliance on multiple patented pharmacological agents, the cost of treatment has also become a real concern. The cost of metformin and sulfonylureas is much lower than for repaglinide, thiazolidinediones and GLP-1 analogues (DeFronzo 1999). The ability of developing countries, such as Vietnam, to afford this level of treatment is dubious. As a
consequence, there continues to be a high demand for new oral antidiabetic drugs. In search for novel therapeutic targets in type 2 diabetes, this research has focused on molecular defects in insulin-sensitive tissues, such as skeletal muscle, fat and liver, as well as in the improving of the insulin secretion capacity of the pancreatic B-cell.

2. Treatment of diabetes in traditional medicine.

Diabetes mellitus has been described in the early history. Symptoms that included polyuria and polydipsia were described in the Egyptian Ebers papyri, Greek Epidemics Book of Hippocrates, and the Chinese Nei Ching (Vuksan and Sievenpiper 2005). Plant derivates with hypoglycemic properties have been used in folk medicine and traditional healing systems around the world e.g. in Native American Indian, Jewish (Wang et al. 2003; Yeh et al. 2003), Chinese (Kimura et al. 1999) and Vietnamese cultures (Thuy 1992). Surveys of the literature have shown that a large variety of compounds obtained from several plant families were found to be responsible for the hypoglycemic action (Atta Ur and Zaman 1989; Ivorra et al. 1989; Yeh et al. 2003). For instance, glycosides isolated from the families **Caesalpinaceae**, **Compositae**, **Convolvulaceae**, **Ericaceae**, **Moraceae**, **Myrataceae**, **Papaveraceae**, **Ranunculaceae**, **Rhamnaceae** and **Scrophulaceae** afforded active principles which lowered blood sugar in test animals (Atta Ur and Zaman 1989; Ivorra et al. 1989; Kimura et al. 1999). Especially traditional Chinese medicine has demonstrated an interesting practice in the therapy of diabetes because of distinctive traditional medical theory and Chinese herbal medicines (Li et al. 2004).

In Vietnamese literature of traditional medicine, diabetes mellitus is called “Duong Tri benh” and has been treated by several prescriptions that involve some of the following traditional medicines: Sinh dia (**Rehmannia glutinosa** - **Scrophulariaceae**), Huyen sam (**Scrophularia buergeriana** - **Scrophulariaceae**), Tri mau (**Anemarrhena asphodeloides** - **Liliaceae**) (Thuy 1992), Cam thao nam (**Seoparia dulcis**), Do Trong (**Eucommia ulmoides** - **Eucommiaceae**), Morinda citrifolia (Nhau) (Loi 1999). Therefore,
the ability to develop novel therapy of diabetes from traditional medicinal plants may be useful.

3. Gynnostemma pentaphyllum

*Gynnostemma pentaphyllum* (G.p.) Makino (Cucurbitaceae) is a medicinal herb that is presently promoted in e.g. Europe as a herbal tea (Cui et al. 1999). The plant is a perennial liana which grows wild in southern China, Japan, India, Korea and northern Vietnam (Hu et al. 1996). Phytochemical studies of *G.p.* plant have identified about 90 dammarane-type glycosides (called gypenosides) closely related to the ginseng saponins (Cui et al. 1999). There are also studies showing that ginseng and *G.p.* decreased blood glucose levels of animals and human beings (Jang et al. 2001; Attele et al. 2002). There is no previous study to define the structures or mechanism of the hypoglycemic effect of *G.p.*

4. Anemarrhena asphodeloides

The rhizome of *A.a.* has been used for treatment of diabetes mellitus in Chinese and Vietnamese traditional medicine (Thuy 1992). Normally the diabetic patients were given the water or ethanol extract of the root of *A.a.* for several weeks (Thuy 1992). In addition, several mixes of herbal drugs containing extract of *A.a.*, have been shown to exert hypoglycemic effect (Miura et al. 1997; Kimura et al. 1999; Cheng et al. 2001). Miura et al. discovered the hypoglycemic effect of methanol extract of *A.a.* in KK-Ay mice (Miura et al. 2001a). This effect was referred to a substance, mangiferin, in the extract and the mechanism of the hypoglycemic effect was suggested to be increased insulin sensitivity of the mice (Ichiki et al. 1998; Miura et al. 2001a; Miura et al. 2001b). In another study hot water extract of rhizomes of *A.a.* lowered the blood glucose levels in alloxan-diabetic mice and the hypoglycemic effect was accounted for by pseudoprototimosaponin AIII (Nakashima et al. 1993). The prototimosaponin and pseudoprototimosaponin have no effect on glucose uptake and insulin release, suggesting the hypoglycemic mechanism to be due to inhibition of hepatic gluconeogenesis and/or glycogenolysis (Nakashima et al. 1993).
5. Modulation of insulin secretion in pancreatic B-cells.

Glucose stimulated insulin secretion.

Figure 1. Schematic model of the glucose induced-insulin secretion in the pancreatic B-cell.

Glucose-stimulated biphasic insulin secretion involves at least two signaling pathways, the K-ATP channel-dependent and K-ATP channel-independent pathways, respectively (Chow et al. 1995; Straub and Sharp 2002). In the former, enhanced glucose metabolism increases the cellular adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio, which closes K-ATP channels leading to depolarization of the cell and activation of the voltage-dependent $\text{Ca}^{2+}$ channels. Activation of voltage-dependent $\text{Ca}^{2+}$ channels increases $\text{Ca}^{2+}$ entry (Yang and Gillis 2004) and stimulates insulin release (Hellman et al. 1994). The latter is also modulated by other secretagogues that act via second messengers such as cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG) to exert stimulatory effect on exocytosis of insulin (Jones et al. 1991; Zawalich
and Zawalich 2001; Straub and Sharp 2002; Quynh et al. 2005). Cyclic AMP enhances Ca^{2+}-stimulated exocytosis while having no effect in the absence of stimulated exocytosis (Tamagawa et al. 1985; Bjorklund and Grill 1999; Bjorklund et al. 2000). Under standard whole-cell conditions, cAMP augmented insulin secretion induced by a single depolarization, and this was unaffected by inhibition of protein kinase A (PKA). When exocytosis was stimulated by the release of caged Ca^{2+}, cAMP had PKA-dependent and PKA-independent effects (Renstrom et al. 1997; Thams et al. 2005). There was a rapid effect that was PKA-independent and a late effect that was PKA-dependent. It was concluded that cAMP potentiates insulin secretion by increasing the release probability of granules in the immediately releasable pool and by increasing the rate at which the pool is refilled (Straub and Sharp 2002). Therefore, its major effects appear to be immediately prior to exocytosis per se. Glucose also stimulates the production of DAG (Straub and Sharp 2002). Increased DAG results in augmentation of Ca^{2+}-stimulated insulin exocytosis that could be mediated by protein kinase C (PKC) isoforms or other DAG-binding proteins. Thus, PKA-, PKC-, and DAG-binding proteins are involved in the augmentation of exocytosis, and they act via several different mechanisms (Straub and Sharp 2002).

Other pathways stimulate insulin secretion

The in vitro sensitivity to glucose as a stimulator of insulin secretion is amplified in vivo by hormones and neurotransmitters. The hormones gastric inhibitory or glucose-dependent insulino tropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are released from the gastrointestinal tract upon food ingestion and act as ‘incretins’, i.e. potentiators of insulin secretion by increasing cAMP by interaction with adenylate cyclase after food ingestion (Nauck 1998). Parasympathetic stimulation potentiates insulin secretion due to the binding of acetylcholin to muscarinic receptors in pancreatic islets. The mechanism of this potentiation include the activation of phospholipase C in B-cell. The latter event gives rise to inositoltriphosphate (IP_{3}) and DAG. IP_{3} liberates Ca^{2+} from the endoplasmic reticulum, and DAG activates protein kinase C (Zawalich and Zawalich 1996; Barker et al. 2002). In addition, some other peptides such as
cholecystokinin (CCK) and other nutrients e.g. amino acids and free fatty acids also regulate insulin secretion. The amino acid L-leucine is metabolized in a glucose like-manner and generates ATP. However, other amino acids like L-arginine, L-lysine and L-histidine stimulate insulin release by elevated $[\text{Ca}^{2+}]_i$, which is caused by the inward transport of positive charge carried by amino acids (Smith et al. 1997).

**Figure 2. Schematic diagram of other pathways regulating insulin secretion of pancreatic beta cell.** GLP-1 can interact by binding to its receptor, promotion of cAMP synthesis and stimulation of protein kinase A. Largely by promoting phosphorylation of proteins, protein kinase A is able to interact with (1) ATP dependent K+ channels; (2) L-type Ca$^{2+}$ channels; (3) mobilization of Ca$^{2+}$ from intracellular stores; and (4) augmentation of storage granule exocytosis.

Activation of $\alpha_2A$-adrenoreceptors as well as binding of somatostatin and galanin to B-cell receptors inhibits insulin secretion. Mechanisms of inhibition include interaction with inhibitory G proteins in the cell membrane, leading, for instance, to inhibition of B-cell adenylylcyclase (Chan 1993; Strowski et al. 2000).
G protein in exocytosis of insulin granules

GTP-binding proteins (G-proteins) play functional roles in the process of mammalian cell activation (Robertson et al. 1991). In endocrine cells, G-protein mediated processes form important branches of signal transduction for hormone release (Robertson et al. 1991). Some G-proteins are inhibited by pertussis toxin such as Gi (the protein that inhibits adenylcyclase), Gt (which opens ligand-gated K+ channels), Go (function uncertain), Gpla (which activates phospholipase A2), Gplc (which activates phospholipase C) (Birnbaumer et al. 1987) and Ge (which is directly coupled with exocytosis) (Komatsu et al. 1993). Regarding the subclass of G-protein, pertussis toxin sensitive Gi-proteins and Ge-proteins are present in the B-cell (Vallar et al. 1987). Pertussis toxin treatment of islets reverses the inhibition of insulin secretion by epinephrine and somatostatin (Robertson et al. 1991; Sharp 1996). Furthermore, the possible involvement of the putative exocytosis-linked Ge protein in mastoparan induced insulin secretion from B-cell was reported (Komatsu et al. 1993).

6. Goto-Kakizaki (GK) rats

Type 2 diabetes is associated with disturbances of secretory functions of the B-cells precipitated by the inability of glucose to stimulate insulin secretion (Efendic et al. 1984). Animal models of diabetes have been created to study the mechanism of type 2 diabetes. Goto-Kakizaki (GK) rats is a rat model of hereditary type 2 diabetes, developed by inbreeding of Wistar (W) rat and showing the majority of attributes of type 2 diabetes (Goto et al. 1976; Portha et al. 1991; Ostenson et al. 1993a).

In GK rats, impaired glucose-stimulated insulin secretion has been demonstrated in vivo (Portha et al. 1991; Gauguier et al. 1996; Salehi et al. 1999), in the perfused isolated pancreas (Portha et al. 1991; Ostenson et al. 1993b) and in isolated pancreatic islets (Ostenson et al. 1993b; Hughes 1994). It’s hypothesised that the defective insulin response to glucose in GK rat B-cells is accounted for by an impaired ATP production and closure of K-ATP channels (Tsuura et al. 1993). However the rate of ATP
production in islet mitochondria was similar in Stockholm GK and control rats (Ling et al. 1998). Also in GK rats of Paris and Seattle colonies, overall ATP/ADP levels were found to be normal (Giroix et al. 1993; Metz et al. 1999). Other defects in GK islet glucose metabolism include increased glucose cycling, due to increased glucose-6-phosphatase activity in B-cells (Ostenson et al. 1993b; Ling et al. 1998), impaired glycerol phosphate shuttle due to markedly reduced activity of the FAD-linked glycerol phosphate dehydrogenase (Ostenson et al. 1993c) and decreased pyruvate carboxylase activity (MacDonald et al. 1996). Abnormalities in the function of the K-ATP channels and L-type Ca\(^{2+}\) channels (Kato et al. 1996) do not seem to account for the major impairment in insulin release in GK rat (Hughes 1994). Indeed, glucose-stimulated insulin secretion was markedly impaired in GK rat islets also when the islets were depolarised by a high concentration of potassium chloride and the K-ATP channels kept open by diazoxide (Abdel-Halim et al. 1996). Similar results were obtained when insulin release was induced by exogenous calcium in electrically permeabilized GK rat islets (Okamoto et al. 1995). These findings, together with recent studies, indicate that important defect(s) resides late in signal transduction, i.e. in the exocytotic machinery (Metz et al. 1999; Nagamatsu et al. 1999; Gaisano et al. 2002; Zhang et al. 2002).
Aims

The aims of the present thesis were to
1. Study the hypoglycemic effects of ethanol extract from 8 Vietnamese herbal drugs.
2. Isolate and characterize new anti-diabetic agents from hypoglycemic extracts.
3. Study the mechanism of hypoglycemic effect of the extracts or the new substances which have been found.
Materials and Methods

<table>
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<tr>
<th>No</th>
<th>Name of the plant</th>
<th>Used parts</th>
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<tr>
<td>1</td>
<td><em>Anemarrhena asphodeloides</em> - Liliaceae (<em>A.a.</em>)</td>
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<td>2</td>
<td><em>Angiopteris evecta</em> - Marattiaceae (<em>A.e.</em>)</td>
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</tr>
<tr>
<td>3</td>
<td><em>Gynostemma pentaphyllum</em> - Cucurbitaceae (<em>G.p.</em>)</td>
<td>Branch and leave</td>
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<tr>
<td>4</td>
<td><em>Eucommia ulmoides</em> - Eucommiaceae (<em>E.u.</em>)</td>
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<td>5</td>
<td><em>Hydrangea macrophylla</em> - Hydrangeaceae (<em>H.m.</em>)</td>
<td>Leave</td>
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<td>6</td>
<td><em>Morinda citrifolia</em> L. - Rubiaceae (<em>M.c.</em>)</td>
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<td>7</td>
<td><em>Rehmannia glutinosa</em> - Scrophulariaceae (<em>R.g.</em>)</td>
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<td>8</td>
<td><em>Scrophularia buergeriana</em> - Scrophulariaceae (<em>S.b.</em>)</td>
<td>Root</td>
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</table>

**Table 1:** Plants studied in screening experiments

1. **Methods of extraction**

The eight plants that have been used in traditional medicine for treatment of diabetes are listed in Table 1. The plants were collected from the mountains, dried in the light sun before pounding into powder. The powders were extracted in 70% ethanol in soxhlet for 4 cycle times. Ethanol was evaporated under low pressure, then the extracts were diluted in water, and again evaporated under low pressure until they contained nearly 30% water. When administered to mice each extract was diluted in water and filtered to get
the soluble parts. Animals were given the extracts orally by gavage or by injection intraperitoneally (i.p.).

2. Purification and characterization of compound structures.

Fifteen gram of the ethanol extract of *G. pentaphyllum* was dissolved in 200 ml water and centrifuged for 20 min at 3000g. The supernatant was collected, diluted with 200 ml water, and extracted with 400 ml n-butanol, with a magnetic stirrer for 10 min. Water and n-butanol were allowed to separate for 30 min in a separating funnel. The butanol phase was collected and the solvent was evaporated until most of the butanol had been removed. The residue from the butanol extraction was diluted with 1000 ml water. Portions of 500 ml were mixed with 200 g Amberlite XAD-16 HP (Supelco, Bellefonte, PA) and stirred for 30 min. The unabsorbed material was removed by filtration and the Amberlite was collected and washed with water. The adsorbed material was eluted from the Amberlite with aqueous acetonitrile at different concentrations (25%, 60% and 80% acetonitrile). The bioactivity was located in the 60% acetonitrile fraction (total volume 500 ml). In running the first HPLC, portions (150 ml) of this fraction were diluted with 400 ml water, applied to a Vydac C18 column (22 x 250 mm; 218TP152022) and eluted with a linear gradient of 20-57.5% B (eluent A, water; eluent B, 95% acetonitrile) in 4.5 column volumes (CV) at a flow rate of 25 ml/min. Fractions were analyzed for insulin-
releasing activity. In the second chromatographic step, the active fraction from the first reverse phase HPLC separation was rechromatographed on a SOURCE 15RPC 16/90 column in a linear gradient of 32-40% B in 9.33 CV at a flow rate of 8 ml/min. In the last chromatographic step, the active fraction was purified on an ÄKTApurifier HPLC system using a C18 column (ODS-AP, 10 x 100 mm, 5 µm, YMC, Germany) and the same solvent system as above, employing a gradient of 40-41% B in 4 CV at 6 ml/min. The structure of the pure component was elucidated using \(^1\)H-\(^1\)H and \(^1\)H-\(^{13}\)C 2D-nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry.

**Mass spectrometry**

Positive-ion mass spectra were recorded on an Ettan ESI-ToF electrospray time-of-flight mass spectrometer (Amersham Biosciences, Uppsala, Sweden) using a capillary exit voltage of 150 V. Samples of chromatographic fractions were infused at 10-50 µl/min via the ESI source using a syringe pump. For alkali metal-adduct measurements, a 50-µl aliquot of the chromatographic fraction was mixed with 2 µl of 5 µM LiCl, NaCl or KCl solution in water and sprayed into the mass spectrometer.

**Nuclear magnetic resonance (NMR)**

NMR spectra were recorded with a Bruker DMX-600 spectrometer equipped with a cryoprobe in DMSO-d\(_6\) solution at 25°C. Chemical shifts are reported in ppm relative to residual solvent signal (\(\delta\) (H) 2.50 ppm, \(\delta\) (C) 39.5 ppm). Two-dimensional spectra recorded included DQF-COSY, ROESY, TOCSY, sensitivity-enhanced \(^{13}\)C-HSQC, and \(^{13}\)C-\(^1\)H HMBC. Pulsed-field gradients were used for all \(^{13}\)C correlation spectra. \(^{13}\)C-HMBC spectra were recorded with coupling evolution delay for the generation of multiple-bond correlations set to 62.5 ms. All 2D spectra were run with a 4096x1024 points data matrix, giving \(\tau_{2\text{max}} = 250\) ms for the \(^1\)H nucleus in the acquisition dimension and \(\tau_{1\text{max}} = 200\) ms for \(^1\)H or \(\tau_{1\text{max}} = 50\) ms for \(^{13}\)C for indirect dimension. Prior to Fourier transform the data matrix was zero-filled twice and a multiplication by shifted
sine-bell window function was applied. For $^{13}$C-HMBC, the magnitude spectra were calculated.

3. Screening study

In the screening study, 380 Swiss mice, weighing $20 \pm 2$g were purchased from National Hygiene Institute, Hanoi, Vietnam and kept in Department of Pharmacology, Hanoi Medical University for 5 days before doing the experiments. The mice had free access to food and fresh water before and during the experiments. The hypoglycemic effects of the extracted compounds were tested by oral and intraperitoneal administration to mice with the volume 0.1 ml/10g body weight. Control animals received NaCl 0.9% (0.1 ml/10g body weight). The amounts of extract were 200 and 300 mg/kg b.w. orally, or 1000 and 1500 mg/kg b.w. i.p.. The hypoglycemic effect was predicted if the blood glucose of the mice decreased significantly compared to the control group.

4. Glucose tolerance test

An oral glucose tolerance test was used to continue studying the herbs that had shown hypoglycemic effect in screening tests. In mice fasted over-night, extracts were given to the animals (0.1 ml/10g b. w., 1000 mg/kg) (the control group was given NaCl 0.9%) orally 210 min before the oral administration of glucose (3 g/kg b.w.). Blood glucose concentrations were measured before oral administration of the extract and then after 210 min. After this time point oral glucose (3 g/kg b.w.) was given and blood glucose levels were measured after 30, 60 and 120 min.

Blood was obtained by tail snipping of mice, and blood glucose concentration was measured by a glucose oxidase method using reagent strips and absorbance in a reflectance meter (One Touch Glucose meter; Johnson & Johnson, USA).

To study the effect of 10, 40 and 80 mg/kg phanoside in vivo, normal male Wistar rats (120 ± 32g) were used in an i.p. glucose tolerance test. In rats fasted over-night, phanoside in DMSO (2.5%, 1 ml/100g b.w.) was given to the animals orally 90 minutes
before the i.p. injection of glucose (3 g/kg b.w.). The control group was given only DMSO orally. Blood glucose concentrations were measured in blood taken after a small tail vein incision at 90 min and immediately before (0 min), and 15, 30, 60 and 120 minutes after injection of glucose.

To study the effect of phanoside (40 and 80 mg/kg b.w.) on insulin secretion in vivo, vein plasma samples were drawn before administration of the compound (-90 min), before injection of glucose (0 min) and 30 min thereafter for measurement of immunoreactive insulin (Norberg et al. 2004).

5. Isolation of pancreatic islets

The experiments were performed with islets isolated by collagenase digestion of the pancreas of male Wistar and GK rats (250-280g), followed by hand-picking (Lacy and Kostianovsky 1967). After isolation, the islets were cultured for 24h in RPMI 1640 medium (Flow lab. Ltd.), containing 11 mM glucose, 10% heat inactivated fetal calf serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin.

6. Batch incubation of islets

The medium used for islet batch incubations was Krebs-Ringer bicarbonate (KRB) buffer solution containing 118.4 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4 and 25 mM NaHCO3 (equilibrated with 5% CO2-95% O2, pH 7.4) and 0.2% bovine serum albumin (BSA), 10 mM HEPES and 3.3 or 16.7 mM glucose. Insulin release was assessed in batch incubations of islets following preincubation for 30 min at 3.3 mM glucose. Batches of 3 islets were incubated for 60 min in KRB with 3.3 or 16.7 mM glucose, and different concentrations of extract or phanoside. For comparison, batches of islets were also incubated with another stimulating agent of insulin release, 0.15 mM tolbutamide or 2 µM glibenclamide (Hellman et al. 1971).
7. Perifusion of islets

To investigate how phanoside or A.a. extract affect the kinetics of insulin release, perifusion of islets was used. Batches of 30 isolated W rat islets were perifused first with KRB medium with 0.2 % BSA and 10 mM HEPES, containing 3.3 mM glucose. Perifusion medium was collected in fractions every 2 min to establish the basal insulin secretion rate at 3.3mM glucose. Then the glucose concentration was increased to 16.7 mM glucose and decreased to 3.3 mM again to get back the basal insulin secretion.

8. Effect of pharmacological agents on insulin secretion stimulated by active substances.

To investigate whether extract or phanoside exert direct effect on the insulin exocytosis, islets were incubated in KRB with high concentration (30 mM or 50 mM) of KCl to depolarize the B-cells, and 0.25 mM diazoxide to keep the K-ATP channels open (Sato et al. 1999) or just 0.25 mM diazoxide with or without active substances.

To investigate the effect of phanoside on the L-type Ca$^{2+}$ channels on insulin secretion, a L-type Ca$^{2+}$ channel inhibitor, nimodipine, was used.

To evaluate the effect of protein kinase A (PKA) and protein kinase C (PKC) on phanoside-induced insulin release, normal W rat islets were incubated with or without phanoside and the PKA-inhibitor, H89 (10 µM) (Filipsson and Ahren 1998) or the PKC inhibitor, calphostin-C (1.5 µM) (Thams and Capito 2001) for 60 min in KRB containing 3.3 or 16.7 mM glucose.

To evaluate the effect of pertussis toxin on A.a. extract -or phanoside-induced insulin release, normal W rat islets were pretreated for 24h at 37 °C in RPMI-1640 culture medium containing 11 mM glucose, 10% heat-inactivated fetal calf serum, 100 ng/ml pertussis toxin, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin and then incubated with or without active substances for 1h.
9. Measurement of insulin secretion from islets

After batch incubations or perifusions, aliquots of the medium were analyzed for insulin content by radioimmunoassay (RIA) (Herbert et al. 1965). The sensitivity of the RIA was 3.9 mU/l, the inter-assay coefficient of variation was <3.8% and the intra-assay coefficient of variation was <3.1%.

10. Cell viability

Trypan blue assay

After incubation in the absence (control group) or presence of 150 µM phanoside, islet cells prepared as previously described (Pipeleers and Pipeleers-Marichal 1981), were exposed to the membrane-impermeant dye, trypan blue (0.1% w/v) for 15 min at 37 °C. The presence of dye was determined by light microscopy and the number of unstained and stained cells in the field were counted to obtain an estimate of the percentage of the cells taking up the dye (Persaud et al. 1999).

Measurement of lactate dehydrogenase (LDH) release

Batches of 100 pancreatic islets were incubated with phanoside (150 µM) for 60 min. LDH release from islets was measured by determining LDH activity (cytotoxicity detection kit-LDH Roche Applied Science). The amount of color formed in the assay is proportional to the number of lysed islet cells. The LDH activity in the total of dead islet cells (high control) was measured after solubilization of islet cells with 5% (v/v) Triton X-100 (Lash et al. 2001). To determine the percentage cytotoxicity, the absorbance at 490 nm was measured in duplicate samples with subtraction of values obtained in control incubation (low control with islets but without phanoside), using the following equation:
Cytotoxicity (%) = \( \frac{\text{Exp. value} - \text{low control}}{\text{High control} - \text{low control}} \times 100 \)

11. Data analysis.

The results have been calculated as mean \( \pm \) SEM and comparisons of the data have been done by Student’s t-test, or one way and two way repeated measures ANOVA test, as appropriate (one factor repeated) with all pairwise multiple comparison procedures (Student-Newman-Keuls Method). Significance of comparison test would be accepted if the p value of the test was smaller than 0.05.
Results and discussion

1. Screening of the hypoglycemic effect of eight herbs (Paper I)

After i.p. injection of mice with 200 and 300 mg/kg Anemarrhena asphodeloides (A.a), blood glucose levels were reduced after 2h. By oral route, A.a. (1500 mg/kg) reduced blood glucose 2h and 4h after administration. However the blood glucose increased again 6h after administration orally.

After i.p.injection of mice with Angiopteris evecta (A.e.) at the dose of 200 mg/kg blood glucose was reduced at 2h and 3h. Increasing the i.p. dose to 300 mg/kg, the blood glucose was reduced from 1-3h after administration. By oral route A.e. (1000 mg/kg) reduced blood glucose route after 4 and 6h. A.e. 1500 mg/kg had even stronger effect starting from 2h and still remaining 6h after administration.

After 2 and 3h following treatment of mice with Gynostemma pentaphyllum (G.p.) at the dose of 200 mg/kg i.p., blood glucose levels decreased compared to the control group. Similar effects were obtained when using G.p. 300mg/kg i.p. When administered orally G.p. (1500 mg/kg) reduced blood glucose after 6h.

After administration i.p./orally of extracts of Rehmannia glutinosa (R.g.), Hydrangea macrophylla (H.m.), Eucommia ulmoides (E.u.), Scrophularia buergeriana (S.b.), blood glucose levels of mice did not differ from control. Morinda citrifolia (M.c.) (200 and 300 mg/kg i.p.), reduced blood glucose of the mice after 2 and 3h but had no effect when given orally.

After oral administration of glucose (3 g/kg), A.a., A.e. and G.p. (1000 mg/kg orally) inhibited the increase in blood glucose of the mice (Fig. 4). At 30 min after intake of glucose, the blood glucose levels of the control group was 11.2 ± 1.4 mM, and the blood glucose levels of the groups given A.a., A.e. and G.p. were 7.5 ± 1.3, 9.2 ± 0.8 and 8.8 ± 0.9 mM respectively. (p< 0.05 for all compared to control group). At 60 min after
loading glucose orally, the blood glucose of the control group was still high (10.5 ± 0.7 mM) but the blood glucose levels of the group given A.a., A.e. and G.p. were 4.9 ± 0.8, 7.3 ± 0.9 and 7.1 ± 1.0, mM respectively (p< 0.001, p< 0.01 and p< 0.05 respectively compared to the control group). At 120 min after intake of glucose, A.a. and A.e. still had a hypoglycemic effect, the blood glucose levels of the mice being 4.0 ± 0.3 and 4.5 ± 0.3 mM respectively, as compared to 6.7 ± 0.3 mM in control group (p< 0.05 for both) (Fig. 4).

Figure 4. The hypoglycemic effect of G.p., A.a. and A.e. on glucose induced hyperglycemia in mice. Normal male Swiss mice (20 ± 2g), were fasted overnight, then the extracts (1000 mg/kg) were given to the animals (the control group was given NaCl 0.9%) orally 210 min before the oral administration of glucose (3 g/kg b.w.). Blood glucose concentrations were measured after administration of the extracts 210 min and immediately before (0 min), and 30, 60 and 120 min after administration of glucose. (Data are mean ± SE, n = 5 for each group, *p< 0.05, **p<0.01 ***p< 0.001 compared to control group).

This study demonstrated that the ethanol extracts of G.p., A.a. and A.e administrated i.p. and orally, decreased blood glucose levels in mice. In addition, 0.5 U/kg insulin i.p. or 50 mg/kg tolbutamide orally decreased blood glucose levels, showing that the mice were sensitive to the hypoglycemic agents and the method of measurement of blood glucose was sensitive enough to study the hypoglycemic effect of the agents.
In East-Asian folk medicine, *G.p.* is widely used and is gaining popularity as a herbal medicine. *G.p.* extracts reportedly have many effects, such as cholesterol-lowering, immunopotentiating, antitumor, antioxidant, and hypoglycemic effect (Li et al. 1993; Lin et al. 1993; Zhou et al. 1998). Our experiments showed that *G.p.* exerts an hypoglycemic effect in mice following oral administration and i.p. injection. Thus, the compound that has the hypoglycemic effect in *G.p.* extract could be absorbed via the gut. The hypoglycemic effect of *G.p.* was not strong in normal mice but significantly improved glucose tolerance in the mice challenged with 3 g/kg glucose.

The rhizome of *A.a.* has been used for treatment of diabetes mellitus in Chinese and Vietnamese traditional medicine (Miura et al. 1997). Normally the diabetic patients were given the water or ethanol extract of the root of *A.a.* (Thuy 1992). In addition, several mixes of herbal drugs containing extract of *A. a.* have been shown to exert hypoglycemic effect (Miura et al. 1997; Kimura et al. 1999; Cheng et al. 2001). Miura et al. discovered the hypoglycemic effect of methanol extract of *A. a.* in KK-Ay mice (Miura et al. 2001a), the mechanism of which involved inhibition of hepatic gluconeogenesis and/or glycogenolysis (Nakashima et al. 1993). Our results showed that ethanol extract of *A.a.* decreased the blood glucose of normal mice when administered both orally and i.p. *A.a.* also inhibited the increase in blood glucose in mice injected with glucose. Thus *G.p.* and *A.a.* should be studied to find compounds that have hypoglycemic effect.

*A.e.* has been used to treat the symptoms resembling those of diabetes in some description of traditional medicine from ethnic people in Vietnam. In this study, we have demonstrated that *A.e.* decreased blood glucose when given via both oral route and i.p. However, with higher doses of the ethanol extract of *A.e.* mice suffered from liver damage and death. Thus, *A.e.* should not be used in man until its toxicity has been studied carefully.

Thus, after screening studies, we have found 3 herbs (*Gynostemma pentaphyllum* Makino, *Anemarrhena asphodeloides* Bunge, *Angiopteris evecta* Forst) that exert
hypoglycemic effect in normal mice. Those herbs also reduced the increase in blood glucose in mice after loading with glucose.

2. **Insulin secretion is stimulated by ethanol extract of Anemarrhena asphodeloides (TH2) in isolated islet of healthy Wistar and diabetic Goto-Kakizaki rats (Paper II)**

   After the screening study, we further investigated the effects of ethanol extract of *A.a.* (TH2) in rat islets. When Wistar rat islets were incubated in KRB with 3.3 mM glucose, 2, 4 and 8 mg/ml TH2 dose-dependently stimulated insulin secretion 2.5-fold, 4.1-fold and 5.7-fold, respectively (p<0.01, p<0.05, p<0.001 respectively) (Fig. 5). Tolbutamide 0.15 mM showed similar effect as 2 mg/ml TH2, (Fig. 5). The insulin response also increased after incubation of islets at 16.7 mM glucose with 2, 4 and 8 mg/ml TH2 1.5-fold, 2.2-fold and 3.8-fold (p<0.05, p<0.05, p<0.001, respectively). Also at 16.7 mM glucose, insulin response to 0.15 mM tolbutamide was similar to the effect of 2 mg/ml TH2 (Fig. 5).

   At 3.3 mM glucose TH2 (2, 4 and 8 mg/ml) stimulated insulin secretion of GK rat islets 1.7-fold, 3.0-fold and 6.3-fold respectively compared to the control group (p< 0.01, p<0.001, p<0.001 respectively) (Fig. 6).

   At 16.7 mM glucose TH2 (2, 4 and 8 mg/ml) also augmented insulin secretion of GK rat islets 2.5-fold, 4.2-fold and 11.9-fold respectively compared to the control group. Especially 8 mg/ml TH2 at 16.7 mM glucose increased insulin secretion 1190 ± 92% compared to control group and at 3.3 mM glucose that value was 630 ± 70% (p< 0.01) (Fig. 6).

   Type 2 diabetes is characterized by profound abnormalities in insulin secretion (Taylor et al. 1994; Grill and Bjorklund 2000; Ostenson 2001). Treatment of diabetes aims at controlling the blood glucose in the normal range. Using the agents that stimulate insulin secretion from islets is an effective way to decrease blood glucose. Our results show that TH2 stimulated insulin secretion from isolated islets of normal Wistar and
diabetic GK rats both at 3.3 mM and 16.7 mM glucose. The stimulatory effect of *A. a.* on insulin secretion is in agreement with the report on Die-Huang-Wan, an herbal mixture containing *A. a.* used in Chinese traditional medicine, which stimulated insulin release in normal rats in vivo (Cheng et al. 2001). Thus *A. a.* could be a promising antidiabetogenic agent.

![Figure 5](image1.png)  
**Figure 5:** Effects of TH2 and tolbutamide on basal (o–o) and glucose-stimulated (•−•) insulin secretion from isolated Wistar rat islets. Results of insulin release (µU/islet/h) are the mean ± SEM of 6-7 batch incubations at each condition. * p< 0.05, ** p< 0.01, *** p< 0.001 compared to control group with no addition.

![Figure 6](image2.png)  
**Figure 6:** Effects of TH2 on basal (o–o) and glucose-stimulated (•−•) insulin secretion from isolated GK rat islets. Results of insulin release (µU/islet/h) are the mean ± SEM of 6-7 batch incubations at each condition. ** p< 0.01, *** p< 0.001 compared to control group with no addition.

At both 3.3 and 16.7 mM glucose, depolarization of Wistar islet B-cells by exposure to 30 mM KCl + 0.25 mM diazoxide increased insulin release 1.5-fold and 6.0-fold, respectively. When islets were incubated at 3.3 mM glucose with 30 mM KCl, 0.25 mM diazoxide and 8 mg/ml TH2, insulin secretion was higher than when islets were incubated in 8 mg/ml TH2 alone (56.0 ± 2.8 µU/islet/h vs. 43 ± 2 µU/islet/h p< 0.01) and also higher than when islets were incubated in 30 mM KCl + 0.25 mM diazoxide (10.0 ±
0.8 µU/islet/h, p<0.001). At 16.7 mM glucose, however, the insulin response to 30 mM KCl, 0.25 mM diazoxide and 8 mg/ml *A.a.* was not significantly higher than to 8 mg/ml TH2 or to 30 mM KCl + 0.25 mM diazoxide. Thus, *A.a.* potentiated insulin secretion in depolarized islets at 3.3 mM glucose but not at 16.7 mM glucose.

It is known that in GK rats the glucose-induced insulin secretion is impaired and this impairment is associated with reduced sensitivity of K-ATP channels to glucose (Tsuura et al. 1993). The closure of almost all K-ATP channels in pancreatic B-cells has been recognized as an essential step of the insulin secretory response to glucose (Cook and Hales 1984; Cook et al. 1988). The resulting depolarization brings about Ca\(^{2+}\) influx through voltage-dependent L-type Ca\(^{2+}\) channels, thereby triggering insulin secretion (Okamoto et al. 1995; Straub and Sharp 2002). Our results show that TH2 stimulates insulin secretion of diabetic GK rat islets at both 3.3 mM and 16.7 mM glucose to the same extent or even more than in control Wistar rat islets. In addition, a second pathway of regulation of insulin secretion by glucose has been identified. By treating B-cells with diazoxide to keep K-ATP channels open and with high extracellular K\(^+\) to depolarize the membrane and enhance the intracellular calcium concentration (Aizawa et al. 1992), glucose does not increase [Ca\(^{2+}\)]\(_i\) but amplifies the action of the ion on the releasing process (Aizawa et al. 1992). At a low glucose concentration (3.3 mM) the insulin response to 8 mg/ml TH2 in the presence of 30 mM KCl and 0.25 mM diazoxide was higher than that to either 8 mg/ml TH2 or 30 mM KCl and 0.25 mM diazoxide, indicating an effect of TH2 distal to the K-ATP channels. At a high glucose concentration (16.7 mM), 8 mg/ml TH2 alone had similar effect as TH2 + KCl + diazoxide as well as KCl + diazoxide. Thus, TH2 did not seem to interact with exocytosis of insulin at high glucose levels. So, the mechanism of TH2 induced insulin secretion does not involve the K-ATP channels.

In the control group without pretreatment of islets with pertussis toxin, 2 and 8 mg/ml TH2 stimulated release of insulin. When islets were pretreated with pertussis toxin, the insulin responses to 2 and 8 mg/ml TH2 were reduced by 35-47% after pertussis toxin treatment. Thus, the TH2 induced-insulin secretion was suppressed by pertussis toxin.
GTP-binding proteins (G-proteins) play functional roles in the process of mammalian cell activation (Robertson et al. 1991). In endocrine cells, G-protein mediated processes form important branches of signal transduction for hormone release (Robertson et al. 1991). In the pancreatic B-cell, pertussis toxin sensitive G_i-proteins and G_e (exocytosis) proteins are present (Vallar et al. 1987). Pertussis toxin sensitive G_e proteins may also be directly linked to exocytosis of insulin (Komatsu et al. 1993). Our experiments have shown that TH2 induced-insulin secretion was suppressed by pertussis toxin, similar to the effect of pertussis toxin on stimulation of insulin secretion action by mastoparan (Komatsu et al. 1993). Thus, the mechanism by which A.a. modulates insulin secretion seems to involve exocytotic G_e-proteins.

3. Phanosome, a novel hypoglycemic substance from Gynostemma pentaphyllum (Paper III - IV)

An ethanol extract of G.p. increased insulin secretion from isolated rat islets at both basal (3.3 mM) and stimulating (16.7 mM) glucose concentrations. The activity depended on the concentration of the extract.

The hypoglycemic effect of G.p. had been proven in the screening study (paper I). Since G.p. stimulated insulin secretion, the pancreatic islets is a possible target of G.p.

Fig. 7. Structural analysis of the active compound, phanosome.
Fig. 8. Effect of phanoside on insulin secretion from isolated rat pancreatic islets. Phanoside was added at indicated concentrations (7.8, 15.6, 31.3, 62.5, 125, 250, 500 µM) in the presence of 16.7 mM glucose (filled dots) or 3.3 mM glucose (unfilled squares). For comparison, glibenclamide was assayed at 2 µM concentration without addition of phanoside. The leftmost points indicate control values at each glucose concentration without addition of phanoside. Results (mean ± SEM) of 5-6 incubations in each condition. All values over 50 µU insulin/islet/h are significant vs no addition at p<0.001, (*p< 0.05, **p< 0.01).

By using the combination of HPLC, mass spectrometry and NMR with bioactivity of the fractions on islets from the plant G. p. we have isolated a novel, biologically active substance with the molecular mass 914.5 Da, a gypenoside, which we have named phanoside (Fig. 7). At 3.3 mM glucose, 125 µM and higher concentrations of the phanoside were stimulatory, whereas at 16.7 mM glucose significant stimulation of insulin release was noted already at 31 µM of the compound (p<0.001). At the highest concentration tested, 0.5 mM, the phanoside stimulated insulin release 10-fold at 3.3 mM glucose and almost 4-fold at 16.7 mM glucose. These effects were several-fold stronger than the insulin release induced by a sulfonylurea, glibenclamide, added at a nearly maximally effective concentration (Fig. 8).

Extracts of G. p. and/or isolated gypenosides have been reported to exert a variety of effects, such as inhibition of inflammation (Lin et al. 1993), anti-oxidant (Li et al. 1993) and lipid-lowering effects (la Cour et al. 1995), as well as anti-carcinogenic (Zhou et al. 1998) and pro-apoptopic properties (Wang et al. 2002). In previous investigations
of *G. p.*, Hu et al. (Hu et al. 1996) have isolated dammarane-type glycosides, structurally related to the ginseng saponins. *Panax ginseng* berry extract has been shown to reduce blood glucose and body weight in mice (Xie et al. 2002). In fact, saponin fractions of *P. ginseng* and *G. p.* were demonstrated to have similar effects on hyperglycemia and hyperlipidemia (Jang et al. 2001). In the *P. ginseng* berry extract, an antihyperglycemic component, ginsenoside Re, was identified (Attele et al. 2002). Several other saponins such as ginsenoside Re (Attele et al. 2002), panaxans I, J, K, L, Q, R, S, T, U (Konno et al. 1985; Oshima et al. 1985) were isolated and experimentally or clinically confirmed to be bioactive for anti-diabetes or anti-diabetic complications. The mechanism of action of these saponins is to regulate the activity of enzymes related to glucose metabolism directly and/or indirectly, inhibit the renal disorder and promote insulin secretion (Wang et al. 2003; Vuksan and Sievenpiper 2005). Interestingly crude saponin fractions isolated from *G. p.* significantly decreased the plasma glucose level and increased plasma triglyceride level to diabetic rats at a dose of 1 mg/kg, compared with glibenclamide-treated or normal rats (Jang et al. 2001). A large number of saponins, called gypenosides, have been isolated previously from *G. p.*. The gypenoside isolated in this study has a relatively unusual structure compared to the majority of *G. p.* saponins known. However, a quite similar compound has been isolated previously from this plant grown in China (Shen et al. 1993), but the biological activity has not been reported.

Our studies with purified phanoside demonstrated a potent insulin-releasing activity, that is both initiatory, i.e. eliciting insulin release at a non-stimulatory glucose concentration (3.3 mM), and potentiatory, i.e. enhancing the stimulatory effect of 16.7 mM glucose. We compared these effects with a sulfonylurea, glibenclamide that is known to initiate insulin secretion also at low glucose levels (Malaisse and Lebrun 1990). At 2 µM concentration glibenclamide has been shown to stimulate insulin release in vitro to a nearly maximal extent (Östenson et al. 1983). It seems that phanoside at the highest tested concentration is a several-fold more potent initiator than the sulfonylurea.
Kinetics of insulin secretion of isolated islets (Paper IV)

Glucose (16.7 mM) induced a biphasic insulin secretion from the perifused islets (Fig. 9). When glucose was decreased to 3.3 mM, insulin release gradually returned to basal levels. Addition of 150 or 75 µM of phanoside to 16.7 mM glucose markedly enhanced insulin secretion from the perifused islets as compared to that of islets perifused only with 16.7 mM glucose, and the effect of phanoside was dose-dependent. When phanoside was omitted from the perifusate, the insulin secretion decreased to basal levels (Fig. 9).

Fig. 9. Effect of phanoside on kinetics of insulin secretion of Wistar rat islets. Batches of 30 isolated Wistar rat islets were perifused with medium containing 3.3 mM glucose for 20 min. After this time (t = -20 min), perfusion medium was collected in fractions every 2 min to establish the basal insulin secretion rate at 3.3 mM glucose. From min 0 to min 20 the glucose concentration was increased to 16.7 mM glucose and then decreased to 3.3 mM. Phanoside (75 or 150 µM) was added from min 0 to min 20. Aliquots of the medium were collected and their insulin content determined by radioimmunoassay. Results represent mean values ± SEM from 3-5 separate experiments.

Cells viability assay (Paper IV)

Exposure of islet cells to phanoside for 60 min did not significantly affect the number of cells to which trypan blue dye gained access, with 9.6 ± 1.7% of the cells, taking up the dye at 150 µM phanoside and 6.2 ± 1.0 % in the control group (p = 0.157).
According to measurements of LDH release from islets, the percentage of dead islet cells after 60 min incubation with 150 µM phanoside was 8.7 ± 1.3 %.

Phanoside is a gypenoside, related to saponins, that may be cytotoxic (Persaud et al. 1999). Previously, the herbal extract of *Gymnema sylvestre*, containing several saponins or surfactants, was shown to induce insulin release from rat islets and several pancreatic B-cell lines by increased membrane permeability (Persaud et al. 1999). The number of cells to which trypan blue dye gained access, was 98% of MIN6 cells, 95% of RINm5F cells and 88% of HIT-T15 at 0.25 mg/ml GS4-a compound extracted from *Gymnema sylvestre* that stimulated insulin secretion (Persaud et al. 1999). Thus, a similar mechanism could explain phanoside-induced insulin release. However, several observations speak against such an explanation and rather favor a specific effect of phanoside on the B-cell secretion. First of all phanoside at concentrations used in islets incubations only slightly increased the release of lactate dehydrogenase (LDH) from islets exposed to the compound for 60 min (< 10%) and did not increase uptake of trypan blue. Secondly, in the perifusion experiments, insulin secretion returned to basal levels when phanoside was omitted from the perifusate, indicating that exposure to the compound did not cause leakage of insulin from the islets.

**In vivo effects of phanoside (paper III)**

Before administration of phanoside and 90 min thereafter (0 min), blood glucose levels were similar in all groups of rats injected with 10, 40 or 80 mg/kg phanoside, and control rats (Fig. 10). Fifteen min after i.p. injection of glucose, blood glucose levels were increased to 15-20 mmol/l in all rats. However, phanoside-treatment (40 and 80, but not 10 mg/kg) decreased significantly the blood glucose concentrations compared to controls (p<0.001 and p<0.05, respectively, n=10 in each group). This difference remained also at 30 min (p<0.01 and p<0.05, respectively). Thus, at the higher doses, 40 and 80 mg/kg, phanoside improved glucose tolerance in healthy rats. In a smaller number of rats (n=4), plasma insulin were similar in phanoside-treated rats (40 and 80 mg/kg) and control rats at -90 min and 0 min (Fig. 11). Thirty min after glucose loading,
the increment in plasma insulin levels was greater in phanoside-treated than in control rats (p< 0.05 for both).

Figure 10. The hypoglycemic effect of phanoside on normal Wistar rats after loading of glucose. Normal Wistar rats were given phanoside at 10, 40 and 80 mg/kg (indicated as P10, P40, P80 respectively) in DMSO (2.5% p.o.), control group was given only DMSO (indicated as Ctrl). Ninety minutes after drug administration, glucose (3 g/kg) was injected i.p. Blood glucose levels were measured at -90, 0, 15, 30, 60 and 120 minutes after glucose loading. Data represent mean ± SEM (n = 10); SEM is indicated for control and phanoside 40 mg/kg groups only (*p< 0.05, **p< 0.01,***p< 0.001 compared to control group)

Figure 11. The effect of phanoside on insulin secretion of normal Wistar rats after loading of glucose. Normal Wistar rats were given phanoside in DMSO (2.5% p.o.), control group was given only DMSO. Ninety min after drug administration, glucose (3 g/kg) was injected i.p. Plasma insulin concentrations were measured at -90, 0 and 30 minutes after glucose loading. Data represent mean ± SEM (n = 4). (*p< 0.05 compared to control group)

These results show that phanoside not only stimulates insulin secretion in vitro but also purified phanoside given orally significantly improved glucose tolerance in normal
rats. Interestingly, this effect was only obvious in the presence of hyperglycemia. The decrease in blood glucose concentrations after administration of phanoside was paralleled by augmented incremental insulin responses. Thus, it appears that the hypoglycemic effect in vivo is mainly attributed to stimulation of insulin secretion.

4. The mechanism by which phanoside stimulates insulin secretion from rat islets (Paper IV)

Effects of phanoside on insulin secretion in Wistar rat islets with K-ATP channel opened by diazoxide.

At 3.3 mM glucose, diazoxide (0.25 mM) did not affect basal insulin release or insulin response to phanoside (Table 2). At 16.7 mM glucose, diazoxide abolished the glucose-induced insulin release, and decreased insulin response to phanoside by almost 50% (p< 0.01).

Effects of phanoside on insulin secretion in W rat islets depolarized by KCl

<table>
<thead>
<tr>
<th>Addition to the medium</th>
<th>3.3M glucose</th>
<th>16.7M glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.5 ± 0.4</td>
<td>14.5 ± 1.0</td>
</tr>
<tr>
<td>Phanoside (150 µM)</td>
<td>27.2 ± 3.0 ***</td>
<td>61.2 ± 6.6 ***</td>
</tr>
<tr>
<td>Diazoxide (0.25 mM)</td>
<td>3.1 ± 0.7</td>
<td>4.7 ± 0.4 ***</td>
</tr>
<tr>
<td>Diazoxide (0.25 mM) + KCl (50 mM)</td>
<td>24.1 ± 3.2 ***</td>
<td>101.1 ± 9.9 ***###</td>
</tr>
<tr>
<td>Phanoside (150 µM) + diazoxide (0.25 mM)</td>
<td>29.1 ± 2.5 ***</td>
<td>33.7 ± 4.6 ***##</td>
</tr>
<tr>
<td>Phanoside (150 µM) + diazoxide (0.25 mM) + KCl (50 mM)</td>
<td>55.3 ± 5.1 ***###</td>
<td>94.3 ± 8.0 ***###</td>
</tr>
</tbody>
</table>

Table 2. Effect of phanoside with or without diazoxide and potassium chloride on glucose-stimulated insulin secretion from isolated Wistar rat islets. Results of insulin release (µU/islet/h) are the mean ± SEM of 6-7 batch incubations at each condition. (*** p< 0.001 compared to control group with no addition.) (## p< 0.01, ### p< 0.001 compared to group with only phanoside).

At both 3.3 and 16.7 mM glucose, depolarization of W rat islet B-cells by exposure to 50 mM KCl + 0.25 mM diazoxide increased insulin release 6.9-fold and 6.1-fold,
respectively (p<0.001) (Table 2). When islets were incubated at 3.3 mM glucose with 50 mM KCl, 0.25 mM diazoxide and 150 µM phanoside, insulin secretion was 2.0-fold higher than when islets were incubated in 150 µM phanoside alone (p< 0.001) and 2.3-fold higher than when islets were incubated in 50 mM KCl + 0.25 mM diazoxide (p<0.001) (Table 2). At 16.7 mM glucose, however, the insulin response to 50 mM KCl, 0.25 mM diazoxide and 150 µM phanoside was higher than to 150 µM phanoside (p<0.001) but not significantly higher than to 50 mM KCl + 0.25 mM diazoxide.

**Effect of nimodipine on phanoside-induced insulin secretion from isolated W rat islets**

When using nimodipine to block L-type Ca\(^{2+}\) channels in B-cell membrane, the phanoside-induced insulin secretion of islets was not affected at 3.3 mM glucose (Table 3). However, at 16.7 mM glucose, insulin secretion was decreased by nimodipine from 16.0 ± 0.8 to 3.8 ± 1.0 µU/islet/h (p< 0.001). In addition, nimodipine decreased phanoside-induced insulin release from 58.5 ± 8.0 to 34.2 ± 3.7 µU/islet/h (p< 0.01) (Table 3).

<table>
<thead>
<tr>
<th>Addition to the medium</th>
<th>3.3 mM glucose</th>
<th>16.7 mM glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.5 ± 0.4</td>
<td>16.0 ± 0.8</td>
</tr>
<tr>
<td>Phanoside (150 µM)</td>
<td>34.5 ± 2.9 ***</td>
<td>58.5 ± 8.0 ***</td>
</tr>
<tr>
<td>Nimodipine (5 mM)</td>
<td>2.4 ± 0.1</td>
<td>3.8 ± 1.0 ***</td>
</tr>
<tr>
<td>Phanoside (150 µM) + Nimodipine (5 mM)</td>
<td>28.3 ± 2.3 ***</td>
<td>34.2 ± 3.7 ***###</td>
</tr>
</tbody>
</table>

Table 3. *Effect of nimodipine on phanoside-induced insulin secretion from isolated Wistar rat islets.*

Results of insulin release (µU/islet/h) are the mean ± SEM of 6-7 batch incubations at each condition. (***) *p< 0.001 compared to control group with no addition.* (#) *p< 0.01 compared to group with only phanoside*.

K-ATP channels play an important role in modulation of insulin secretion from islets. Rises in circulating glucose concentrations increase intracellular ATP and decrease intracellular ADP, closing ATP-sensitive K\(^{+}\) (K-ATP) channels in the B-cells. This results in membrane depolarization, opening of voltage-dependent Ca\(^{2+}\) channels...
and a rise in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that is the main trigger for insulin secretion (Straub and Sharp 2002; MacDonald and Wheeler 2003). When the K-ATP channels are kept open by diazoxide, glucose induced-insulin secretion is decreased (Trube et al. 1986). Phanoside stimulated insulin secretion at both 3.3 and 16.7 mM glucose. Diazoxide decreased the insulin response to phanoside at 16.7 mM, but not at all at 3.3 mM glucose. Thus the effect of phanoside did not seem to involve the closure of B-cell K-ATP channels. In the presence of a high concentration of K\(^+\) (50 mM) and diazoxide (0.25 mM) the B-cells are depolarized and the cytosolic Ca\(^{2+}\) increasing, leading to insulin release (Quynh et al. 2005). At 3.3 mM glucose phanoside stimulated insulin secretion from depolarized islets, suggesting that the effect of phanoside resides in the distal part of the B-cell stimulus-secretion coupling for glucose, i.e on the exocytotic machinery. However, at 16.7 mM glucose phanoside did not further enhance insulin secretion from depolarized islets, suggesting that islets have a near-maximal exocytosis of insulin under the conditions.

In addition of K-ATP channels, L-type Ca\(^{2+}\) channels are also involved in regulation of insulin secretion. Opening of voltage-dependent Ca\(^{2+}\) channels caused a rise in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that is the main trigger for insulin secretion (Straub and Sharp 2002; MacDonald and Wheeler 2003). By using nimodipine, an L-type Ca\(^{2+}\) channel blocker, the Ca\(^{2+}\) channels will be closed and, thus the insulin secretion due to influx of Ca\(^{2+}\) from outside the cell is blocked (Keahey et al. 1989; Hellman et al. 1994; Chow et al. 1995; Straub and Sharp 2002). In our experiments, in presence of nimodipine at 3.3 mM glucose, the phanoside-induced insulin secretion was not blocked, indicating that the effect of phanoside does not involve L-type Ca\(^{2+}\) channels. At 16.7 mM glucose, the insulin secretion of islets incubated with nimodipine plus phanoside was lower than that of the islets incubated with phanoside alone but still higher than that of the islets incubated with 16.7 mM glucose. Thus, it is likely that nimodipine blocks glucose-induced insulin secretion but does not affect the phanoside effect.
Effects of PKA and PKC inhibition on phanoside-induced insulin release

An increase in intracellular Ca^{2+} in the B-cell in response to insulin secretagogues, including glucose, directly triggers exocytosis of the insulin granules (Hellman et al. 1994). Second messengers, such as cAMP and DAG, increase insulin release through protein phosphorylation events mediated by PKA (Thams et al. 2005) and PKC, respectively (Jones et al. 1991). Using the PKA inhibitor-H89 (Thams et al. 2005) and the PKC inhibitor-calphostin C (Thams and Capito 2001), it was not possible to block the effect of phanoside in rat islets. This indicates that the effect of phanoside is not involving the PKA or PKC systems.

GTP-binding proteins (G-proteins) play functional roles in the process of signal transduction for hormone release (Robertson et al. 1991). In the pancreatic B-cell, G_{\alpha}-proteins have been functionally linked to insulin exocytosis (Komatsu et al. 1993). In our study, pretreatment of islets with pertussis toxin increased glucose-induced insulin secretion. This effect can be explained by the fact that pertussis toxin treatment of islets reverses the inhibition of insulin secretion by e.g. epinephrine and somatostatin via G_{i} protein. Phanoside-induced insulin secretion was not suppressed by pertussis toxin, thus the mechanism by which phanoside modulates insulin secretion seems not to involve exocytotic G_{\alpha}-proteins.
Conclusions.

1. In the screening study, we have found 3 herbs (*Gynostemma pentaphyllum* Makino, *Anemarrhena asphodeloides* Bunge, *Angiopteris evecta* Forst) that exert hypoglycemic effect in normal mice. Those herbs reduced blood glucose levels in mice when administered both i.p. and orally. Furthermore, they inhibited the increase in blood glucose in mice 30, 60 and 90 minutes after loading glucose.

2. Ethanol extract of the roots of *Anemarrhena asphodeloides* contains a substance, TH2, that stimulated insulin secretion both at 3.3 and 16.7 mM glucose in islets of normal Wistar and diabetic GK rats. The mechanism behind TH2-stimulated insulin secretion involves an effect on the exocytic machinery of the B-cell, mediated via pertussis toxin-sensitive G<sub>e</sub>-proteins.

3. From the plant *Gynostemma pentaphyllum* we have isolated a novel, biologically active substance with the molecular mass 914.5 Da, a gypenoside, which we have named phanoside. Phanoside is a potent initiator and potentiator of insulin secretion both *in vivo* and *in vitro* in the rat. The compound stimulated insulin secretion from W and GK rat islets. This effect seems to be exerted distal to K-ATP channels and L-type Ca<sup>2+</sup> channels, that is on the exocytotic machinery of the B-cells. Thereby, the mechanism behind phanoside’s effect on the B-cells differs from that of sulfonyleurea that act by closing the K-ATP channels.
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References


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