HYPOGLYCEMIC EFFECTS OF STILBENE GLYCOSIDE FROM *POLYGONUM MULTIFLORUM* IN TYPE 2 DIABETES AND ITS MECHANISM OF ACTION

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ABSTRACT OF THE DISSERTATION

Hypoglycemic effects of stilbene glycoside from *Polygonum multiflorum* in type 2 diabetes and its mechanisms of action

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Diabetes is one of the leading causes of death in the U. S. The number of people with diabetes has been increasing dramatically, and is expected to reach 366 million worldwide by 2030. Type 2 diabetes, affecting more than 90% of diabetes patients, is strongly associated with oxidative stress and is characterized by insulin resistance. There has been a strong need for safe and effective hypoglycemic agents due to side effects from anti-diabetic drugs.

Polygonum multiflorum (PM) has long been used as a tonic in traditional Chinese medicine. The medicinal effects of PM are believed to be mediated through its strong antioxidant activity. Our objective is to test if PM extract has anti-diabetic effect, and to identify its active compounds. We gave 0.075% PM extract to KK CgAy/J type 2 diabetic mice in drinking water and after 7 weeks, PM-treated mice had significantly lower glucose level than control mice ($p<0.003$). We explored possible mechanisms with Elisa and Western Blotting and suggested that the effect was through maintaining

β cell function.

The major peak in the extract, also the major active compound in PM, *trans*-2,3,5,4'-tetrahydroxystilbene 2-O-β-glucopyranoside (*trans*-SG) was evaluated using the same animal model, however, no hypoglycemic effect was found. In exploring efficacious compounds, we identified *cis*-SG in PM extract and induced isomerization from *trans*- to *cis*- SG with UV light. In a high fat-induced type 2 diabetic mice model, crude extract containing *cis*- and *trans*- SG with the ratio of 2:3 exhibited significant hypoglycemic capacity, while pure *trans*-SG and crude extract with the ratio of 1:20 did not.

The mechanism of differential anti-diabetic activities of *trans*- and *cis*- SG are investigated using PEPCK assay with HepG2 cell culture, and both isomers could effectively suppress Dex/cAMP induced PEPCK transcription, with *cis*-SG offering slightly better results. We also utilized high fat-induced CF-1 male mice to study the anti-diabetic effect of these two isomers. Both SGs were effective in lowering blood glucose in this model, and *cis*-SG improved glucose intolerance while *trans*-SG did not. And from the HOMA model the hypoglycemic effect was found to be through ameliorating insulin resistance.

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1. Literature Review

1.1. Diabetes

Diabetes is defined as a state in which carbohydrate and lipid metabolism is improperly regulated by insulin. This results in elevated fasting and postprandial serum glucose that leads to complications if left untreated. Diabetes is the 7th leading cause of death in the U.S. (American Diabetes Association); the number and percentage of US population with diabetes is rapidly increasing. Today, 25.8 million children and adults in the United States - 8.3% of the population - have diabetes, a third of whom are undiagnosed (American Diabetes Association). The number will reach 366 million by 2030, according to the World Health Organization (WHO). Although diabetes is more prevalent in developed countries, it is likely that the developing world will bear the brunt of the epidemic in the future [1]. The disease is considerably more common among the elderly, although there's recently a trend of youth and juvenile diabetes as well. African-, Mexican- and Native Americans are at a higher risk (1.7-3 times) for diabetes than non-Hispanic whites, and the total medical costs annually in the U.S. for diabetes exceeded $100 billion[1].

Insulin stimulates glucose uptake, utilization, and storage, while suppressing hepatic glucose production, thus reducing plasma glucose levels. Glucagon promotes the release of stored and newly synthesized glucose into the bloodstream. These two hormones act in concert to ensure that glucose homeostasis is maintained throughout a wide variety of physiological conditions[2]. In response to an elevation in plasma glucose

and amino acids (after consumption of a meal), insulin is released from the β cells of the islets of Langerhans in the pancreas. The glucose is transported into β cells via the Glut2 transporter, which has a relatively low affinity for glucose, such that the rate of glucose transport changes with fluctuations in blood glucose concentration[3]. After phosphorylation, glucose is oxidized and ATP is generated. The increased ATP/ADP ratio closes potassium channels, which will alter the membrane potential and trigger the release of preformed insulin-containing granules, and insulin is released [3]. When plasma glucose falls, glucagon is secreted by α cells, which surround the β cells in the pancreas. Both α and β cells are extremely sensitive to glucose concentrations and can regulate hormone synthesis and release in response to small changes in glucose concentrations.

1.1.1. Different types of diabetes

The current classification and diagnosis of diabetes used in the U.S. was developed by the National Diabetes Data Group (NDDG) and published in 1979[4], WHO Expert Committee on Diabetes in 1980[5] and later the WHO Study Group on Diabetes Mellitus endorsed the substantive recommendations of the NDDG. According to them, there are mainly three types of diabetes: type 1, type 2 and gestational diabetes [6]. They are discussed in much detail in Diabetes books[7].

1.1.1.1. Type 1 diabetes

Type 1 diabetes (β cell destruction, usually leading to absolute insulin deficiency) has two forms: Immune-mediated diabetes and Idiopathic diabetes[6].

Immune-mediated diabetes, previously encompassed by the term insulin-dependent diabetes mellitus (IDDM), type 1, or juvenile-onset diabetes, results from a cellular mediated autoimmune destruction of the β-cells of the pancreas. At the later stage of the disease there is litter or no insulin secretion as manifested by low or undetectable levels or plasm C-peptide[8]. Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age. Individuals at increased risk of this type of diabetes can often be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers[9]. This form of the disease may account for 5% - 10% of all cases of diabetes[10].

The other form of type 1 diabtetes is Idiopathic diabetes which has no known etiologies. Although only a minority of patients with type 1 diabetes fall into this category, of those who do, most are of African or Asian origin. This form of diabetes is strongly inherited and lacks immunological evidence for β cell autoimmunity [6].

1.1.1.2. Type 2 diabetes

Type 2 diabetes (ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance), responsible for more than 90% of all diabetes patients and previously referred to as non-insulin dependent diabetes mellitus (NIDDM), or adult-onset diabetes, is a term used for individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency[6]. At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive. A degree of hyperglycemia sufficient to cause pathologic and functional changes in various target tissue, but

without clinical symptoms, may be preesent for a long period of time before diabetes is detected. During this asymptomatic period, it is possible to demonstrate an abnormality in carbohydrate metabolism by measurement of plama glucose in the fasting state or after a challenge with an oral glucose load[11]. The risk of developing this form of diabetes increases with age, obesity and lack of physical activity. Obesity and type 2 diabetes are closely correlated.

Type 2 diabetes is characterized with insulin resistance, which is a diminished ability of insulin to exert its biologic action across a broad range of concentrations. In Western societies, insulin resistance is common and is associated with a variety of abnormalities, including obesity, hypertension, hyperlipidemia, and hyperuricemia, as well as a sedentary lifestyle[12] .

Insulin concentrations are increased in persons with insulin resistance who have normal glucose tolerance. Obesity is probably the commonest cause of insulin resistance. Bergman and coworkers have demonstrated a hyperbolic relation between the sensitivity index, a measure of insulin sensitivity, and the acute insulin response to glucose - that is, as insulin sensitivity falls, insulin secretion must increase substantially if glucose concentrations are to remain normal[13]. The failure of the β cell to continue to hyper-secrete insulin thus underlies the transition from insulin resistance with compensatory hyperinsulinemia to impaired glucose tolerance with mild increases in postprandial glucose concentrations and then to clinical diabetes with overt hyperglycemia[14].

1.1.1.3. Gestational diabetes mellitus (GDM)

GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. The definition applies regardless of whether insulin or only diet modification is used for treatment or of whether the condition persists after pregnancy[15].

While the complications that arise from type 1 and type 2 diabetes are similar, the diseases are completely different entities in terms of pathophysiology. The scope of the current study will be restricted to type 2 diabetes, which is the most common type.

1.1.2. Mechanisms of type 2 diabetes

Increasing studies have confirmed that the pathogenesis of diabetes is related to various signaling pathways, such as insulin signaling pathway, AMPK, ER Stress related pathway, PPAR regulation, etc. This section aims to comprehensively discuss the well validated, as well as putative mechanisms involved in the development of diabetes. In addition, new fields of research, which warrant further investigation as potential therapeutic targets of the future, will be highlighted.

1.1.2.1. Insulin signaling pathway

Insulin is the major hormone controlling critical energy functions such as glucose and lipid metabolism. The primary targets for insulin are skeletal and cardiac muscle, adipose tissue and liver. The first step of insulin signaling pathway is insulin binding to insulin receptor (IR) and activating it. IR is a heterotetrameric membrane protein consisting of two identical α and β subunits. Insulin binds to the α subunits of IR,

thereby activating the intrinsic kinase activity in the β subunit. This results in an intramolecular trans-autophosphorylation reaction whereby one β subunit tyrosine phopshorylates the adjacent β subunit. The insulin receptor substrate (IRS) family of proteins specifically interacts with the phosphorylated IR through a phosphotyrosine binding (PTB) module, which then facilitates phosphorylation of IRS on a number of tyrosine residues by the activated IR[16]. These phosphotyrosine residues on IRS proteins provide docking sites for phosphatidylinositol 3' kinase (PI3K) which exists in the cytosol as a dimer of a regulatory p85 subunit and a catalytic p110 subunit[17]. Recruitment of the regulatory subunit brings the catalytic p110α subunit to the plasma membrane, where it catalyzes the formation of PI(3,4,5)-trisphosphate from PI(4,5)-bisphosphate, and PI(3,4)-bisphosphate from PI(4)-phosphate, thereby recruiting the 3' phosphoinositide-dependent kinase-1 (PDK-1)[18]. PDK-1 phosphorylates and activates both protein kinase B (PKB/Akt) and the atypical PKC λ/ζ (aPKCs)[19]. PKB and the atypical PKCs promote Glut4 translocation to the plasma membrane, which stimulates glucose uptake in muscle and adipocytes[20, 21].

Glucose uptake is the rate-limiting step in glucose utilization and storage. Insulin stimulates the transport of glucose into muscle and fat cells by increasing the concentration of a specific glucose transporter isoform, Glut4, at the cell surface. This action of insulin causes a 10- to 40- fold increase in cellular glucose uptake[22]. Upon entering the muscle cell, glucose is rapidly phosphorylated by hexokinase and either subsequently stored as glycogen due to the activation of glycogen synthase, or oxidized to generate ATP synthesis, via activation of enzymes such as pyruvate kinase[23]. In

adipocytes, glucose is stored primarily as lipid, due to increased uptake of glucose and activation of lipid synthetic enzymes. Most if not all of these insulin-dependent changes in enzyme activities are mediated by attenuation of their phosphorylation state, due to a combination of protein kinase inhibition and phophatase activation[14].

In addition to tyrosine phosphorylation, both the IR and IRS proteins undergo serine phosphorylation, which may attenuate signaling by decreasing insulin-stimulated tyrosine phosphorylation[24]. These inhibitory phosphorylations provide negative feedback to insulin signaling and serve as a mechanism for cross-talk from other pathways that produce insulin resistance. Several kinases have been implicated in this process, including PI3K, Akt, glycogen synthase kinase (GSK)-3 and mammalian target of rapamycin (mTOR)[25].

Insulin action is attenuated by protein tyrosine phosphatases (PTPases), which catalyze the rapid dephosphorylation of the receptor and its substrates[26]. A number of PTPases have been identified including the *trans*membrane, receptor-type PTPase LAR and the intracellular, non-receptor enzyme PTP1B[27]. Most attention has focused on the cytoplasmic phosphatase PTP1B, which is a negative regulator of insulin signaling, and inhibits insulin signaling by dephosphorylating the activated IRTK and IRS[28]. PTP 1B inhibitors would increase insulin sensitivity by blocking the PTP 1B-mediated negative insulin signaling pathway and might be an attractive target for type 2 diabetes mellitus and obesity[26].

1.1.2.2. Oxidative stress

Increased production of high levels of free radicals can generate reactive oxygen

species (ROS), ultimately leading to increased oxidative stress in a variety of tissues[29-32]. Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals[33]. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of *trans*ition metals, can lead to production of extremely reactive hydroxyl radicals [34]. Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals[34]. Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals [35]. Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and then advanced glycation endproducts (AGEs)[32]. These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions, promote free radical formation , and quench and block anti-proliferative effects of nitric oxide[36].

In the absence of an appropriate compensatory response from the endogenous antioxidant network, the system becomes overwhelmed (redox imbalance), leading to the activation of stress-sensitive intracellular signaling pathways such as nuclear factor-κB (NF-κB), p38 mitogen-activated protein kinases (MAPK), stress-activated protein kinases (SAPK), sorbitol, and others[29]. The consequence is the production of gene products which cause cellular damage and are ultimately responsible for the long-term complications of diabetes[37].

In addition to playing a key role in late diabetic complications, activation of the same or similar signaling pathways also appears to play a role in mediating insulin resistance and impaired insulin secretion. ROS interfere with insulin signaling at various levels and are able to inhibit the translocation of Glut4 in the plasma membrane. Another mechanism is that oxidative stress decreases the expression and activity of key transcription factors such as PDX-1 and MafA, which regulate multiple genes involved in β-cell function, including proin-sulin[38]. As an example, a β-cell microarray analysis of PDX-1-bound chromatin identified almost 600 novel PDX- 1 target genes, many of which contribute to energy sensing and insulin secretion[39]. In addition, PDX-1 has anti-apoptotic and proliferative activities that help maintain the β-cell mass [40]. Therefore, impaired activity of PDX-1 and most probably other *trans*cription factors in response to oxidative stress is detrimental to β-cell function and survival.

Evidence for a protective effect of antioxidants has been presented in experimental studies[36, 41, 42], but conclusive evidence from patient studies is missing. Large-scale clinical trials are needed to evaluate the long-term effects of antioxidants in diabetic patients and their potential to reduce the medical and socio-economic burden of diabetes and its complications.

1.1.2.3. Inflammation

Obesity and Type 2 diabetes are characterized by a state of chronic inflammation associated with changes in adipose tissue T-cell subsets, and the infiltration and activation of macrophages, which results in increased production of pro-inflammatory cytokines by both immune cells and fat cells[43, 44]. There is evidence implicating

pro-inflammatory cytokines in the development of insulin resistance. Adipose tissue and circulating tumor necrosis factor-α (TNF-α) levels are elevated in obese rodents, and in vivo neutralization of TNF-α dramatically improves the sensitivity of these obese diabetic animals to insulin-stimulated peripheral glucose uptake[45]. In addition, plasma levels of TNF-α, interlukin-1β (IL-1β) and interleukin-6 (IL-6) are elevated among individuals both with features of the insulin resistance syndrome and with clinically overt type 2 diabetes mellitus diabetes[46-48]. A prospective study found that two circulating markers of systemic inflammation, C-reactive protein and IL-6 were determinant of risk for development of type 2 diabetes mellitus in apparently healthy middle-aged women[49].

One mechanism by which these agents could cause insulin resistance is by inducing the expression of cellular proteins that inhibit insulin receptor signaling[50]. TNF-α can have direct effects on insulin-stimulated tyrosine phosphorylation cascades and can cause dose-dependent decreases in insulin-stimulated tyrosine phosphorylation of IR and IRS-1, reducing insulin sensitivity[51]. A close connection between insulin resistance and classic inflammatory signaling pathways has also recently been identified. NF-κB is held in an inactive state in resting conditions by binding to an inhibitory partner, IκB. Phosphorylation of IκB by its kinase (IKK) leads to IκB degradation, releasing NF-κB for translocation to the nucleus where it can affect the transcription of diverse genes involved in the inflammatory response[52]. It was found that genetic disruption of IKK returned skeletal muscle insulin resistance to normal, through improvement in IRS1 tyrosine phosphorylation and activation of its

downstream signal cascade[53].

1.1.2.4. 5'-AMP-activated protein kinase (AMPK)

5'-AMP-activated protein kinase (AMPK) is a major cellular energy sensor and a master regulator of metabolic homeostasis. AMPK is a heterotrimeric enzyme comprised of two regulatory subunits (β, 30 kDa; γ, 38-63 kDa) and a catalytic subunit (α, 63 kDa). Several isoforms of each subunit have been discovered: α1, α2, β1, β2, γ1, γ2 and γ3, making it possible to form a total of 12 complexes[54].

The AMPK system is activated by two distinct signals: a Ca^{2+}-dependent pathway and an AMP-dependent pathway mediated by LKB1[55]. When triggered by stresses that deplete cellular ATP supplies such as low glucose, hypoxia, ischemia, and heat shock, AMPK activation causes a switch from an anabolic state promoting increased synthesis and storage of glucose, glycogen, fatty acids, cholesterol and triglycerides, together with increased cell growth and/or proliferation, to a catabolic state involving oxidation of glucose, fatty acids and triglycerides, and inhibition of cell growth and proliferation[55]. AMPK thus is a key player in regulating energy balance at both the cellular and whole-body levels, placing it at centre stage in studies of obesity, diabetes and the metabolic syndrome and novel drugs activating AMPK may also have potential for the treatment of obesity and diabetes.

Role of AMPK in the liver

The role of AMPK in liver has been widely studied. In the liver, Activated AMPK acts on multiple substrates affecting both glucose and lipid metabolism[56].

Liver AMPK controls glucose homeostasis mainly through the inhibition of

gluconeogenic gene expression and hepatic glucose production[57]. In primary hepatocytes, AMPK activation mediated by both AICAR (5-aminoimidazole-4-carboxamide-1-b-D-ribo- furanoside, antagonist of AMPK) and metformin has been demonstrated to down-regulate phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase)[57]. In addition, AMPK activation increases phosphorylation of glycogen synthase kinase 3b (GSK-3b) and thereby reduces CRE *trans*criptional activity and PEPCK-C gene expression in the liver, reducing gluconeogenesis[58].

To regulate lipid metabolism, AMPK interacts with and directly phosphorylates sterol regulatory element binding proteins (SREBP-1c and -2)[59]. AMPK stimulates Ser372 phosphorylation, suppresses SREBP-1c cleavage and nuclear *trans*location, and represses SREBP-1c target gene expression in hepatocytes exposed to high glucose, leading to reduced lipogenesis and lipid accumulation[59]. In a recent study, hepatic activation of AMPK by the synthetic polyphenol S17834 protects against hepatic steatosis, hyperlipidemia, and accelerated atherosclerosis in diet-induced insulin-resistant LDL receptor- deficient mice in part through phosphorylation of SREBP-1c Ser372 and suppression of SREBP-1c- and -2-dependent lipogenesis[60]. AMPK-dependent phosphorylation of SREBP may offer therapeutic strategies to combat insulin resistance, dyslipidemia, and atherosclerosis.

Role of AMPK in muscle

AMPK-mediated mitochondrial improvement may overcome the metabolic inflexibility as well as insulin resistance, since both are associated with muscle

mitochondrial dysfunction in type 2 diabetic and obese subjects[54, 61]. Acute muscle AMPK activation increases fatty acid oxidation by decreasing malonyl-CoA concentrations, which results in decreased intramyocyte lipid accumulation and increased muscle insulin sensitivity[61]. Chronic AMPK activation has been shown to increase muscle Glut4, hexokinase, and glycogen content in skeletal muscle and further enhance muscle lipid breakdown[62]. In muscle-specific transgenic mice expressing an inactive form of the AMPKα2 catalytic subunit, the lack of skeletal muscle AMPKα2 activity exacerbated the development of glucose intolerance and insulin resistance caused by high-fat feeding, suggesting a key role of AMPKα2 in glucose homeostasis[63]. Subcutaneous injection of AICAR into rats was shown to activate skeletal muscle AMPK, to induce an increase in muscle Glut4 and hexokinase, and to cause an increase in glycogen content of the muscle[64].

Role of AMPK in β cells

Early evidence that AMPK regulation may be important for the maintenance of β-cell function, including insulin secretion, came from the observation that high passage hamster insulinoma (HIT) cells have lost their 'glucose responsiveness', i.e. the capacity to secrete insulin and reduce AMPK activity in response to glucose[65]. In addition, an increase in K^+/ATP channel trafficking to the cell surface in cells treated with AICAR under high glucose conditions suggested a possible link between AICAR, AMPK and inhibition of insulin secretion via the K^+/ATP channel[66]. Recent studies on the role of AMPK in the regulation of insulin secretion in β-cells have associated it with the mTOR pathway, energy availability, protein synthesis, cell

growth and apoptosis[67]. Collectively, these results demonstrate the complexity of AMPK function in regulating insulin secretion in β-cells and the need for thorough investigations of the complexity of such direct and/or indirect interactions.

Role of AMPK in adipose tissue

The role of AMPK in the other principal metabolic and insulin-sensitive tissue, adipose, remains poorly characterized in comparison, yet increasing evidence supports an important role for AMPK in adipose tissue function. White and brown fat development is differentially regulated by AMPK: white adipogenesis is negatively controlled by AMPK but this kinase is required for brown adipocyte differentiation. AMPK activation has also been reported to influence lipolysis[54]. AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC) in adipocytes, reducing fatty acid synthesis, and may inhibit the expression of ACC and fatty acid synthase (FAS) genes[55]. Exciting recent studies indicate that AMPK may not only influence metabolism in adipocytes, but also act to suppress this pro-inflammatory environment[68], such that targeting AMPK in adipose tissue may be desirable to normalize adipose dysfunction and inflammation.

1.1.2.5. Peroxisome proliferator-activated receptors (PPAR)

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors belonging to the nuclear receptor super-family of regulatory factors. They have complex biological effects, resulting from the trans-activation or trans-repression of dozens of genes that play important roles in glucose and lipid homeostasis[69]. Transcriptional regulation by PPARs requires hetero-dimerization with

the retinoid X receptor (RXR). When activated by a ligand, the dimer modulates transcription via binding to a specific DNA sequence element called a peroxisome proliferator response element (PPRE) in the promoter region of target genes. A wide variety of natural or synthetic compounds was identified as PPAR ligands. There are three known isoforms of PPARs: PPAR-*α*, PPAR-*γ*, and PPAR-*β*/*δ*, each with different tissue specificity and physiological function[69]. A summary of how each isoform is involved in diabetes development is presented below.

PPAR-α is a target of the lipid-lowering fibrates and is highly expressed in tissues that are metabolically very active, such as liver, heart, kidney and skeletal muscle[70]. The central theme for PPAR-α is fatty acid metabolism and transport. Activation of PPAR-α leads to modulation of lipid metabolism, including transcription of apolipoprotein A1 (apoA1) and apolipoprotein AII[71]. PPAR-α is corrective of dyslipidemias (increase HDL, decrease LDL, and TG) and in atherosclerosis (via anti-inflammatory effects)[72]. It is activated in fasting states by free fatty acid (FFA) released from adipocytes. Direct activation of PPAR-α leads to the induction of adipocyte genes such as those for lipoprotein lipase and fatty-acid *trans*porter 1, those associated with fatty acid uptake, β oxidation (medium-chain acyl CoA dehydrogenease, carnitine palmitoyltransferase I), and transport into peroxisomes, which in turn contribute to lowering triglyceride and FFA levels, respectively[72].

In contrast, PPAR-β/δ is expressed at comparable levels in virtually all tissues and is less well studied[73]. Recent work suggests that selective agonists of PPAR-β/δ improve insulin sensitivity in both murine models and in humans[74]. PPAR-β/δ

expression is reduced in cardiac muscle in experimental diabetes[75]. Furthermore, over-expression of PPAR-β/δ in mouse heart protects against lipid accumulation in the presence of a HFD, increases glucose metabolism and protects against ischaemia-reperfusion injury, providing preliminary evidence that activation of this receptor might be protective in diabetic cardiomyopathy [76].

PPAR-γ is highly expressed in adipocytes and is involved in adipocyte differentiation, lipid storage, glucose homeostasis, and adipocytokine regulation, which can improve insulin sensitivity and glucose tolerance[77]. PPAR-*γ* promotes cell differentiation, particularly as a potent master gene, as an inducer and regulator of adipocyte growth and differentiation, promoting their transition from small, quiescent adipocytes to large, activated adipocytes by a transcriptional cascade that controls the expression of a number of genes that are essential in lipid accumulation in adipocytes during the differentiation and also in mature adipocytes[78]. The main hypothesis for the anti-diabetic effects of TZDs is that insulin sensitization is achieved by activation of PPAR-γ in the adipose tissue which allows to redirect lipids away from muscle and liver where fat accumulation causes detrimental effects[79]. PPAR-γ is involved in adipocyte differentiation and survival and has favorable effects on insulin resistance and metabolic syndrome via its effects on lipid metabolism (CD36, aFABP, LPL, acyl CoA synthetase), and energy expenditure (glycerol kinase, UCP-2, UCP-3) [80]. Moreover, it is a key gene involved in the control of hepatic peroxisomal *β*-oxidation of fatty acids[80].

In addition to its role in adipocyte differentiation and lipid metabolism, PPAR-γ is

also crucial for controlling gene networks involved in glucose homeostasis, including increasing the expression of Glut4 and c-Cbl–associated protein (CAP)[81]. PPAR-γ activation has also been shown to rejuvenate pancreatic β cell function resulting in their improved function and regulate directly key β cell genes involved in glucose sensing, insulin secretion and insulin gene *trans*cription[82]. PPAR-γ improves muscle insulin action and prevents apoptosis of pancreatic β cells by sequestering lipids in adipose tissue or controlling the expression of numerous factors secreted from adipose tissue, such as adiponectin, resistin, leptin and TNF-α, which also influence insulin sensitivity[82].

1.1.2.6. Endoplasmic reticulum (ER) stress

The endoplasmic reticulum (ER) is an organelle which synthesizes various secretory and membrane proteins. These proteins are correctly folded and assembled by chaperones in the ER. Under stressful conditions such as an increase in the level of misfolded proteins, the chaperones become overloaded and the ER fails to fold and export newly synthesized proteins, leading to ER stress[83]. It is the most intensively studied in pancreatic β cells where the ER is the crucial site for insulin biosynthesis.

A high demand for insulin secretion can lead to an imbalance in protein homeostasis and ER stress. ER stress is actually increased under diabetic conditions as evidenced from the increase of ER stress markers such as the immunoglobulin binding protein (Bip) and Lys-Asp-Glu-Leu (KDEL) in diabetic mice, both genetic and dietary[84]. It was also evident that ER density and volume are over two-fold higher in human diabetic β cells than non-diabetic β cells[85].

ER stress in pancreatic β cells is likely to contribute to β cell failure in type 2 and other forms of diabetes, and our understanding of ER stress signaling in β cells has significantly progressed in recent years[86-89]. Once ER stress is provoked in the cells, it can be mitigated by an adaptive, cellular response, the Unfolded Protein Response (UPR)[90]. It comprises mechanisms to both regulate new protein translation and a transcriptional program to allow adaptation to the stress. The core of this response is a triad of stress-sensing proteins: protein kinase R-like endoplasmic reticulum kinase (PERK) which is an ER trans-membrane protein kinase that phosphorylates the α subunit of translation initiation factor 2 (eIF2α) in response to ER stress, inositol-requiring enzyme 1 (IRE1) and activating *trans*cription factor 6 (AKF6)[90]. Recent findings suggest that the UPR is an integral component of the ER machinery, regulating pro-insulin biosynthesis in β cells[91]. Thus, collectively, the UPR seems indispensable for β cell function, proliferation and survival. However, in some cases, this response is not sufficient to relieve stress, and β cells may become susceptible to ER stress-induced cell death (apoptosis) or potentially dysfunction and a state of de-differentiation [87]. Both of these can contribute to the development of diabetes. There are three UPR apoptotic pathways which culminate in the cleavage of caspase-3 leading to apoptosis: (1) CHOP induction by the ATF6 and PERK pathways, (2) activation of the ER-resident caspase, caspase-12, and (3) activation of JNK by IRE1-dependent recruitment of glycogen synthase kinase 3 (GSK3b)[92, 93]. Therapeutic approaches that can preferentially activate cell-protective aspects of the UPR or reduce cell-destructive effects may be useful approaches in maintaining functional β-cells

during chronic ER stress.

ER stress response has been shown to be implicated in diabetes development not only by affecting insulin production by pancreatic β-cells, but also by affecting insulin sensitivity in peripheral tissues and impairing insulin action. A recent report throws more light on the link between obesity, ER stress, insulin action and type 2 diabetes[94]. Obesity causes ER stress, which leads to a significant increase in JNK-mediated serine phosphorylation of IRS-1 and thereby inhibited insulin action[94]. Indeed, suppression of the JNK pathway in obese diabetic mice markedly improves insulin resistance and β cell function, leading to an amelioration of glucose tolerance[94]. Taken together, efforts should be made to develop orally active chemical chaperones which could alleviate ER stress thereby result in normalization of hyperglycemia, restoration of systemic insulin sensitivity, resolution of fatty liver disease, and enhancement of insulin action in liver, muscle, and adipose tissues.

1.1.2.7. Wnt

The Wnts are a family of secreted glycoproteins that influence cell development via autocrine and paracrine mechanisms. There are three known pathways of Wnt signaling; the Wnt/β-catenin (canonical), the Wnt/Ca^{2+} and the Wnt/polarity pathway, which activate distinct intracellular signaling cascades[95]. The canonical pathway is the most well-characterized pathway, in which Wnt ligands regulate gene transcription by controlling protein levels and localization of the multi-functional protein β-catenin.The major effector of the canonical Wnt signaling pathway is the bipartite transcription factor β-catenin/T cell *trans*cription factor (β-cat/TCF), formed by free

β-cat and one of the four TCFs[96].

The Wnt signaling pathway was initially discovered for its role in tumorigenesis and the development of Drosophila and other eukaryotic organisms. More recently, it was discovered to be involved in lipid metabolism and glucose homeostasis. In 2006, a large scale genome wide association study (GWAS) revealed that certain single nucleotide polymorphisms (SNPs) in TCF7L2 gene (also known as TCF-4) are strongly associated with the susceptibility of type 2 diabetes[97]. This important finding was subsequently replicated numerous times globally in different ethnic groups in the last few years[96, 98-104]. Molecular mechanisms underlying this association, however, are far from understood at this time. Furthermore, β-cat is able to interact with forkhead box *trans*cription factor subgroup O (FOXO) proteins[95]. Since FOXO and TCF proteins compete for a limited pool of β-cat, enhanced FOXO activity during ageing and oxidative stress may attenuate WNT-mediated activities.

Extensive investigations have shown that the Wnt signaling pathway controls hormone gene expression and mediates the function of certain hormones including GLP-1, GIP and insulin, which are critically important in glucose and energy homeostasis[105]. It was found that expression of the proglucagon gene (gcg) and production of GLP-1 were stimulated in intestinal endocrine L cells by lithium, which mimics the activation of Wnt signaling by Wnt ligands[106]. The function of GIP is also indirectly regulated by TCF-4, although more detailed mechanisms need to be explored, along with the recognition of the role of the Wnt signaling in the production and function of incretin hormones and blood glucose homeostasis[107].

The discovery of the association between certain SNPs of TCF7L2 and T2D susceptibility has also fueled great efforts to explore the role of Wnt signaling in the function of pancreatic β-cells. According to a study by Haytham Aly of the Washington University School of Medicine in St. Louis, MO, USA, activation of the Wnt/GSK-3/β-catenin pathway by pharmacologic inhibition of GSK-3 in combination with nutrient activation of mTOR, modestly enhanced human β-cell proliferation in vitro[108]. Further mechanistic exploration of the underlying mechanisms for these linkages will lead to the discovery of novel therapeutic targets of type 2 diabetes and other metabolic disorders.

1.1.3. Long term complications of diabetes

It is increasingly apparent that not only is a cure for the current worldwide diabetes epidemic required, but also for its major complications, which occur in the majority of individuals with both type 1 and type 2 diabetes. These complications are wide ranging and are due at least in part to chronic elevation of blood glucose levels, which leads to damage of blood vessels. In diabetes, the resulting complications are grouped under “micro-vascular disease” (due to damage to small blood vessels) and “macro-vascular disease” (due to damage to the arteries). Micro-vascular complications include eye disease or “retinopathy,” kidney disease termed “nephropathy,” and neural damage or “neuropathy". The major macro-vascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes[109].

Clinical studies have demonstrated that chronic diabetic complications occur late

after disease onset, with the appearance strongly correlated with the duration of the diabetes and the level of glycemic control, which correlates with formation of AGEs[110]. The AGE concept proposes that chemical modification and cross-linking of tissue proteins, lipids and DNA affect their structure, function and turnover, contributing to a gradual decline in tissue function and to the pathogenesis of diabetic complications[111].

AGE-modified proteins are formed from the covalent reaction between free amino groups of amino acids, such as lysine, arginine or protein terminal amino acids and oxo group of sugars (glucose, fructose, ribose etc.) to create, first, the Schiff base and then Amadori products of which the best known is fructosamine (fructoselysine)[112]. It has been well documented that AGEs progressively accumulate on the tissues and organs which develop chronic complications of diabetes mellitus, such as retinopathy, nephropathy, neuropathy and also macrovascular disease atherosclerosis[113].

Recent studies have shown that increased formation of serum AGEs exists in diabetic children and adolescents with or without vascular complications[114]. Furthermore, the presence of diabetic complications in children correlates with elevated serum AGEs[115]. The level of serum AGEs could be considered as a marker of later developments of vascular complications in children with Type 1 and 2 diabetes mellitus[115]. The careful metabolic monitoring of young diabetics together with monitoring of serum AGEs can provide useful information about impending AGE-related diabetic complications[116]. Advanced glycation end-product inhibitors (AGEIs) appear to show beneficial effects against diabetic complications in tissues[117]. It is becoming clear that these anti-AGE strategies play an important role in the

treatment of diabetic patients.

1.1.4. Treatment for diabetes

In making therapeutic choices in the management of type 2 diabetes, the major goal of protecting patients from the long-term complications of the disease must be considered. Because insulin resistance plays a fundamental role in the pathogenesis of type 2 diabetes and especially its adverse cardiovascular outcomes, interventions should initially be aimed towards improvement in tissue insulin sensitivity. This often involves lifestyle intervention, with modest exercise and weight loss, which clearly reduces the risk of progression of impaired glucose tolerance to overt diabetes and can improve many of the cardiovascular risk parameters of the metabolic syndrome[118].

1.1.4.1. Traditional anti-diabetic drugs

Generally, current therapeutic strategies for type 2 diabetes are limited and involve insulin and four main classes of oral anti-diabetic agents that stimulate pancreatic insulin secretion (sulphonylureas and rapid-acting secretagogues/insulinotropics *e.g.*, glibenclamide, glipizide, rapaglinide), reduce hepatic glucose production (biguanides *e.g.*, metformin), delay digestion and absorption of intestinal carbohydrate (α-glucosidase inhibitors *e.g.*, acarbose) or improve insulin action (TZDs, *e.g.*, pioglitazone, rosiglitazone)[119]. In 2014 a new anti-diabetic drug was approved by FDA called Empagliflozin, which could offer an insulin-independent mechanism for improving blood glucose levels by serving as sodium-glucose co-transporter 2 (SGLT2) inhibitors, since they promote urinary glucose excretion (UGE) by inhibiting glucose

reabsorption in the kidney[120]. Each of above agents suffers from generally inadequate efficacy and most of them have side effects, such as severe hypoglycemia, lactic acidosis, idiosyncratic liver cell injury, permanent neurological deficit, digestive discomfort, headache, dizziness and even death[121]. For instance, a major adverse effect associated with clinical use of the TZD is weight gain, which seems to be coupled to the effects of the drugs on adipose cell differentiation and triglyceride storage[122]. One of the side effects with sulfonylurea is that it causes a decreased amount of insulin production by putting too great a strain on the insulin producing beta cells[123].

1.1.4.2. Traditional Chinese Medicine (TCM) as intervention for diabetes

There is a strong need for safe and effective oral hypoglycemic agents that provide the clinician with a wider range of options for preventing, treating and managing diabetes. The treatment of diabetic patients with naturally derived agents has the advantage that it does not cause the significant side effects as do chemical agents[124].

Over the centuries, Chinese herbal drugs have served as a major source of medicines for the prevention and treatment of diseases including diabetes mellitus. It is estimated that more than 200 species of plants exhibit hypoglycemic properties, including many common plants, such as pumpkin, wheat, celery, wax guard, lotus root and bitter melon. To date, hundreds of herbs and traditional Chinese medicine formulas have been reported to have been used for the treatment of diabetes mellitus, and some of them have been approved for anti-diabetic formulations[123]. It was believed, through pharmacological studies, that medicinal herbs were meticulously

organized in these anti-diabetic drug formulas such that polysaccharide containing herbs restore the functions of pancreatic tissues and cause an increase in insulin output by the functional β cells, while other ingredients enhance the microcirculation, increase the availability of insulin and facilitate the metabolism in insulin-dependent processes[125]. Pharmacological and clinical evaluations indicated that the long-term use of these agents may be advantageous over chemical drugs in alleviating some of the chronic diseases and complications caused by diabetes. Additionally, the use of these natural agents in conjunction with conventional drug treatments, such as a chemical agent or insulin, permits the use of lower doses of the drug and/or decreased frequency of administration which decreases the side effects most commonly observed[125].

1.1.5. Animal models for type 2 diabetes

Due to the high prevalence of diabetes worldwide, extensive research is being performed to develop new anti-diabetic agents and determine their mechanisms of action. Consequently, a number of diabetic animal models have been developed and improved over the years, of which rodent models are most thoroughly described[126]. These rodent models can be classified into three broad categories: 1) genetically induced spontaneous diabetes models; and 2) experimentally induced nonspontaneous diabetes models; and 3) nutrition induced nonspontaneous diabetes models[126].

1.1.5.1. Genetically derived models

Spontaneously diabetic animals of type 2 diabetes may be obtained from the

animals with one or several genetic mutations *trans*mitted from generation to generation (*e.g.*, *ob/ob*, *db/db* mice)[127]. These animals generally inherited diabetes either as single or multigene defects. The metabolic peculiarities result from single gene defect (monogenic) which may be due to dominant gene (*e.g.*, Yellow obese or KK/Ay mouse) or recessive gene (diabetic or db/db mouse, Zucker fatty rat) or it can be of polygenic origin [*e.g.*, New Zealand obese (NZO) mouse][128]. Advantages of genetically derived models include: characteristics resembling human type 2 diabetes, small variability, small sample size, homogeneous genetic background and others. However, diabetes from this model is highly genetic and homogeneous unlike in human. Also, they are expensive and have limited availability, which is among other setbacks of this model[129].

1.1.5.2. Experimentally induced models

The popularity of using experimentally induced models for diabetes research over that of genetically induced is due to their comparatively lower cost, ease of diabetes induction, maintenance and wider availability. The various experimentally induced type 2 rodent models developed over the last 30- plus years for both routine pharmacological screening and mechanistic diabetes-linked research trials include: adult streptozotocin (STZ)/alloxan rat models, neonatal STZ/alloxan models, partial pancreotectomy models, etc[130]. The use of these models, however, is not without limitations. Disadvantages include: hyperglycemia develops primarily by direct cytotoxic action on the β cells and insulin deficiency rather than consequence of insulin resistance; less stable and sometimes reversible because of the spontaneous

regeneration of β cells; chemical produces toxic actions other organs; variability is high[129].

1.1.5.3. Nutrition-induced models

When evaluating this approach for the induction of diabetes as a whole, long-term high fat diet (HFD)-fed type 2 diabetes models develop hyperglycemia, hyperinsulinemia, glucose intolerance, insulin resistance and dyslipidemia, all recognized conditions associated with type 2 diabetes, induced by the same mechanism as that in humans[131]. The time required to develop this model, however, could be considered a limiting factor. Toxicity on other body organs can be avoided; however, one of the drawbacks is that no frank hyperglycemia develops upon genetically normal animals.

Many of the animal models described apparently share similar characteristic features of type 2 diabetes and have allowed experimentation that would be impossible in humans. None of the known single species is exactly equivalent to human diabetes, but each model act as essential tool for investigating genetic, endocrine, metabolic, morphologic changes and underlying mechanisms that could also operate during the evolution of type 2 diabetes in humans[129]. One has to carefully select which model to use depending on the nature of his study.

1.2. Health benefits of *Polygonum multiflorum* (PM)

Polygonum multiflorum (PM), other names He Shou Wu or Fo-ti, is a longevity tonic in China used for graying hair, premature aging, weakness and other dysfunctions.

The root of PM is used as a tonic and an anti-aging agent in many remedies in traditional Chinese medicine[132-134].

According to the literature (The State Pharmacopoeia Commission of the People's Republic of China, 2005), the major constituents in PM are several natural product groups including stilbene glycosides, anthraquinone glycosides and anthraquinone derivates. The anthraquinone-containing fraction provides a dose dependent protection against myocardial ischemia– reperfusion injury, and reduces cerebral ischemia induced infarct volume[135, 136]. Recent research has provided evidence that stilbene containing fractions are effective as anti-aging and anti atherosclerotic factors, reducing brain pathologic atrophy and promoting learning and memory ability[137-139]. Components identified in PM extracts so far include 2,3,5,4-tetrahydroxystilbene-2-O-β-D-glucoside (SG), emodin-8-O-β-D-glucoside, physcion-8-O-β-Dglucoside, emodin, chrysophanol, rhaponticoside, torachrysone-8-O-β-D-glucoside, chrysophanol- 8-O-β-D-glucoside, physcion and so on. The determination of stilbene glycoside, emodin and physcion in this medicinal plant has been previously carried out by TLC, HPLC and LC-MS[140-142].

The medicinal effects of PM in the treatment of these age-related diseases are possibly mediated by the antioxidant capacity of this plant because free-radical-involved oxidative stress has been implicated in the aging process[143]. In 2005 the extracts of 30 Chinese medicinal plants were studied systematically for their antioxidant activities and PM root was found to be among the highest for both aqueous and methanol extracts[144]. PM extract has been found both in vitro and in vivo to possess

antioxidant activity[145]. Research indicates that PM enhances the cellular antioxidant activity[146], increases the function of superoxide dismutase (SOD), significantly inhibits the formation of oxidized lipids[145], represses lipid peroxidation in rat heart mitochondria and enhances antioxidant enzymes in the liver[147].

2. Experimental Part I: Anti-diabetic effect of PM extract and its mechanism

2.1. Introduction

KK CgAy/j mouse (also known as Yellow KK obese mouse) carries both lethal yellow obese (Ay) and diabetic gene unlike KK mouse where it carries only diabetic gene[148]. Mice homozygous for the yellow spontaneous mutation (Ay) die before implantation or shortly thereafter. KK CgAy/j mouse is heterozygous which shows severe obesity, hyperglycemia, hyperinsulinemia and glucose intolerance by 8 wks of age[148]. The strain KK CgAy/j mice serves as a good model for obesity and type 2 diabetes and for screening various classes of anti-diabetic agents. Studies using isolated adipocytes indicate that tissue responsiveness to insulin is decreased progressively from 5 wk of age (Jackson Labs). Histo- and immunochemical studies show that pancreatic islets are hypertrophied and β cells are degranulated. These findings suggest that principal cause of diabetes in these mice is insulin resistance which may be due to defects in both insulin receptor and post receptor signaling systems[149].

As previously discussed, oxidative stress is currently suggested as a mechanism underlying diabetes and diabetic complications. In diabetes, protein glycation and glucose autoxidation may generate free radicals, which in turn catalyze lipid peroxidation[150]. Moreover, disturbances of antioxidant defense systems in diabetes were shown: alteration in antioxidant enzymes, impaired glutathione metabolism and decreased ascorbic acid levels[151, 152]. In light of the association of diabetes and oxidative stress, the pharmacological properties of PM prompted us to hypothesize that

PM may have a beneficial effect on diabetes. The objective of the current study is to prepare an extract from the root of this plant, to confirm its anti-diabetic activities with KK CgAy/j mice, and find out about the mechanism.

2.2. Material and method

2.2.1. Preparation of extract from PM Roots

Dried root powder of PM was purchased from Anguo Mayway Herb Company Ltd., An Guo, Heibei Province, China, and followed extraction procedure of Lishuang Lv with slight modification[153] . Briefly, the dried roots of PM were crushed and extracted with 60% ethanol, at a ratio of solution to solid of 1:10 (v/w), at room temperature for 2 days. The plant material was filtered off, and the ethanolic extracts were combined and concentrated under reduced pressure using a rotary evaporator. The dry extract obtained was then subjected to open column chromatography (CC) packed with macroporous resin. The column was eluted stepwise with each of 9 different concentrations of ethanol (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%). The 40% aqueous-ethanol fraction was then concentrated under reduced pressure using a rotary evaporator. The powder obtained was subjected to HPLC for analysis, and used for animal study later.

2.2.2. HPLC analysis of PM extract

PM extract was analyzed by a Waters Acquity HPLC system coupled with a UV detector (Waters, Milford, MA). A 250mm×4.6 mm inner diameter, 5 μm, HYPERSIL-C18 column was used. For binary gradient elution, mobile phases A (100%

water with 0.2% formic acid) and B (acetonitrile) were used. The flow rate was maintained at 1 mL/min, and the mobile phase began with 8% B. It was followed by progressive linear increases in B to 35% at 17 min, to 90% at 18 min, and maintained at 90% until 21 min. The injection volume was 10 µL for each sample.

2.2.3. KK CgAy/J type 2 diabetic mouse model

Female KK CgAy/J diabetic mice were purchased from Jackson labs (Barr Harbor, ME) and were housed in stainless steel wire-bottomed cages and acclimatized under laboratory conditions (19-23 °C, humidity 60%, 12 h light/dark cycle). The mice were divided into two groups with 10 each, and fed with Western HFD (Research Diets, New Brunswick, NJ) composed of 20 kcal % protein, 20 kcal % carbohydrate, and 60 kcal % fat (from butter). Group I: Diabetic control which had free access to drinking water; Group II: Treatment group which had free access to drinking water with 0.075% of PM extract. Body weight, food and water uptake of the mice were taken on a regular basis. After 7 weeks, all the experimental mice were sacrificed. Body weight, blood glucose level and lipid profile were recorded. Blood glucose was measured with blood glucose test strips from Contour, and lipid profile was measured with PTS Panels test strips. The weight of parametrial fat, retro-peritoneal fat and brown fat was recorded. Liver, spleen and kidney were removed and weighed as well.

2.2.4. Biochemical assays

Blood sample was collected and centrifuged at 12,500 rpm for an hour. Serum

insulin level was measured with a commercial kit (Cayman Chemical, Detroit, MI), and performed according to the protocol. Liver and fat tissues were homogenized, lysed with lysis buffer [(0.5% (w/v) sodium lauryl sarkosinate + 10 mM EDTA + 0.5 mg/ml proteinase K + 0.1 mg/ml RNase A in 50 mM Tris-Base, pH 8.0)], centrifuged and protein of homogenates quantified using a BCA protein assay kit (Pierce Chemical, Rockford, IL).

ELISA assay

The levels of Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the liver homogenates of control and experimental groups of KK CgAy/J mice were determined by specific ELISA kits according to the manufacturer' s instructions (Camarillo, CA). The capture antibody, diluted with PBS, was used to coat a 96-well plate overnight at room temperature. The plate was then washed, blocked (1% BSA, 5% sucrose in PBS with 0.05% NaN_3), and washed again. The standards were added to the plate leaving at least one zero concentration well and one blank well. The diluted samples (1:5-1:20) were then added to the plate. After incubating for 2h, the plates were washed and the detection antibody was added. After incubating for another 2h the plates were washed and Streptavidin-HRP was added. After 20min incubation, the plates were washed, and substrate (H_2O_2) and tetramethylbenzidine were added. After another 20 min incubation, the stop solution (2N of H_2SO_4) was added and then, plates were read with a microplate reader at a wavelength of 450 nm. Standard plots were constructed by using standard cytokines and the concentrations for unknown samples were calculated from the standard plot.

Western blotting

The sample of liver or fat homogenates (60 μg of protein) in 4× loading buffer was denatured at 95°C for 5 min, and subjected to SDS-polyacrylamide gel (4–10%) electrophoresis. The gel then was *trans*ferred onto a polyvinylidene difluoride membrane (Bio Rad, Hercules, CA), and the membrane was blocked with TBS-T (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4), containing 5-7% nonfat dried milk. The blocked membrane was incubated at 4°C overnight with 1:500 dilution of monoclonal antibody for IR-α, IRS-1 (liver) and Glut4 (fat) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblotted membrane was incubated at room temperature for 2 hr with secondary anti-rabbit or anti-mouse IgG antibodies conjugated with horseradish peroxidase and then exposed on X-ray film with ECL detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Bands were quantified using the Adobe Photoshop program with scanning process.

All experiments and analyses were performed at least in triplicate. Results are expressed as means ± SE. Statistical analyses were performed using the Student's T-test. * denotes the difference was statistically significant ($p < 0.05$).

2.3. Results

2.3.1. Hypoglycemic effect of PM in KK CgAy/J diabetic mice

After 7 weeks of PM extract administration in drinking, body weight of KK Cg/Ay mice was comparable between control group and treatment group after 7 weeks test period. Triglyceride level was significantly higher in the treatment group, but the other

lipid parameters, such as HDL, LDL and total cholesterol almost stayed the same in the two groups. Weight of liver and kidney also showed no difference in the two groups, but spleen showed a significant weight loss. At the same time, the hyperglycemia of diabetic KK Cg/Ay mice was almost completely reverted to normal by SG treatment, since the glucose level in the control group and treatment group is 233.6 mg/dl and 121.6 mg/dl, respectively (Table 1). That indicates that PM-SG extract has a strong hypoglycemic effect.

2.3.2. Pro-inflammatory cytokine levels in the liver

Pro-inflammatory cytokine (IL-6, IL-1 β, and TNF-α) levels in the liver were measured with commercial Elisa kits after seven weeks of PM treatment. From Figure 2, levels of IL-6 and IL-1b didn't differ significantly between diabetic control group and PM group. However, for TNF-α the level slightly increased in PM treated group ($p<0.05$). The level of IL-6 was the highest among all three cytokines，at around 200 pg/mg.

2.3.3. Markers for insulin signaling pathway

The levels of selected marker from insulin signaling pathway including IR-α and IRS-1 from the liver and Glut4 from the fat, did not show any significant difference from diabetic control and PM group (Figure 3), which is consistent with our previous findings. Taken together, these results suggest that the hypoglycemic effect of PM extract was not mediated through insulin resistance.

2.3.4. Serum insulin levels

Serum insulin level significantly increased from 1 μU/ml in diabetic group to around 7 μU/ml in PM extract (Figure 4), indicating that PM extract had a potent effect in stimulating insulin secretion.

2.3.5. HOMA-β and HOMA-IR

Based on the calculation from Homeostatic Model Assessment (HOMA) model, HOMA-IR = Glucose* Insulin/405 and HOMA-β = (360*Insulin)/(Glucose-63); with fasting blood glucose and fasting blood insulin levels， insulin resistance increased slightly in PM group, but what's more significant is β cell function, which improved by nearly 20 fold from 2 to 45 after PM treatment.

2.4. Discussion

Macroporous resin is widely used in the industrial production due to its high efficiency and low-cost. Moreover, the advantages of macroporous resins, such as ideal pore structure, unique adsorption properties, less solvent consumption and easy regeneration, make them popular in the separation and purification of many pharmacologically important natural products[154]. Macroporous resin is also regarded as a dual-task method which can be established to simultaneously remove the anthraquinones and enrich the TSG, since TSG is considered to be responsible for the curative effects of PM root while anthraquinones have been ascribed to the hepatotoxic compounds[155]. In the present study, extraction procedure using macroporous resin was modified according to that of Lishuang Lv[153].

From the HPLC chromatogram of the 40% aqueous-ethanol fraction of PM

extract from macroporous resin column (Figure 1)，the major peak has a retention time of 13.8 min, and the extract contains many other components as well. With a standard compound, the 13.8 min peak was identified to be 2,3,5,4-tetrahydroxystilbene-2-O-β-D-glucoside (SG) (see Part II), which is the major active compound in PM roots. The level of SG in the PM extract was quantified in the PM extracts with a standard. A standard curve was plotted using nine SG standard solutions prepared from serious dilution, whose concentration ranged from 2 mg/ml to 0.007813 mg/ml. 2 mg/ml sample solution was used for HPLC analysis, and the concentration of SG in it was calculated from the standard curve to be 0.162 mg/ml. Thus the content of SG in the extract was quantified to be 0.081 mg/mg extract powder.

Polygonum multiflorum has been used in Chinese medicine for thousands of years. It is considered a tonic to increase vitality and energy, strengthen the blood, kidneys and liver. There is evidence that PM can lower serum cholesterol, decrease hardening of the arteries, and improve immune function[134, 156, 157]. In recent years, due to its strong antioxidant activity, PM has attracted a lot of interest and become extensively studied. From previous studies, the 40% ethanol extract of *Polygonum multiflorum* roots has high 2,2-Diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging effect[158]. Lishuang Lv et al showed that the antioxidant capacity of relatively impure RF-PM (40% aqueous-ethanol fraction eluted from macroporous resin column) has strong protection effect against age-related oxidative stress[159]. Since oxidative stress is currently suggested as mechanism underlying diabetes and diabetic complications, 40% RF-PM was utilized for the anti-diabetic study.

From the animal study, the high glucose level in the KK Cg/Ay mice was almost completely reversed by 7-week treatment of 0.075% PM extract in drinking water. The strong hypoglycemic effect of PM extract was demonstrated for the first time with a type 2 diabetic mouse model. The model used in the current study, KK Cg/Ay mouse is an animal model of type 2 diabetes, characterized by hyperglycaemia, hyperinsulinemia and glucose intolerance.

Several cytokines, including IL-6, IL-1β and TNF-α, have been shown to cause insulin resistance and one mechanism by which these agents could cause insulin resistance is by inducing the expression of cellular proteins that inhibit insulin receptor signaling. Since pro-inflammatory cytokines are closely related to insulin resistance, the mechanism of anti-diabetic effect of PM extract was explored first by evaluating if PM can suppress production of cytokine protein levels in the liver, especially those cytokines most related to diabetes. No significant difference was found in the levels of IL-6 and IL-1β from liver between diabetic control and PM group, while TNF-α increased slightly in PM group. Since TNF-α could interfere with insulin signaling pathway, we next evaluated if the pathway was affected by this increase in TNF-α. Several markers were selected from insulin signaling pathway, including IR-α and IRS-1 from the liver and Glut4 from the fat. Consistent with the previous findings, these markers are not significantly different in the two groups, suggesting that insulin resistance is not the pathway by which the hypoglycemic effect of PM extract is mediated.

After measuring the insulin levels in the serum of the two groups, it was found that

there was a significant increase in the level of insulin from the PM-treated group. And with fasting glucose and fasting insulin levels, we calculated the insulin resistance and β cell function for the two groups based on a model called HOMA, which is based on the assumption that the relationship between glucose and insulin in the basal state reflects the balance between hepatic glucose output and insulin secretion[160], and it has been shown to correlate well with experimental method[161]. From the results insulin resistance increased slightly in PM group, but β cell function improved by more than 20 fold, which was much more significant. So with this a possible mechanism for PM extract's hypoglycemic effect was proposed: in the diabetic mice, insulin resistance did not decrease, however, failure was either prevented or delayed, so β cells still function to produce insulin, and by the time the mice were sacrificed there was still considerable amount of insulin in the blood to compensate for insulin resistance, as a result blood glucose levels are lowered.

2.5. Summary

PM extracts were obtained with macroporous resin column extraction and for the first time, they were found to have anti-hyperglycemic effects in a female KK CgAy/j diabetic mouse model. To investigate the mechanism of action, pro-inflammatory cytokines and insulin signaling pathway was studied, and it was found that the mechanism was not through insulin signaling or insulin resistance. With HOMA model, it was proposed that PM extract achieved this effect through stimulating insulin secretion, thereby maintaining β cell function. The current study suggests potential use of PM roots into functional beverages for pre-diabetics. And the next

phase of study is to identify functional ingredient(s) in the extract which contributes to this effect.

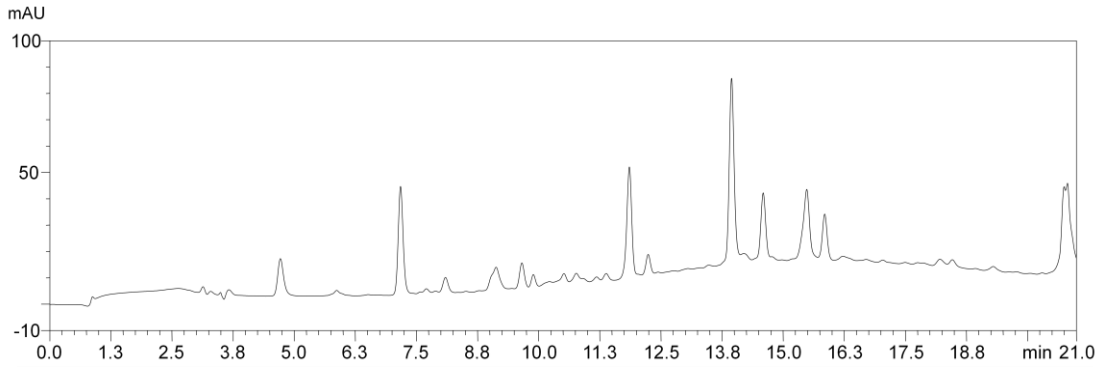


Figure 1. HPLC chromatogram for PM extract. PM root powder was extracted with 60% ethanol and eluted with different concentrations of ethanol in macroporous resin column. The 40% resin fractionate was used for animal study.

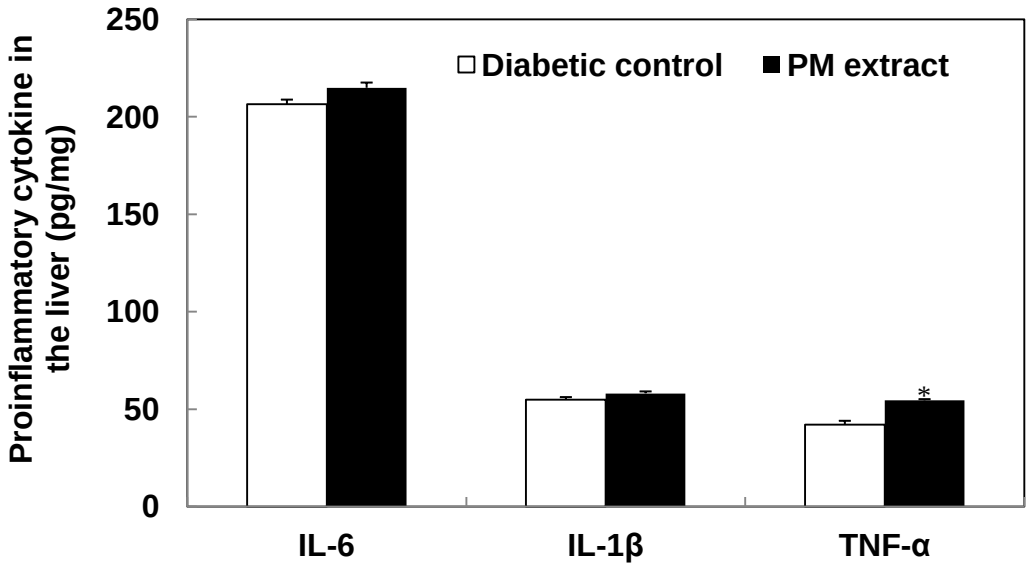


Figure 2. Effect of PM extract on levels of IL-6, IL-1β, and TNF-α in liver samples. After 7 weeks of treatment mice were sacrificed and liver samples were homogenized with homogenate buffer. Protein of liver homogenate was quantified and the levels of IL-6, IL-1β and TNF-α were measured with ELISA described before. *$p<0.05$.

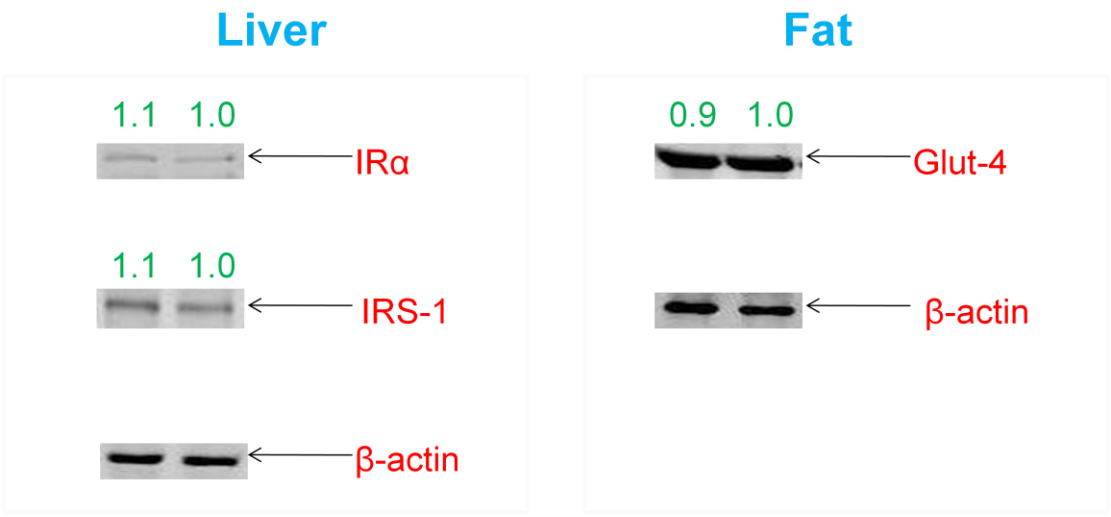


Figure 3. Effect of PM extract on selected markers from insulin signaling pathway. Liver and fat tissues from mice in diabetic control and in PM group were homogenized, lysed, centrifuged, quantified with protein content, and applied to western blotting as described before. For liver sample antibodies IR-α and IRS-1 were used, and for fat sample antibody Glut4 was used. Left band shows PM group while right band shows diabetic control.

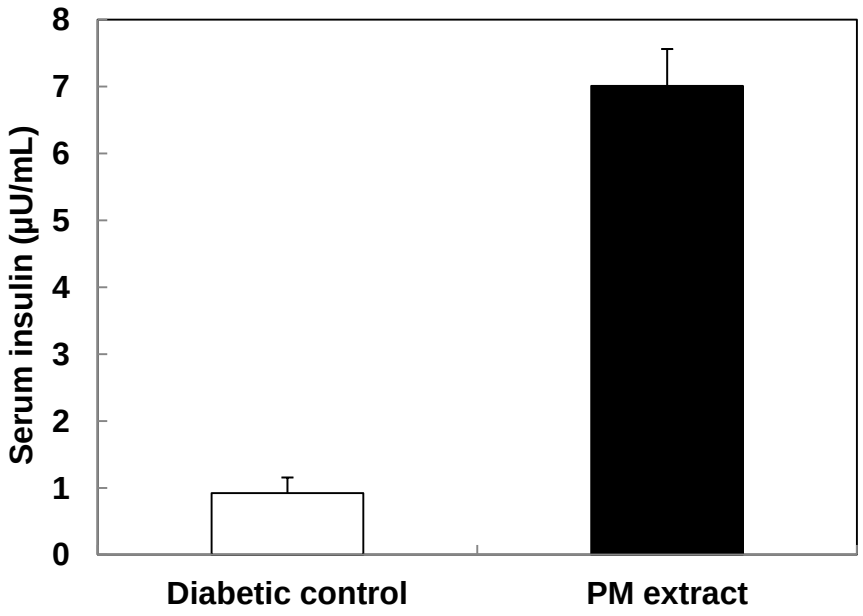


Figure 4. Effect of PM extract on serum insulin levels of KK CgAy/J mice. After 7 weeks of treatment mice were sacrificed and liver samples were homogenized with homogenate buffer. Blood samples were collected and centrifuged for an hour at 12, 500 rpm. Serum was measured for insulin levels with a commercial insulin kit.

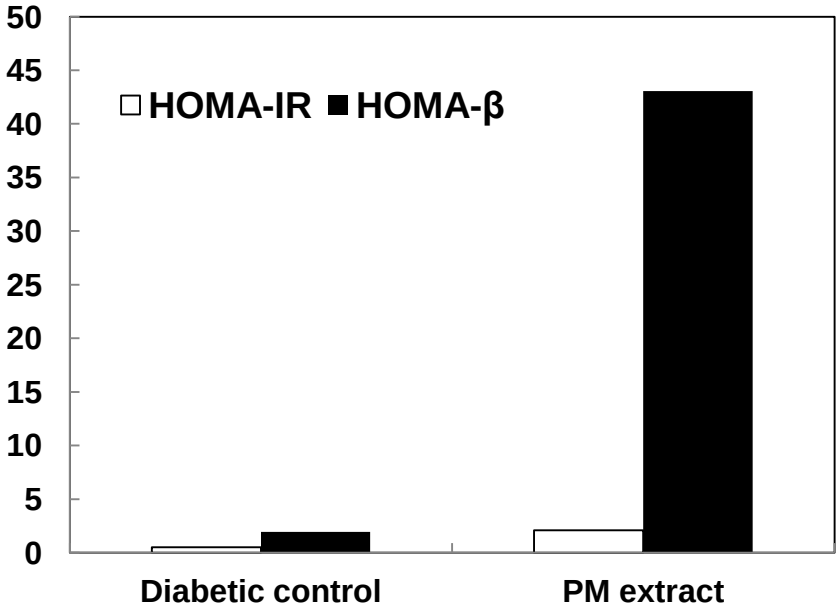


Figure 5. Approximated insulin resistance (IR) and β cell function (β) from HOMA model. Calculation was based on the following two equations: HOMA-IR = Glucose* Insulin/405 and HOMA-β = (360*Insulin)/(Glucose-63); fasting blood glucose and fasting blood insulin levels were used.

Table 1. Anti-diabetic effects of feeding PM extract in the drinking water to female KK CgAy/J mice

List of Assays	Group 1	Group 2
Body weight(g)	40.46±1.91	41.06±0.75
Glucose(mg/dl)	233.56±33.18	121.60±13.07 **
Triglyceride(mg/dl)	238.44±13.80	271.70±34.59
Total cholesterol (mg/dl)	173.33±3.38	179.30±4.53
HDL (mg/dl)	90.00±2.38	86.00±3.07
LDL (mg/dl)	35.62±5.77	39.00±5.96
Parametrial fat(g)	4.73±0.34	4.96±0.31
Retroperitoneal fat(g)	0.58±0.06	0.75±0.04 *
Brown fat(g)	0.80±0.07	0.78±0.06
Liver(g)	2.1±0.17	2.09±0.11
Spleen(g)	0.15±0.01	0.12±0.01*
Kidney(g)	0.34±0.02	0.37±0.01

** $P<0.003$, * $P<0.05$ as determined by the Student' T-test. Group 1 (drinking water); group 2 (0.075 % PM extract in drinking water) for 7 weeks.

3. Experimental Part II: Evaluation of stilbene glycoside for its anti-diabetic effect

3.1. Introduction

2,3,5,4-tetrahydroxystilbene 2-O-b-glucopyranoside (stilbene glycoside, SG, Fig 2.1 A) with the chemical structure $C_{20}H_{22}O_9$ and molecular weight 406.39, is a white amorphous powder, soluble in water, methanol and ethanol. It is stable in water solutions, but high temperature (>80 °C) might affect its stability; it's very unstable in acid. SG is the major active compound in PM, and the concentration in the roots of PM can reach 3%-6%. According to Chinese Food and Drug Administration, SG is used as an index of quality control for PM products and the concentration of SG in commercial PM products has to be higher than 1%.

SG is a type of stilbene derivative, and other well-known stilbene compounds in stilbene family include resveratrol (Fig 2.1 B) and pterostilbene. The only difference between structures of SG and resveratrol is the glycoside portion. Because of the additional hydroxyl group at the iso- position, SG has higher antioxidant activity than resveratrol[159].

Using Ultra-performance liquid chromatography–time-of-flight mass spectrometry (UPLC-Q-TOF/MS) and HPLC-UV, the pharmacokinetics, bioavailability, absorption, and metabolism of SG were studied in rats following a single intravenous or oral administration[162]. The metabolites (M1 and M2) were identified in plasma by UPLC-Q-TOF/MS. SG was rapidly distributed (within 30 min) and then eliminated from rat plasma. Absolute bioavailability of SG was 40%. Total

recovery of unchanged SG within 24 hr were low (0.041% in bile, 0.06% in feces), whereas the amount of unchanged SG excreted in the urine within 24 hr was lower than Lower Limit of Quantification (LLOQ). SG was excreted mainly in the forms of metabolites, including monoglucuronide and the aglycone form which is more stable. This study suggests that SG may be absorbed quickly into circulation and may trigger a pharmacological effect for possible biological activity at extra-hepatic sites[162]. The correlation between pharmacokinetic parameters and biological activities of SG needs to be further investigated.

SG has been shown by a plethora of cell and animal studies to have anti-neurodegenerative[163] , anti-angiogenesis[137] and anti-antherogenic[164] effects. The radical scavenging activities of SG are well known. A study found that SG could efficiently inhibit the formation of AGEs which play a major pathogenic role in diabetes and its complications, in a dose-dependent manner by trapping reactive methylglyoxal under physiological conditions (pH 7.4, 37 °C), making it a potential natural inhibitor of AGEs[165].

The strong antioxidant activity of SG has been also widely researched. It was found to be the DPPH radical-scavenging active components from PM roots[166]. In 2007, Lv induced a rat model for aging by injecting them with D-galactose which could cause oxidative stress. She treated the aged rats with either RF-PM or pure SG, and found that they both could bring antioxidant enzymes in serum and in the liver to levels closer to normal rats[159]. She concluded that SG had a protection effect against age-related oxidative stress.

In searching for the efficacious component in PM extract which is responsible for its anti-diabetic effect, we first evaluated the potential hypoglycemic effect of SG, by first purifying SG from PM extract, and then conducting a series of experiments to explored the anti-inflammatory effects of SG; after that the anti-diabetic effect was studied with the same animal model as in Part I for PM extract.

3.2. Material and method

3.2.1. Purification of SG

PM roots were extracted in the same manner as described in 1.2.1, and macroporous resin column was also used for separation. The column was eluted with 2 L of 40% ethanol to get rid of impurities, and then with 1 L of 50% ethanol. Fractions collected were monitored with HPLC analysis and the ones with pure SG were piled and concentrated under reduced pressure using a rotary evaporator. SG was analyzed with the same HPLC program as in 1.2.2.

3.2.2. Anti-inflammatory activity of SG

We first investigated the anti-inflammatory effects of SG, in macrophage cell cultures and in mouse ear edema model with CD-1 mice.

3.2.2.1. Macrophage cell culture studies

Nitrate assay and ELISA for pro-inflammatory cytokines

RAW 264.7 macrophage cells were cultured in DMEM supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100

mg/mL streptomycin. When the cells reached a density of 2-3 $\times 10^6$ cells/mL, they were activated by incubation in medium containing E. coli LPS (10 µg/mL). Various concentrations of test compounds dissolved in DMSO were added together with LPS. Cells were treated with 0.05% DMSO as vehicle control. The nitrite concentration in the culture medium was measured as an indicator of NO production, with Griess reagent (Sigma Aldrich, St. Louis, MO) following manufacturer's instructions. Absorbance of the mixture at 540nm was measured with an ELISA plate reader. Cell supernatant was also used for evaluation of Pro-inflammatory cytokine levels and same ELISA procedure as in mouse ear model was followed. IL-6 cytokine levels were evaluated for cell supernatant samples treated with various concentrations of SG.

Cell viability by MTT assay

The determination of cell viability is a common assay to evaluate the in vitro cytotoxicity of biomaterials. In the present study, cell viability was assessed by MTT[167]. The MTT assay is a quantitative and rapid colorimetric method based on the cleavage of a yellow tetrazolium salt (MTT) to insoluble purple formazan crystals by the mitochondrial dehydrogenase of viable cells[167].

Raw cells were seeded onto 96-well plates at a density of 1×10^5 cells/well and cultured in 100 µl of DMEM for 24 h in the CO_2 incubator. The spent medium was replaced with SG solution diluted with culture medium to give a concentration from 2.5 µM to 80 µM. After overnight incubation at 37°C, the SG solution was replaced with 100 µl of MTT (0.5 mg/ml in PBS, pH 7.4) solutions, and the cells were incubated for a further 2.5 h at 37°C. The test solution was decanted, and 50 µl of DMSO was added to

solubilize the cells. The resultant solutions were measured in a microplate reader at λ_{570}. Cell viability was expressed as percentage of absorbance relative to control, the control comprising cells only exposed to 0.05% DMSO.

3.2.2.2. Mouse ear edema model

Seven-week-old female CD-1 mice were purchased from Charles River Laboratories (Kingston, NY), and kept in our animal facility at least 1 week before use. Mice were fed a Purina Laboratory Chow 5001 diet ad libitum (Ralston-Purina Co., St. Louis, MO) and kept on a 12-h light, 12-h dark cycle. Mice were provided drinking water ad libitum. Each group was comprised of four mice. The persistent skin inflammation was induced in both ears of the mice by topical treatment of 15µl of acetone (vehicle) or 2 µM PM-SG in acetone or 0.8 nmol TPA in acetone. The treatment was done twice a day for 4 days, one 10 minutes before TPA and the other one 1 hr after TPA. The mice were sacrificed 4h after the last TPA treatment. Ears punches (6 mm in diameter) were taken and weighed. The increased weight of ear punch was used as ear edema (inflammation). Ear samples from each group were pooled and homogenized in PBS containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1mM PMSF, 0.1mM bensethonium, 10mM EDTA, and 20 U aprotinin per each mL. The homogenates were centrifuged at 12,000 rpm for 60 min at 4°C. The supernatants were used to assay for the levels of cytokines IL-1β, IL-6 and TNF-α.

The levels of Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the ear homogenates of control and experimental groups of CD-1 mice were determined by specific ELISA kits according to the manufacturer’ s instructions (Camarillo, CA,). The

concentration of Pro-inflammatory cytokines was determined spectrophotometrically at 450 nm. Standard plots were constructed by using standard cytokines and the concentrations for unknown samples were calculated from the standard plot.

3.2.3. Anti-diabetic effect of SG in animal study

Female KK CgAy/J mice were also utilized in this experiment and handled with the same protocol as in 1.2.3. The mice were divided into four groups with 10 each, and fed with Western HFD. Group I: Normal KK control mice which had free access to drinking water and normal chow; Group II: Diabetic control which had free access to drinking water and HFD; Group III: Treatment group which had free access to drinking water with 0.075% of PM-SG and HFD. Group IV: Positive control group which had free access to metformin at 300 mg/Kg and HFD. Body weight, food and water uptake of the mice were taken on a regular basis. After 12 weeks, all the experimental mice were sacrificed. Similar measurements were taken to the previous experiment with PM extract.

Before sacrifice, all the experimental mice were subjected to glucose and insulin tolerance tests. For glucose tolerance test, mice were fasted overnight and injected with glucose solution at 1 g/10 ml and 10 ml/kg body weight. The change in blood glucose level was monitored during the following two hours. For insulin tolerance test, mice were fasted for 5 hours and injected with insulin solution at 1 u/ml and 10 ml/kg body weight. The change in blood glucose level was monitored during the following two hours.

3.3. Results

3.3.1. Anti-inflammatory effects of SG

The anti-inflammatory effects of SG were evaluated both in cell culture and in mouse ear edema model. For cell culture, the level of nitrite in the culture media of RAW 264.7 cells were determined 24 h after co-treatment with 100 ng/mL LPS and various concentrations of SG. The doses used to evaluate the anti-inflammatory effects of SG are not toxic, judging from the results of MTT assay. As shown in Figure 7A, SG inhibited LPS-induced nitrite production in a dose-dependent manner with IC_{50} at around 15 μM. The results from ELISA (Figure 7B) indicated that SG can significantly suppress the production of cytokines IL-6; SG concentration higher than 10 μM can almost completely suppress the increase in protein level induced by LPS.

For the mouse ear edema model, persistent inflammation in ears of female CD-1 mice was evaluated by measuring average weight of ear punches (6 mm in diameter). Topical application of 2 μmol of PM-SG inhibited TPA-induced persistent inflammation in ears by 63% ($P<0.01$). In addition, topical application of TPA (0.8 nmol) to ears of the mice once a day for 4 days induced persistent inflammation and up-expression of IL-1β, TNF-α and IL-6 protein levels in ears. 2 μmol of PM-SG also blocked TPA-induced up-expression of those cytokines by 77.2%, 71.5%, 48% and 60%, respectively. These results manifested that SG markedly inhibited TPA induced persistent inflammation and up-expression of pro-inflammatory cytokines in CD-1 mouse ears.

3.3.2. Anti-diabetic effects of SG

At the end of the feeding experiment, mice were sacrificed and measured for blood glucose and blood insulin levels. Diabetic control KK CgAy/j mice had significantly higher level of blood glucose compared to normal KK mice, and it was effectively brought down by anti-diabetic drug metformin (300 mg/kg); however, diabetic mice treated with 0.075% SG in drinking water had elevated levels of serum glucose as well as serum insulin compared to diabetic control (Figure 10), suggesting it did not exert any hypoglycemic effect.

To confirm the absence of hypoglycemic effect of SG, glucose tolerance test and insulin tolerance test were also carried out right before sacrifice. From the result of glucose tolerance test (Figure 11A), 30 minutes after glucose injection (1 g/kg), the blood glucose levels in all experimental groups increased drastically, with SG group being the most elevated, and the blood glucose in SG group remained much higher than normal control and metformin groups at the end of the test, suggesting the diabetic mice in SG group were glucose intolerant. On the other hand, the insulin tolerance test displayed that both SG and metformin groups had higher level of glucose 30 minutes after insulin injection, however, two hours after injection, the blood glucose in metformin group dropped to a lower level than that of SG group (Figure 11B). The positive control metformin had glucose tolerance and insulin tolerance similar to normal control, while the diabetic control had a high level of intolerance to both glucose and insulin, validating both models.

3.4. Discussion

In Part I, the anti-diabetic effect of PM extract from macroporous resin was confirmed in a KK CgAy/j mouse model. And in an effort to identify the efficacious compound leading to this effect, SG, which is the major active component in PM extract, was purified and tested. The strong antioxidant activity of SG was confirmed previously in a rat model for aging, and in this study we conducted research to investigate its anti-inflammatory effects. In RAW 264.7 cell, SG suppressed LPS-induced NO production, which is one of the mechanisms how cytokines affect insulin resistance, in a dose-dependent manner, and it also inhibited IL-6 production at concentrations higher than 10 μM. In the mouse ear edema model, topical application of 2 μM of SG could effectively suppress persistent inflammation induced by TPA, and also reversed the up-regulation of cytokine levels of IL-6, IL-1β and TNF-α. These results confirmed our hypothesis that SG has significant anti-inflammatory activities.

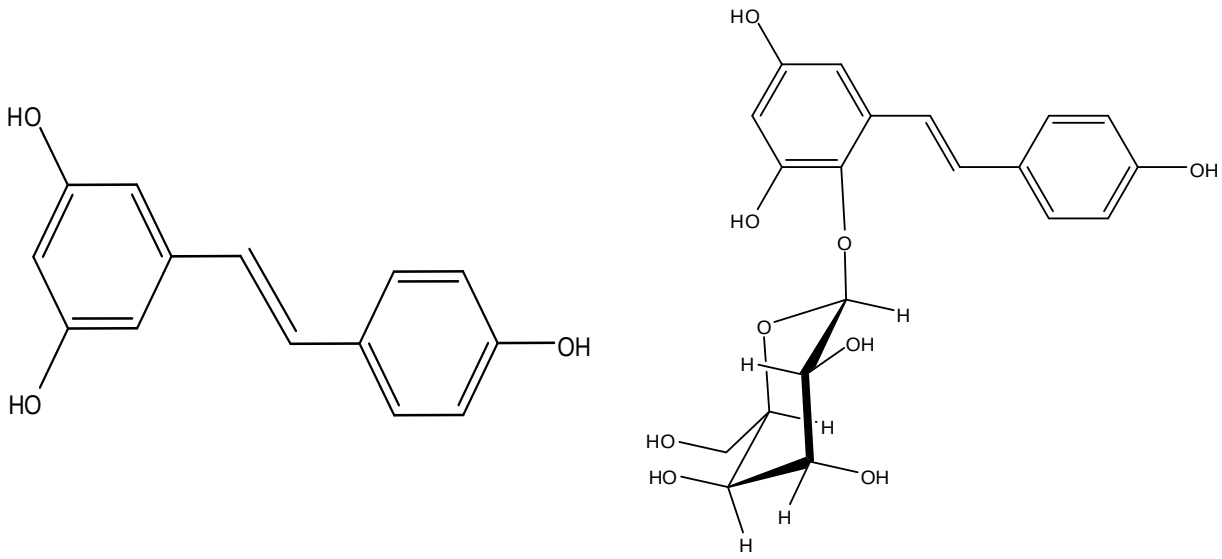
In a previous study by Cairong Li et al, 20 mg/kg SG for 8 weeks ameliorated diabetic nephropathy in rats, but didn't affect the hyperglycemia in the diabetic rats. However, in that study diabetes in the rats was induced with 60 mg/kg STZ. Streptozotocin is an antibiotic derived from *Streptomyces achromogenes* and structurally is a glucosamine derivative of nitrosourea. It causes hyperglycemia mainly by its direct cytotoxic action on the pancreatic β cells[130]. Therefore, STZ-induced diabetic rat was used as the severe insulin-deficient diabetic model, which is similar to type 1 diabetes. However, the effect of SG in type 2 diabetes was largely unknown.

Therefore, we utilized the same animal model and experimental design as that for PM extract in Part I. However, according to the results of the animal study no

hypoglycemic effect was observed from SG in KK CgAy/J mice after 12 weeks of SG treatment in drinking water at the dose of 0.075%. Furthermore, based on the glucose tolerance in SG-treated group. Therefore, SG could be eliminated for being responsible for the anti-diabetic effect of PM extract.

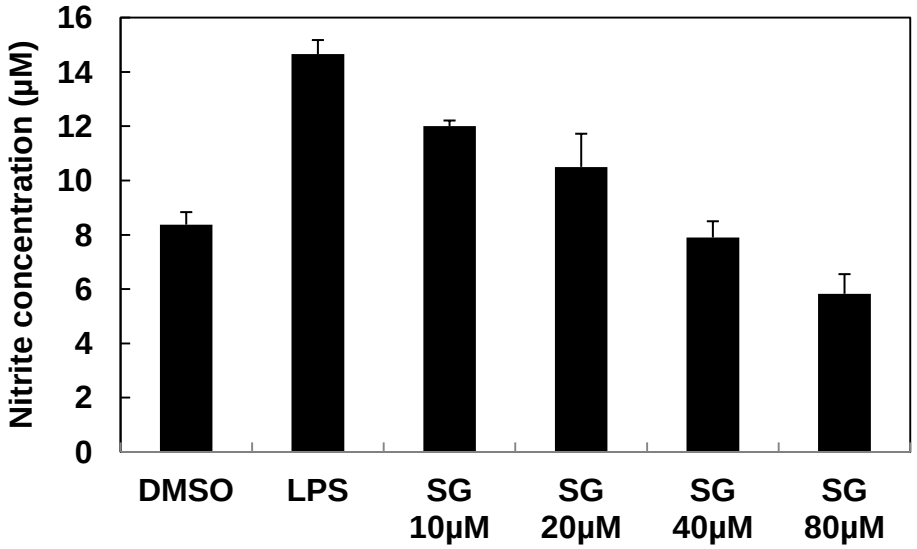
3.5. Summary

SG was isolated from PM roots and purified. It was found to have potent anti-inflammatory effect in RAW 246.7 macrophage cells and in TPA-induced mouse edema model, by lowering the level of anti-inflammatory cytokines including IL-6, IL-1β and TNF-α. However, it did not have any hypoglycemic effect as shown in the KK CgAy/j diabetic mice model.



(A) (B)

Figure 6. Chemical structures of (A) resveratrol and (B) stilbene glycoside.



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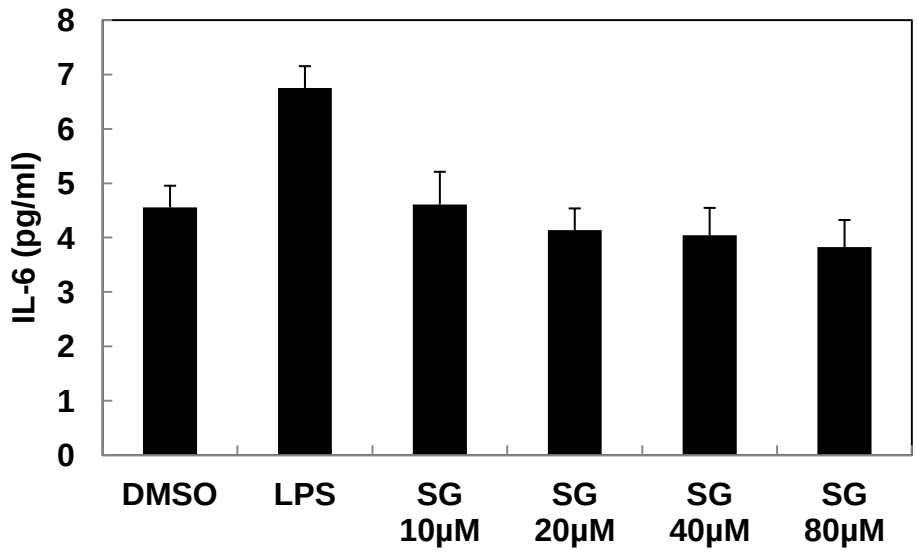


(B)

Figure 7. Effects of SG on LPS-induced NO and cytokine production in RAW 264.7 macrophage cells. The cells were treated with 100 ng/mL LPS only or with different concentrations of SG. After incubation for 24 h, 100 μL of culture media were collected for (A) nitrite assay, (B) IL-6 Elisa assay.

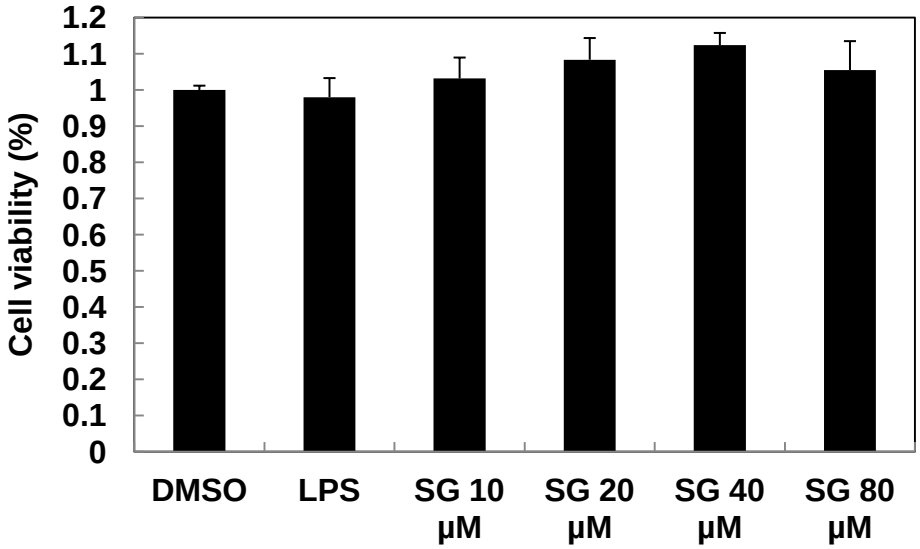
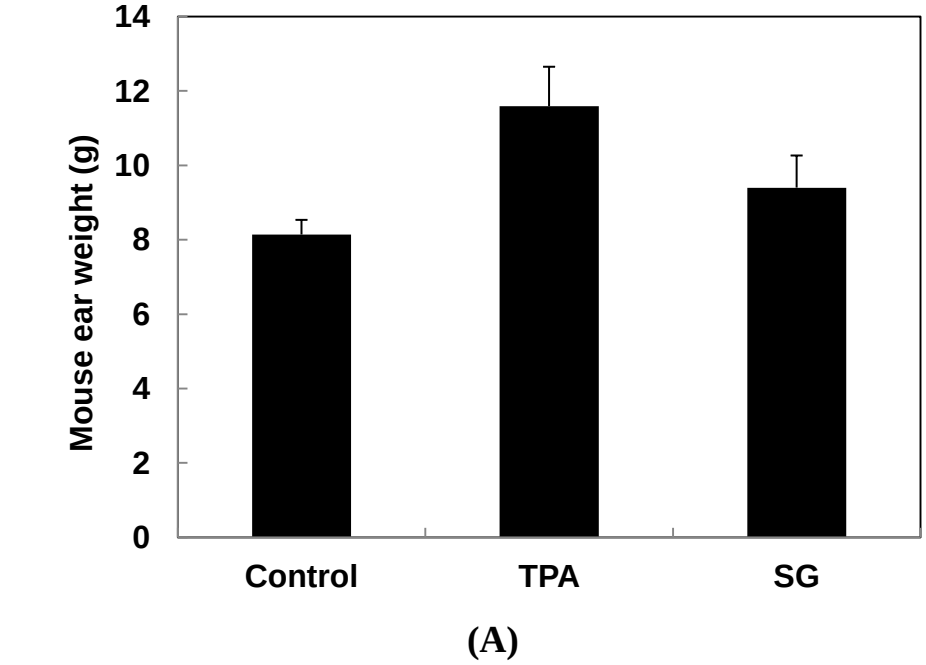


Figure 8. Cytotoxic effects of SG in RAW 264.7 cells as determined by MTT assay. Raw cells were seeded at a density of 1×10^5 cells/well and cultured in 100 μl of DMEM for 24 h in the CO_2 incubator. The spent medium was replaced with SG solution diluted with culture medium to give a concentration from 10 μM to 80 μM. After overnight incubation at 37°C, the SG solution was replaced with 100 μl of MTT (0.5 mg/ml in PBS, pH 7.4) solutions, and the cells were incubated for a further 2.5 h at 37°C. The test solution was replaced by 50 μl of DMSO and resultant solutions were measured in a microplate reader at λ_{570}. Cell viability was expressed as percentage of absorbance relative to 0.05% DMSO control.



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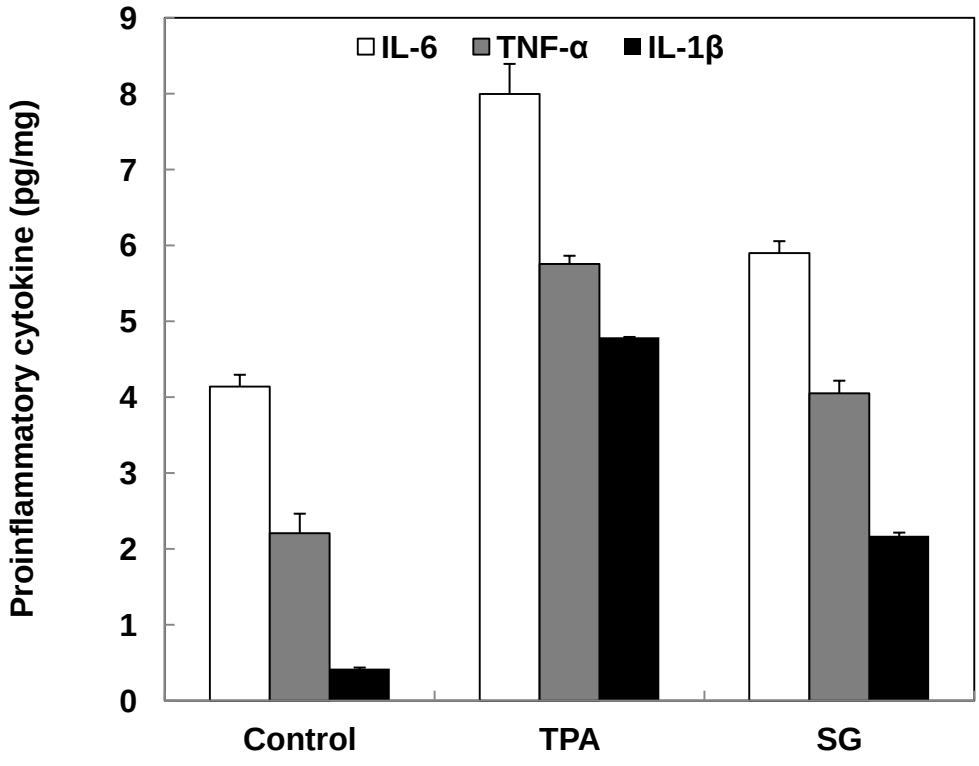
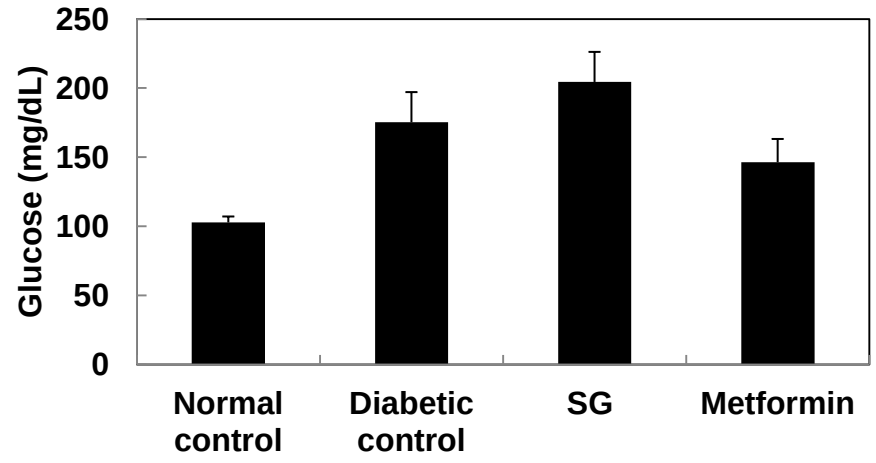


(B)

Figure 9. Inhibitory effect of SG on TPA-induced persistent inflammation and up-expression of pro-inflammatory cytokines in the ears of female CD-1 mice. (A) mouse ear edema; (B) ELISA of IL-6, IL-1β and TNF-α.



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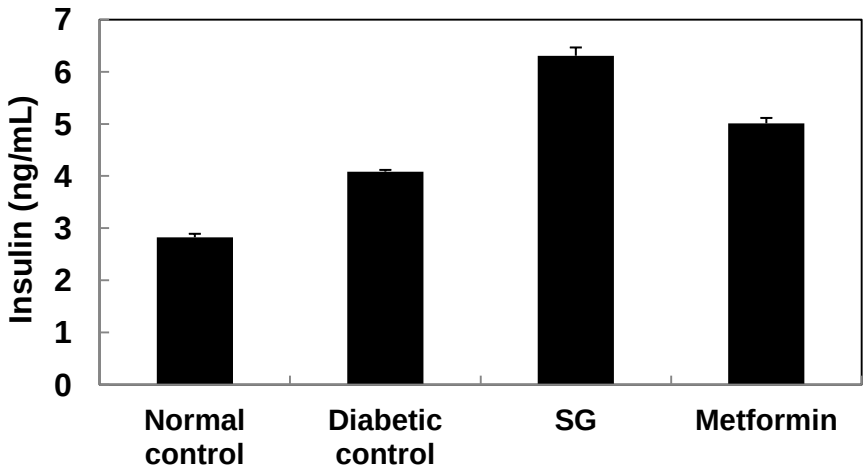
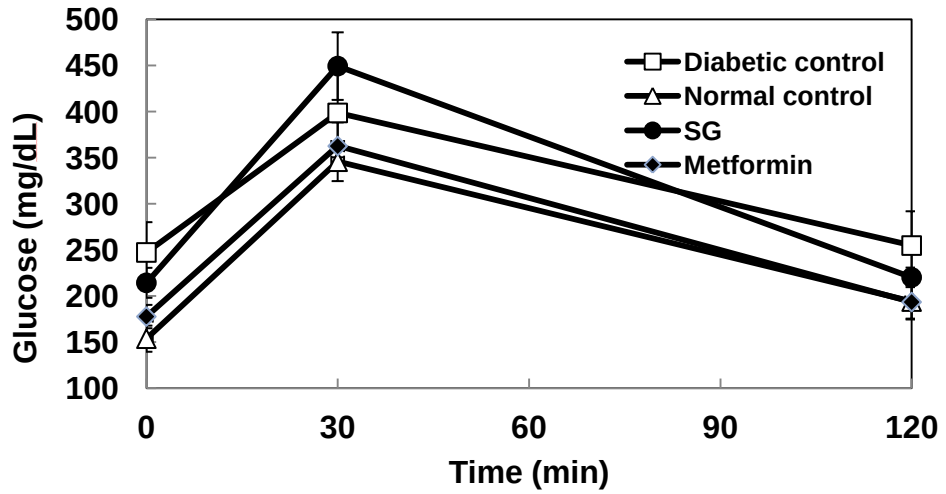
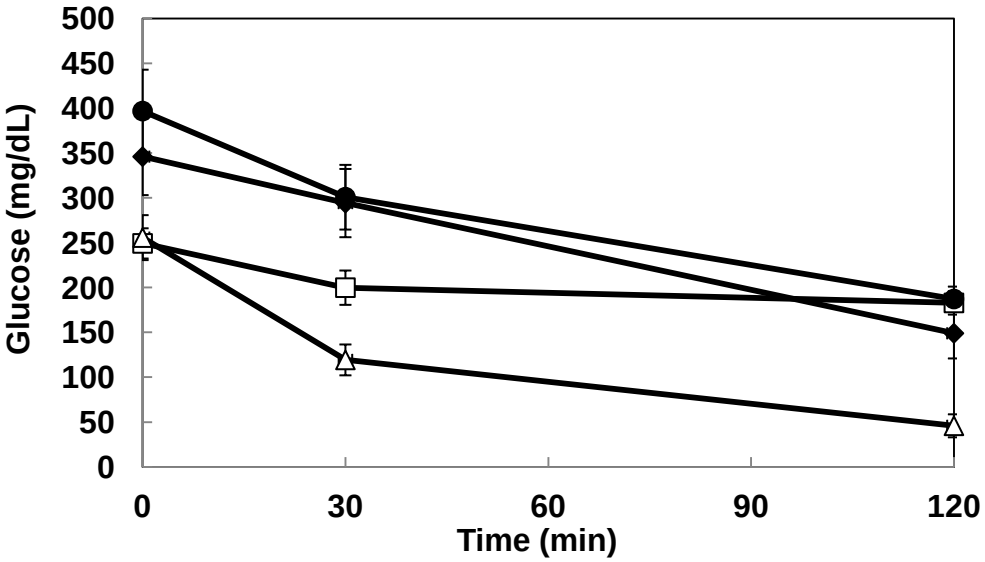


(B)

Figure 10. Effect of SG on serum glucose and insulin levels. KK CgAy/J mice were divided into four groups: normal control, diabetic, SG (0.075% in drinking water) and metformin (300 mg/kg, positive control). After 12 weeks, blood samples were collected and centrifuged for 1 h at 12,500 rpm. Serum glucose was measured with a commercial meter from Contour and serum insulin levels were measured with a kit from Cayman.



(A)



(B)

Figure 11. Effect of SG on (A) glucose tolerance and (B) insulin tolerance. KK CgAy/J mice were divided into four groups: normal control, diabetic, SG (0.075% in water) and metformin (300 mg/kg). Either glucose or insulin solution was injected and the changes in blood glucose levels were monitored for the following two hours.

4. Experimental Part III: *Cis*-SG as possible anti-diabetic agent from PM

4.1. Introduction

Cis-stilbene polyphenols are found to always have higher activities than their *trans*- isomers[168]. In a report which studied over 20 *trans*-stilbene derivatives and their *cis*-isomers to explore structure activity relationship by evaluating their antiproliferative activity in cell culture, the authors found that the *cis*- isomers are always 50-200 times more potent than their *trans*- counterparts, and sometimes activities of these analogues can vary by as much as 3 to 4 orders of magnitude [168]. *Cis*-SG was first discovered in PM roots in 2002[169] and the structure was identified with NMR. The level of *cis*-SG was found to be very low in PM, making it very hard to isolate and enrich. To this date there is rarely any endeavor which enriches the level of *cis*-SG in PM and studies its activity. From literature, *cis*- stilbene polyphenols could be induced with isomerization from *trans*- stilbenes with UV-light[170]. And the objective of this section is first to purify *cis*-SG after isomerization from *trans*-SG, and then using diabetic models to evaluate if *cis*-SG is the efficacious compound in PM responsible for its anti-diabetic activities.

Aside from animal study, both *trans*- and *cis*- SG will be evaluated for their effects on phosphoenolpyruvate carboxykinase (PEPCK), which is the key enzyme catalyzing the first step in hepatic gluconeogenesis[171]. Glucagon and stress hormones, such as

glucocorticoids, upregulate PEPCK gene expression in hepatocytes via a cyclic AMP (cAMP)-dependent pathway. Alternatively, insulin strongly represses PEPCK *trans*cription through the activation of the phosphoinositide-3 kinase (PI3K) pathway[172]. Normally, the increase in blood glucose levels after food intake stimulates the secretion of insulin from the pancreas. This increase in blood insulin concentration then leads to the down-regulation of PEPCK gene expression and, subsequently, the cessation of gluconeogenesis by the liver. Insulin resistant hepatocytes, however, are unable to effectively convey the insulin signal, leading to an increase in PEPCK mRNA transcription[173]. Thus, the glucose synthesis persists despite a high blood glucose concentration. The compounds that are able to repress PEPCK expression and overcome insulin resistance could constitute a new class of glucose lowering agents[174]. To study the effect of *cis*- and *trans*- SG on PEPCK regulation, we will utilize in vitro assay with HepG-2 cancer cell line, and use real-time PCR to track changes in PEPCK gene expression in response to the treatment.

As summarized in Section 1.5, existing animal models each have their advantages and disadvantages. We aim to develop a model which is convenient, stable, fast and inexpensive, and resembles human type 2 diabetes. The closer it came to sharing the metabolic characteristics of patients with type 2 diabetes, the more relevant and useful it would be in screening potential anti-diabetic agents. In the literature C57BL/6 (C57) mice are widely used in a diet induced model for type 2 diabetes. This strain becomes obese, hyperglycemic and insulin resistant when fed a HFD[175] and it was concluded that the high-fat diet-fed C57 mouse model is a robust model for impaired glucose tolerance

(IGT) and early type 2 diabetes, which may be used for studies on pathophysiology and development of new treatment[176]. On the other hand, CF-1 mouse is an excellent model for obesity and it has been traditionally employed by our lab to study the anti-obesity effect of a variety of nutraceuticals[177, 178]. However, the suitability of HFD-induced CF-1 mouse as a model for type 2 diabetes was never explored in literature. Therefore, we compared C57BL/6J and CF-1 strains in terms of resembling human type 2 diabetes, and explored gender difference as well. After the most appropriate model is identified, it will be used to test the anti-diabetic effects of pure *trans*- and *cis*- SG, and to confirm if the hypoglycemic effect of PM extract stems from *cis*-SG.

4.2. Material and method

4.2.1. Purification of *cis*-SG with isomerization from *trans*-SG

Trans-SG was generated as described in Section 2.2.1. Pure *trans*-SG was dissolved in large volume of water and placed under UV-light over night. The reaction was monitored with HPLC analysis (same HPLC program as in Section 2.2.2) and the solution after UV exposure was concentrated, filtered, and subjected to Preparative HPLC. A HYPERSIL-C18 column was used and mobile phase gradient of 40% to 65% B (acetonitrile) over 15 min was chosen. The flow rate was maintained at 8 mL/min. Pure *cis*-SG generated was piled, concentrated with a rotary evaporator and dried with a freeze drier.

4.2.2. Identification with Nuclear Magnetic Resonance Spectroscopy (NMR) and Liquid Chromatography-Mass Spectroscopy (LC-MS)

NMR spectra were recorded on a Varian 500 Spectrometer (Varian Inc., Palo Alto, CA). With TMS serving as an internal standard, ^{1}H NMR was recorded at 500 MHz and ^{13}C NMR at 125 MHz.

LC-MS data were obtained from an HPLC-MS system being composed of an auto-sampler injector (Switzerland), an HP1090 system controller, with a variable UV wavelength 190-500 nm) detector, an ELSD (Evaporizing Laser Scattered Deposition) detector and an ESI-MS detector from Micromass VG Platform II mass analyzer (Micromass, Beverly, MA). Octadecyl (C_{18}) derivatized silica gel (60Å) reverse phase analytical HPLC column was purchased from Waters Corporation (Milford, MA, USA). ESI-MS conditions were as following: acquisition mode, ESI-positive; mass scan range, 100-800 amu; scan rate, 0.4 sec; cone voltage, 25 volts; source temperature: 150 °C; probe temperature: 550 °C. Analytical HPLC conditions on HPLC-MS: column: Chromeabond WR C_{18}, 3 μm, 120 Å; length and OD: 30 x 3.2 mm; injection volume, 15 μL; flow rate: 2 mL / min; run time: 3 min. Mobile phase consisted of acetonitrile and H_2O with 0.05% TFA, typical gradient of 10 - 90 % acetonitrile and the gradient varied.

4.2.3. Animal study with *trans*- and *cis*- SG extracts

Female KK mice were purchased from Jackson labs (Barr Harbor, ME) and were housed in stainless steel wire-bottomed cages and acclimatized under laboratory conditions (19-23 °C, humidity 60%, 12 h light/dark cycle). Normal control mice (n=10) were fed with control diet which was normal Chow 5001 from LabDiet (St. Louis, MO) and was composed of 28.5 kcal % protein, 58 kcal % carbohydrate and 13.5

kcal % fat. Diabetic control mice (n=10) were induced with HFD (Research Diets, New Brunswick, NJ) composed of 20 kcal % protein, 20 kcal % carbohydrate, and 60 kcal % fat (from butter) for 18 weeks. Three solutions were administered to mice (n=10 each) on HFD *ad libitum* and the compositions are shown below. Solution 1: pure *trans*-SG (0.05% in drinking water), HPLC chromatogram in Fig 3.2 (A); Solution 2: 60% ethanol extract of PM root powders, obtained according to procedure in Section 1.2.1 (proportion of *cis*-SG to *trans*-SG is approximately 1:20, 0.075% in drinking water), HPLC chromatogram in Fig 3.2 (B); Solutions 3: obtained from exposing solution 2 under UV-light overnight (proportion of *cis*-SG to *trans*-SG is approximately 2:3, 0.075% in drinking water), HPLC chromatogram in Fig 3.2 (C). After 18 weeks, body weight and blood glucose of all mice were measured.

3.2.4 Screening of mice model for type 2 diabetes

CF-1 mice and C57BL/6 (C57) mice (male and female) were purchased from Charles River laboratories (Horsham, PA) and were housed in stainless steel wire-bottomed cages and acclimatized under laboratory conditions (19-23 °C, humidity 60%, 12 h light/dark cycle). Each strain was divided into four groups with ten mice each: M-control, male mice on normal diet; M-HF, male mice on Western HFD; F-control, female mice on normal diet; F-HF, female mice on Western HFD. All the mice had access to drinking water *ad libitum.* Body weight and nonfasting glucose levels were taken at week 5, 8 and 12. At the end of week 12, all the experimental mice were sacrificed. Lipid profile was measured with PTS Panels test strips; parametrial fat, retro-peritoneal fat and brown fat tissues were harvested and weighed;

liver, spleen and kidney were harvested and weighed as well. Before sacrifice, glucose tolerance test was performed on all mice as described before.

4.2.4. Animal study with pure *trans*-SG and pure *cis*-SG with male CF-1 mice

Male CF-1 mice were selected as the model for type 2 diabetes. The mice were kept on Western HFD for 12 weeks and were divided into five groups with 10 each. Group I: Normal control which had free access to drinking water and normal Chow; Group II: Diabetic control which had free access to drinking water and Western HFD; Group III: Treatment group which had free access to drinking water with 0.01% of pure *trans*-SG and HFD; Group IV: Treatment group which had free access to drinking water with 0.01% of pure *cis*-SG and Western HFD. Group V: Positive control group which had free access to drinking water with 0.01% of caffeine and Western HFD. Body weight, food and water uptake of the mice were taken on a regular basis. After 12 weeks, all the experimental mice were sacrificed. Body weight, blood glucose and blood insulin levels were evaluated as described before. Glucose tolerance test was also carried out at the end of the study.

4.2.5. PEPCK assay with pure *trans*-SG and pure *cis*-SG

The HepG2 cells were plated in 24-well tissue culture plates and were grown to near confluence in Dulbecco's modified Eagle's medium containing 2.5% (vol/vol) newborn calf serum and 2.5% (vol/vol) fetal calf serum. Cells were treated for 8 h with 500 nM dexamethasone and 0.1 mM 8-CTP- cAMP (Dex-cAMP) to induce PEPCK gene expression together with test compounds (5μM *trans*-SG and 5μM

cis-SG).

Total RNA was extracted from HepG2 cells using Trizol reagent, following the manufacturer's instructions. RNA was quantified spectrophotometrically by absorbance measurements at 260 and 280 nm. Quality of RNA was assessed by separation in gel electrophoresis. RNA was then treated with DnaseI (Invitrogen), following the manufacturer's guidelines, to remove any traces of DNA contamination. The cDNAs were synthesized with 2.5 g of RNA for each sample, using Stratascript reverse *trans*criptase (Stratagene, La Jolla, California), following the manufacturer's protocol. The synthesized cDNAs were diluted fourfold. Five microliters of each of these diluted samples was used for PCR reactions of 25 ηL final volume. The other components of the PCR reactions were 0.5 ηL of 6 ηM gene-specific primers and 12.5 ηL of Brilliant SYBR Green PCR master mix (containing green jump-start Taq ready mix). ROX (Stratagene, La Jolla, California) was used as a reference dye. The primers were selected using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) as follows: β-actin: forward primer 5'-GGGAAATCGTGCGTGACATT-3', reverse primer 5'-GCGGCAGTGGCCATCTC-3'; PEPCK: forward primer 5'-GCAGAGCATAAGGGCAAGGT-3', reverse primer 5'-TTGCCGAAGTTGTAGCCAAA-3'. These primers generated a 76-bp product from β-actin mRNA. The intron-spanning forward primer was selected to cover the exon 9–10 boundary. The reverse primer was selected from exon 10. The oligos were synthesized by IDT. These primers generated a 74-bp product from PEPCK mRNA

and a 207-bp product from genomic DNA.

Quantitative PCR (qPCR) amplifications were performed on an MX3000p system (Stratagene, La Jolla, California) using one cycle at 50°C for 2 min and one cycle of 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The dissociation curve was completed with one cycle of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. Non-RT control and no-template control were included in each experiment as quality control steps.

PEPCK mRNA expressions were analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin house keeping gene. The ΔΔCT Values obtained from these methods reflect the relative mRNA quantities for a specific gene in response to a treatment as relative to a calibrator. The Dex-cAMP treatment (positive control) served as the calibrator sample in this study. The PECPK gene expression of the calibrator sample was assigned to a value of 1.0. A value of <1.0 indicates transcriptional down-regulation (inhibition of gene expression) relative to the calibrator. Amplification of specific transcripts was further confirmed by obtaining melting curve profiles. All samples were run in duplicate.

4.3. Results

4.3.1. NMR and MS for *trans*- and *cis*-SG

Trans-SG and *cis*-SG were identified with ^{1}H and ^{13}C NMR spectra as well as LC-MC spectrum by comparing to literature [140, 169, 179, 180], structures shown in Fig. 3.1. For *trans*-SG, ^{1}H-NMR (CD_3OD, 500 MHz) δ: 3.34-3.79 (6H, m, sugar H), 4.50 (1H,

d, J = 7.8 Hz, H-1''), 6.24 (1H, d, J = 2.8 Hz, H-6), 6.61 (1H, d, J = 2.8 Hz, H-4), 6.75 (2H, d, J = 6.7 Hz, H-3',5'), 6.91 (1H, d, J = 16.4 Hz, H-b), 7.44 (2H, dd, J = 1.9, 6.7 Hz, H-2',6'), 7.70 (1H, d, J = 16.4 Hz, H-a); ^{13}C-NMR (CD_3OD, 500 MHz); see Table 2.

For *cis*-SG, ^{1}H-NMR (CD_3OD, 500 MHz) δ: 3.39-3.82 (6H, m, sugar H), 4.58 (1H, d, J = 7.6 Hz, H-1''), 6.15 (1H, d, J = 2.8Hz, H-6), 6.24 (1H, d, J = 2.8 Hz, H-4), 6.50 (1H, d, J = 12.2 Hz, H-α), 6.62 (2H, d, J = 8.5 Hz, H-3',5'), 6.73 (1H, d, J = 12.2 Hz, H-b), 7.08 (2H, d, J = 8.5 Hz, H-2',6'); ^{13}C-NMR (CD_3OD, 500 MHz); see Table 2.

The ^{1}H-NMR spectrum of *cis*-SG was very similar to that of *trans*-SG, with the exception of the coupling constants of the vinylic protons signals (H-a and H-b), indicating the presence of two *cis*-coupled vinylic protons at δ 6.71 (H-b) and 6.47 (H-a). The ^{13}C-NMR spectrum of *cis*-SG exhibited two chemically equivalent aromatic carbons at δ_c 131.4 (C-2'/C-6') and δ_c 115.9 (C-3'/C-5').

The negative mass spectrum of *trans*-SG and *cis*-SG showed a $[M-1]^-$ ion at m/z 405. The fragment ion peak m/z 405 generated a main fragment ion $[M\text{-glc }-1]^-$ at m/z 243, which can be considered characteristic of the presence of an aglycone moiety.

4.3.2. Anti-diabetic effect of extracts of *trans*- and *cis*-SG

Female KK mice were fed with Western HFD for 18 weeks to induce a model for type 2 diabetes. Three solutions were employed in the study: pure *trans*-SG (0.05% in drinking water), PM extract with *trans*-SG/*cis*-SG 1:20 (0.075% in drinking water) and PM extract enriched with *cis*-SG (*trans*-SG/*cis*-SG 2:3, 0.075% in drinking water),

HPLC chromatograms as shown in Figure 14. Body weight and blood glucose levels were monitored. From the results, none of the three solutions significantly decreased high fat induced body weight gain compared to diabetic control. As for blood glucose, it showed a decreasing trend of blood glucose with increasing level of *cis*-SG in the extract. And the only solution which showed a significant hypoglycemic effect was the *cis*-SG enriched extract ($p<0.05$), while the other two did not. Considering the solution in group 3 was obtained from overnight exposure of solution in group 2 under UV-light and the only difference between the two extracts is the level of *cis*-SG, this data suggested possible anti-diabetic effect of *cis*-SG. However this effect needs to be confirmed by carrying out animal studies with pure *trans*- and *cis*- SG. In the present study HFD induced female KK mouse model was utilized to reduce cost of KK CgAy/j mice, however due to limited supply of KK mice and the length of time it takes for KK mice to develop obesity and hyperglycemia we explored other options for a suitable type 2 diabetes animal model which is economical, reliable and mimics human type 2 diabetes.

4.3.3. Identifying a model for type 2 diabetes

Two strains of mice were identified as possible candidates for diet induced diabetes model: C57BL/6J (C57) mice and CF-1 mice, and both male and female mice were evaluated. All mice were fed either a high-fat diet (58% energy by fat) or a normal diet (11% energy by fat), and body weight and blood glucose levels were taken at weeks 5, 8 and 12; glucose tolerance test also evaluated at the end of the study.

For C57 strain, both female and male mice had higher body weight at week 12

when fed a HFD compared to those on normal diet: F-HF group was 40.2% heavier than F-control while M-HF group was 11.8% heavier compared with M-control. Circulating blood glucose displayed a slight increasing trend from week 5 to week 8 except for M-HF, which decreased from 172.10 to 156.33 mg/dL; not much difference in blood glucose was shown from week 8 to week 12 except for M-control. For glucose tolerance test M-control group was missing because male C57 mice on normal diet displayed aggressive behaviors and by the end of study they were either injured or dead. Both male and female mice on HF diet displayed significant glucose intolerance since 30 minutes after glucose injection blood glucose rapidly rose to 266.78 and 256.50 mg/dL, respectively, nearly doubling the level before glucose injection. For lipid profile, not much difference was shown in all groups for the three parameters: triglycerides, HDL and LDL, except for triglycerides in M-HF group which was slightly higher than M-control, 64.67 versus 50 mg/dL. Body fat including parametrial fat and retro-peritoneal fat were much heavier in HF groups compared to control groups, and greater fat accumulation was observed in female mice. Weight of important organs, including pancreas, liver and kidney, were slightly higher in HF groups.

CF-1 mice became very obese at the end of 12 weeks, with the weight of M-HF mice being the highest of all, 53.4% heavier than F-control and 13.4% heavier than M-control. For nonfasting glucose, M-HF displayed a high level as early as week 5 and remained the highest throughout the course of 12 weeks, while F-HF had no significant difference in blood glucose from the control group. It was also M-HF group that displayed the highest degree of glucose intolerance: 30 minutes after glucose injection,

blood glucose jumped to 358.2 mg/dL compared to an initial reading of 134.7 mg/dL, significantly higher than other three groups as well; after 120 minutes, it remained elevated at 192 mg/dL. According to Figure 17D, the trend for lipid profile, fat mass and organ mass in CF-1 mice was similar to that of C57 mice. All information taken together, we selected male CF-1 mice on HF diet as the model for type 2 diabetes, as it is characterized with obesity, elevated blood sugar and a high level of glucose intolerance, which is very similar to human type 2 diabetes, and is absent for any aggressive behaviors.

4.3.4. Anti-diabetic effect of pure *trans*- and *cis*-SG

After identifying diet-induced male CF-1 mice as the model animal, and obtaining *cis*-SG from isomerization of *trans*-SG and purification with Prep-HPLC, we tested the anti-diabetic effects of pure *cis*- and *trans*- SG in the new model, and caffeine was used as a positive control. About 5 grams of each treatment was consumed during this 12-week study. As evident in Figure 18, both isomers of SG (0.01% in drinking water) could significantly decrease the serum glucose level in the male CF-1 mice after HFD treatment for 12 weeks, similar to that of caffeine. In GTT, HF group showed glucose intolerance test since blood glucose increased sharply 30 min after the intraperitoneal injection of glucose solution and remained at a very high level after 120 min. In contrast, the rise in blood glucose level was greatly suppressed by *cis*-SG; however, the effect on glucose intolerance was absent in *trans*-SG treated group. HF mice had a much higher level of serum insulin than normal control at the end of the study and in both SG treatment groups insulin levels were significantly lowered. After calculating the

HOMA-IR with the equation HOMA-IR = Glucose* Insulin/405 [161], insulin resistance was found to be greatly elevated in HF group but reduced in all experimental groups. Calculated HOMA-IR in *cis*-SG group was 102.7% lower than that in *trans*-SG group, indicating *cis*-SG had a much stronger effect in alleviating insulin resistance than *trans*-SG.

4.3.5. PEPCK assay

Real-time PCR, also called quantitative PCR or qPCR, can provide a simple and elegant method for determining the amount of a target sequence or gene that is present. PEPCK assay was performed with liver HepG2 cells and the level of PEPCK mRNA from real-time PCR is normalized with β-actin. Dex/cAMP could induce the *trans*cription of PEPCK gene and as seen in Figure 19A, both *cis*-SG and *trans*-SG could effectively suppress the up-regulation. However no dose-dependent pattern was observed, as *cis*-SG suppressed more efficiently at 30 μM than at 100 μM, while for *trans*-SG higher concentration resulted in lower level of PEPCK *trans*cription. This agrees with the results from the comparative Ct method, where *cis*-SG (30 μM) had a 61.7% reduction compared to Dex/cAMP group while *trans*-SG (100 μM) had 53.3% reduction. Therefore *cis*-SG was found to be more effective than *trans*-SG in ameliorating Dex/cAMP- induced PEPCK *trans*cription in HepG2 cells.

4.4. Discussion

Stilbene polyphenols are natural products of biomedical significance. It has been well documented that *cis*-stilbenes have stronger activities than *trans*- isomers. The

effects of *cis*- and *trans*-isomers of 3,5-dihydroxystilbene on the activity of mushroom tyrosinase have been studied and the IC_{50} values for *cis*- and *trans*- compounds were estimated as 0.405 and 0.705 mM, respectively[181]. The inhibitory capacity of *cis*-isomer was stronger than that of corresponding *trans*-isomer. In another study, *cis*-SG showed cytotoxicities against the HT29 and MCF-7 cell lines (IC_{50}: 49 and 50 μM, respectively), but *trans*-SG was not effective[180].

Isomerization of *trans*-stilbenes is known to be induced by UV light. Resveratrol is the most widely studied stilbene compound and it exists in two isoforms, *cis*- and tran-sresveratrol (and their glycosidic forms)[182], the later being most widely studied. UV irradiation of *trans*-resveratrol solutions induces partial isomeric conversion into the highly fluorescent *cis*-resveratrol, resulting in a mixture of *cis*- and *trans*-resveratrol[183]. *Trans*-resveratrol is reported to remain stable for several months (except in high pH buffers) when completely protected from light[184]. In most studies, the *cis* isomer has not been detected in grapes, unlike the *trans*- counterpart, but is present in wines at variable concentrations, suggesting that it may be produced from the *trans* isomer by yeast isomerases during fermentation, or released from resveratrol polymers or from resveratrol glucosides[185, 186]. Similarly, *trans*-SG was discovered in 1975 [140] and as the major component in PM roots, it was widely studied. On the other hand, *cis*-SG was not reported in PM roots until 2002[169], and it exists in PM roots only in trace amount. To the best of our knowledge, the current study is the first endeavor to enrich the level of *cis*-SG in PM roots from isomerization of *trans*-SG. UV-light was employed as the method of isomerization, and the structures of *cis*- and

trans- SG were identified by comparing NMR and LC-MS data to literature.

To get the preliminary anti-diabetic effect of *cis*-SG, we first tested three extract solutions including pure *trans*-SG, PM extract containing 1:20 *cis* : *trans* SG and PM extract containing 2:3 *cis*:*trans*, and found that blood glucose levels of tested animals were reversely related to the level of *cis*-SG in the extract, suggesting potential anti-diabetic effect of *cis*-SG, which was confirmed in proceeding animal study with pure *trans*- and *cis*-SG, where *cis*-SG not only reversed diet-induced hyperglycemia, ameliorated glucose intolerance, but also displayed better results in HOMA-IR.

To minimize cost in diabetic KK CgAy/j mice, we utilized diet induced KK mice as the model for type 2 diabetes for our experiment with extracts. However, the problem with KK mice is reliability and the long time it takes to induce hyperglycemic, which presented a necessity for another model which is reliable, cost-efficient and mimics human type 2 diabetes. From literature and from the experience based on our laboratory, two strains were identified as candidates: CF-1 and C57 mice. After placing the mice on HFD for 12 weeks and carefully examining body weight change, blood glucose, glucose tolerance and exploring gender difference, we finally selected diet induced male CF-1 mice as the model for type 2 diabetes.

Interestingly, during the course of the study, it was discovered that male C57 mice on control diet displayed aggressive behavior, which was absent in male C57 mice on HF diet. The effect of fat intake on aggression was contradicting in literature, since both positive and negative results have been reported. One study found that

consumption of a diet containing high quantities of n-6 polyunsaturated fats dietary fat lead to aggressive behavior in male mice and rats, possibly by elevating circulating estradiol levels[187], while a decrease in n-3 fatty acids and inflammation have been linked with irritability and aggression in humans[188]. Further, increased aggression has been associated with low concentrations of n-3 polyunsaturated fat and low plasma cholesterol in dogs and a high intake of n-3 polyunsaturated fat has been associated with less hostility in humans[189]. The inconsistent effects of dietary fat on behavior might arise from variations in actions of the different types of fat consumed, blood cholesterol levels and/or variations in the specific behavioral traits investigated[190]. The advantage of the male CF-1 mice selected as a type 2 diabetic model to study the anti-diabetic effect of *cis*-SG is that they don't display aggressive behaviors, whether on regular diet or HF diet.

Hepatic gluconeogenesis accounts for approximately 60%-97% of the hepatic glucose production during periods of prolonged fasting or starvation[191]. Phosphoenopyruvate carboxykinase (PEPCK) is a key rate-limiting enzyme of gluconeogenesis and is tightly controlled at the transcriptional level[191]. In various diabetic animal models, both hepatic gluconeogenesis and PEPCK expression are upregulated[192]. In addition, overexpression of PEPCK can result in fasting hyperglycemia, hyperinsulinemia, and impaired glucose tolerance[193]. Knocking-down PEPCK expression either in vitro or in vivo was found to ameliorate both hyperglycemia and insulin resistance[194]. As a result, any defects from either insulin secretion or insulin action can lead to abnormal PEPCK expression. In the present

study, qPCR and comparative Ct method revealed that both *trans*-SG and *cis*-SG could significantly suppress PEPCK *trans*cription, although not dose dependent, and *cis*-SG is a more efficient suppressor than *trans*-SG. The results suggest that SG, especially *cis*-SG, could serve a good diabetes therapy by targeting restoration of elevated PEPCK expression.

Consumption of He Shou Wu (HSW) was first recorded in the book The Secret Recipes of the Immortals for Treating Wounds and Fractures (*Xian Shou Li Shang Xu Duan Mi Fang*, 846 A.D.), written during the Tang Dynasty by Lin Daoren. Processing of HSW was also described in herbal "Kaibao Bencao" issued by the imperial court of the Song Dynasty in 974. In traditional Chinese medicine, processing is a very important procedure and it is believed that processing could decrease the toxicity and change therapeutic efficacy of Chinese herbal medicine[195]. PM roots have been used in two forms in traditional Chinese medicine: dried, called He Shou Wu (HSW) in Chinese, and processed with the steaming method, called Zhi-He Shou Wu (ZHSW) in Chinese[196].

According to Pharmacopoeia of the People's Republic of China, the processing of HSW is as follows: the freshly picked tubers are sliced, stewed in black bean soup (in a proportion of 10 parts HSW to 1 part black beans) until the soup is exhausted. The "prepared" roots are then dried. This is a technique that is still used today. The black soy bean juice is prepared by boiling black soybeans in water for about 4 hours; the liquid that is left after the cooking is poured off and the beans are cooked again with less water for 3 hours; the resulting extract is combined with the former extract to make the

juice. The alchemical version of this processing is to repeat the stewing or steaming 9 times. Thus, after preparing with the black soybean (overnight) and drying, it is again prepared with black soybean on the next day, and so on, until nine cycles have been completed.

Pharmacological studies have shown that these two preparations have different activities, which must be a result of their different chemical components. Unprepared HSW does not possess the tonic effects and can have unwanted side effects. During processing, some compounds decomposed or degraded, while other compounds increased, and new compounds were produced from reactions between the native components of HSW. There are studies aiming to elucidate the chemical difference between the processed and unprocessed forms of HSW. HPLC analyses indicated that the contents of characteristic compounds in HSW were changed by processing: the content of *trans*-SG was decreased by 55.8%, whereas, the content of emodin was increased by 34.0%[197]. A group proposed that the Maillard reaction is involved in the steaming process of the root of PM based on the comparative analysis of the components of HSW and ZHSW[198]. Another group found that 16 batches of HSW and 15 batches of ZHSW samples shared eight compounds, including gallic acid; 3,5,4′-tetrahydroxylstilbene-2,3-di-O-glucoside, *cis*-2,3,5,4′-tetrahydroxylstilbene-2-O-β-D-glucoside, *trans*-2,3,5,4′-tetrahydroxylstilbene-2-O-β-D-glucoside, emodin-8-O-β-D-glucoside, physcion-8-O- β-D-glucoside, emodin, and physcion. Nevertheless, the relative amounts of gallic acid, emodin, and physcion were very high in ZHSW

samples compared to those in HSW samples. Six compounds disappeared after processing and were unique for HSW: catechin, flavanol gallate dimer, polygonimitin B, emodin-1-O-glucoside, emodin-8-O-(6′-O-malonyl)-glucoside, and physcion-8-O-(6′-O-malonyl)-glucoside. Three compounds were unique for ZHSW: hydroxymaltol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one, and 5-hydroxymethyl furfural[199].

However, most published reports have focused on steaming as the sole parameter for the process of ZhiHSW, while neglected drying, especially drying in the sunlight, which might be a crucial step in the chemical components therefore biological activities of PM roots. Photoisomerizations of active compounds are not unique in nature; light-induced events include *cis-trans* isomerization of carotenes (β-carotene, lutein, lycopene, phytoein, zeaxanthin)[200, 201]; *cis-trans* isomerizations of double bonds of fatty acids such linoleic acid[202] and postharvest synthesis of chlorophyll and glycoalkaloids in potatoes[203]. It was found that mixtures of geometric isomers were formed after 20 days following exposure of piperine sunlight that provides UV radiation, and the kinetics were studied[204]. Another study detected formation of *cis*-resveratrol from *trans*-resveratrol between 10 under the D65 fluorescent lamp, whose spectral distribution is similar to that of natural sunlight[205]. Therefore, drying for a prolonged time in the sun could induce the isomerization of the major component, *trans*-SG, in the root of PM into *cis*-SG, therefore boosting its biological efficacy. It is not known whether trace amount of *cis*-SG present in PM roots are formed naturally or by light-induced postharvest isomerizations. We also do not know whether and to what

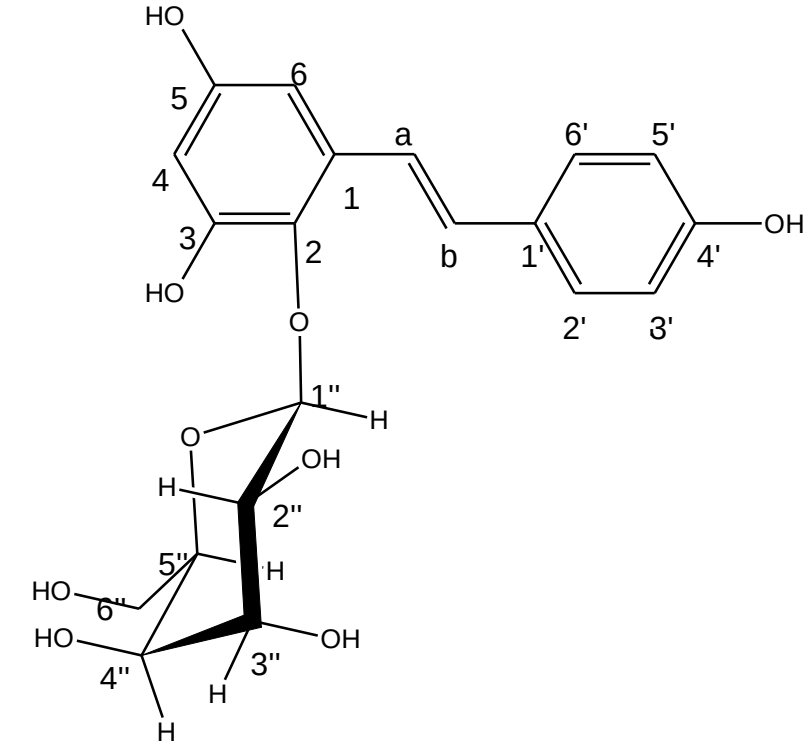
extent light-induced isomerizations of *cis*-SG also occur with *trans*-SG located within the complex matrices of vegetative plant tissues during growth and/or postharvest. These aspects merit further study. And another future research target is to find out if *cis*-SG has any effect against skin cancer; if it does, *trans*-SG could be conveniently isolated and applied in sunscreen products, so that *cis*-SG could be induced over exposure to sun and exert its protective effect on skin.

4.5. Summary

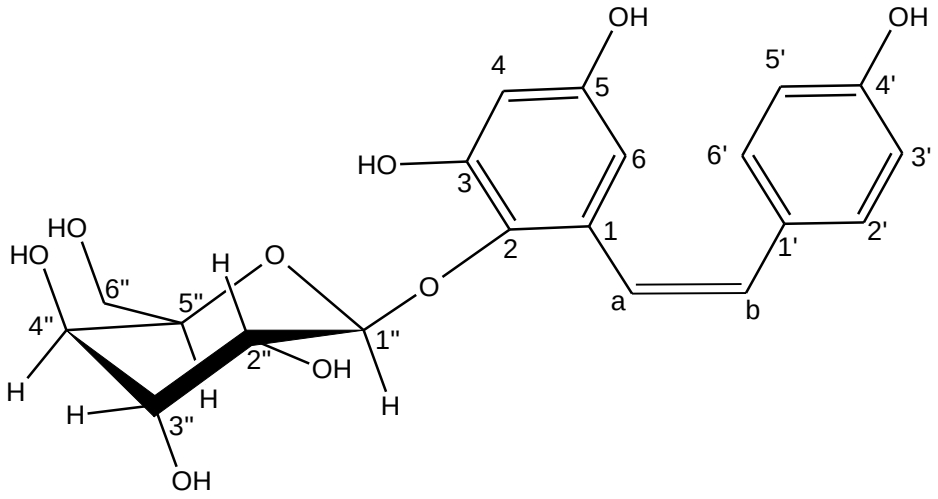
Cis-SG was induced by UV-light from *trans*-SG in PM roots, purified with Preparative HPLC and identified by NMR and LC-MS. The preliminary anti-diabetic effect of *cis*-SG was studied in female KK mice by comparing *trans*-SG and two *cis*-SG containing PM extracts, and it was found that hypoglycemic effect was reversely correlated with the level of *cis*-SG. After selecting diet-induced male CF-1mice as a good model for type 2 diabetes, we tested the effect of pure *trans*-SG and *cis*-SG (0.01% in drinking water) with this model and used caffeine as a positive control. *Cis*-SG was found to be a better anti-diabetic agent than *trans*-SG as it exerted hypoglycemic effect and ameliorated glucose intolerance as well as insulin resistance. In HepG2 cells, *cis*-SG was also more potent than *trans*-SG in suppressing the *trans*cription of PEPCK, which is an important enzyme in gluconeogenesis.

Table 2. ^{13}C NMR spectra of *trans*- and *cis*- SG; 500 mHz, CD_3OD

C	*Trans*-SG	*Cis*-SG
1	133.8	133.9
2	138	137.8
3	152.1	151.8
4	103.7	108.2
5	156	155.3
6	102.8	103.6
1'	130.9	129.8
2',6'	129.3	131.4
3',5'	116.5	115.9
4'	158.3	157.7
a	**121.7**	**125.5**
b	**130.2**	**131.3**
1"	108.2	107.6
2"	75.5	75.4
3"	78	77.9
4"	70.8	71
5"	78.2	78.3
6"	62.1	62.2

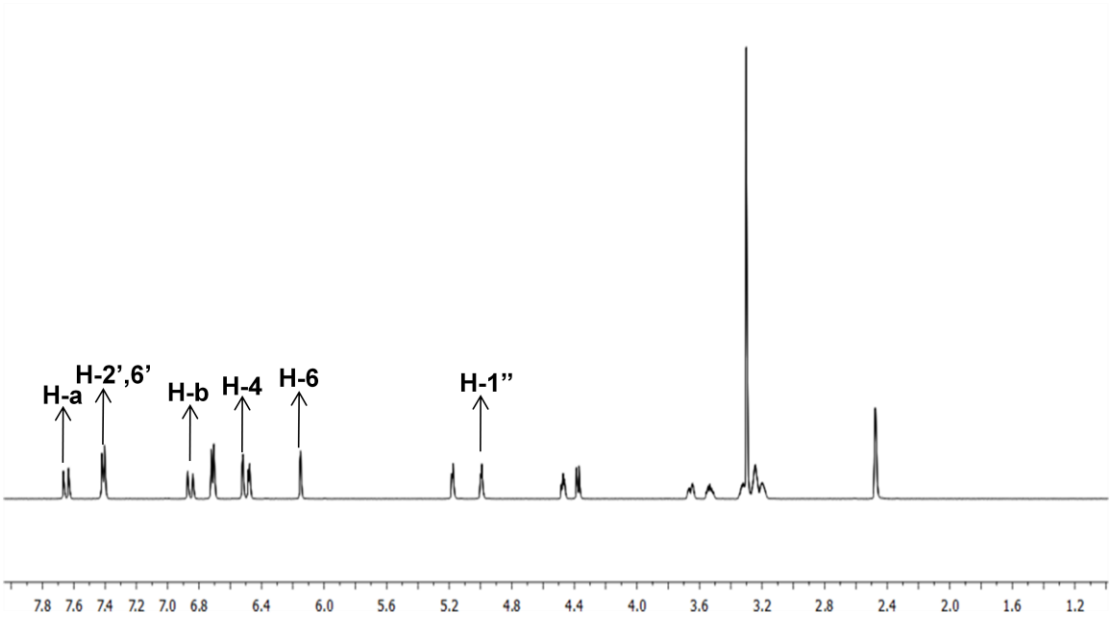


(A)

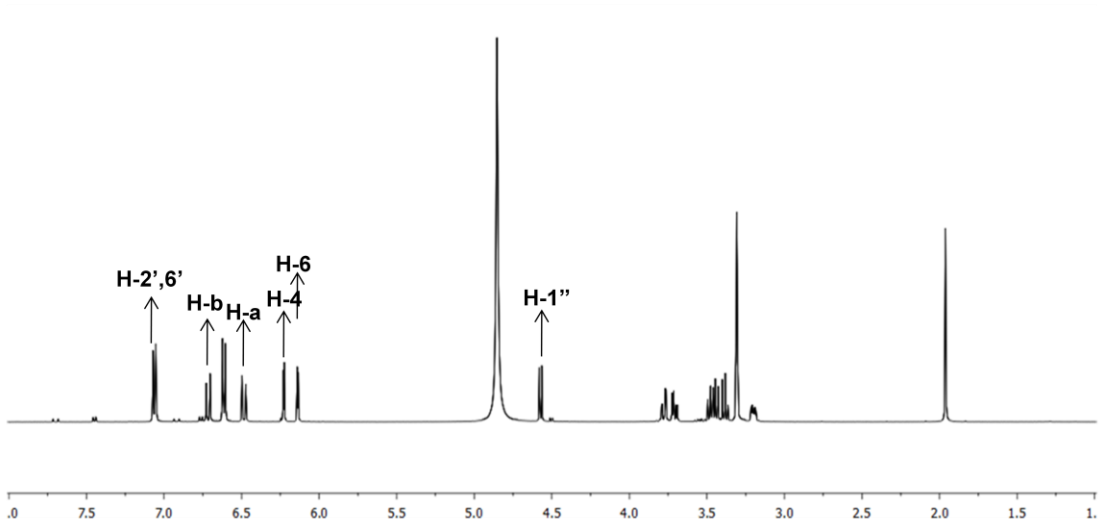


(B)

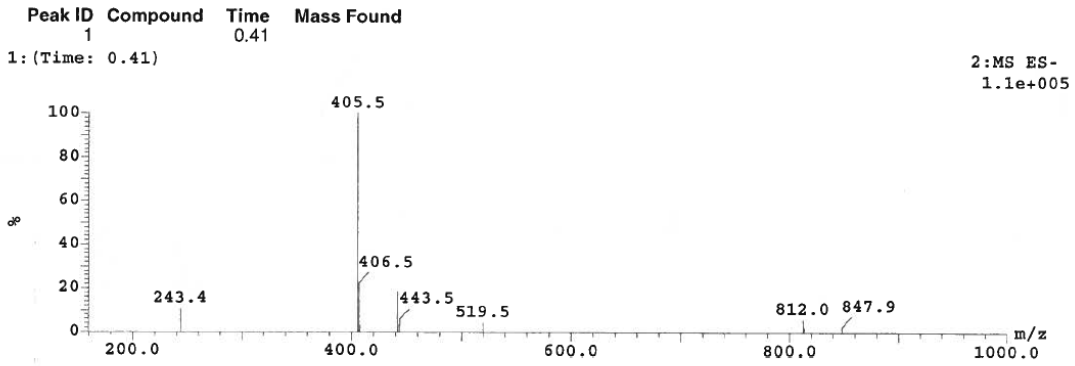
Figure 12. Chemical structures of (A) *trans*-SG and (B) *cis*-SG.


H-a
H-2',6'
H-b
H-4
H-6
H-1"
7.8 7.6 7.4 7.2 7.0 6.8 6.4 6.0 5.6 5.2 4.8 4.4 4.0 3.6 3.2 2.8 2.4 2.0 1.6 1.2

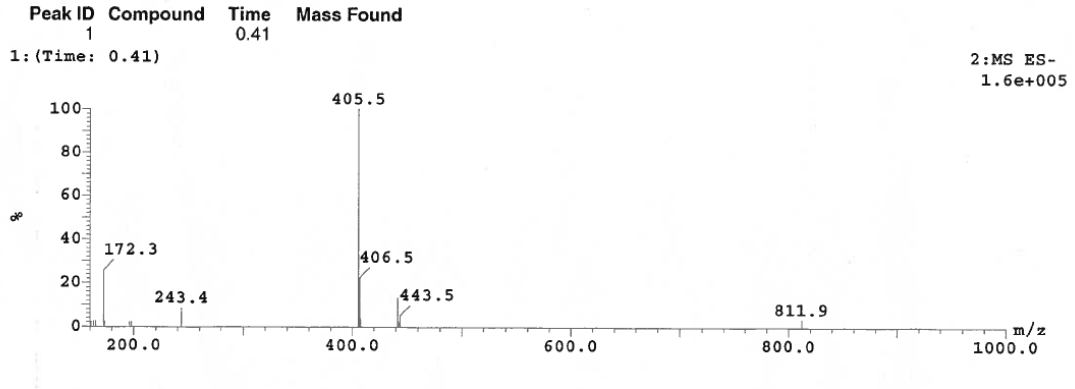
(A)


H-2',6'
H-b
H-a
H-4
H-6
H-1"
.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.

(B)

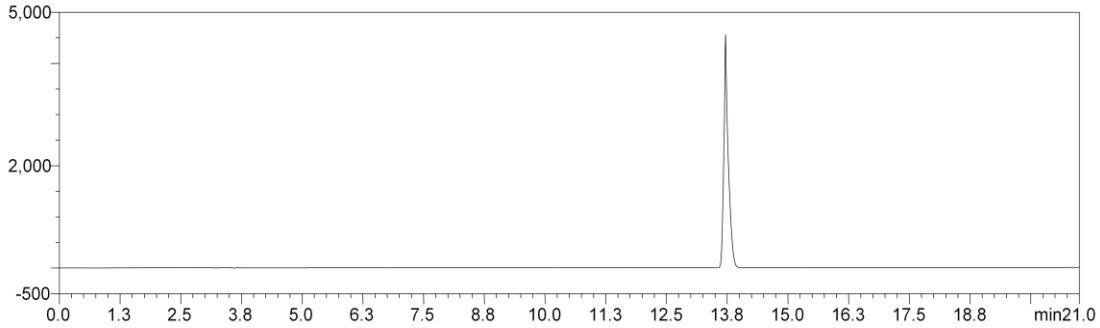


(C)

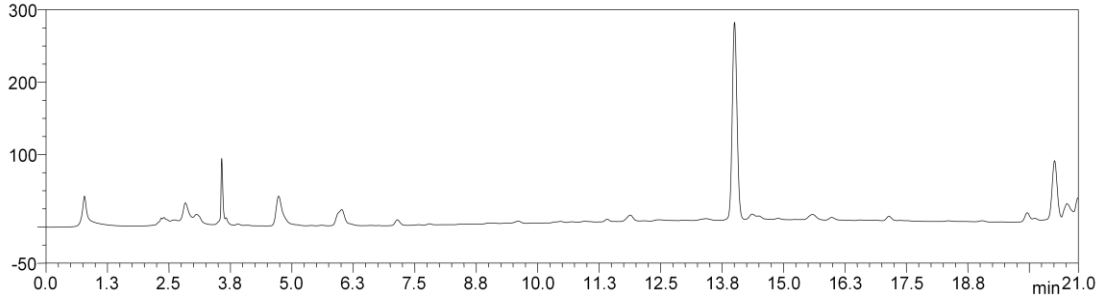


(D)

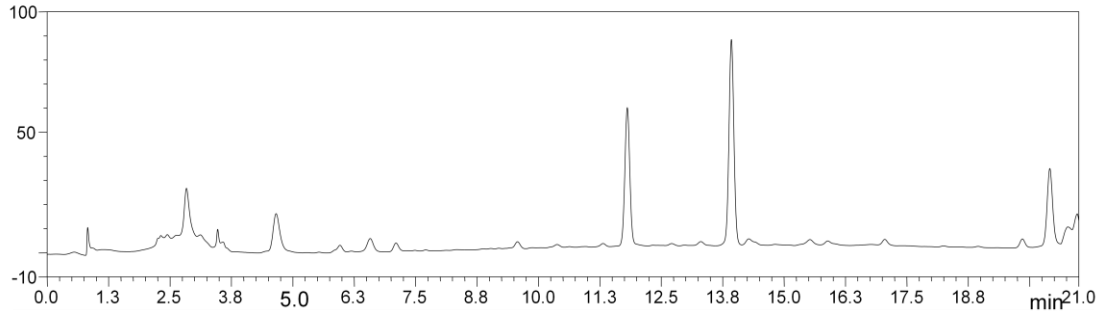
Figure 13. NMR and LC-MS spectrums of *trans*-SG and *cis*-SG. (A) ^{1}H-NMR spectrum for *trans*-SG; (B) ^{1}H-NMR spectrum for *cis*-SG; (C) LC-MS spectrum for *trans*-SG; (D) LC-MS spectrum for *cis*-SG; For ^{1}H-NMR, both *trans*-SG and *cis*-SG were dissolved in CD_3OD; For LC-MS analysis, negative mode was used**.**



(A)



(B)



(C)

Figure 14. HPLC chromatograms for the three solutions used in the animal study to investigate possible anti-diabetic effect of *cis*-SG. (A) pure *trans*-SG; (B) 60% ethanol extract of PM root powder with proportion of *cis*-SG to *trans*-SG approximately 1:20; (C) Extract B enriched with *cis*-SG under UV light; proportion of *cis*-SG to *trans*-SG approximately 2:3.

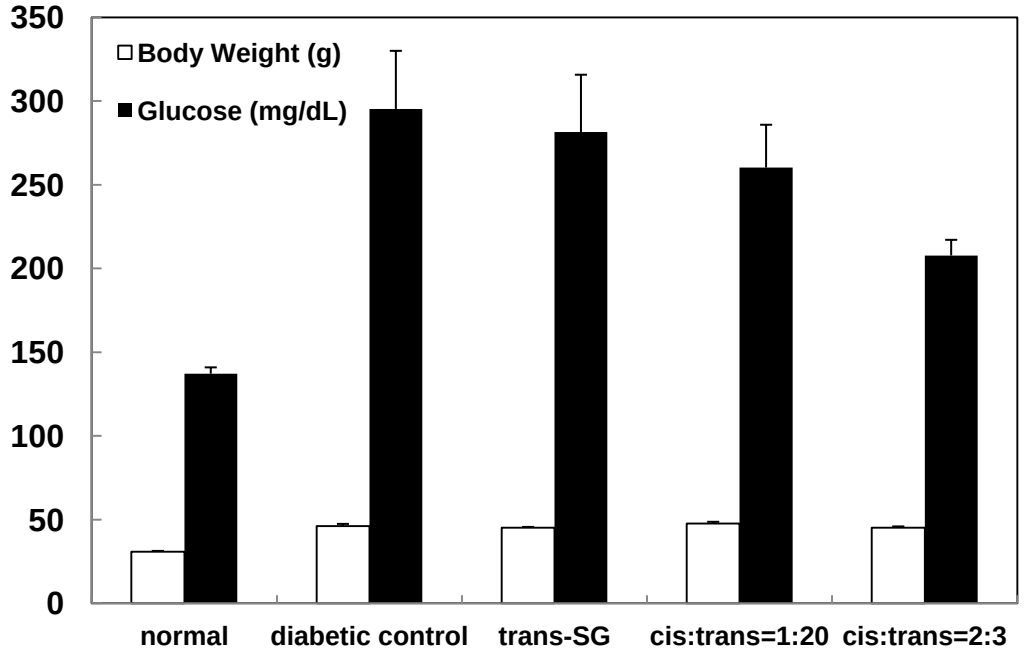
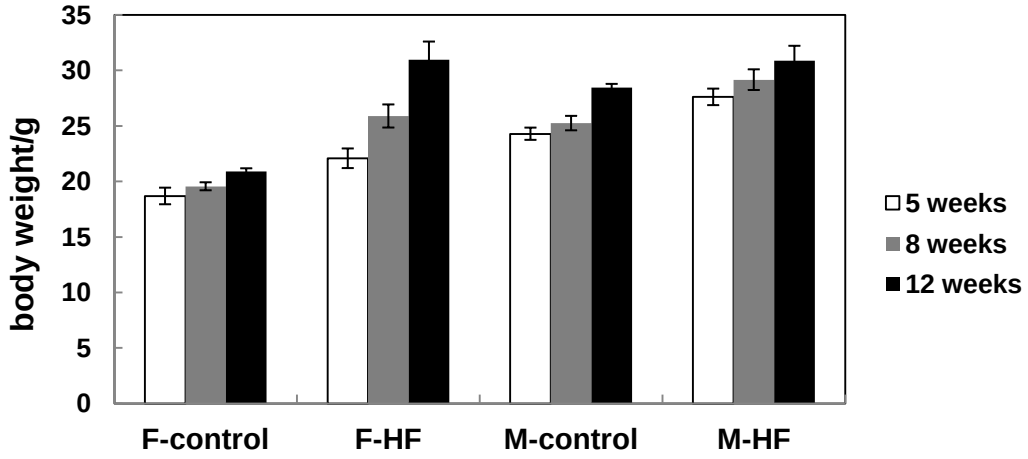
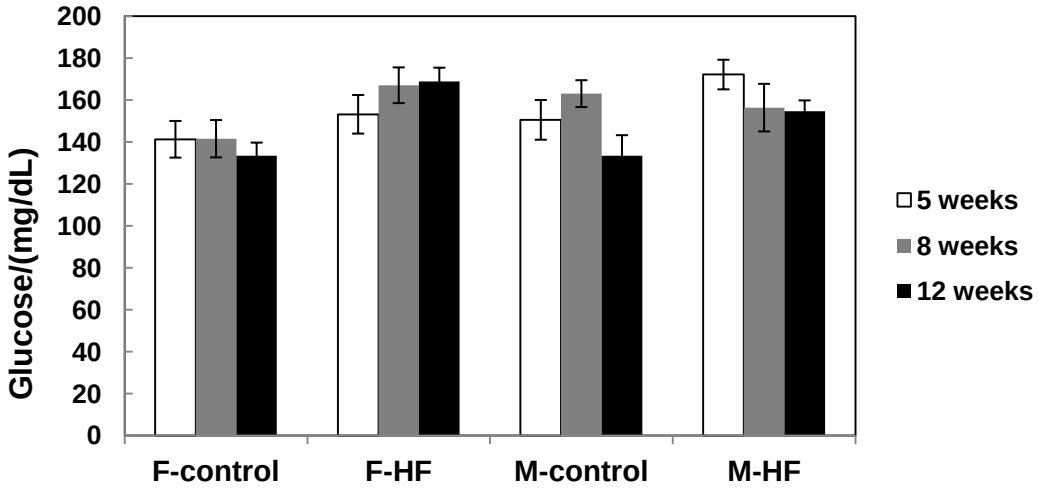


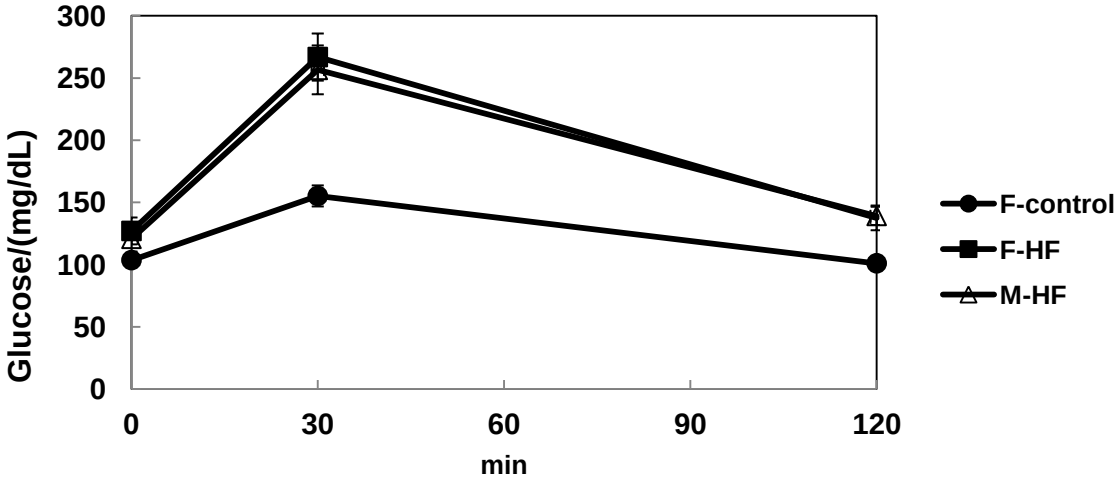
Figure 15. Anti-diabetic effects of *trans*-SG and *cis*-SG in different proportions. Female KK mice were induced with HFD for 18 weeks and were administered with the three solutions as in Fig. 3.4., with the level of 0.05%, 0.075% and 0.075%, respectively. After 18 weeks, body weight and blood glucose levels were measured and recorded for all groups.


35
30
25
20
15
10
5
0
body weight/g
F-control
F-HF
M-control
M-HF
5 weeks
8 weeks
12 weeks

(A)


200
180
160
140
120
100
80
60
40
20
0
Glucose/(mg/dL)
F-control
F-HF
M-control
M-HF
5 weeks
8 weeks
12 weeks

(B)

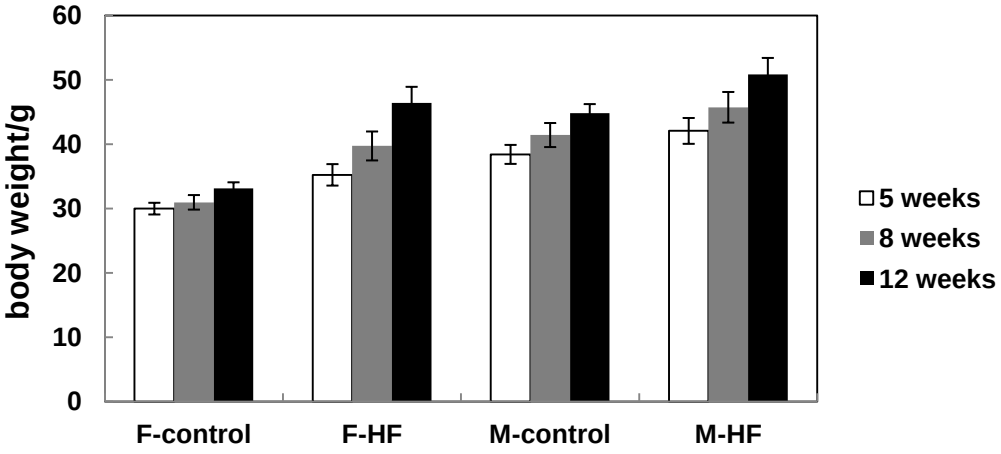

300
250
200
150
100
50
0
Glucose/(mg/dL)
0
30
60
90
120
min
F-control
F-HF
M-HF

(C)

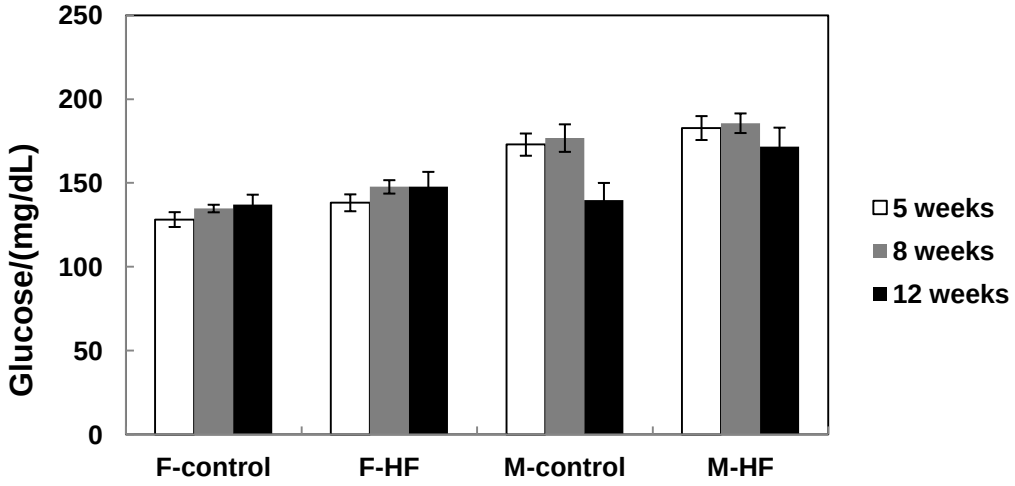
C57 mice	F-control	F-HF	M-control	M-HF
Triglyceride/ (mg/dL)	**50.00±0.00**	**53.00±1.47**	**64.67±10.09**	**54.20±2.46**
Total cholesterol/ (mg/dL)	**100.00±0.00**	**100.50±0.50**	**101.33±1.33**	**104.40±3.49**
HDL/ (mg/dL)	**50.75±4.66**	**45.00±7.68**	**42.67±12.20**	**49.80±3.79**
Parametrial fat/g	**0.08±0.01**	**1.24±0.25**	**0.27±0.01**	**0.61±0.06**
Retro-peritoneal fat/g	**0.01±0.00**	**0.23±0.05**	**0.08±0.02**	**0.13±0.02**
Pancreas/g	**0.09±0.01**	**0.20±0.02**	**0.15±0.03**	**0.15±0.02**
Liver/g	**0.79±0.02**	**0.81±0.03**	**0.97±0.03**	**0.95±0.03**
Spleen/g	**0.05±0.00**	**0.10±0.01**	**0.15±0.02**	**0.16±0.02**
Kidney/g	**0.25±0.01**	**0.34±0.02**	**0.19±0.06**	**0.41±0.02**

(D)

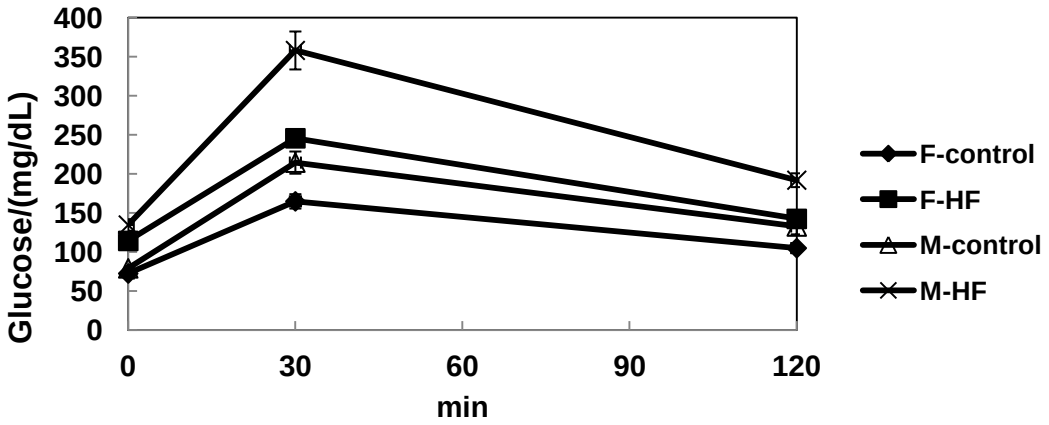
Figure 16. Developing type 2 diabetes model with C57 mice. Male and female C57 mice were induced with HFD for 12 weeks. All experimental mice were divided into four groups with ten each: M-control, male mice on normal diet; M-HF, male mice on Western HFD; F-control, female mice on normal diet; F-HF, female mice on Western HFD. All the mice had access to drinking water *ad libitum.* At week 5, 8 and 12, the mice were tested for (A) Body weight; (B) Nonfasting glucose; at the end of week 12, all the experimental mice were sacrificed and tested for (C) GTT; (D) Lipid profile and organ weight.


60
50
40
30
20
10
0
body weight/g
F-control
F-HF
M-control
M-HF
5 weeks
8 weeks
12 weeks

(A)


250
200
150
100
50
0
Glucose/(mg/dL)
F-control
F-HF
M-control
M-HF
5 weeks
8 weeks
12 weeks

(B)

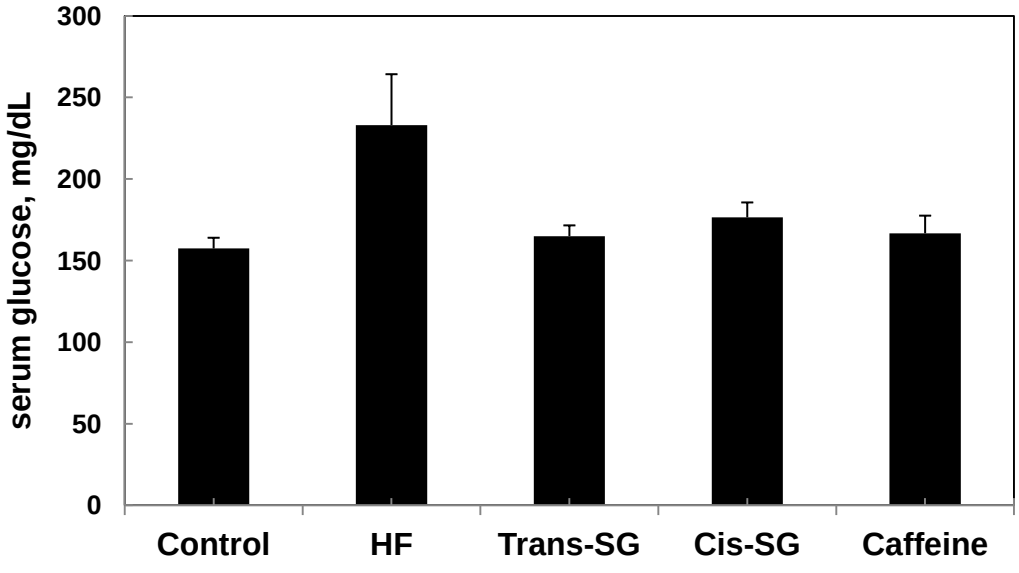

400
350
300
250
200
150
100
50
0
Glucose/(mg/dL)
0
30
60
90
120
min
F-control
F-HF
M-control
M-HF

(C)

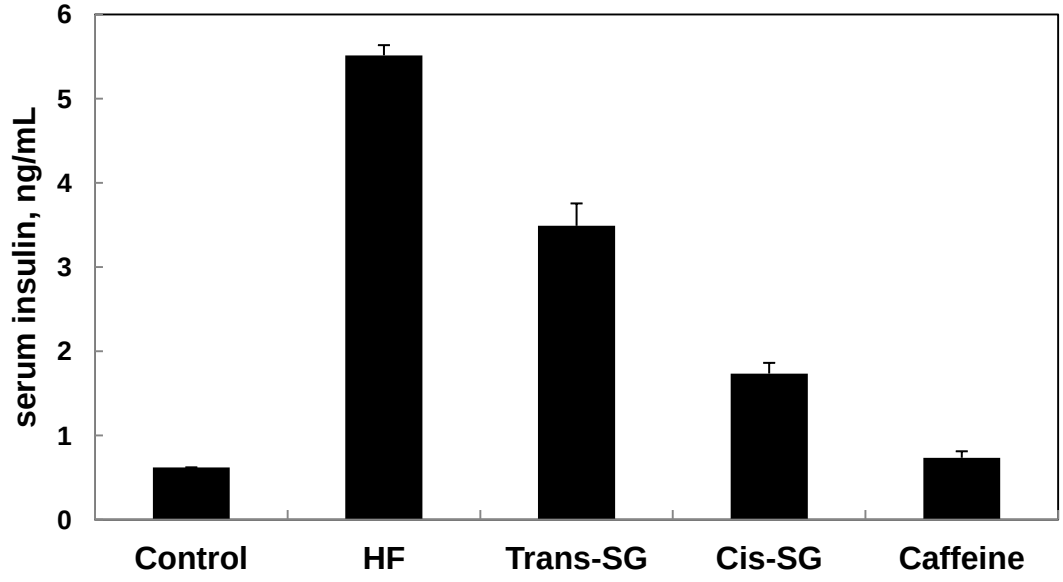
CF-1 mice	F-control	F-HF	M-control	M-HF
Triglyceride/ (mg/dL)	**55.60±5.60**	**50.00±0.00**	**87.00±9.20**	**69.40±7.64**
Total cholesterol/ (mg/dL)	**100.00±0.00**	**100.00±0.00**	**100.00±0.00**	**108.60±8.60**
HDL/ (mg/dL)	**15.80±0.80**	**27.20±3.60**	**40.40±5.01**	**76.40±5.07**
Parametrial fat/g	**0.44±0.09**	**2.83±0.47**	**1.15±0.14**	**3.11±0.31**
Retro-peritoneal fat/g	**0.16±0.03**	**0.64±0.10**	**0.30±0.03**	**0.80±0.06**
Pancreas/g	**0.15±0.01**	**0.20±0.02**	**0.19±0.02**	**0.21±0.01**
Liver/g	**1.22±0.07**	**1.53±0.06**	**1.45±0.05**	**1.54±0.09**
Spleen/g	**0.15±0.01**	**0.16±0.01**	**0.15±0.02**	**0.14±0.01**
Kidney/g	**0.43±0.02**	**0.53±0.02**	**0.73±0.05**	**0.74±0.06**

(D)

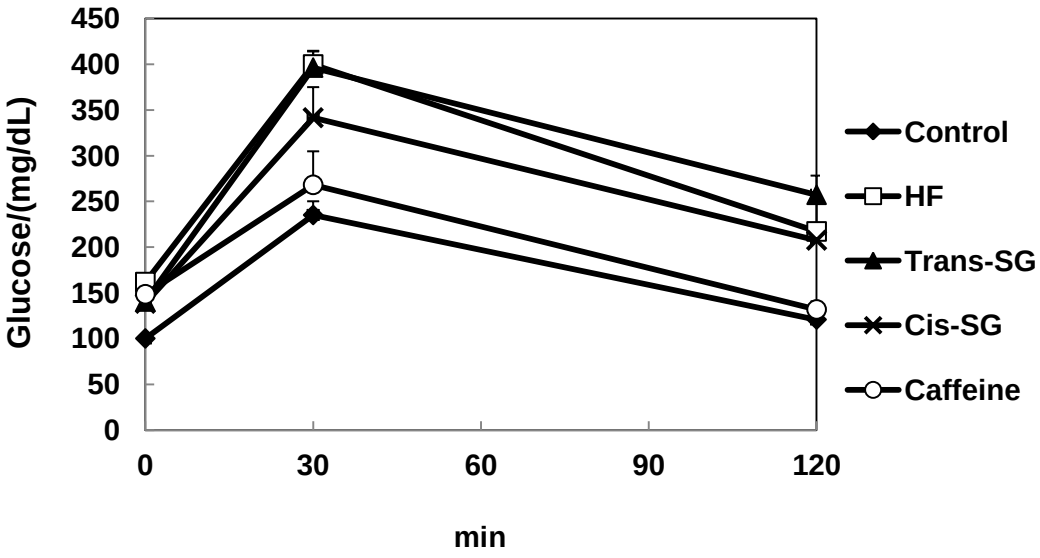
Figure 17. Developing type 2 diabetes model with CF-1 mice. Male and female C57 mice were induced with HFD for 12 weeks. All experimental mice were divided into four groups with ten each: M-control, male mice on normal diet; M-HF, male mice on Western HFD; F-control, female mice on normal diet; F-HF, female mice on Western HFD. All the mice had access to drinking water *ad libitum.* At week 5, 8 and 12, the mice were tested for (A) Body weight; (B) Nonfasting glucose; at the end of week 12, all the experimental mice were sacrificed and tested for (C) GTT; (D) Lipid profile and organ weight.


serum glucose, mg/dL
300
250
200
150
100
50
0
Control
HF
Trans-SG
Cis-SG
Caffeine

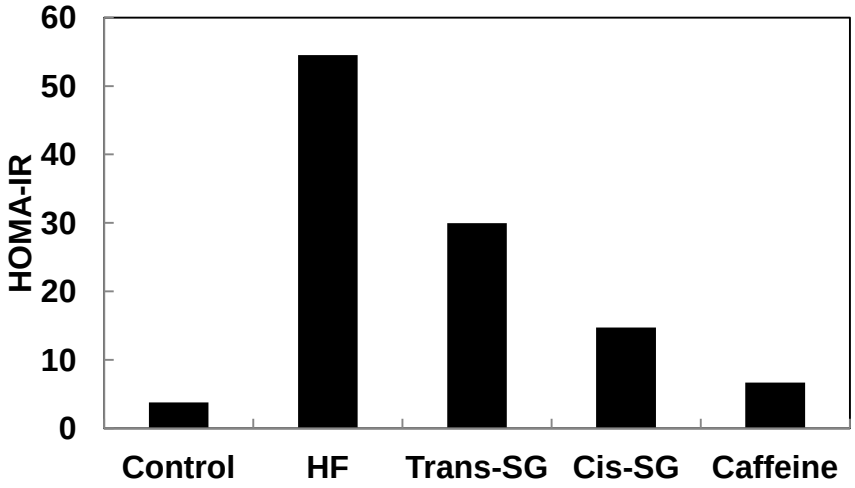
(A)


serum insulin, ng/mL
6
5
4
3
2
1
0
Control
HF
Trans-SG
Cis-SG
Caffeine

(B)

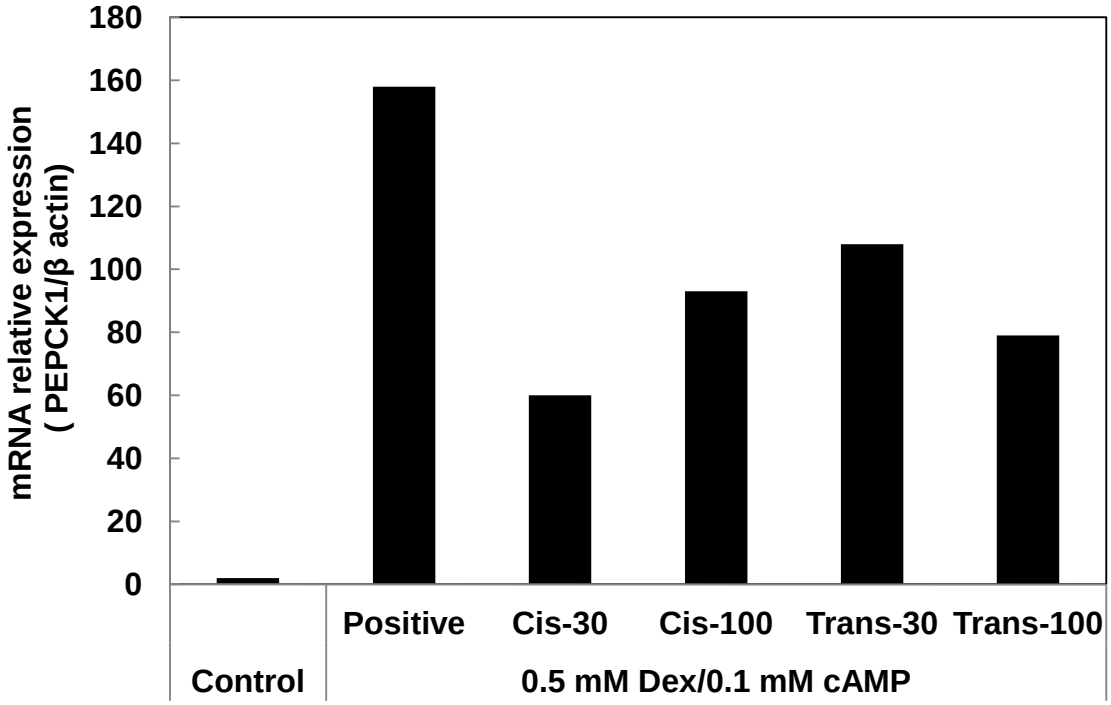

Glucose/(mg/dL)
450
400
350
300
250
200
150
100
50
0
0
30
60
90
120
min
Control
HF
Trans-SG
Cis-SG
Caffeine

(C)


HOMA-IR
60
50
40
30
20
10
0
Control
HF
Trans-SG
Cis-SG
Caffeine

(D)

Figure 18. Anti-diabetic effects of pure *trans*-SG and *cis*-SG in high fat diet induced male CF-1 mice. Level of pure *trans*-SG and *cis*-SG was 0.01% in drinking water and caffeine was used as a control. (A) Serum glucose levels after 12 weeks of HFD treatment; (B) Serum insulin levels after 12 weeks of HFD treatment; (C) Effect of *trans*-SG and *cis*-SG treatments on glucose tolerance of male CF-1 mice; (D) Effect of *trans*-SG and *cis*-SG on approximated insulin resistance (IR) from HOMA model. Calculation was based on the following two equations: HOMA-IR = Glucose* Insulin/405, fasting blood glucose and fasting blood insulin levels were used. Serum glucose levels after 12 weeks of HFD treatment.



(A)

	β-actin	PEPCK			
	C_t	C_t	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
Control	33.33	35.92	2.59	0.00	1.0
Positive	34.98	30.30	-4.68	-7.27	154.3
Cis-30	33.52	30.21	-3.31	-5.90	59.7
Cis-100	33.01	29.14	-3.87	-6.46	88.0
Trans-30	32.58	28.47	-4.11	-6.70	104.0
Trans-100	33.31	29.72	-3.59	-6.18	72.5

(B)

Figure 19. PEPCK mRNA expression normalized to β-actin. HepG2 cells were treated for 8 h with 500 nM dexamethasone and 0.1 mM 8-CTP cAMP (Dex-cAMP) to induce PEPCK gene expression together with 5 ηM *trans*-SG or *cis*-SG. (A) Effect of different concentrations of *cis*- and *trans*- SG on PEPCK mRNA expression from Real-Time PCR; (B) Results from comparative CT method.

Conclusion

The current project successfully confirmed the hypoglycemic effect of PM extract and suggested that the mechanism was through maintaining β cell functions; it aslo suggested that the component that was responsible for this effect was cis-SG. Using KK CgAy/J female type 2 diabetic mice, we evaluated the anti-diabetic effect of 40% resin fractionate of PM root extract from macroporous resin column and found that the hyperglycemia in diabetic mice was successfully reverted by PM extract treatment, indicating a remarkable hypoglycemic effect in type 2 diabetes. The mechanism of this effect of PM extract was investigated, by studying levels of Pro-inflammatory cytokines and markers from insulin signaling pathway; however, the experimental results as well as approximates from HOMA model suggested that the effect was not mediated through insulin resistant. On the other hand, serum insulin level in PM group significantly increased; therefore it was proposed that stimulated insulin secretion from pancreatic β cells plays a key role in lowering blood glucose of diabetic mice. The major peak as well as the main active compound in PM extract, *trans*-SG, was excluded for possible anti-diabetic effect using the same animal model and experimental design, since it did not have any hypoglycemic effect. It was also found that *trans*-SG could be induced to *cis*-SG with UV-light, so *cis*-SG was purified with Preparative HPLC and identified with NMR and LC-MS. Preliminary anti-diabetic effect of *cis*-SG was confirmed in a study with pure *trans*-SG, extract containing mainly *trans*-SG, and extract containing *cis*-SG and *trans*-SG, since only the latest solution had significant hypoglycemic effect in HF diet-induced KK mice.

Furthermore, another animal study with CF-1 male mice on HF diet and HepG2 cell culture study with PEPCK assay both agree with the conclusion that *cis*-SG has stronger anti-diabetic effect than *trans*-SG. Taken together, our study shows that PM root extract has great potential as an anti-diabetic agent, and more activity could be obtained by inducing isomerization of *trans*-SG to *cis*-SG.

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