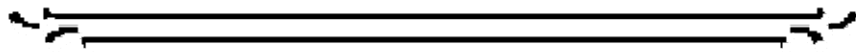


1 General Introduction



1.1 Introduction

Type 2 diabetes (T2D) is a chronic and progressive metabolic disorder associated with a number of clinical complications. In normal individuals, blood glucose concentrations are strictly maintained by various complex interactions of genetic, environmental, immunological and lifestyle factors. T2D is characterised by impaired insulin secretion by the pancreas and/or resistance to the insulin action on the target tissues resulting in impaired glucose uptake. The elevated glucose concentrations in the blood damage the micro and macro vascular tissues leading to neuropathy, nephropathy and peripheral vascular complications (DeFronzo, 1992; Yki-Jarvinen, 1994).

During the last few decades, the incidence of T2D has increased to epidemic proportions with more than 170 million individuals globally estimated to be affected and this estimate is predicted continue to increase in the future (Zimmet *et al.*, 2001). Furthermore, the World Health Organization (WHO, 1999) estimated that approximately 4 million deaths per year could be attributed to diabetes. The prevalence of diabetes rises from 6% in people 45-64 years of age to 12% for those above 65 years of age. The WHO reports that the population of annual health care cost ranges from 2.5% to 15% per diabetic person, depending upon local prevalence and sophistication of the treatment (WHO, 2002). The current treatment of T2D patients is mainly through oral

hypoglycemic agents and insulin which all have limitations, such as possible side effects of chloestatic jaundice, agranulocytosis, dermatological reactions, abdominal discomfort, anorexia and metallic taste (Rang & Dale, 1991) and although preventative are not a cure.

Recent trends in diabetes treatment show an increasing interest in traditional systems of medicine. Traditional knowledge can form the basis for the development of new health care products. Every human society has evolved an indigenous health care system to cope with various types of illness, with ethnomedical information from Ayurveda, Unani, Kampo, and Chinese traditional medicines having contributed extensively in the development of novel health care compounds. A recent review by Fabricant and Farnsworth (2001), reported that the uses of 80% of 122 plant-derived drugs were related to their original ethnopharmacological purposes. These systems are still in practice because of their long tradition and effectiveness. However, a major drawback of these traditional medicines is a lack of robust scientific data and few or no records of any adverse effects. Thus, the scientific examination of these treatments using powerful modern technologies to understand their physiological mechanism will greatly facilitate the development of many novel treatment options.

The plant kingdom has been described as “mankind’s pharmacy”(Newman *et al.*, 2003; Balunas & Kinghorn, 2005). During the last few decades, there has been a resurgence of interest in plants as a possible source of medicines (Kinghorn, 1994; Fabricant & Farnsworth, 2001; Butler, 2005). The importance of plant derived pharmaceuticals has

been acknowledged in detail in recent reviews and reports (Newman *et al.*, 2000, 2003; Chin *et al.*, 2006; Jones *et al.*, 2006). The goals of investigating natural products are:

- 1) To elucidate a novel bioactive chemical entity either as a direct drug or as a template to develop more active and less toxic synthetic or semi synthetic molecules;
- 2) To use whole or parts of plants as an alternative herbal remedy.

The following sections in this chapter briefly review the scientific background of glucose homeostasis and its impairment by various regulating factors, pathogenesis of T2D, and the treatments currently available. In addition to these, the role of various Ayurvedic medicinal plants in the treatment of T2D is reviewed, thereby providing the foundation of the hypothesis of the research presented in the subsequent chapters.

1.2 Glucose Homeostasis

Glucose is the vital metabolic fuel required for the existence of most living organisms. The concentration of glucose in the plasma is a reflection of the peripheral glucose uptake and hepatic glucose production (Cryer, 1981). The appearance of glucose in the circulating system is caused mainly by intestinal absorption, glycogenolysis (the breakdown of glycogen to glucose) and gluconeogenesis (the formation of glucose from lactates and amino acids).

1.2.1 Glucose transport

Glucose, a major energy source in mammals is derived from the meal, following the hydrolysis of carbohydrates and also by hepatic production. Dietary glucose and glucose synthesised within the body is mainly transported across the lipid bilayers through specific membrane-associated carrier proteins. Under normal physiological conditions, glucose transport is rate-limited for insulin-mediated glucose utilisation in many tissues (Kaltz *et al.*, 1988). They are broadly classified in two categories; sodium (Na^+) dependent glucose transporters (SGLT), and facilitative glucose transporters (GLUT).

1.2.1.1 SGLT

The SGLT is largely restricted to the luminal membrane of the cell lining of the intestine and the proximal tubules of the kidneys to transport glucose against a glucose concentration gradient via a secondary active transport mechanism (Wright *et al.*, 1997). SGLT1 (high affinity, low capacity) is mainly expressed on the apical membranes of the small intestine absorptive cells (enterocytes) and renal proximal straight tubules (S₃-cells) and its main function is to recover glucose by preventing loss in the urine. SGLT2 (low affinity, high capacity) is expressed in the S₁ and S₂-cells of renal proximal tubules and transports the large amount of plasma glucose from the globular filtrate. However, the mechanisms of the SGLT are not understood fully (Heidiger *et al.*, 1995; Wright, 2001).

1.2.1.2 GLUT

The mechanism of the facilitative glucose transporter (GLUT) family is not fully understood. GLUT proteins transport glucose by diffusion gradient or facilitative diffusion across the plasma membrane. The group of members of the GLUT protein family exhibit different substrate specificities, kinetic properties and tissue expression profiles (Thorens *et al.*, 1990; Mueckler, 1994; Wood & Trayhurn, 2003).

GLUT1 proteins are widely distributed in many cells, largely expressed in brain and erythrocytes but they also have expression on the adipose, muscle and liver tissues. GLUT1 protein expression in the brain barrier enables the glucose to reach the cranial

neuronal cytoplasm (Wood & Trayhurn, 2003). GLUT1 protein is also responsible for the transportation of glucose across endothelial and epithelial tissues (Gould *et al.*, 1991). The activation of protein expression of GLUT1 after glucose starvation suggests it may also play a partial role in the basal glucose uptake (Cusi & DeFronzo, 2001).

GLUT2 proteins are primarily expressed in the pancreatic beta-cells (β -cells), the liver, and the kidneys. The expression of GLUT2 on the sinusoidal membranes of hepatocytes mediates the bi-directional transportation of glucose under hormonal control (Wood & Trayhurn, 2003) and also in the bilateral surface of proximal tubules and enterocytes, thus forming the transcellular pathway for glucose and fructose transportation (Thorens *et al.*, 1990). This isoform is largely expressed within the β -cells of the pancreas and plays a vital role in the glucose sensing mechanisms because of its transportation rate being higher than other GLUT proteins (Gould *et al.*, 1991). GLUT2 proteins possess an added advantage of net release and absorption of glucose. During the postabsorptive fasting state, intracellular glucose concentration can exceed blood glucose level, at this stage the exportation of glucose from the liver and kidneys occurs through GLUT2 proteins (Mueckler, 1994).

GLUT3 protein has a high affinity for glucose and is expressed in the neurons (Gould *et al.*, 1991). The neuronal plasma membrane with expression of GLUT3 enables the glucose transport across the cytoplasm of neurons in the brain (Vannucci *et al.*, 1998).

The insulin responsive glucose transporter, GLUT4 proteins are found in the skeletal muscle, the liver, and the adipose tissues and are stimulated by insulin. They are responsible for the reduction of the postprandial plasma glucose rise by increasing glucose uptake (Marshall *et al.*, 1993). Insulin exerts its action by stimulating the translocation of vesicles containing GLUT4 proteins from intracellular stores to the plasma membrane resulting in an increase in glucose transport (Shepherd & Kahn, 1999; Bryant *et al.*, 2002). Insulin also causes the translocation of a small numbers of GLUT1 proteins, but this effect is said to be minimal (Pilch *et al.*, 1993). Exercise also stimulates the GLUT4 protein translocation to plasma membranes in an insulin-independent manner (Thorell *et al.*, 1999). These experimental findings illustrate that GLUT4 proteins have a key role in the process of glucose homeostasis (Marshall *et al.*, 1993).

1.2.2 Glucose regulation

Under normal physiological conditions, the blood glucose concentration during food consumption or food deprivation is tightly regulated by various glucoregulatory hormones including glucagon, secreted by the alpha-cells (α -cells) of the pancreas; insulin and amylin, secreted by β -cells of the pancreas; glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP) (previously known as gastric inhibitory polypeptide), secreted by the L-cells of the intestine; epinephrine; cortisol and some growth hormones (Chang *et al.*, 1996; DeFronzo, 1997) (Figure 1-1). In the fasting state, most of the glucose is supplied by the liver and it is utilised by the insulin-independent tissues such as red blood cells, skin, smooth muscles, and the brain (Aronoff *et al.*, 2004). After a meal, when blood glucose concentration increases above 5 mM, the

pancreas rapidly stimulates the secretion of insulin, resulting in an increased rate of glucose transportation, metabolism and uptake of excess glucose by skeletal muscle, adipose and liver tissues (Pilkis & Granner, 1992).

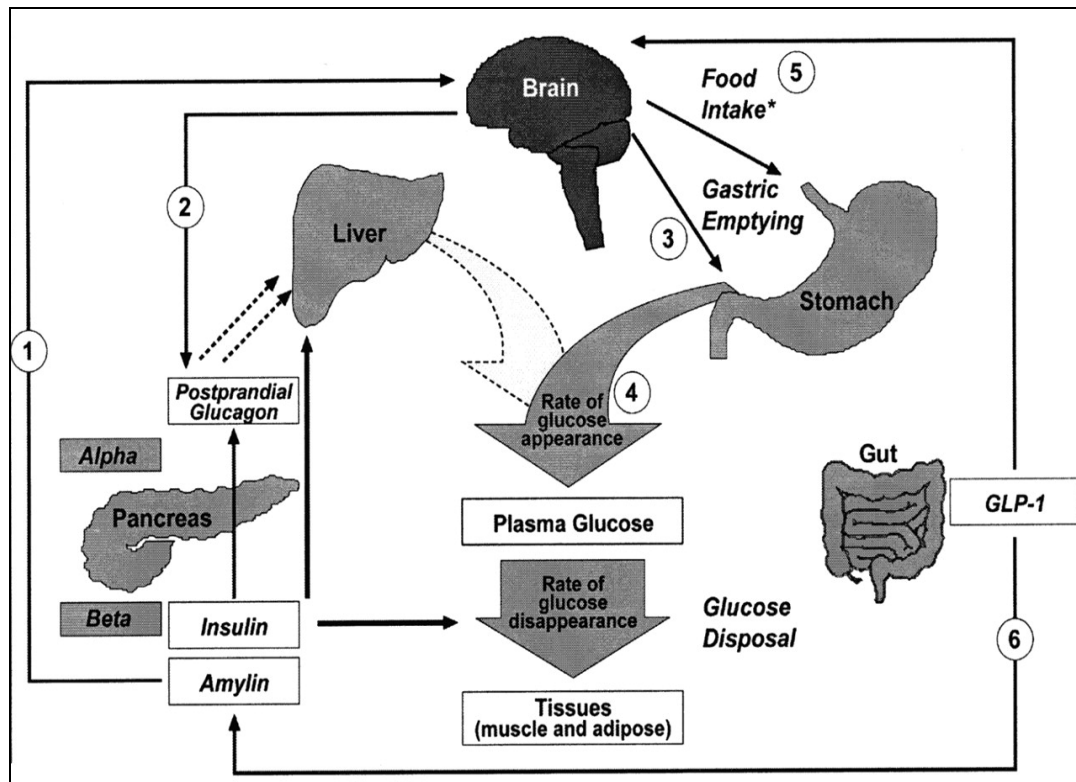


Figure 1-1 Glucose homeostasis: roles of insulin, glucagon, amylin, and GLP-1.
The multi-hormonal model of glucose homeostasis (non-diabetic individuals): in the fed state, amylin communicates through neural pathways (1) to suppress postprandial glucagon secretion (2) while helping to slow the rate of gastric emptying (3). These actions regulate the rate of glucose appearance in the circulation (4). *In animal models, amylin has been shown to dose-dependently reduce food intake and body weight (5). In addition, incretin hormones, such as GLP-1, glucose-dependently enhance insulin secretion (6) and suppress glucagon secretion (2) and, via neural pathways, help slow gastric emptying and reduce food intake and body weight (5) (Aronoff *et al.*, 2004).

The endocrine cells of the pancreas play a prominent role in glucose homeostasis by secreting various glucoregulatory hormones such as insulin, amylin, glucagon, somatostatin and some pancreatic polypeptides. The clustered endocrine glands, the islets of Langerhans, consist of four different cell types α , β , δ , and γ -cells (Figure 1-2). The

hormones secreted by these cells modulate the cellular nutrition or metabolism from the rate of absorption to storage. Endocrine pancreas dysfunction, or abnormal responsiveness of secreted hormones, results in disturbances in the glucose homeostasis (Orci *et al.*, 1975a; Orci & Unger, 1975b).

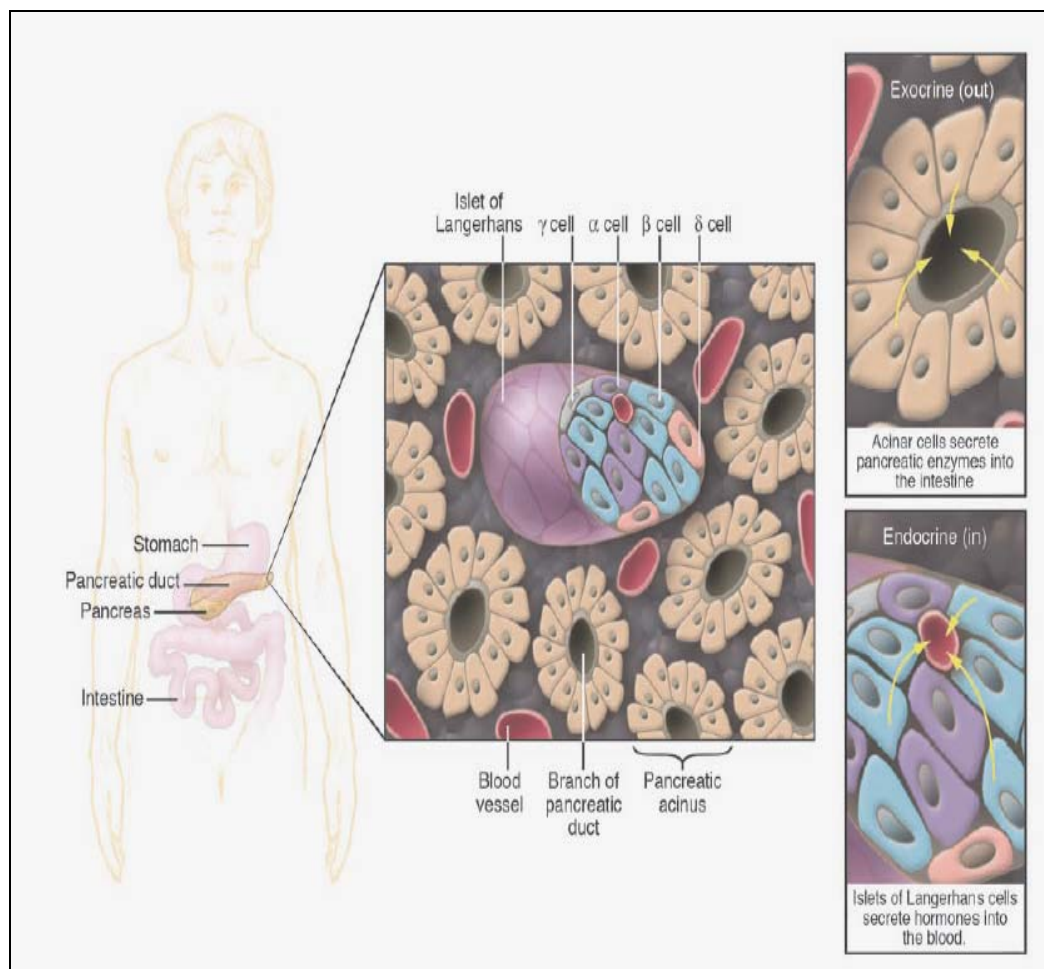


Figure 1-2 Cross section of the pancreas

The pancreas houses 2 distinctly different tissues. Its bulk comprises exocrine tissue, which is made up of acinar cells that secrete pancreatic enzymes delivered to the intestine to facilitate the digestion of food. Scattered throughout the exocrine tissue are many thousands of clusters of endocrine cells known as islets of Langerhans. Within the islet, α cells produce glucagon; β cells, insulin; δ cells, somatostatin; and γ cells, pancreatic polypeptide — all of which are delivered to the blood. (Trucco, 2005).

1.2.2.1 *Insulin*

Insulin is a vital anabolic hormone in the control of glucose homeostasis. It is a small protein composed of two polypeptide chains containing 51 amino acids and is derived biosynthetically from the precursor molecule, preproinsulin; a long polypeptide produced by the endoplasmic reticulum of the pancreatic β -cells. Proteolytic cleavage of preproinsulin by the microsomal enzymes forms proinsulin which is transported by golgi bodies and packed as clathrine-coated secretory granules. It breaks down further into equimolar amount of C peptide and insulin (Wollheim & Sharp, 1981). Insulin is released by the process of exocytosis. Insulin secretion by pancreatic β -cells are regulated by three mechanisms, neural, hormonal, and nutrients (Wollheim *et al.*, 1996).

The autonomic innervation of islets plays an important role in the modulation of insulin release from the islet cells of Langerhans. Insulin is secreted at the smell, sight and expectation of food. This is known as the cephalic phase of insulin secretion and is due hypothalamo-entero-insularaxis which is mediated by vagal nerve (While & Khan, 1994). During feeding the parasympathetic nerves are activated and are instrumental for the cephalic phase of insulin release. The cephalic phase of insulin secretion minimizes the early rise in postprandial blood sugar (Havarankova *et al.*, 1978; Cheatham & Kahn, 1995). Vagotomy and pancreatic transplantation (Islet denervation) results in early rise in postprandial blood sugar. Sympathetic activity partly mediates inhibition of insulin secretion during stress and trauma.

Many peptides modulate insulin release. They are co-localised in islet cells with their major secretory products. Insulin release is stimulated by TRH (Thyrotropin-releasing hormone), GHRH (Growth hormone releasing hormone), ACTH (Adrenocorticotrophic hormone) and opiodis. Insulin secretion is inhibited by many hormones including pancrestatin, islet amyloid polypeptide (IAPP), Diazepam-binding inhibitor (DBI) Peptide YY(PYY), Corticotrophin releasing factor (CRF), atrial natriuritic peptic (ANP) and biogenic amines (Araki *et al.*, 1994).

Glucose is the primary regulator of insulin synthesis and secretion (Goodman, 2001). After a meal, when the extracellular glucose concentration increases, β -cell metabolism accelerates and stimulates insulin release. Insulin secretion has been demonstrated to have dual hierarchal control by triggering and amplifying pathways (Henquin, 2000; Henquin *et al.*, 2003). However, the mechanism underlying this multifactorial regulation (Figure 1-3) is still not understood fully. Glucose enters the pancreatic β -cells by passive diffusion which is facilitated by GLUT2, leading to an acceleration of β -cell metabolism. In diabetic animals, a decreased expression of GLUT2 suggests normal glucose sensing is impaired (Unger, 1991). Glucose transported to β -cells is phosphorylated by glucokinase (Newgard, 1996), a glucose sensing enzyme expressed in β -cells (Iynedijian, 1993). The low expression of glucokinase (Bali *et al.*, 1995; Grupe *et al.*, 1995) and of the glucose transporters (Accili *et al.*, 1996; DeFronzo, 1997) alters glucose sensing and the regulation of insulin secretion. In the β -cells, glucose-6-phosphate (glucose phosphorylated by glucokinase), undergoes further metabolism through glycolytic pathway, and releases ATP (an energy molecule).

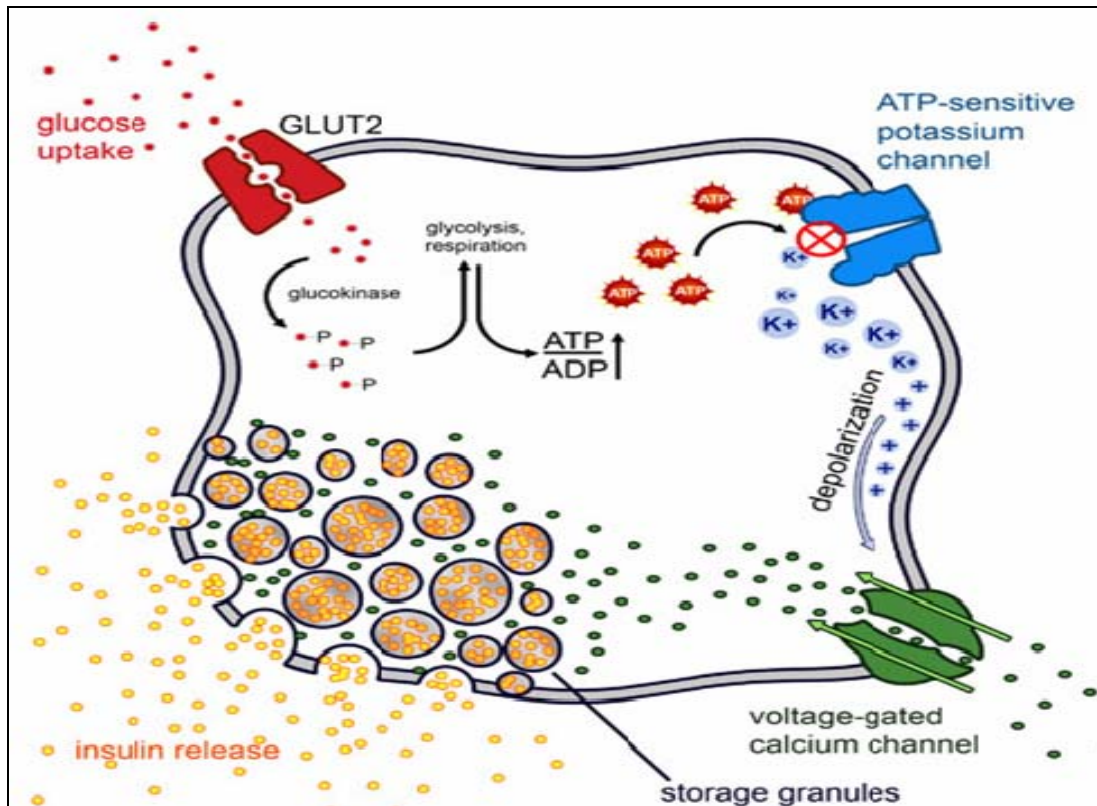


Figure 1-3 Metabolic hypothesis of glucose-stimulated insulin release from β -cells (Cartailler, 2004)

Glucose enters the pancreatic β -cells by passive diffusion which is facilitated by GLUT2, leading to an acceleration of β -cell metabolism. In diabetic animals, a decreased expression of GLUT2 suggests normal glucose sensing is impaired (Unger, 1991). Glucose transported to β -cells is phosphorylated by glucokinase (Newgard, 1996), a glucose sensing enzyme expressed in β -cells (Iynedijian, 1993). The low expression of glucokinase (Bali *et al.*, 1995; Grupe *et al.*, 1995) and of the glucose transporters (Accili *et al.*, 1996; DeFronzo, 1997) alters glucose sensing and the regulation of insulin secretion. In the β -cells, glucose-6-phosphate (glucose phosphorylated by glucokinase),

undergoes further metabolism through glycolytic pathway, and releases ATP (an energy molecule). The increased ATP/ADP ratio closes the [K.sub.ATP] channel and leads to the depolarization of the plasma membrane with the opening of calcium (Ca^{2+}) ions channels. The increased concentration of Ca^{2+} ions triggers the exocytosis of insulin (Aguilar-Bryan & Bryan, 1999; Ashcroft & Gribble, 1999; Seino *et al.*, 2000; Gilon & Henquin, 2001). Some hormones and neurotransmitters, that are independent of [K.sub.ATP] channels, can mobilize Ca^{2+} ions directly from the intracellular stores (Gromada *et al.*, 1998; Gilon & Henquin, 2001) and also some cationic amino acids can depolarize β -cells directly because of their positive form and poor metabolism (Henquin *et al.*, 2003). The nature and role of signals from glucose metabolism to the amplification pathway of insulin stimuli are still unclear (Sato & Henquin, 1998; Aizawa *et al.*, 2002; Straub & Sharp, 2002). Recent experimental studies suggest that in T2D both triggering and amplifying pathways were impaired in β -cells (Henquin, 2000). Insulin secretion from pancreatic β -cells is a biphasic process; when the blood glucose concentration exceeds 5 mM, an initial rapid exocytosis of insulin (first phase) is followed by a steady and long term release of insulin (second phase) (Gerich, 1998; Prato *et al.*, 2002). In T2D patients, the first phase of insulin secretion is found to be low or absent and the second phase is decreased (Rang *et al.*, 2000).

Although insulin has many physiological effects, the primary metabolic effect of insulin is to stimulate glucose disappearance, by binding the glucose to insulin receptors on the surface of target cell membranes such as the liver, fat and muscle cells (White & Kahn, 1994; Guyton & Hall, 2000; Saltiel & Kahn, 2001)(Figure 1-4).

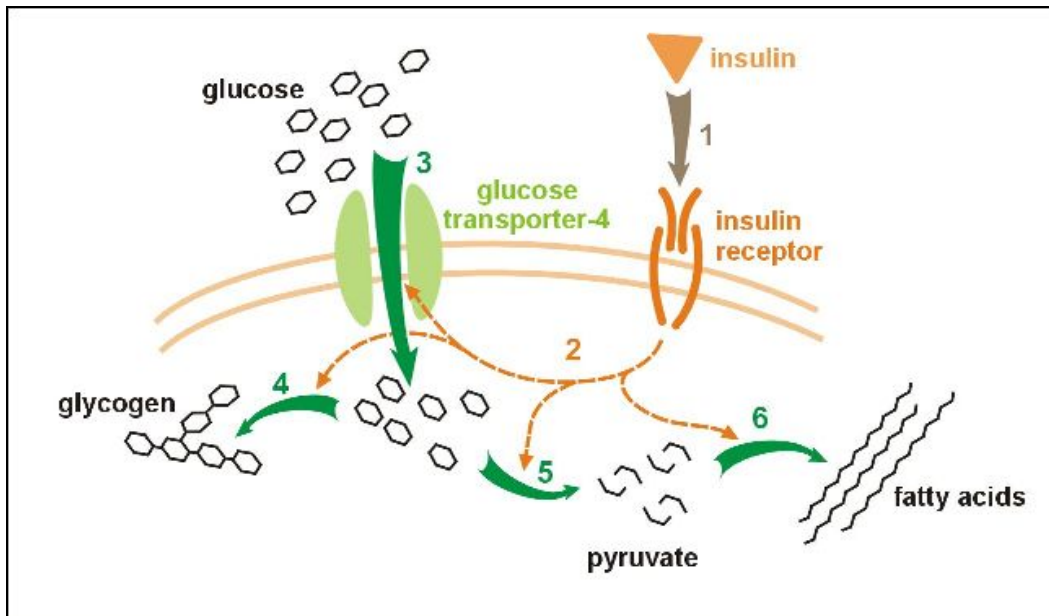


Figure 1-4 Effect of insulin on glucose uptake and metabolism

Insulin binds to its receptor (1) which in turn starts many protein activation cascades (2). These include: translocation of GLUT4 transporter to the plasma membrane and influx of glucose (3), glycogen synthesis (4), glycolysis (5) and fatty acid synthesis (6) (Meiquer, 2006).

In the skeletal muscles, glucose uptake is stimulated by the activation of the translocation of GLUT4 proteins by the presence of insulin (Shepherd & Kahn, 1999). Insulin acts by binding to an insulin receptor (IR) which is a heterotetrameric glycoprotein consisting of two α and two β subunits linked by disulfide bonds, a member of the tyrosine kinase receptor family. Insulin exerts its effect by binding to the alpha subunits of the insulin receptor and activates the autophosphorylation of beta subunits (Ross *et al.*, 2004), leading to the activation of a number of ‘docking’ proteins (Kasuga *et al.*, 1982) (see Figure 1-5).

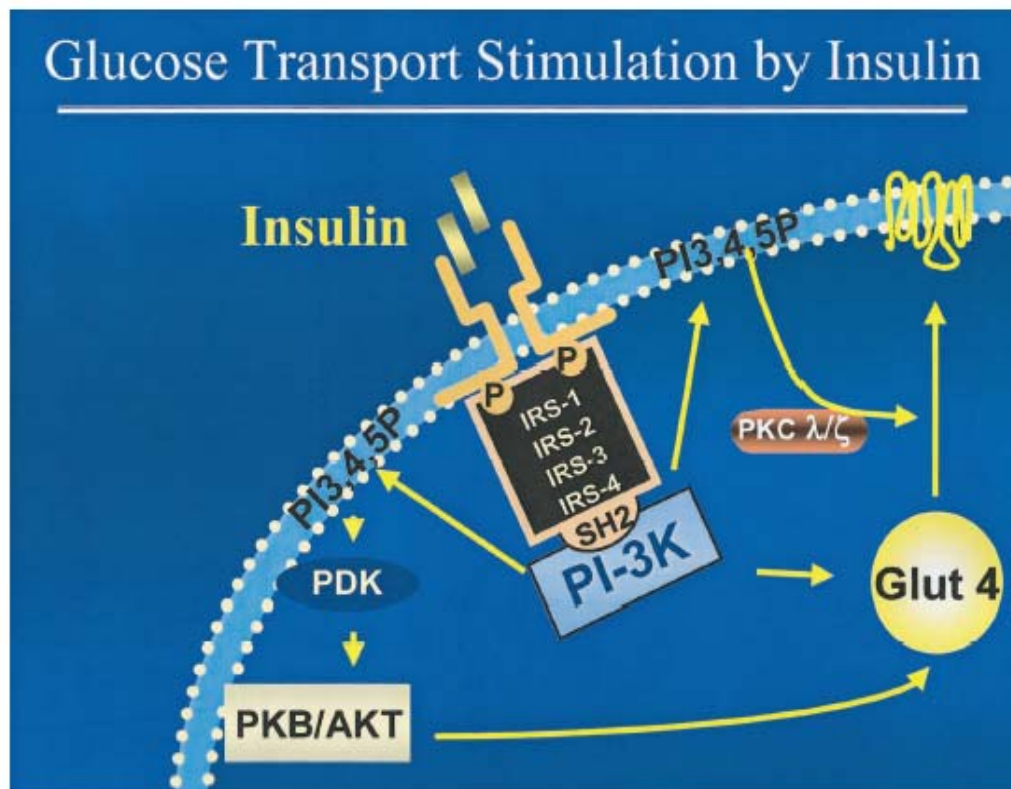


Figure 1-5 Molecular mechanism of insulin-stimulated glucose transport

The insulin-dependent glucose transporter 4 (GLUT4) is translocated by a phosphatidylinositol 3-kinase (PI3-kinase)-dependent pathway including PKB/AKT and PKC stimulation downstream of PI3K. (Matthaei *et al.*, 2000)

The insulin receptor substrates (IRS) are vital proteins involved in the insulin-mediated glucose uptake (Zierath & Wallberg-Henriksson, 2002). IRS proteins modulate the activities of the phosphatidylinositol 3-kinase (PI3-kinase). Insulin-mediated activation of PI3-kinase is necessary for translocation of GLUT4 vesicle from an intracellular site to the plasma membrane to facilitate glucose uptake (Despres & Marette, 1999). Scientific findings suggest that the use of two separate pharmacological inhibitors of PI3-kinase, wortmannin or LY294002, are sufficient to completely block the insulin-mediated glucose uptake and transport (Kanai *et al.*, 1993; Hara *et al.*, 1994; Zhou *et al.*, 1997)

However, the downstream effectors which link PI3-kinase to glucose transport are as yet undefined. Some more recent studies indicate that the activation of PI3-kinase alone does not result in glucose transportation in muscle and adipose tissues, suggesting there may be an alternative PI3-kinase independent pathway involved in the signal transduction at this level (Despres & Marette, 1999). This is demonstrated by the chronic treatment of muscle cells with 5-amino imidazole-4-carboxamide-1- β -D-ribofuranoside (AICR); an activator of adenosine monophosphate-activated protein kinase (AMPK) stimulating glucose transport independently of PI3-kinase (Hayashi *et al.*, 1998). In a recent study, it was established that troglitazone has a direct effect in stimulating glucose transport and GLUT4 translocation, independent to PI3-kinase and protein kinase B (PKB) and C (PKC) isoform (Yonemitsu *et al.*, 2001). Recently, a p38 mediated glucose uptake and GLUT4 translocation by alpha-lipoic acid (Konrad *et al.*, 2001) and a c-Cbl/CAP signaling pathway (Ross *et al.*, 2004) have been recognized as alternative pathways for insulin-mediated glucose uptake in the skeletal muscle and adipocytes.

In the postprandial state, the majority of insulin released is absorbed in the liver and promotes glycogen synthesis, storage and also inhibits glycogenolysis (Duckworth *et al.*, 1988; Sato *et al.*, 1991). Insulin increases protein and triglyceride synthesis in muscle by increasing amino acid transport and by stimulating ribosomal protein synthesis. Insulin also increases glucose transportation in the fat cells and inhibits intracellular lipolysis (Cryer, 1992) which may partially explain the decreased insulin clearance rates that are seen in obesity and diabetes (Bonora *et al.*, 1983; Trischitta *et al.*, 1989).

1.2.2.2 *Amylin*

Amylin, or islet amyloid polypeptide (IAPP), is a complementary polypeptide of insulin secreted by the pancreatic β -cells in response to extracellular nutrient stimuli (Cooper *et al.*, 1987). It plays a vital role in the regulation of the glucose appearance from both endogenous (liver-derived) and exogenous (meal-derived) glucose sources (Cooper *et al.*, 1987; Moore & Cooper, 1991; Koda *et al.*, 1992). The secretory and the plasma concentration profile of amylin resemble insulin during both the fasting and fed state (Koda *et al.*, 1995; Fineman *et al.*, 1996). Amylin exerts its action through a calcitonin-like receptor, in the area postrema (a part of the dorsal vagal complex in the brain stem). A notable feature of the area postrema is that it lacks a blood-brain barrier, allowing exposure to rapid changes in plasma glucose concentrations as well as circulating peptides, including amylin (Beaumont *et al.*, 1993; Wimalawansa, 1997). Research findings suggest that amylin works with insulin in the maintenance of glucose homeostasis (Pehling *et al.*, 1984) primarily by suppressing glucagon secretion in the postprandial state (Gedulin *et al.*, 1997) and slowing gastric emptying rate (Sampson *et al.*, 2000). Interestingly, in subjects with diabetes, amylin is deficient in type 1 diabetes (T1D) and impaired in T2D (Young, 1997; Kruger *et al.*, 1999).

1.2.2.3 *Glucagon*

Glucagon, a single chain polypeptide consisting of 29 amino acids, is a key catabolic hormone secreted by pancreatic α -cells. It plays a vital role in glucose homeostasis during

the fasting state by opposing the effects of insulin (Unger, 1971). When plasma glucose concentration falls below the normal range, glucagon secretion increases, which acts rapidly on the liver to stimulate glycogenolysis and inhibit glycogen synthesis, glycolysis and lipogenesis (Orci *et al.*, 1975a; Gerich *et al.*, 1979). Glucagon acts through hepatic cell surface G-protein coupled receptors by various mechanisms. In the diabetic condition, the inadequate suppression of postprandial glucagon secretion actually causes an increase in hepatic glucose production leading to elevation of plasma glucose (Cryer, 1981; Dinneen *et al.*, 1995). The elevated glucose and ketone production that characterises uncontrolled diabetes appears to require both an excess of glucagon and deficiency of insulin. Overall ratio of insulin to glucagon appears to control the glucose metabolism (Jiang & Zhang, 2003).

1.2.2.4 *Incretin hormones*

Perley and Kipinis (1967) demonstrated in people that ingested food caused a more potent release of insulin than glucose infused intravenously. This effect termed, “the incretin effect,” suggested that signals from the gut are important in the hormonal regulation of glucose metabolism. GIP and GLP-1 are the dominant two gut peptides or incretin hormones which play important roles in the hormonal regulation of glucose homeostasis. GIP stimulates insulin secretion and the regulation of fat metabolism, but it does not inhibit glucagon secretion or gastric emptying rate (Yip & Wolfe, 2000).

In T2D patients, GIP levels are normal or slightly elevated (Vilsboll *et al.*, 2001). Both GIP and GLP-1 are stimulated by the ingestion of a mixed meal enriched with fat and carbohydrates (Elliott *et al.*, 1993; Herrmann *et al.*, 1995), but GLP-1 is secreted in greater concentrations and is considered to be a more physiologically relevant hormone in humans (Kreymann *et al.*, 1987; Nauck *et al.*, 1993; Holst, 1994).

GLP-1 also stimulates glucose-dependent insulin secretion (Lugari *et al.*, 2002), but in contrast to GIP, it inhibits glucagon secretion and slows the gastric emptying rate (Matsuyama *et al.*, 1988). GLP-1 is derived from the proglucagon molecule in the intestine and synthesised and secreted by L-cells in the ileum and colon. It stimulates insulin secretion in a glucose-dependent manner, while inhibiting glucagon secretion through activation of GLP-1 receptors on the pancreatic β -cells and indirectly through sensory nerves (Burcelin *et al.*, 2001). GLP-1 is significantly reduced postprandially in T2D (Vilsboll *et al.*, 2001; Lugari *et al.*, 2002). Infusion of GLP-1 significantly lowers postprandial glucose as well as overnight plasma glucose concentrations; this effect of GLP-1 occurs due to its partial inhibitory effect on glucagon secretion, and also its effect on regulation of gastric emptying and gastric acid secretion. In addition, experimental findings indicate that administration of GLP-1 improves insulin sensitivity, enhances glucose disposal and is also associated with the regulation of feeding behavior and body weight (Turton *et al.*, 1996; Zander *et al.*, 2002). An experimental study using animal models suggests that the role of GLP-1 may enhance the β -cells mass by the preservation of its function and proliferation (Drucker, 2003). GLP-1 has a very low plasma half-life

of about 2 minutes, and it is rapidly inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4). In this context, clinical developments of potential compounds that elicit similar glucoregulatory effects either by mimicking the action of GLP-1 (incretin mimetics) with a longer plasma half life, or by inhibiting DPP-4 are being investigated (Drucker, 2003).

1.2.3 Endogenous glucose production

Glucose is produced endogenously by both the liver and kidneys. The majority of the glucose produced by the kidneys is being utilized by themselves. This illustrates the vital role the liver plays in the maintenance of glucose homeostasis, through providing glucose to insulin dependent and independent tissues throughout the body. Endogenous glucose production is accelerated in T2D (Meyer *et al.*, 1998; Weyer *et al.*, 1999)

In the fasting state, when the plasma glucose level falls below the basal level, the glucose production by the liver accelerates by glycogenolysis and gluconeogenesis, thus preventing hypoglycemia (DeFronzo, 1997). When blood glucose is low, α -cells of the pancreas trigger glucagon secretion, which has the dual effect of turning on glycogen breakdown and turning off glycogen synthesis. However, glycogenolysis is limited. Fatty acid oxidation and protein degradation in the liver promotes gluconeogenesis, which is the alternative glucose producing pathway mediated by the liver very early in a fasting state, usually within 12 hours. Alterations in the hepatic glucose production may induce insulin resistance (Valera *et al.*, 1994) and elevated hepatic glucose production by both glycogenolysis and gluconeogenesis is found to be responsible for increased plasma glucose concentration T2D with overt fasting hyperglycemia (DeFronzo, 1997).

1.2.4 Hyperglycemia

As reviewed earlier, impairment in glucose homeostasis causes chronic hyperglycemia, which is an independent factor that regulates insulin secretion and action (Bergman, 1989). Hyperglycemia inhibits insulin secretion and glucose utilization, resulting in deleterious metabolic complications which is commonly referred as ‘Glucose toxicity’ (Yki-Jarvinen, 1992). In β -cells, hyperglycemia leads to the production of more reactive oxygen species that damages the cellular components (Robertson *et al.*, 2003). When insulin action decreases, the β -cell function increases but over time it results in β -cell dysfunction because of the glucose toxicity (Robertson *et al.*, 2004; Stumvall *et al.*, 2005).

The information thus far provided explains the vital physiological and biochemical processes of glucose homeostasis and its relevance to T2D. Further discussion on the pathophysiology of diabetes mellitus, possible complications and its treatment will be reviewed in detail in the following sections.

1.3 Diabetes mellitus

Diabetes is a wonderful affection.....being a melting down of the flesh and limbs into urine the patients never stop making water, but the flow is incessant as if from the opening of aqueducts....the patient is short livedfor melting is rapid, the death is speedy.

**ARETAEUS
2nd CENTURY.**

Diabetes mellitus comprises a heterogeneous group of disorders characterised by high blood glucose. Therapeutically, diabetes can be classified in two categories: type 1 diabetes (T1D), formerly known as childhood, juvenile, or insulin-dependent diabetes mellitus (IDDM), and type 2 diabetes (T2D), formerly known as obesity related diabetes, adult-onset diabetes or non insulin-dependent diabetes mellitus (NIDDM).

T1D is most often observed in young people, although it may present at any age, and the incidence of T1D is increasing (Pitkaniemi *et al.*, 2004). It is an autoimmune disease in which the pancreatic β -cells are destroyed or damaged by the body's own immune system leading to decreased or complete depletion of insulin production. Blood samples from patients with T1D have been found to contain a number of autoantibodies to components of the pancreas, including islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies against glutamic acid decarboxylase (GAD), and insulinoma-associated autoantigen-2 (IA-2) (Petrovsky & Schatz, 2003). In T1D patients, depletion of insulin products causes hyperglycemia leading to lipolysis, excess ketone bodies, ketoacidosis

and other peripheral complications. In addition, an elevated level of glucagon is observed and therefore requires exogenous injections of insulin to maintain nutrient homeostasis (Guyton & Hall, 2000).

Of all diabetic patients, 80-90% have T2D (McCarty & Zimmet, 1994; Zimmet *et al.*, 2001). Currently T2D is diagnosed on the basis of WHO recommendations from 1999, when the underlying metabolic disorder consists of reduced β -cell function and insulin resistance, which cause elevation of plasma cell glucose above 7 mM (126 mg/dl) in the fasting state and/or above 11.1 (200 mg/dl) 2 hours after a 75 g of glucose load (WHO, 1999).

The inherited quality of T2D has been known for centuries and statistics indicate that those with a family history of diabetes have a higher (25-30%) risk of T2D than those without such a background. Approximately 90-95% of monozygotic twins are concordant for T2D (Newman *et al.*, 1987). First degree relatives of the type 2 diabetic patients have a 15-25% chance to develop T2D (Pierce *et al.*, 1995). The life-time risk for T2D is 60% by the age of 60 years if both parents are affected and around 38% at the age of 80 years if one parent had T2D (Tattersal & Fajans, 1975; Pierce *et al.*, 1995). T2D is genetically and environmentally heterogeneous because in most individuals, genetic defects in insulin secretion itself are not sufficient to cause T2D without acquired factors such as pregnancy, weight gain, glucose toxicity or physical inactivity (Gerich, 1998).

1.3.1 Pathophysiology

T2D is a heterogeneous syndrome with two basic etiologic lesions, one involves an impaired β -cell function and is characterised by insulin deficiency, the other involves a resistance to the effectiveness of insulin action on peripheral tissues such as muscle, liver and adipose tissues and is characterised by elevated insulin, caused by both genetic and acquired environmental factors (DeFronzo, 1988; DeFronzo *et al.*, 1992; DeFronzo, 1997) (Figure 1-6).

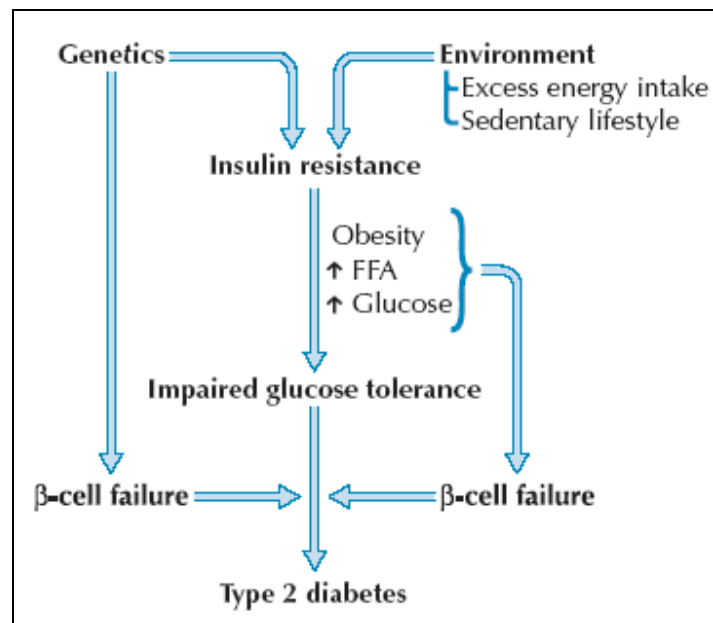


Figure 1-6 Overview of the pathogenesis of type 2 diabetes mellitus (Cheng & Fantus, 2005)

1.3.1.1 *Insulin resistance*

T2D patients sometimes present with hyperglycemia, together with an ‘inappropriately’ high plasma insulin concentration; this apparent inconsistency is due to tissue insensitivity to insulin, known as insulin resistance (Reaven, 1988). In such a condition, the biological effects of insulin are less than expected for glucose clearance by the skeletal muscle and the suppression of hepatic glucose production (Dinneen *et al.*, 1992). Insulin resistance is asymptomatic and may be found in subjects years before they develop T2D (Reaven, 1988). The primary factors that influence the insulin sensitivity includes genetic background (Barroso *et al.*, 1999), age (DeFronzo, 1979; Chen *et al.*, 1985), obesity (Olefsky *et al.*, 1973; Kolterman *et al.*, 1980; Lillioja & Bogardus, 1988; Bjorntorp, 1993), physical inactivity (Kahn & Flier, 1990; Prigeon *et al.*, 1995), body fat index (Peiris *et al.*, 1986; Peiris *et al.*, 1989; Pouliot *et al.*, 1992; Bjorntorp, 1993) and dietary nutrients (Peiris *et al.*, 1986; Chen *et al.*, 1987). In type 2 diabetic patients with insulin resistance, six weeks of exercise increased the insulin sensitivity by increasing peripheral glucose disposal and glycogenesis (Perseghin *et al.*, 1996). Exercise effects on glucose transport molecules include increases in blood flow and availability of insulin to the target tissues such as skeletal muscles (Hespel *et al.*, 1995), liver (DeFronzo *et al.*, 1987), and adipocytes (Koivisto & Yki-Jarvinen, 1987). Dietary nutrients play a unique role in the insulin sensitivity. Consumption of fat-rich diet tends to promote insulin resistance which is typically associated with the aging (Chen *et al.*, 1987; Chen *et al.*, 1988). In contrast, carbohydrate-rich diet increases the insulin sensitivity in T2D (Brunzell *et al.*, 1971).

Abnormalities in insulin signaling may induce insulin resistance (Kellerer *et al.*, 1999). In T2D, the reduced autoactivation of insulin receptors has been described by many but not all investigators (Caro *et al.*, 1987; Freidenberg *et al.*, 1987; Obermaier-Kusser *et al.*, 1989; Nyomba *et al.*, 1990; Theis *et al.*, 1990; Maegawa *et al.*, 1991; Bak *et al.*, 1992; Nolan *et al.*, 1994; Klein *et al.*, 1995). Obesity was the major contributory factor for the development of reduced insulin receptor activity (Freidenberg *et al.*, 1988), suggesting defective insulin receptor kinase activity is secondarily acquired due to obesity and metabolic disturbances. The role of insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) in the pathogenesis of insulin resistance has been studied recently in the respective gene knockout models. IRS1 knockout mice were insulin resistant but not hyperglycemic, suggesting a mild and non-diabetic phenotype of IRS1 (Araki *et al.*, 1994). In contrast, IRS2 knockout mice developed severe hyperglycemia due to β -cell dysfunction and peripheral insulin action which illustrates many similarities to T2D (Withers *et al.*, 1998). Insulin-mediated activation of PI3-kinase is necessary for translocation of GLUT4 vesicles from an intracellular site to the plasma membrane to facilitate glucose uptake. In T2D, decreased activation of PKB in skeletal muscle has been described (Krook *et al.*, 1998). However, a recent study which describes normal activation of PKB in skeletal muscles of lean and obese diabetic patients (Kim *et al.*, 1999), remains controversial. Thus a decreased expression and phosphorylation level of early insulin signaling elements may contribute to insulin resistance in T2D.

Many obese individuals have mildly increased plasma glucose levels together with normal or even increased plasma insulin concentrations. Obesity and body fat distribution are strongly associated with insulin resistance (Olefsky *et al.*, 1973; Kolterman *et al.*, 1980; Beard *et al.*, 1987; Lillioja & Bogardus, 1988; Peiris *et al.*, 1989; Pouliot *et al.*, 1992; Bjorntorp, 1993). In obesity, the accumulation of fat in the intra abdominal and subcutaneous region is responsible for insulin resistance and increased plasma leptin concentrations. The role of leptin in obesity and insulin resistance gained much attention recently after its identification by Friedman and colleagues (1998). Leptin is secreted from adipocytes which are dependent on body fat index (Friedman & Halaas, 1998). Leptin deficiency and impaired receptors in rodents resulted in hyperglycemia and insulin resistance (Chen *et al.*, 1996). In obese and insulin resistant individuals an elevated plasma level of non-esterified free fatty acids (NEFA) has been documented (Boden, 1997; Stumvoll & Jacob, 1999). The increased mass of triglycerides leads to a reduction of the antilipolytic effect of insulin. The elevated plasma level of NEFA suppresses the biological effects of insulin by increasing hepatic gluconeogenesis and decreasing peripheral glucose utilization (Randle *et al.*, 1963; Felley *et al.*, 1989; Gonzalez *et al.*, 1989). The NEFA induced insulin resistance results in increased acetyl CoA production and the inhibition of glucose oxidation. The elevated levels of inflammatory cytokines such as Tumor Necrosis Factor- α (TNF- α) and Interleukin-6 (IL-6) secreted by visceral adipocytes adversely modulates the insulin-mediated glucose metabolism (Hotamisligil, 2000; Ravussin & Smith, 2002; Rajala & Scherer, 2003). In obesity, a decreased amount of adiponectin (adipocyte derived protein) has been documented (Ravussin & Smith, 2002; Rajala & Scherer, 2003). Adiponectin signals via AMP-kinase to suppress hepatic

glucose production, peripheral glucose uptake, fatty acid oxidation, and lipolysis inhibition. This evidence suggests adiponectin's potential role in T2D and insulin resistance (Yamauchi *et al.*, 2002; Goldstein & Scalia, 2004).

1.3.1.2 *β-cell dysfunction*

Progressive insulin deficiency is another major factor which has a prominent role in the pathogenesis of T2D. Experimental data from the United Kingdom Prospective Diabetes Society (UKPDS) suggests that β-cell dysfunction commences years before hyperglycemia develops (Holman, 1998). The insulin deficiency is due to β-cell exhaustion by the hypersecretion of insulin, lipid toxicity and/or genetic factors. In T2D, the β-cell dysfunction will be manifested by the inappropriate secretion of insulin in response to intravenous glucose (Bagdade *et al.*, 1967; Perley & Kipinis, 1967; Brunzell *et al.*, 1976), alterations in the pulsatile insulin release (O'Rahilly *et al.*, 1988; Porksen, 2002), inefficient proinsulin conversion to insulin (Mako *et al.*, 1977; Kahn *et al.*, 1995; Kahn & Halban, 1997), reduced amylin secretion (Sanke *et al.*, 1991; Enoki *et al.*, 1992), and ultradian oscillatory insulin secretion (Polonsky *et al.*, 1988).

This progression of diabetes is also marked by important changes in the β-cell mass, phenotype and function (Gordon & Susan, 2004). Hyperglycemia increases oxidative glucose metabolism in the β-cells resulting in an increased number of reactive oxygen species, which damages cellular components and enhances NF [kappa] B activity, which in turn induces apoptosis of the β-cell. The increased plasma NEFA is shown to cause

adverse effects on β -cells, by decreasing insulin release and also decreasing proinsulin to insulin conversion (Zhou & Grill, 1994).

The mass of β -cells is strictly maintained through a balance of β -cell birth and death (apoptosis) (Bonner-Weir, 2000). It has been estimated that around 50% of β -cell insulin production is lost by the time the diagnosis is made. Thus, in the majority of type 2 diabetic patients insulin therapy will be needed within 5 years after diagnosis.

1.3.2 Complications

T2D is one of the major causes of morbidity and mortality (WHO, 2002). T2D diabetic patients have higher risk of coronary heart failure, stroke and peripheral vascular diseases (Kannel & McGee, 1979). In the diabetic state, the elevated blood glucose damages the micro and macro vascular tissues leading to angiopathy, retinopathy, nephropathy, coronary heart disease, and various acute complications. Non-enzymatic modification of intracellular proteins by glucose, glycoxidation and abnormal polyol pathway are regarded as important factors for the development of chronic diabetic complications (DeFronzo, 1997; Rang *et al.*, 2000; Davis & Granner, 2001).

1.3.3 Treatments

The primary line of treatment for T2D is lifestyle intervention, comprising a combination of diet and exercise which delays the progression from impaired glucose tolerance (IGT) to T2D (Tuomilehto *et al.*, 2001). However, in many individuals, compliance is a significant problem and also changes to diet and exercise may not be practical (Bailey & Flatt, 1995). Thus, medication-based interventions to control hyperglycemia and T2D have been prescribed and scientific testing is continuing with a number of novel compounds. Currently, oral antihyperglycemic agents are a vital therapeutic option available for the T2D patients. These are broadly classified into five categories that include: sulfonyl and non-sulfonylureas, biguanides, thiazolidinediones, peroxisome proliferators activated receptor-gamma (PPAR- γ) activators, and alpha-glucosidase inhibitors.

1.3.3.1 Sulfonyl and non-sulfonylureas

As reviewed in the earlier sections, hyperglycemia occurs due to impaired insulin secretion, often in terms of an increased requirement of insulin due to insulin resistance. Until recently, sulfonylureas were the only drug used in the treatment of T2D to stimulate insulin secretion. The sulfonylureas are hydrophobic weak acids which bind with the sulfonylurea receptor 1 (SUR1), thus activating the closure of the [K sub.ATP] channel, depolarization of membrane, Ca^{2+} influx, rise of intercellular Ca^{2+} and triggering of insulin secretion in the pancreatic β -cells (Proks *et al.*, 2002; Henquin, 2004; Rendell,

2004). Sulfonylureas mimic the effect of glucose on the insulin secretory pathway. However, in contrast to glucose, sulfonylureas do not promote proinsulin biosynthesis (Henquin, 2004) (Figure 1-7).

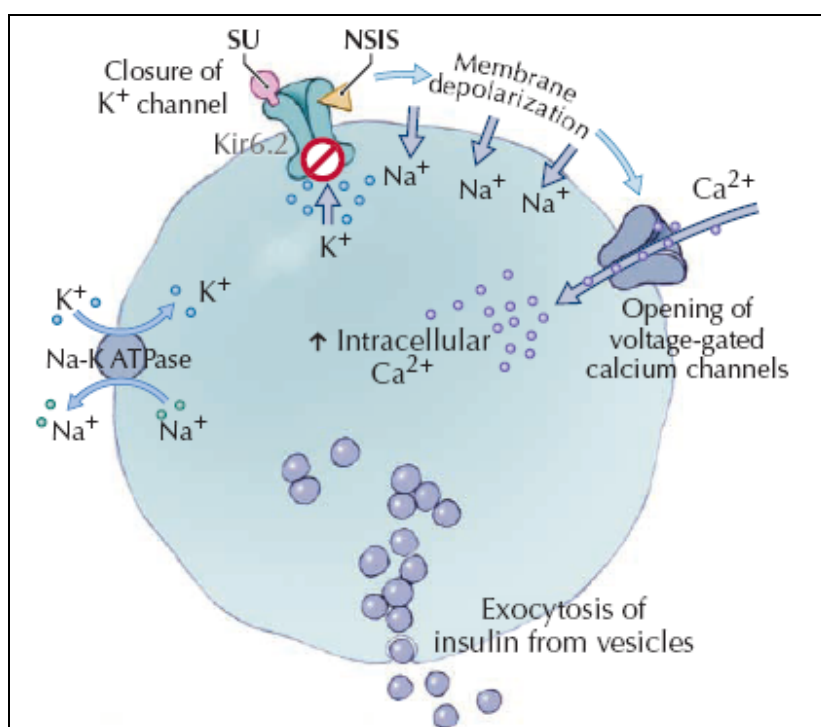


Figure 1-7 The site of action of sulfonylurea and non-sulfonylurea insulin secretagogues
Insulin secretagogues mimic glucose to close adenosine triphosphate sensitive potassium channels (kir6.2) and stimulate insulin secretion. (Cheng & Fantus, 2005)

First generation sulfonylureas such as tolbutamide and chlorpropamide block channels containing SUR1 (β -cell type) but not SUR2 (cardiac and smooth muscles type), whereas some second generation drugs such as glibenclamide, repaglinide and glimepiride, block both the channels (Proks *et al.*, 2002). In brief, tolbutamide and the half of the glibenclamide molecule containing the sulfonylurea group bind with site A in SUR1 and

the non-sulfonyl part of the glibenclamide binds with the site B in SUR 1. Thus, binding to one of these two sites is sufficient to produce the exocytosis of insulin (Henquin, 2004). Second generation sulfonylureas are most commonly used because of their high affinity to SUR1 and lower effective plasma levels, which lowers the risk of drug-drug interaction. However, there is no difference between the first and second generation sulfonylureas in their maximum hypoglycemic effect (Henquin, 2000; Henquin *et al.*, 2003; Henquin, 2004).

Experimental results of the UKPDS group (1998) suggest that there is a reduction in risk of occurrence of micro vascular complications by the use of sulfonylureas and a reduction of 16% in macro vascular complications with no increase in mortality being reported (Goldner *et al.*, 1971). The primary side effects of sulfonylureas are weight gain and hypoglycemia (Zimmerman, 1997). However, the University Group Diabetes Project (UGDP) reported a two fold increase in cardiovascular (CV) mortality among patients receiving tolbutamide compared with those receiving insulin or a placebo (Davis & Granner, 2001). In contrast to this, a recent study by UKPDS 33, did not demonstrate any increased CV mortality among patients receiving glypuride or chlorpropamide (UKPDS, 1998) and so this area remains controversial. Sulfonylureas are contraindicated in patients with moderate to severe liver dysfunction, whereas chlorpropamide and glyburide are contraindicated in patients with severe renal complication. They are more hydrophilic and have active metabolites that are eliminated partially through renal excretion and may cause renal failure (Rang *et al.*, 2003).

Like sulfonylureas, the non-sulfonylurea (benzamido) moiety of glyburide is also able to stimulate insulin release *in vitro* and also to some extent *in vivo* (Dornhost, 2001), suggesting the sulfonyl urea moiety may be vital for SUR1 binding rather than for SUR1 activation. Recently, newer classes of insulin-releasing meglitinide or glinides such as repaglinide and nateglinide have been developed. This group of drugs stimulate insulin release by binding with a different yet adjacent site on the SUR and they are glucose dependent and therefore they may have less risk of hypoglycemia several hours after meals (Gribble & Reimann, 2003; Rendell, 2004).

1.3.3.2 *Biguanide*

The second major class of oral antihyperglycemic agents presently used in the treatment of T2D is biguanide, in particular metformin (Bailey, 1992; Dunn & Peters, 1995; Bailey & Turner, 1996). The UKPDS data suggest that metformin is effective in overweight type 2 diabetic subjects (UKPDS, 1998). Metformin acts primarily by suppressing hepatic glucose production mostly by gluconeogenesis (Nosadini *et al.*, 1987; DeFronzo *et al.*, 1991; Stumvoll *et al.*, 1995; Cusi *et al.*, 1996) and to a small extent enhancing the insulin sensitivity in peripheral tissues (Inzucchi *et al.*, 1998; Johansen, 1999). In the liver, metformin acts by activating hepatic adenosine monophosphate-activated protein kinase (AMPK), inhibiting lipogenesis, increasing fatty acid oxidation, and by increasing insulin sensitivity. In addition, the activation of AMPK decreases expression of sterol-regulatory-element-binding-protein-1 (SREBP-1), which is a transcription factor involved in the pathogenesis of insulin resistance, dyslipidemia, and diabetes (Zhou *et al.*, 2001;

Kirpichnikov *et al.*, 2002) (Figure 1-8). In contrast to sulfonylureas, metformin does not stimulate insulin secretion. However, it can cause hypoglycemia in combination with insulin or insulin secretagogues.

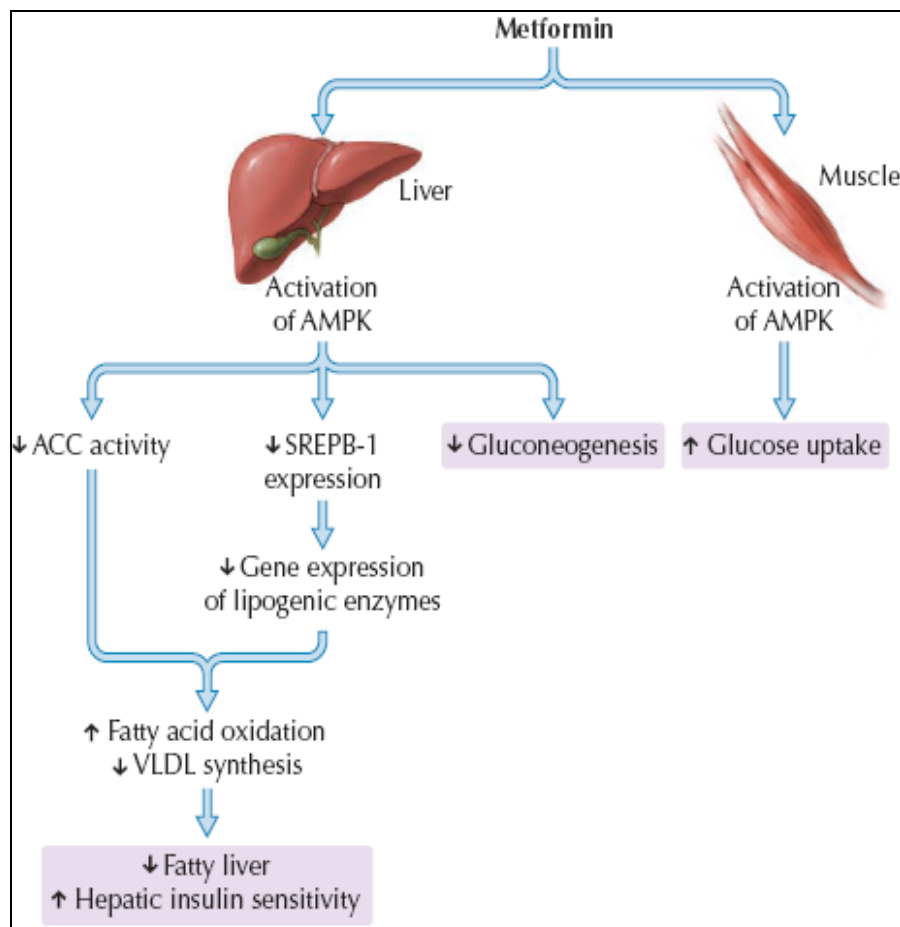


Figure 1-8 Metformin activates AMPK in liver and muscle to improve glucose and lipid metabolism in type 2 diabetes.

AMPK = adenosine monophosphate-activated protein kinase; ACC = acetyl-CoA carboxylase; SREPB-1 = sterol-regulatory-element-binding-protein-1; VLDL = very low density lipoprotein. (Cheng & Fantus, 2005)

Metformin is well distributed, with maximum accumulation in the small intestinal wall, it is eliminated without any change by the kidneys (Kirpichnikov *et al.*, 2002). The

beneficial biological effects of metformin beyond glycemic control includes: weight loss, lipid profile improvement, reduced free fatty acids (Abbasi *et al.*, 1997), triglycerides and very low density proteins (Landin *et al.*, 1991). The adverse effects of metformin includes decreased intestinal absorption (Ikeda *et al.*, 2000), weight loss (DeFronzo *et al.*, 1995), abdominal discomfort, anorexia and diarrhea. Lactic acidosis is a major adverse effect of biguanides, in particular phenformin, but it is said to be very minimal for metformin (Dunn & Peters, 1995). However, it has been contraindicated in patients with risk factors for lactic acidosis or drug accumulation (Hundal & Inzucchi, 2003)

1.3.3.3 Peroxisome proliferators-activated receptor-gamma (PPAR- γ) activators (Thiazolidinediones)

The new class of oral antihyperglycemic agents, thiazolidinediones, which were developed in the early 1980s, enhance insulin sensitivity thereby controlling glycemia in the absence of increasing insulin, and referred to as “insulin sensitisers” (Yki-Jarvinen, 2004). Thiazolidinediones include rosiglitazone and pioglitazone that do not increase insulin secretion but are dependent on the presence of insulin for their effects. It acts as ligands for the PPAR- γ in adipocytes (Figure 1-9). The activation of PPAR- γ suppresses lipolysis, decreases the plasma free fatty acids (FFA), leptin and TNF- α and increases adiponectin, which all lead to increases in insulin sensitivity (Aldhahi & Hamdy, 2003). The beneficial effect of thiazolidinediones on β -cell function has been reported in animal models (Lister *et al.*, 1999; Finegood *et al.*, 2001) but has not been demonstrated definitely in humans (Bell, 2003).

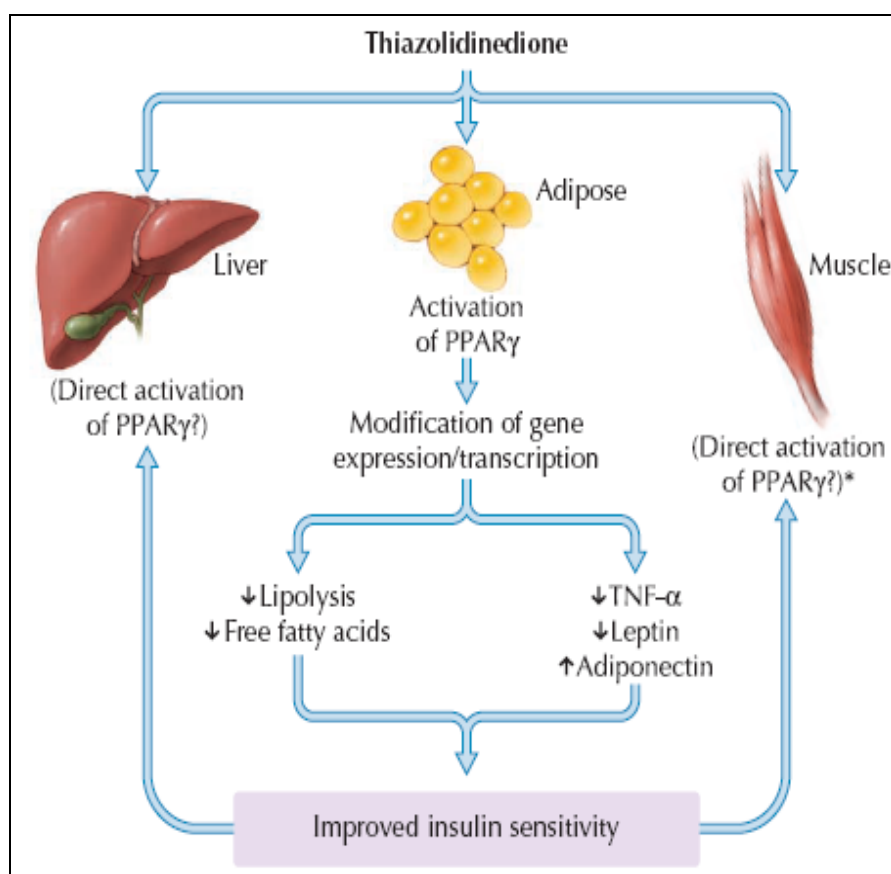


Figure 1-9 Mechanism of action of thiazolidinediones

PPAR γ - peroxisome proliferator-activated receptor-gamma; TNF- α - tumor necrosis factor- α .
(Cheng & Fantus, 2005)

The major side effects of this class of drugs are: weight gain, edema, anemia, and congestive heart failure (Kelly *et al.*, 1999; Nesto *et al.*, 2003). It is generally contraindicated in patients with congestive heart failure or with hepatic dysfunction.

1.3.3.4 *Alpha-glucosidase inhibitors*

The alpha-glucosidase inhibitors, acarbose and miglitol inhibit the membrane-bound intestinal alpha-glucosidase, which is responsible for the hydrolysis of complex carbohydrates to glucose and other monosaccharides; therefore the intestinal absorption is delayed. This delay decreases the development of postprandial hyperglycemia. (Lebovitz, 1997; Chiasson *et al.*, 1998), as the alpha-glucosidase inhibitors are less effective when compared to other antidiabetic drugs. The main adverse effects of this class of drugs include abdominal pain, diarrhoea, bloating, and flatulence.

1.3.4 Limitations

The current oral drug treatments often fail to maintain euglycemia due to progressive loss of β -cell function and these treatments often require multiple combinations of drugs that mostly potentiate the adverse effects. Insulin treatment has its own limitations that include: hypoglycemia, needle phobia, fear of stigmatisation, affordability and availability. The drugs for T2D available currently focus the reduction of hyperglycemia by either increasing insulin secretion or action but none of these drugs have so far shown significant alterations in the natural history of progressive loss of insulin secretion or β -cell dysfunction that leads to insulin injection therapy requirement. Dietary and lifestyle modifications can be impractical or difficult to implement and maintain for those individuals in middle age with longstanding habitually poor diet. Moreover people, especially those in developing countries without access to modern medicines, as well as those in developed countries looking for alternative options to avoid available

conventional medicines because of their side effects, will always look to accept naturally occurring, plant based therapies and traditional medicines.

1.4 Traditional antidiabetic plants

The use of plants in the treatment of conditions associated with diabetes dates back to 1550 B.C. (Bailey & Day, 1989). Even the most commonly used antidiabetic drug (metformin) was developed from the ‘French lilac’ (*Galega officinalis*), a herb used in Europe to treat hyperglycemia (Schafer, 1983; Bailey & Day, 1989). The review by Marles and Farnsworth (1995), reported an overview of the species of plants to be used to treat T2D, and remains a classical reference for researchers using ethnobotanical data to study treatment options for T2D. Over 800 plants have been documented for their antidiabetic potential, but most of them still warrant rigorous scientific examination (Gray & Flatt, 1997; Alarcon-Aguilara *et al.*, 1998).

Ayurveda, a traditional Indian system of medicine, advocates a wide range of medicinal plants to treat T2D. This system was described in scripts written in the time of *Charaka* and *Sushruta* in 6th century B.C (Grover & Vats, 2001). It advocates more than 100 plant species either singly or in combinations. Most of these Ayurvedic medicines exhibit antidiabetic properties but the data are inconclusive (Trivedi, 1963; Nagarajan *et al.*, 1982; Grover *et al.*, 2002b; Kar *et al.*, 2003). In most cases, the only parameter measured was the effect of extract on blood glucose, but these data alone are not sufficient to prove their antidiabetic property, thus it demands further examination. Marles and Farnsworth

(1995), suggest that the selection of plant species for screening novel antidiabetic molecules should give preference to species which have proven activity. Hence considering the importance of discovering lead molecules and their molecular mechanism from proven antidiabetic plant species, some of the extensively advocated Ayurvedic antidiabetic plants were selected for the research, presented in this thesis.

1.4.1 *Gymnema sylvestre* R.Br (GS)

GS is a woody, climbing shrub native to India, belonging to the family Asclepiadaceae (Figure 1-10). The leaves of this plant have been used traditionally in India for more than 2000 years to treat diseases (Grover & Vats, 2001; Grover *et al.*, 2002b). Chewing these leaves abolishes the sweet taste giving a Hindi name ‘gurmar’ meaning ‘sugar destroyer’. In recent years, the dried leaf extract have been studied extensively by various researchers for its hypoglycemic principles, activity and also for a number of other biological effects in both experimental animal and human models. Experimental reports have indicated that GS leaf extract decreases hyperglycemia in diabetic rats, rabbits and humans (Shanmugasundaram & Paneerselvam, 1981; Shanmugasundaram *et al.*, 1983; Srivastava *et al.*, 1985; Shanmugasundaram *et al.*, 1990a; Shanmugasundaram *et al.*, 1990b).



Figure 1-10 *Gymnema sylvestre* R. Br.

Chattopadhyay (1999) showed a significant dose-dependent blood glucose lowering activity of GS leaf extract in normal and streptozotocin (STZ)-induced diabetic rats. They also examined the effect of aqueous soluble portions of an alcoholic extract of GS leaves on glycogen content in normal and glucose-fed hyperglycemic rats and reported that the extract administration had significantly lowered glycogen content (Chattopadhyay, 1998). The water soluble extract of leaves of GS at a dose of 20mg/day for 20-60 days to STZ-induced diabetic rats significantly normalised the blood glucose concentration (Shanmugasundaram & Paneerselvam, 1981; Shanmugasundaram *et al.*, 1990a) and this result indicates that the glucose lowering effect of GS leaf extract may be mediated by an increase in insulin secretion.

The aqueous alcoholic extract of leaves of GS provided two potentially active principles: an acid soluble polyol-polyhydroxy cyclic compound, designated as conduritol A (Miyatake *et al.*, 1993); acid-insoluble triterpenoid saponins (gymnemic acids), designated as GS₃ and GS₄ (Shanmugasundaram *et al.*, 1990a). Conduritol A has been reported to have stimulatory effect on basal insulin secretion, but the mechanism remains undefined (Billington *et al.*, 1994). GS₃ and GS₄ have been reported to have an insulin-releasing activity in both *in vivo* and *in vitro* study models (Okabayashi *et al.*, 1990; Shanmugasundaram *et al.*, 1990a). In STZ-induced diabetic rats, administration of dried leaf powder of GS for 30-32 days significantly lowered the blood glucose concentration (Okabayashi *et al.*, 1990). In a similar experiment, GS₃ (60 days) and GS₄ (20 days) treatments significantly normalised the blood glucose and serum insulin concentrations and were also able to double the islet and β -cell number in the diabetic pancreas. An alcoholic extract of GS stimulated the insulin secretion from rat islets of Langerhans and various β -cell lines *in vitro*. This effect of GS on insulin secretion could be possibly by increased membrane permeability leading to an unregulated loss of insulin and also partly by channel-independent Ca^{2+} influx into the β -cells (Persaud *et al.*, 1999).

In humans, the reports of antidiabetic effects of GS leaf extract are minimal. Oral administration of GS leaf extract at a dose of 400 mg/day to 22 T2D patients for 18-20 months showed a significant reduction in blood glucose, glycosylated hemoglobin (HbA1c), plasma proteins and an increased serum insulin concentration, suggesting insulin-releasing effect either through β -cells regeneration or repair by extract treatment (Baskaran *et al.*, 1990). In T1D patients, oral administration of GS extract (400 mg/day)

lowered the fasting blood glucose, HbA1c, plasma proteins and insulin requirements as well as decreasing serum lipids (Shanmugasundaram *et al.*, 1990b).

Additionally to this hypoglycemic activity, the dried leaf powder of GS has been demonstrated to reverse the pathological changes in the liver, kidney and muscle tissues which were initiated during alloxan-induced hyperglycemic phase in rabbits. GS extract also decreased the gluconeogenesis enzyme activity, and increased the peripheral glucose uptake and the activity of enzymes involved in insulin-dependent glucose utilization including: phosphorylase; glycolytic enzymes; sorbitol dehydrogenase concentrations (Shanmugasundaram *et al.*, 1983). The peripheral glucose uptake activity of GS leaf extract was also evident in T1D patients. Administration of GS₄ to T1D patients resulted in a significant reduction in fasting blood glucose concentration (Shanmugasundaram *et al.*, 1990b), and further supported findings by Khare and colleagues (1983) where the dried leaf powder significantly lowered fasting blood glucose in T1D.

Overall, these experimental findings suggest that GS leaf extract treatment in diabetic conditions lowers the blood glucose concentration back to the normal physiological level, possibly due to its ability to stimulate endogenous insulin production mediated either by regeneration of pancreatic β -cells or by increased β -cell membrane permeability. However, the underlying mechanisms of the hypoglycemic effect of GS are still controversial because of limited knowledge of the effects that aglycones of triterpenoidal saponins have on insulin secretion (Persaud *et al.*, 1999). In contrast to all these findings, a few reports failed to demonstrate hypoglycemic effect of GS in STZ-induced diabetic

rats (Tominaga, 1996) as well as in normal subjects (Prakash *et al.*, 1986; Yoshikawa *et al.*, 1997). Yeh and colleagues (2003) reported that the glucose lowering effect of GS only exists in animals with residual pancreatic function and not in total pancreatectomised animals and this evidence supports the previous finding where it has been suggested that GS leaf extract acts mainly via stimulating insulin secretion rather than increasing peripheral glucose uptake (Shanmugasundaram *et al.*, 1990a). Over all these interesting findings suggests the potential importance of hypoglycemic constituents of GS and therefore demands further scientific investigations.

1.4.2 *Trigonella foenum-graecum* Linn (TFG)

TFG is an annual herb from the family Papilionaceae-Leguminosae, widely used as a remedy for treating diabetes mellitus over the ages (Moissides, 1939; Fourier, 1948). It is well known as a spice throughout the world and it is mostly used in many curry preparations for its pungent and aromatic properties (Max, 1992). Ayurveda advocates TFG seeds (Figure 1-11) as a potent treatment option for T2D and its associated complications (Vats *et al.*, 2002; Grover *et al.*, 2002b). The antidiabetic effect of TFG seed extract has been demonstrated in various experimental animal models as well as in humans (Moissides, 1939; Fourier, 1948; Khosla *et al.*, 1995; Abdel-Barry *et al.*, 1997; Vats *et al.*, 2002). Supplements of TFG seeds were shown to lower serum cholesterol, triglyceride and low-density lipoproteins in human patients and experimental models of hypercholesterolemia and hypertriglyceridemia (Basch *et al.*, 2003).



Figure 1-11 *Trigonella foenum-graecum* Linn (TFG) Seeds

Several clinical trials have demonstrated that the antidiabetic effects of TFG seeds ameliorate most metabolic symptoms associated with T1D and T2D diabetes in both humans and relevant animal models (Basch *et al.*, 2003). TFG is currently available commercially in encapsulated forms and is being prescribed as dietary supplements for the control of hypercholesterolemia and diabetes by practitioners of complementary and alternative medicine.

Supplementation of 20% TFG in the diet prior to STZ injection to mice showed a significant effect on blood glucose, free fatty acids, cholesterol, and triglycerides concentrations, suggesting a positive preventive role of TFG seeds against chemically-induced T2D (Amin *et al.*, 1988). In healthy, T1D, and T2D human volunteers the supplementation of TFG seed powder showed a significant reduction in postprandial

glucose (Sharma, 1986b; Madar *et al.*, 1988; Sharma & Raghuram, 1990; Sharma *et al.*, 1990). Despite this reduction in postprandial glucose, no significant change in plasma insulin was observed in T2D patients (Madar *et al.*, 1988), and also in some animal models (Madar, 1984; Ribes *et al.*, 1984). In contrast, some studies with alloxan-induced diabetic rats suggest that administration of TFG seeds significantly raised the plasma insulin concentration this effect was suggested to be due to its direct stimulatory effect on β -cells (Petit *et al.*, 1995a). In this context, Sauvaire and colleagues (1996) have reported an insulin-secretion stimulating compound, 4-hydroxyisoleucine from TFG seeds.

The hypoglycemic principles and their molecular mechanism responsible for antidiabetic property of TFG seeds were extensively investigated by various researchers. Earlier studies by Mishkinsky and colleagues (1967) and Shani and colleagues (1974) demonstrated that trigonelline, a major alkaloid from TFG, was a molecule responsible for the antidiabetic activity of TFG. Moorthy and colleagues (1989) reported the presence of an orally active antidiabetic fraction from TFG seeds, which was more potent than trigonelline. This fraction improved glucose tolerance in alloxan-induced diabetic rabbits, and reduced the concentrations of fasting blood sugar when administered to severe diabetic rabbits at a dose of 50 mg/kg for 30 days. In addition, they also reported an improvement in HbA1c, serum lipid profile, activation of glycolytic enzymes present in muscle and minimal inhibition of gluconeogenic enzymes in liver and kidney. However, to date there have been no reports on the structural composition of this potent fraction.

Defatted extract of TFG seeds which contains fibre, saponins and proteins showed a significant hypoglycemic activity along with positive effects on pancreatic hormone concentrations such as insulin, glucagon and somatostatin in normal and diabetic dogs, suggesting its beneficial effect on carbohydrate homeostasis (Ribes *et al.*, 1984). Further fractionation of defatted extract yielded two sub-fractions ‘a’ and ‘b’. The sub fraction ‘a’, which contained testa and endosperm and is rich in fibre, showed a potent antidiabetic property (Madar, 1984; Ribes *et al.*, 1986; Sharma, 1986b). An insulin-stimulating substance, 4-hydroxyisoleucine was identified in the seeds of TFG (Hilaire-Buys *et al.*, 1993) and reported to have hypoglycemic activity in both *in vitro* and *in vivo* experiments (Petit *et al.*, 1995a; Sauvaire *et al.*, 1996). 4-hydroxyisoleucine is an unusual amino acid, which represents up to 80% of free amino acids in TFG seeds (Sauvaire *et al.*, 1984). It was first isolated and identified by Fowden and colleagues (1973) and further structure conformation was described by Alcock and colleagues (1989). During *in vitro* experiments using isolated pancreas perfused with glucose, treatment with 4-hydroxyisoleucine at a concentration of 200 $\mu\text{mol/L}$ evoked a biphasic insulin response (Sauvaire *et al.*, 1996). In a more recent study, the dialyzed aqueous extract (8000 cut off) of TFG was found to have hypoglycemic potential, stimulating insulin signaling pathways in adipocytes and liver cells by activating tyrosine phosphorylation of insulin receptor (IR) (Vijayakumar *et al.*, 2005).

Coumarins, one of the constituents of TFG have demonstrated potent hypoglycemic effect in both normal and alloxan-induced diabetic rats (Shani *et al.*, 1974). Apart from these, other postulated hypoglycemic constituents from TFG seeds are scopoletin (Shani

et al., 1974), and fenugreekine (a steroidal sapogenin ester) (Ghosal *et al.*, 1974). However, no reports were found on relevant hypoglycemic doses and effective TFG concentration needs more investigation.

TFG seed is a rich source of fibre and gums. It is very well known that dietary intake of fibres in diabetic patients results in a reduction of blood glucose (Monnier *et al.*, 1978; Jenkins, 1979; Jenkins & Jenkins, 1984). In this context, Madar and colleagues (1984) postulated that TFG may modulate plasma glucose concentrations by delaying gastric emptying and by directly interfering with glucose absorption at gastrointestinal level. *In vitro* experiments using inverted gut sac of male rats, addition of TFG seed powder to the mucosal side produced a significant inhibition of glucose transport to the serosal side (Madar, 1984) suggesting that dietary fibres in the form of galactomannan (Sharma, 1986a), which resembles guar gum (Ribes *et al.*, 1984), is a major contributing factor for the decreased blood glucose concentration. The insignificant effect of TFG powder on insulin level from this study suggests that reduction of plasma glucose is mediated by either inhibition of diffusion or transport of glucose without involving any intestinal hormonal factor (Sharma, 1986b; Madar *et al.*, 1988). Sharma and colleagues (1986b) reported that extracts excluding non-mucilaginous fibre resulted in very little hypoglycemic effect. Recent investigations in both *in vitro* and *in vivo* experiments have shown that gel fractions of TFG seeds, which are rich in galactomannan, are responsible factors, which reduce plasma glucose by increasing the viscosity of gut contents (Madar, 1990).

TFG seed powder administration in both *in vivo* and *in vitro* experiments decreased glucose absorption and starch digestion, suggesting its possible effect on carbohydrate digestion, which may be postulated as an additional possible mechanism of action of TFG (Madar, 1990).

Ali and colleagues (1995) have shown that methanolic extract and the spent material after methanol extraction of TFG seeds had a significant hypoglycemic effect. Further, the significant hypoglycemic effect of the fibre was observed only when the extracts were fed simultaneously with glucose in normal and T2D model rats, suggesting that fibre might be only responsible for the observed improvements in glucose tolerance and may not have any hypoglycemic effects and thus the association of other components and mechanisms as observed in other experiments should not be ignored.

Apart from these antidiabetic effects, the TFG seed also showed significant hypocholesterolaemic effects in both experimental animals and human (Singhal *et al.*, 1982; Sharma, 1984, 1986a; Madar *et al.*, 1988; Sharma *et al.*, 1990). These effects have been associated mainly with reduced intestinal reabsorption of cholesterol and bile acids and this activity has been speculated to be associated with saponins, sapogenins and galactomannan fibre contents of TFG (Ribes *et al.*, 1987).

Overall, the hypoglycemic and antihyperglycemic effects of TFG seeds have been attributed mainly to gastrointestinal effects of dietary fibres and to pharmacological effects of some active principles such as 4-hydroxyisoleucine, coumarins and saponins

sapogenins, and many unidentified components. The potential effect of TFG seeds demands further investigation in terms of more scientific evaluation on the systemic effect of active principles of TFG and their structural elucidation.

1.4.3 *Curcuma longa* Linn. (CL)

CL rhizomes (Figure 1-12), known as turmeric, have been widely used as a spice for its coloring, flavoring, medicinal and nutritional properties, particularly in Asian countries. In the traditional Ayurvedic system of medicine, CL has been documented as an antiinflammatory, antimicrobial agent and also for its numerous other curative properties (Ammon & Wahl, 1991).



Figure 1-12 *Curcuma longa* Linn. (CL) rhizomes

Supplementation of CL and its active ingredient, curcumin, to diabetic rats has been shown to attenuate hyperglycemia possibly by reducing glucose influx through polyol pathway (Arun & Nalini, 2002b). Administration of CL to alloxan-induced diabetic rats significantly reduced the blood sugar, HbA1c concentrations and oxidative stress encountered by diabetic rats (Arun *et al.*, 2002a). The ethanolic extract of CL showed a potent hypoglycemic effect on genetically diabetic KK-A^y mice (Kuroda *et al.*, 2005). In their study, the extract stimulated adipocyte differentiation in a dose-dependent manner and also showed a PPAR- γ ligand-binding activity.

Curcuminoids (curcumin and its analogues), yellow coloured compounds from CL, have been reported to have cholerectic, hydrocholagogic (Ramprasad & Sirsi, 1956), hypocholesterolemic (SubbaRao *et al.*, 1970; Patil & Srinivasan, 1971), antiinflammatory (Srimal, 1997), anticarcinogenic (Huang *et al.*, 1994; Kuo *et al.*, 1996) and antioxidative properties (Quiles *et al.*, 1998; Asai *et al.*, 1999). In addition, a recent study by Kuroda and colleagues (2005) demonstrated the isolation of curcuminoids and sesquiterpenoids from the ethanolic extract of CL. They reported that curcumin, demethoxycurcumin, bisdemethoxycurcumin and ar-turmerone, were the main active ingredients of CL that contribute to the antidiabetic effect via PPAR- γ activation. In contrast to the above findings, curcumin has been reported to have an inhibitory effect on insulin-induced GLUT4 translocation and glucose transport (Ikonomov *et al.*, 2002) and cyclic AMP protein kinase (cAMP) (Hasmeda & Polya, 1996).

In addition to these hypoglycemic actions, CL treatment was shown to be effective against the development of diabetic cataract in STZ-induced rats (Suryanarayana, 2005). In the same experiment, CL treatment countered the hyperglycemia-induced oxidative stress which was evident through normalisation of lipid peroxidation, reduced glutathione, protein carbonyl content, osmotic stress, and activities of antioxidant enzymes, suggesting that this could be possibly by antioxidant properties of extract (Suryanarayana, 2005). Suresh Babu and Srinivasan (1998) reported that the dietary curcumin ameliorate renal lesions in STZ diabetic rats. Their observations demonstrated that dietary curcumin decreases the leaching of renal tubular enzymes and albuminuria, partially reverses some alterations in the activities of kidney cellular enzymes associated with diabetes, reverses the decrease in membrane integrity ratio and ATPase activity of renal membranes, and lowers nephromegaly in diabetic animals (Suresh Babu & Srinivasan, 1998).

Despite numerous experiments that demonstrated that curcumin and its analogues, are potent antioxidants, have blood glucose lowering effect and various pharmacological effects, no study has examined the effect of the aqueous extract of CL for its antidiabetic effect separate from antioxidant effect. Moreover, the potent antioxidant activity of CL and their beneficial role in the autoimmune diseases such as T1D, has gained a considerable attention in the recent alternative supplementary therapies.

1.4.4 *Pterocarpus marsupium* Roxb. (PM)

PM is a moderate to large, deciduous tree commonly known as Indian Kino, distributed well in hilly regions of India (Figure 1-13). In Ayurveda it is widely used as a magical remedy to treat diabetes and also various other associated complications (Satyavathi *et al.*, 1987; Warriar *et al.*, 1995). Overnight water stored in a wooden tumbler made up of heartwood of PM is advocated by the Ayurvedic physicians as a traditional therapy to treat diabetes especially in central part of India (Chopra *et al.*, 1958; Jain, 1968; Maheshwari *et al.*, 1980). Kino is the dried exudation obtained by incising the trunk and traditionally used as an astringent, antidiarrhoeal. The gum is used for toothache, the bark is used for diarrhoea, heartburn, and management of diabetes and the leaves are used for boils, sores, and skin diseases (Grover *et al.*, 2002b).



Figure 1-13 *Pterocarpus marsupium* Roxb (PM) heartwood.

A number of reports claim that the extract of PM exhibits hypoglycemic action in various experimental animal models (Jogelkar *et al.*, 1959; Gupta, 1963; Trivedi, 1963; Shah, 1967; Saifi *et al.*, 1971; Pandey & Sharma, 1978; Chakravarthy *et al.*, 1980; Ahemed *et al.*, 1991; Dhanabal *et al.*, 2006) and also in humans (Sepaha & Bose, 1956; Pandey & Sharma, 1975; Rajasekharan & Tuli, 1976; Kedar & Chakrabarti, 1981; ICMR, 1998).

The experimental findings by Chakaravarthy and colleagues (1981a; 1981b) reported that the flavanoid fraction from the bark of PM regenerates β -cells in alloxan-induced diabetic rats. They also isolated a hypoglycemic principle, (-)-epicatechin, a benzopyran derivative, from the ethyl acetate soluble portion of alcoholic extract of the bark of PM (Chakravarthy *et al.*, 1980; Chakravarthy *et al.*, 1981a; Chakravarthy *et al.*, 1981b; Chakravarthy & Gode, 1985) and reported that the administration of (-)-epicatechin in alloxanised rats has a prophylactic effect against alloxan-induced necrosis of the β -cell population of the pancreas and suggested that the (-)-epicatechin could provide a new approach in the treatment of diabetes mellitus. However, these claims have been questioned by Kolb and colleagues(1982) and Sheehan and coworkers (1983) . Kolb and colleagues (1982) repeated Chakravarthy's original experiments and also tested the effect of STZ on (-)-epicatechin pre-treated rats; they found that the (-)-epicatechin had no effect on chemically-induced diabetes in rodents. Similarly, in another study, the treatment of (-)-epicatechin (30mg/kg twice daily for 3 days) in alloxan-induced diabetic rats did not show any significant difference to the control and suggests that, at least in alloxan-treated diabetic rats, (-)-epicatechin does not stimulate islet β -cell regeneration or decrease hyperglycemia once the diabetic state has been firmly established (Sheehan *et al.*, 1983). Therefore because of these contrary findings on (-)-epicatechin, further studies

are warranted before it can be considered as a potent antidiabetic agent in T1D or for use in human clinical trials.

Ahmad and coworkers (1991b) studied the effect of (-)-epicatechin on cAMP content, insulin release and conversion of proinsulin to insulin in immature and mature rat islets *in vitro*. They found that (-)-epicatechin causes a significant and dose-dependent increase in islet cAMP content, insulin release and the conversion of proinsulin to insulin at both non-stimulatory (2 mM) and stimulatory (20 mM) glucose concentrations. They also reported that the immature (1 month old) rats respond better to (-)-epicatechin as compared to mature rats (12 months old) and this more pronounced effect of (-)-epicatechin on immature rats than in mature rats may be attributed to the aging factors and configurable changes in β -cells. Further, their studies reported that the (-)-epicatechin stimulates insulin release in both static incubations as well as in perfusion, suggesting its possible effect on Ca^{2+} uptake in the isolated islets of Langerhans *in vitro*. The increase in intracellular Ca^{2+} concentration in the islets treated with (-)-epicatechin may be responsible for an increase in cAMP content. In addition they also reported that the (-)-epicatechin did not have any effects on the release of glucagon. These findings prompted this group to suggest (-)-epicatechin as a potential hypoglycemic agent.

Rizvi and colleagues (1995) demonstrated that the (-)-epicatechin (1 mM) shows a protective effect like insulin on the osmotic fragility of human erythrocytes. Insulin exerts its effects on erythrocytes by the activation of Na/H antiport and stimulation of ouabain sensitive Na/K-ATPase. They also found that the ouabain destroyed the protective

effects of insulin but failed to abolish the (-)-epicatechin activity and suggested that (-)-epicatechin and insulin may act via different mechanisms while eliciting their protective effects on red cell osmotic fragility of human erythrocytes. In this context, Abu Zaid and coworkers (2002) also demonstrated the effect of (-)-epicatechin on calcium-ATPase activity in normal and diabetic human erythrocytes. They found that (-)-epicatechin mimics insulin action on both normal and diabetic human erythrocytes by increasing the activity of calcium-ATPase.

Apart from these findings on hypoglycemic potentials of the bark of PM and its active ingredient (-)-epicatechin, a number of other polyphenolic constituents from the aqueous extract of the heartwood of PM were examined by many investigators. Polyphenols are abundant in plants, and includes other subclasses besides flavanoids such as phenolic acids, stilbenes, lignans, tannins and oxidized polyphenols. Polyphenols have various biological properties and they have been mainly used as antioxidants in the prevention of several chronic diseases involving oxidative stress (Rice-Evans, 1995). PM is a rich source of polyphenols (Seshadri, 1972; Adinarayana & Syamasundar, 1982; Maurya *et al.*, 1982; Mathew & Rao, 1983; Maurya *et al.*, 1984; Bezuidenhoudt *et al.*, 1987), 2004). Manickam and colleagues (1997) examined the antihyperglycemic activity of three major phenolics present in the ethyl acetate soluble portion of the aqueous extract of the heartwood of PM, being marsupsin (a benzofuranone), pterosupin (a dihydrochalcone), and pterostilbene (a stilbene). They found that the treatment with marsupsin and pterostilbene (20 mg/kg for 3 days) to STZ-induced diabetic rats significantly decreased the plasma glucose concentration, whereas treatment with pterosupin (20 mg/kg for 3

days) failed to normalise hyperglycemia. However, all these treatments significantly decreased the bodyweight. In addition, the investigators also found that the effect of pterostilbene was comparable to the effect of metformin and suggest that this compound may have insulin-like effects on several tissues and/or may suppress hepatic gluconeogenesis, stimulate glycolysis, inhibit glucose absorption from the intestine or act by other mechanisms and therefore recommend further investigations on these compounds before they were considered for therapeutical application.

Anandrajan and colleagues (2005) isolated an isoflavone from the methanolic extract of the heartwood of PM and studied the effect on cellular targets such as GLUT4, PPAR- γ and PI3-kinase. They performed a sequential extraction of increasing polarity with various solvents and picked the maximum glucose uptake activity fraction (fraction no-9) using L6 myotubes *in vitro* bioassay. The active fraction was elucidated to be an isoflavone (7-O- α -L-rhamnopyranosyloxy-4'-methoxy-5-hydroxy-isoflavone) using nuclear magnetic resonance and mass spectrometric studies. The treatments with the methanolic extract and an isoflavone significantly increased the glucose uptake, GLUT4 translocation, and PPAR- γ . This effect was comparable with that of insulin. Further they reported that the incubation of the extract with cycloheximide (a protein synthesis inhibitor), completely inhibited troglitazone-mediated glucose uptake, suggesting that new protein synthesis is required for increased glucose transport. Their results also showed that the isoflavones failed to activate PI3-kinase, unlike the methanol extract, prompting these investigators to postulate that an isoflavone from PM may activate glucose transport via a PI3-kinase-independent pathway.

The phenolic constituents also exhibited antihyperlipidemic effects. The earlier findings by Pandey and Sharma (1978) on the hypocholesterolemic activity of the aqueous decoction of the heartwood of PM was substantiated by recent studies by Farboodniai Jahromi and colleagues (1993). In their experiment, they studied the effect of the ethyl acetate soluble fraction of the aqueous decoction of PM along with three other purified principles from the ethyl acetate fraction namely marsupsin, pterosupin, and liquiritigenin, on both diet-induced hyperlipidemic and triton-induced hyperlipidemic rats. They reported that these treatments significantly reduced the serum cholesterol triglycerides, and LDL and VLDL cholesterol without affecting HDL-cholesterol in both hyperlipidemic models. Pterosupin was found to be the most potent hypolipidemic and hypocholesterolemic agent out of the pure principles examined and further suggested that the activity of pterosupin was comparable with cholestyramine, a conventional treatment used to control cholesterol. Pterostilbene, a stilbene derivative has been reported in PM (Maurya *et al.*, 1984) and shown to have a hypoglycemic effect (Manickam *et al.*, 1997). As discussed earlier it also acts as an agonist for PPAR- α , which possesses an activity comparable to clinically used hypolipidemic fibrate drug, providing a possible alternative for the treatment of dyslipidemias (Rimando *et al.*, 2005). Mallavadhani and Sahu (2003) suggested that the relative high content of pterostilbene in heartwood can be used as a quality control marker in the authentication of PM.

In addition to these potential hypoglycemic and hypolipidemic effects, the aqueous extract of PM has also been reported to have peripheral effects. The effect of aqueous

extract of PM on glycogen levels of insulin-dependent (skeletal muscle and liver) and non-insulin-dependent tissues (brain and kidneys) and enzymes such as glucokinase, hexokinase and phosphofructokinase were studied by Grover and colleagues (2002a). They found that feeding the extract to STZ-treated diabetic rats significantly reduced the blood glucose after the 15th and 30th day when compared with the control. PM treatment normalised the kidney weight in diabetic rats and partly prevented the increase in renal glycogen content and the reduction in hepatic and skeletal muscle glycogen. They also found that the PM treatment partially corrected alterations in hexokinase and glucokinase with complete normalisation of phosphofructokinase in diabetic rats compared to controls (Grover *et al.*, 2002a).

There are a few encouraging clinical studies reports suggesting the potential effect of PM in humans (Sepaha & Bose, 1956; Pandey & Sharma, 1975; Rajasekharan & Tuli, 1976; Kedar & Chakrabarti, 1981; ICMR, 1998). An Indian based flexible dose double blind multicentric study assessing PM in the treatment of newly diagnosed patients with T2D demonstrated that the extract controlled fasting and postprandial blood glucose concentrations in 69% of participants after 12 weeks of treatment (ICMR, 1998). Unlike the significant reduction in lipid concentrations in both diet-induced and triton-induced hyperlipidemic rats (Farboodniay *et al.*, 1993) and the significant hypocholesterolemic effect on healthy rabbits (Pandey & Sharma, 1978), no significant change was observed in the mean concentrations of lipids in the PM treated diabetic patients. They also reported that the treatment of PM in T2D patients did not produce any adverse side effects and could be used in the treatment of newly diagnosed or untreated mild T2D

patients (ICMR, 1998). Overall, PM has been found to exert a number of beneficial effects in addition to its hypoglycemic action. Though there are numerous encouraging experimental results with evidence to show that PM has antidiabetic actions, there is no conclusive evidence to reveal the hypoglycemic principles of PM and their mode of action.

1.5 Summary

The increasing prevalence and incidence of diabetes demands novel solutions for its treatment and management in the future. The treatments which are currently used for T2D often require combinations of drugs with an increased potential for adverse effects. Many currently available medications for T2D diabetes reduce hyperglycemia either by increasing insulin secretion, production and sensitivity or reduce hepatic glucose uptake and absorption of dietary carbohydrates or delaying gastric emptying or mimicking insulin action or altering renal glucose handling, but none have so far convincingly demonstrated that they can beneficially alter the natural history of progressive loss of pancreatic insulin that can culminate with exogenous insulin therapy. Therefore research into the novel molecules that could preserve and protect or indeed increase β -cell mass along with other beneficial peripheral effects would be a major advance in the treatment of diabetes. Consequently, scientists are now investigating a number of traditional medicinal plants. Ayurvedic plants such as *Trigonella foenum-graecum* Linn (TFG), *Pterocarpus marsupium* Roxb (PM), *Gymnema sylvestre* R.Br (GS) and *Curcuma longa*

Linn (CL) have been extensively advocated in traditional medicines systems to treat T2D and related complications. All these plants have shown significant antidiabetic effects in both experimental animals and also in T1D and T2D humans, although in most cases their active components have not yet been characterised.

Previously, most work undertaken on these plants to identify and isolate the active constituents has been carried out using a single experimental model such as chemically-induced (alloxan or STZ) diabetic animals or isolated pancreatic islet or various cell lines. Though much of this initial work is essential when identifying biochemical pathways and verifying activities, it still remains unclear what potential effects these drugs may have on tissues primarily involved in glucose homeostasis (*in vitro*) and also on glucose clearance and insulin secretion in normoglycemic, non-diabetic individuals (*in vivo*).

Hence, insulin secretion and action are the two major components of T2D pathogenesis; the identification of more potent hypoglycemic constituents from these plants which exhibit multiple therapeutic actions such as stimulating insulin secretion and action, and also potential effects on glucose clearance and insulin secretion on non-diabetic individuals, would possibly provide rationale therapeutic options for the treatment of T2D.

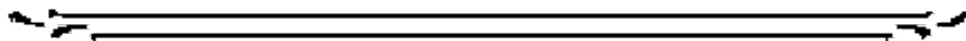
1.6 Aim

The present study aims:

- 1) To investigate the potential effects of some of the extensively advocated Ayurvedic antidiabetic plants such as *Trigonella foenum-graecum* Linn, *Pterocarpus marsupium* Roxb, *Gymnema sylvestre* R.Br and *Curcuma longa* Linn on tissues primarily involved in glucose homeostasis;
- 2) To isolate, purify and identify a potent hypoglycemic constituent(s) from these plants, based on their potential effects on both glucose uptake and insulin secretion, by using *in vitro* bioassay-guided fractionation methods;
- 3) To derive their modes of action using both mouse muscle and pancreatic tissues *in vitro* and non-diabetic normal sheep *in vivo* as experimental model systems.

In this regard, the current study may identify a potent antidiabetic fraction from these sources having beneficial effects on both insulin secretion and action, hopefully providing an alternative in the treatment of T2D.

2 General Methods and Materials



2.1 Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and the antibiotic-antimycotic solution used (10,000 units/ml penicillin G sodium, 10,000 µg/ml streptomycin sulphate, 25 µg/ml amphotercin B, 0.85% saline) were purchased from Gibco, Invitrogen Australia Pty Limited, Mount Waverley, VIC 3149, Australia. Bovine Serum Albumin (BSA), wortmannin, diazoxide, arginine, metformin and tolbutamide were purchased from Sigma Aldrich Pty Limited, Castle hill, NSW 1765, Australia. Human insulin (Actrapid®) was purchased from Novo Nordisk Pharmaceutical Pty Limited, North Rocks, NSW 2151, Australia. Tissue culture plates, Syringe filters (0.45µm and 0.22µm) were purchased from Sarstedt Australia Pty Limited, South Australia 5095, Australia. All of the chemicals used were of analytical grade, unless otherwise specified.

2.2 Plant materials and extract preparation

Gymnema sylvestre R.Br leaves (GS) and *Curcuma longa* Linn (CL) rhizome powders were purchased from Austral Herbs, Uralla, NSW 2358, Australia. *Pterocarpus marsupium* Roxb hard wood (PM) was obtained from Ayurvedic medical store, India. *Trigonella foenum-graecum* Linn (TFG) seeds were purchased from local commercial health food market, Armidale, NSW 2350, Australia.

All extraction and purification procedures were performed at room temperature unless otherwise specified. Aqueous extracts of GS, CL, and TFG were prepared by soaking 10g of these plant materials in 1 litre of distilled water for 10 days. Extraction of PM was accomplished by soaking 118 g of the dried hardwood pieces for 10 days in 10 litre distilled water. After this period, the aqueous extract of PM was removed and the extraction was repeated to reach a total volume of 30 litres. All extracts obtained were then filtered (Whatman no.1) to remove any particles and the filtrate was centrifuged at 5000 rpm for 15 min to remove any fine particles and stored at -20°C . Immediately before the extract was to be used in treatment, the solution was sterilised through a $0.22\text{ }\mu\text{m}$ syringe filter. These extracts were identified as GSE, CLE, TFGE and PME.

2.3 Animal ethics

Animal experimentations using mice and sheep were approved by University of New England Animal ethics committee and are in accordance with NH &MRC guidelines for animal experimentations.

2.4 *In vitro* bioassay

2.4.1 Animals and tissue preparation

Adult male Swiss mice weighing approximately 18 to 22 g were obtained from the Physiology breeding house at the University of New England, Armidale, NSW 2351,

Australia. Mice were maintained in a temperature (21°C) and light (12:12) controlled room with a standard diet of rodent chow and water. They were euthanised via CO₂ asphyxiation. The pancreas and the skeletal muscle from the abdomen were removed and placed on ice in phosphate buffered saline (PBS) of neutral pH 7.4. Muscle and pancreas tissues were dissected into strips approximately 5mm long, 2mm wide, 2mm thick and 2mm long, 2mm wide, 2mm thick respectively and rinsed with phosphate buffer saline (PBS) immediately before the incubation.

2.4.2 Tissue culture assay

An in-house developed tissue culture method was employed to evaluate the effect of experimental treatments on tissues involved in glucose homeostasis. In brief, tissues (5 pieces/well) were separately transferred to the 24 well tissue culture plates with each well containing 1ml of DMEM, supplemented with 0.1% bovine serum albumin and 1% antibiotic and antimycotic solution. Glucose concentrations in the media were maintained at 5 and 12 mM to mimic normal and hyperglycemic culture conditions respectively. Incubation was performed in a humidified atmosphere of 5% CO₂ in air at 37°C for 24 hours. Immediately after the experiment, media and tissue samples were stored at -20°C for further analysis. Dose response (10 fold diluted doses) of all treatments and positive controls on the above tissue culture method were conducted prior to any activity evaluation. A dose that represents a maximal efficacy was chosen for subsequent studies. All the treatments, control and positive control were examined as quadruplicate replicates and all the sample analysis were carried out in duplicate replicates.

2.5 *In vivo* bioassay

2.5.1 Animals and Plasma sample collection

Sheep (Merino wethers) were obtained from the Rural Properties at the University of New England (Armidale, NSW). They were housed in pens (12 m² in area) the day before the experiment and allowed *ab lib* access to feed (blend of White chaff and Lucerne) and water. All the animals were weighed immediately before the experiment and randomly divided into groups with respect to age and weight. An intravenous catheter was inserted into either left or right jugular vein of each sheep. Blood samples, approximately 10 ml were collected in pre-heparinized polypropylene vials with 20 µl of lithium heparin (1000 IU/ml). The inserted catheter tubing was flushed with 3 % sodium citrate after the blood collection to maintain patency. Blood samples were then centrifuged at 3000 rpm, for 20 minutes and the supernatant plasma samples were transferred to a 5 ml sample vial and stored at -20°C until required for analysis.

2.6 Glucose analysis

The frozen samples were thawed at room temperature and 100 µl of culture media/plasma samples were analyzed using DADE clinical analyzer (DADE-XL, Dupont, USA). The glucose method is an adaptation of the hexokinase-glucose-6-phosphate dehydrogenase method and this method is more specific than general reducing sugar methods (Kunst *et al.*, 1983).

2.7 Insulin (Radioimmunoassay)

Pancreas culture media and plasma samples insulin concentrations were determined by radio immunoassay (RIA) specific for insulin. Guinea pig antiserum (Sigma Aldrich Pty Limited) raised against bovine insulin was used as an antibody and bovine insulin (Sigma Aldrich Pty Limited, Castle hill, NSW 1765, Australia) as standard. Insulin concentration of the culture media and plasma samples were calculated in $\mu\text{IU/ml}$ using a linear equation derived from log/logit transformation of the RIA standard curve.

2.7.1 Culture media sample

One hundred micro liter of culture media samples and standards (in DMEM) were added to RIA tubes in duplicate followed by the addition of 100 μl of anti-bovine insulin (1:8000) and 100 μl of radioactive insulin tracer (20,000 cpm/100 μl). All these tubes were vortexed to mix thoroughly and incubated at 4°C overnight. After overnight incubation, 1.2 ml of 22% polyethylene glycol (PEG) and 100 μl of charcoal striped horse serum (CSHS) were added and further incubated for 15-20 min at room temperature then centrifuged at 3000 rpm for 30 minutes followed by aspiration. The pellet containing the bound reactive ligand was immediately counted for γ -decay using an automatic Gamma counter (Wallac, 1470 WIZARD).

2.7.2 Plasma sample

100 µl of plasma samples and standards (in CSHS) were added to RIA tubes in duplicate followed by the addition 100 µl of anti-bovine insulin (1:8000) and 100 µl of radioactive insulin tracer (20,000 cpm/100 µl). All these tubes were vortexed and incubated at 4°C overnight. After overnight incubation, 1.2 ml of 22% polyethylene glycol (PEG) were added to all the tubes and further incubated for 15-20 min at room temperature, then centrifuged at 3000 rpm for 30 minutes followed by aspiration. The pellet containing the bound reactive ligand was immediately counted for γ -decay using an automatic Gamma counter (Wallac, 1470 WIZARD).

2.8 SDS-PAGE

The molecular weight determinations of samples were carried out as described by Laemmli (1970) with a small modification. Electrophoresis of samples along with standard molecular weight markers (BIO-RAD) through 4-15% sodium dodecyl sulphate-polyacrylamide gel was performed (Mighty Small Tail System, Hoefer, San Fransisco, SA, USA). The samples were added to both reducing and non reducing sample buffer (2X) to a dilution of (1:2). All samples were heated to 95°C for 15 minutes and then loaded on the gel and run at 15mA/gel.

2.8.1 Protein detection

Gels were stained in Roeder's stain (0.25mg/ml Coomassie R-250, 25% isopropanol, 10% acetic acid) overnight and de-stained (10% acetic acid in 20% Methanol) until the background was clear.

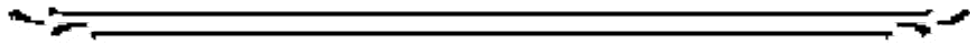
2.8.2 Glycoprotein detection

Gels were stained to detect glycoproteins using a commercially available glycoprotein detection kit (GLYCO-PRO, Sigma Aldrich Pty Limited).

2.9 Data analysis

Basal glucose uptake was calculated based on the difference in glucose concentration between control wells with and without tissues. Glucose uptake in response to various treatments was calculated as a percent variation from basal levels (% glucose uptake). All the experiments were repeated thrice (n=3) unless otherwise specified. Experimental data were analysed statistically, using the general linear model procedure in SAS statistical software (SAS Institute Inc. Cary, NC, USA) . The data were evaluated using 2-way ANOVA followed by Student-Newman Keuls *post hoc* test. Values were considered to be significantly different at $p < 0.05$ and presented as mean $\pm$ standard error ($\pm$ S.E).

3 Preliminary *in vitro* Screening



3.1 Introduction

The increasing prevalence and incidence of diabetes requires the development of novel drug treatment and management of this disease in the future. Plants have been a source of medicines since ancient times and most of the drugs currently available are derived from natural products. The ethnomedical and the traditional knowledge of the plant treatments always have been recognized by millions of the people around the world and the use of plants in the treatment of conditions associated with diabetes was known for centuries

Ayurveda, one of the ancient Indian traditional medicine system advocates many plant preparations in the treatment and management of diabetes. Plants such as *Trigonella foenum-graecum* Linn (TFG), *Pterocarpus marsupium* Roxb (PM), *Gymnema sylvestre* R.Br (GS) and *Curcuma longa* Linn (CL) have been extensively advocated traditional medicines to treat T2D and related complications (Trivedi, 1963; Warriar *et al.*, 1995; Grover *et al.*, 2002b). The antidiabetic effect of these plants has been studied in animals (Shah, 1967; Saifi *et al.*, 1971; Okabayashi *et al.*, 1990; Khosla *et al.*, 1995; Persaud *et al.*, 1999; Zia *et al.*, 2001; Arun & Nalini, 2002b; Kuroda *et al.*, 2005) as well as in humans, especially in T1D patients (Shanmugasundaram *et al.*, 1990a; Shanmugasundaram *et al.*, 1990b; Sharma *et al.*, 1996) and in T2D patients (Rajasekharan & Tuli, 1976; Baskaran *et al.*, 1990; Sharma *et al.*, 1996) and these effects

have been speculated to be due to their various hypoglycemic constituents isolated (Petit *et al.*, 1995; Murakami *et al.*, 1996; Sauvaire *et al.*, 1996; Manickam *et al.*, 1997).

Even though these plants either individually or in combination were used in Ayurvedic remedies, no study to date has examined the evaluation of these treatments on tissues involved in glucose homeostasis using an *in vitro* tissue culture-based bioassay technique in order to understand their comparative biological effects and also to provide a guide for the structural elucidation of potent antidiabetic molecule. Hence, the present study investigated the effect of aqueous extracts of these plants on glucose uptake by mouse muscle tissues and also insulin secretion from mouse pancreas under normal and hyperglycemic conditions *in vitro*.

3.2 Materials and methods

3.2.1 General methods

Plant extract preparation, animal ethics approval, statistical analysis, muscle and pancreas tissue preparations, tissue culture assay and their respective sample analysis for glucose and insulin have been described in Chapter 2.

3.2.2 Effect of plant extract on muscle glucose uptake and pancreas insulin secretion

To investigate the effect of plant treatments on glucose uptake by mouse abdomen muscle tissues and insulin secretion under both basal and hyperglycemic conditions *in vitro*, the plant extracts (GSE, CLE, TFGGE and PME), were examined and compared with control (PBS), metformin (10 mM) as a positive control in muscle culture and tolbutamide (0.1 mM) as positive control in pancreas culture as described in Section 2.4. Samples for analysis were taken after 24 hours and analysed as described in Section 2.6 and 2.7.

3.3 Results

3.3.1 Glucose uptake by mouse muscle tissues *in vitro*

Glucose uptake by muscle tissues in response to various plant treatments at both basal and hyperglycemic culture conditions was compared with the control and positive control and the results summarized in Table 3-1

Table 3-1 Effects of some Ayurvedic plant treatments on glucose uptake by mouse muscle tissues *in vitro*

Treatment (10 µl/well)	Glucose uptake (%)			
	Basal	% of control	Hyperglycemic	% of control
Control	5.6 ± 0.2 ^A	100	15.7 ± 0.2 ^C	100
TFGE	5.5 ± 0.26 ^A	98.21	23.6 ± 1.41 ^A	150.3
GSE	5.7 ± 0.22 ^A	101.7	14.7 ± 0.44 ^C	93
CLE	5.5 ± 0.17 ^A	98.21	26.2 ± 0.8 ^A	166.8
PME	5.5 ± 0.19 ^A	98.21	19.2 ± 0.58 ^B	122
Metformin	6.1 ± 0.09 ^A	108.92	25.3 ± 1.44 ^A	161.6

Data are expressed as Mean ± S.E.

Means without a common letter are significantly different (p<0.05).

All the treatments showed no significant difference in muscle glucose uptake under basal culture conditions (p>0.05). The percentage of glucose uptake activity in decreasing order 26.2 ± 0.8 (CLE); 23.6 ± 1.41 (TFGE); 19.2 ± 0.58 (PME) of plant treatments were significantly different from the control (15.7±.2 %; p<0.05) under hyperglycemic conditions. Treatment with CLE extract showed a peak glucose uptake activity (26.2 ± 0.8 %; p>0.05) among other plant treatments and this effect was significantly different

from the control (15.7 ± 2.2 %; $p < 0.05$) and also directly comparable with the effect of positive control (25.3 ± 1.44 %). However, under similar experimental conditions, GSE (14.7 ± 0.44) failed to produce a significant glucose uptake activity.

3.3.2 Insulin secretion from mouse pancreas tissues *in vitro*

The effects of plant treatments on insulin secretion from pancreas were measured and compared with the control and positive control (Table 3-2).

Table 3-2 Effects of some Ayurvedic plant treatments on insulin secretion from mouse pancreas tissues *in vitro*

Treatment (10 µl/well)	Insulin (µIU/ml)			
	Basal	% of control	Hyperglycemic	% of control
Control	70.1 ± 1.15^{AB}	100	87.2 ± 2.71^E	100
TFGE	68.6 ± 1.31^{AB}	97.86	179.5 ± 6.12^D	205.8
GSE	64.9 ± 1.24^B	92.58	213 ± 6.56^C	244.2
CLE	69.2 ± 1.24^{AB}	98.71	222 ± 7.0^C	254.4
PME	71.6 ± 1.7^{AB}	102.13	328 ± 18.46^A	376.1
Tolbutamide	76.3 ± 2.2^A	108.84	293 ± 6.91^B	336.6

Data are expressed as Mean $\pm$ S.E.

Means without a common letter are significantly different ($p < 0.05$)

Under basal culture conditions, all the treatments showed no significant difference from control in stimulating insulin secretion from pancreas ($p > 0.05$). However, all treatments under hyperglycemic conditions showed a significant increase in the media insulin and they were significantly different from the control ($p < 0.05$). The maximal media insulin

was observed with the PME (328±18.46 μ IU/ml) treatment and it was significantly greater than the media insulin of the positive control treatment (tolbutamide; 293 $\pm$ 6.91 μ IU/ml). All other treatments such as CLE, GSE and TFGE (222 $\pm$ 7.0; 213 $\pm$ 6.56; 179.5 $\pm$ 6.12 μ IU/ml) also significantly elevated the media insulin compared with control (87.2 $\pm$ 2.71 μ IU/ml).

The comparative effect of these treatments on glucose uptake and on insulin secretion under hyperglycemic conditions were converted as folds with respect to control and presented in Figure 3-1.

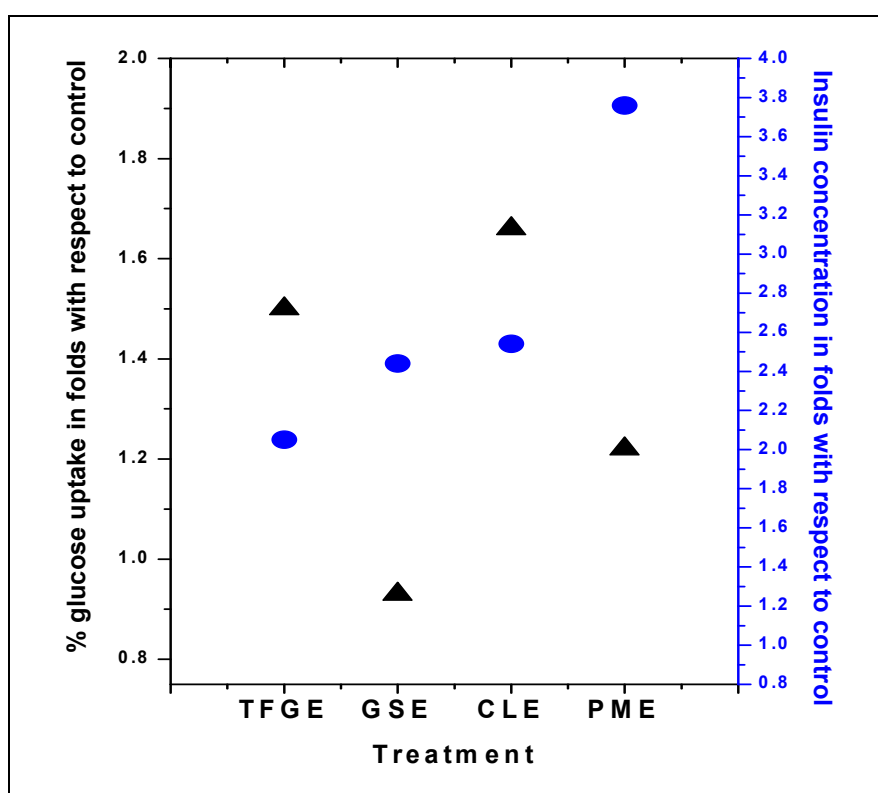


Figure 3-1 Comparative effect of plant treatments on glucose uptake (▲) and on insulin secretion (●) in folds with respect to control under hyperglycemic culture condition *in vitro*

3.4 Discussion

The present study for the first time reports the comparative effects of aqueous extracts of *Trigonella foenum-graecum* Linn (TFG), *Pterocarpus marsupium* Roxb (PM), *Gymnema sylvestre* R.Br (GS) and *Curcuma longa* Linn (CL) treatments on glucose uptake by mouse muscle tissues and insulin secretion from mouse pancreas tissues, under both and basal and hyperglycemic culture conditions *in vitro*.

The tissue culture protocol used in this study was developed based on a number of preliminary experiments (data not shown), mainly focusing on the maintenance of metabolic viability of the tissues throughout the experiment. The basal glucose uptake under basal and non-basal culture conditions were reported to be the one of the most sensitive indicators of *in vitro* functional viability of the tissues (Dohm *et al.*, 1988). In this context, basal glucose uptake was considered as an indicator of metabolic viability in the present experiments. The results of basal glucose uptake from the present experiments, compare favorably with earlier reports in the literature, and moreover significant response of the tissues when treated with metformin substantiates that the tissue culture protocol is functional as well as metabolically viable (Hundal *et al.*, 1992; Klip *et al.*, 1992; Henry *et al.*, 1995; Tsiani *et al.*, 1995) Similarly, the significant response of pancreas with tolbutamide and also their significant stimulatory response from basal to hyperglycemic conditions demonstrate that the said *in vitro* assay technique is metabolically functional and reproducible.

Aqueous extracts of plants mostly have constituents such as sugars, saponins, and polyphenols which may cause interference in diabetes-related *in vitro* bioassays (Soumyanath & Srijayanta, 2006). These interferences may have detrimental effects and also may overshadow a true mechanism-based effect of an extract. The results from the current study indicate that the glucose uptake and insulin secretion under basal culture conditions in response to various treatments were not significantly different from control, suggesting that these treatments at 10 µl/well (10 g/l) to the tissues within the culture conditions *in vitro*, are non-toxic or the extracts may lack or have very minimal *in vitro* assay interfering constituents. These treatments under hyperglycemic culture conditions did not diminish the basal glucose uptake and insulin secretion activity of the tissues, providing further evidence that enhanced activities of these treatments was not due to a detrimental tissue effect.

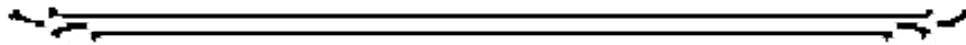
Glucose uptake by the muscle tissues is mostly facilitated by the translocation of GLUT4 vesicles from an intracellular site to plasma membranes mediated by the presence of insulin in hyperglycemic conditions (Shepherd & Kahn, 1999). Of these plant treatments, TFGE, CLE and PME showed a significant glucose uptake activity, suggesting these plant treatments may have either protective effects on tissues to keep them metabolically viable under hyperglycemic conditions or mimic insulin action or act via insulin-mediated enhanced peripheral glucose uptake like metformin (Bailey & Puah, 1986; Matthaei *et al.*, 1991). However, GSE treatment failed to produce enhanced activity under similar conditions, which supports the earlier findings, where it has been suggested

that GSE has no effect on peripheral glucose uptake (Shanmugasundaram *et al.*, 1990a; Shanmugasundaram *et al.*, 1990b; Yeh *et al.*, 2003).

Insulin secretion by pancreatic β -cells in response to glucose homeostasis is regulated by glucose, other nutrients, neurotransmitters and other hormones (Wollheim *et al.*, 1996). However, glucose is the primary regulator of insulin synthesis and secretion (Goodman, 2001). Chronic hyperglycemia was reported to have deleterious effects on insulin secretion and glucose utilization (Yki-Jarvinen, 1992) and in pancreatic β -cells, hyperglycemia leads to the production of greater amounts of reactive oxygen species that damage the cellular components (Robertson *et al.*, 2003). The enhanced insulin secreting activity of plant treatments under hyperglycemic conditions suggest that these treatments have either protective or regenerative activity on β -cell metabolism that leads to impaired insulin secretion.

In conclusion, all these plant treatments enhance glucose homeostasis within the culture conditions either by stimulating insulin secretion (GSE) or enhancing glucose uptake (TFGE) or activating both (CLE and PME). In terms of their comparative effects on tissues that regulate glucose metabolism, the aqueous extracts of plants, PME and CLE, were found to be more potent when compared with other studied Ayurvedic plant treatments, TFGE and GSE, within culture conditions. However, the precise mechanism by which these plant treatments, particularly CLE and PME, enhance glucose homeostasis and the biologically active constituents of these sources remain to be elucidated.

4 Effects of CLE and PME on tissues involved in Glucose Homeostasis



4.1 Introduction

As reviewed in detail in Chapter 1.3, Ayurveda, a traditional Indian system of medicine, advocates a wide range of medicinal plants to treat T2D. Most of these Ayurvedic medicines exhibit antidiabetic properties but the data are inconclusive (Trivedi, 1963; Nagarajan *et al.*, 1982; Grover *et al.*, 2002b; Kar *et al.*, 2003). Reports of our preliminary antidiabetic activity screening study (Chapter 3) of some of the extensively advocated Ayurvedic plants showed that the aqueous extract of *Curcuma longa* Linn (CL) and *Pterocarpus marsupium* Roxb (PM) enhanced insulin secretion and mimicked the effect of insulin on tissues involved in glucose homeostasis. However, the precise mechanism by which these plant treatments enhance glucose homeostasis remains to be elucidated.

Briefly, CL rhizomes have been widely used in the traditional Ayurvedic system of medicine to treat diabetes mellitus either on its own or in a combination with other plants. The potent antioxidant activity of CL and its active principles, such as curcuminoids and their analogues, has gained considerable attention in recent alternative supplementary therapies.

PM commonly known as ‘Indian Kino’ or ‘Vijayasar’ is another extensively advocated traditional Ayurvedic medicine to treat T2D. A significant number of experimental findings support the Ayurvedic claims that the extract of PM exhibits antidiabetic activity

in various experimental animal models (Jogelkar *et al.*, 1959; Gupta, 1963; Trivedi, 1963; Shah, 1967; Pandey & Sharma, 1975; Chakravarthy *et al.*, 1980; Chakravarthy *et al.*, 1981a; Chakravarthy *et al.*, 1981b; Ahmad *et al.*, 1991a; Dhanabal *et al.*, 2006) and also in humans (Sepaha & Bose, 1956; Pandey & Sharma, 1975; Rajasekharan & Tuli, 1976; Kedar & Chakrabarti, 1981; ICMR, 1998).

Despite numerous experiments demonstrating that CL and PM and their constituents have blood glucose lowering effects (section 1.4.3-4), no study has demonstrated their precise molecular mechanistic effects specifically on the tissues involved in glucose homeostasis such as pancreas and skeletal muscle. Moreover, the results from our preliminary investigations on the action of these plants, on tissues which are involved in glucose homeostasis, strongly suggest that these plants have potent hypoglycemic molecules that can enhance both insulin secretion and action (Chapter 3). However, it still remains unclear how these plants precisely act on tissues involved in glucose homeostasis. This has prompted us to further investigate these plants to understand their possible mechanistic effects on tissues involved in glucose homeostasis. Hence, as T2D is a heterogeneous syndrome, which involves impaired insulin secretion and action, the present study investigated the effect of aqueous extract of CL (CLE) and PM (PME) on insulin secretion from pancreatic tissues and also on glucose uptake by skeletal muscle within *in vitro* tissue culture conditions.

4.2 Material and methods

4.2.1 General methods

Plant extract preparation, animal ethics approval, statistical analysis, muscle and pancreas tissue preparations, tissue culture assay and sample analysis for glucose and insulin have been described in Chapter 2.

4.2.2 Insulin secretion *in vitro*

Insulin secretion was evaluated using pancreatic tissues of male Swiss mice as described in Section 2.4 and 2.7. Briefly, the *in vitro* pancreas tissue culture assay was performed with incubations of 15 and 30 minutes (Acute) to study the acute response and 24 hours (Chronic) for the chronic response, with PME and CLE (0.1-100 µl) of increasing final concentration and compared with control (PBS).

4.2.3 Muscle glucose uptake *in vitro*.

The effect of PME and CLE on abdomen muscle glucose uptake was determined as described in Section 2.4. *In vitro* muscle culture assay was performed with incubations of 24 hours with PME (0-100 µl) and CLE (0-5 µl) in the presence or absence of insulin (100 nM).

In a further experiment, chronic incubation of abdominal muscle tissues under hyperglycemic condition with the treatments of PME (10 μ l), CLE (5 μ l), insulin (100 nM), wortmannin (100 nM; a pharmacological inhibitor of PI3-kinase), PME and CLE spiked with wortmannin, and insulin spiked with wortmannin were carried out to investigate the specificity of the signaling pathway.

The concentration of insulin was derived from its dose-dependent response on the abdomen muscle tissue culture experiment. Briefly, chronic incubation of muscle tissues under hyperglycemic condition with insulin of various doses (10 fold diluted doses) was examined, as described in Section 2.4. A dose (100 nM) that represents a maximal efficacy observed was chosen (Data not shown).

4.3 Results

4.3.1 Insulin secretion *in vitro*

4.3.1.1 *Effect of CLE on insulin secretion*

The effect of CLE on insulin secretion from pancreatic tissues following 30 minutes (Acute) incubation (30 minutes) under hyperglycemic condition (Figure 4-1) and 24 hours (Chronic) incubation under both basal and hyperglycemic condition (Figure 4-2) were examined *in vitro*.

Under hyperglycemic culture conditions all the doses of CLE over 30 minutes of incubation showed an inhibited insulin release which was significantly different from the control ($p<0.05$). No significant difference was observed between the 0.1, 1 and 10 μ l doses of CLE ($p>0.05$), but the highest dose of CLE (100 μ l) was significantly different from the control and the other doses of CLE ($p<0.05$) (Figure 4-1). Pancreatic tissues over 15 minutes of incubation with various doses of CLE in hyperglycemic culture condition were not significantly different in insulin secretion from the control (Data not shown).

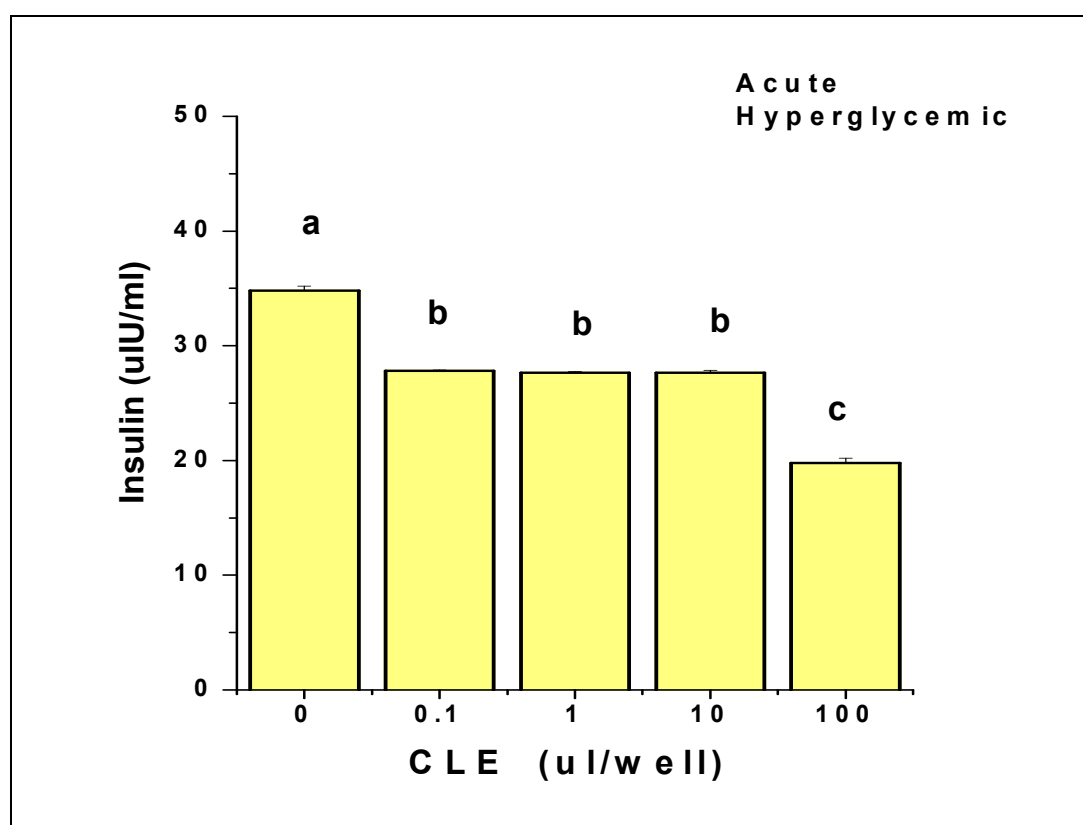


Figure 4-1 Effect of CLE (0-100 μ l) on insulin secretion from mouse pancreatic tissues at hyperglycemic (12 mM glucose) culture condition over 30 minute (Acute) incubation *in vitro*. Values are expressed as mean $\pm$ S.E. Means without a common letter are significantly different ($p<0.05$).

Insulin secretion in response to various doses of CLE during chronic incubation under normoglycemic condition was not significantly different from control (Figure 4-2A). However, insulin secretion in response to various doses of CLE showed a dose-dependent response over chronic incubation under hyperglycemic conditions (Figure 4-2B).

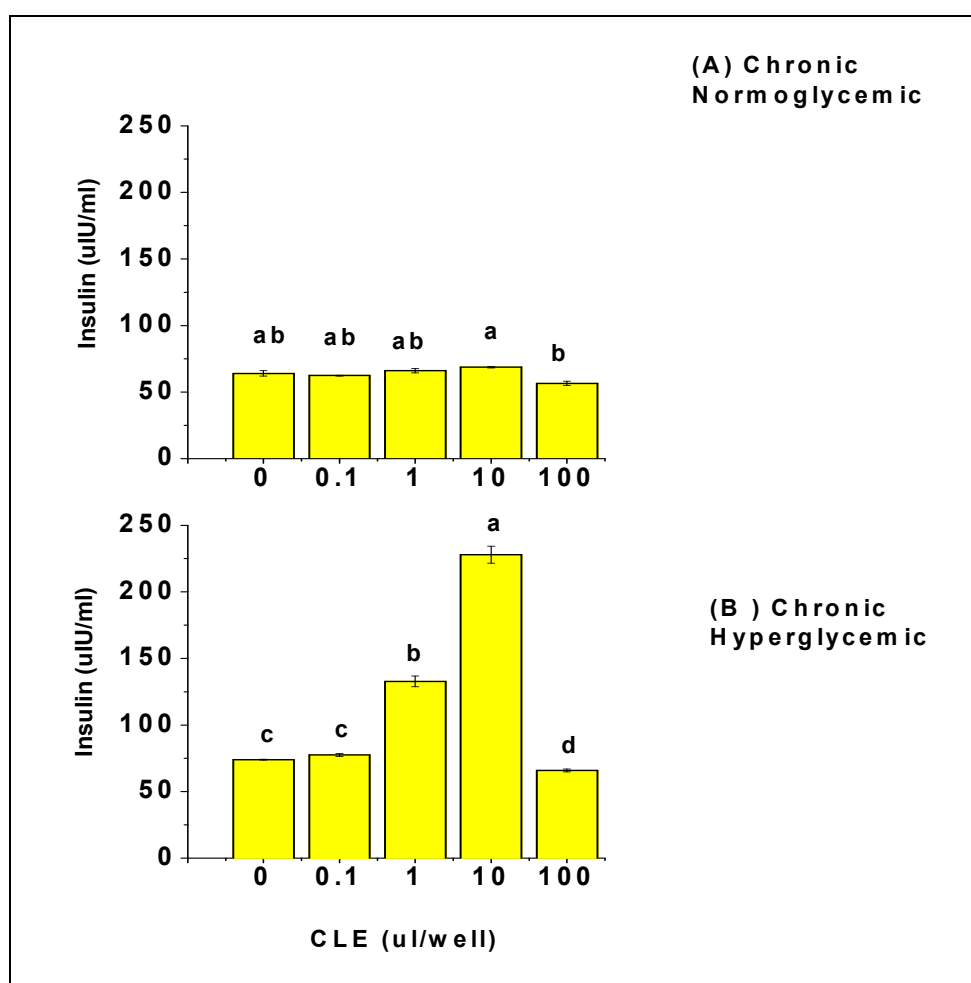


Figure 4-2 Effect of CLE (0-100 µl) on insulin secretion from mouse pancreatic tissues over chronic incubation (24 hours) *in vitro*
A: 5 mM glucose (normoglycemic). **B:** 12 mM glucose (hyperglycemic). Values are expressed as mean $\pm$ S.E Means indicated without a common letter are significantly different ($p < 0.05$).

There was no effect at the lowest dose of 0.1 μ l/well, followed by an increasing response at 1 and 10 μ l/well, with a maximum response (228 ± 6.41 μ IU/ml) at 10 μ l/well. However, at the highest dose of 100 μ l/well less insulin was secreted than in the control (73.95 ± 0.29 μ IU/ml; $p < 0.05$) (Figure 4-2B).

4.3.1.2 Effect of PME on insulin secretion

Insulin secretions during acute incubations (15 and 30 minutes) of pancreatic tissues with PME under both normal and hyperglycemic culture conditions showed no significant difference from the control ($p > 0.05$) (Data not shown).

Chronic incubation of PME at normoglycemic culture condition showed no significant difference from the control (Figure 4-3A).

Chronic incubation of pancreatic tissues with PME of increasing concentration (0-100 μ l) in hyperglycemic culture conditions showed an increase of insulin secretion such that secretion was significantly different at 10 μ l/well (324.2 ± 16.82 μ IU/ml) from control (88.13 ± 10.84 μ IU/ml) and all other treatment groups (Figure 4-3B). Again there was less insulin secreted with 100 μ l/well (75.05 ± 11.90 μ IU/ml) but this was not significantly different from control ($p > 0.05$).

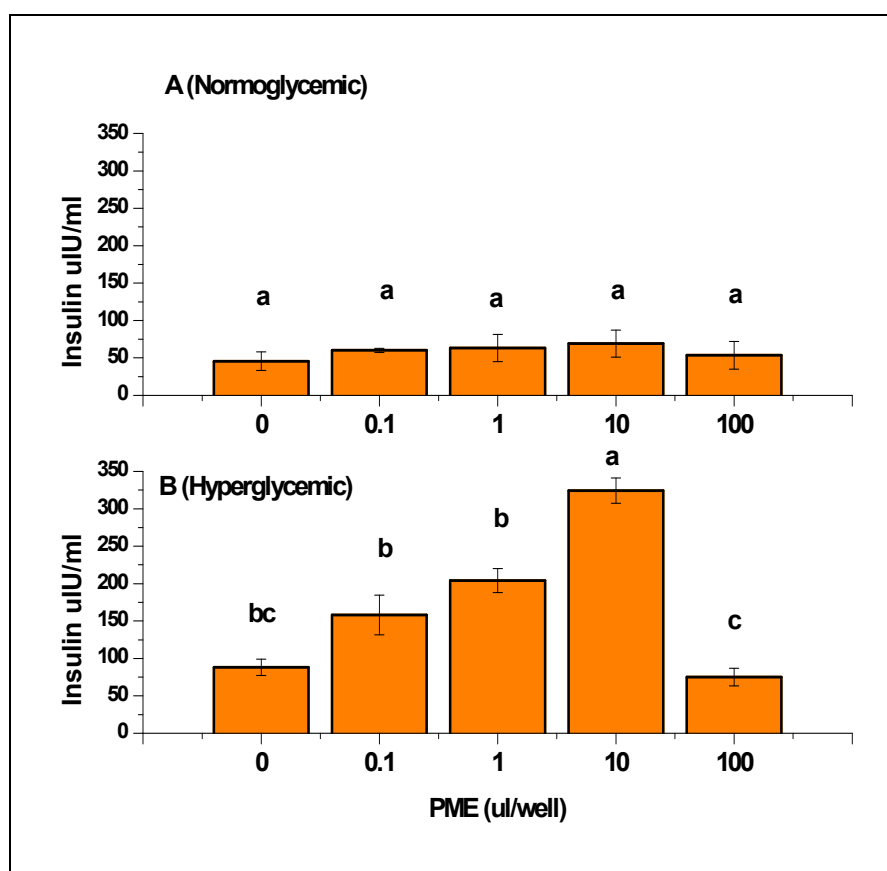


Figure 4-3 Effect of PME on insulin secretion from mouse pancreas tissues over chronic incubation (24 hours) *in vitro*

A: 5 mM glucose (normoglycemic). B: 12 mM glucose (hyperglycemic). Values are expressed as mean $\pm$ S.E Means indicated without a common letter are significantly different ($p < 0.05$).

4.3.2 Muscle glucose uptake *in vitro*

4.3.2.1 Effect of CLE on muscle glucose uptake

Glucose uptake by abdomen muscle tissues in response to treatment with varying doses of CLE in the presence and absence of insulin (100 nM) under hyperglycemic culture condition were measured and compared with the basal glucose uptake (Figure 4-4).

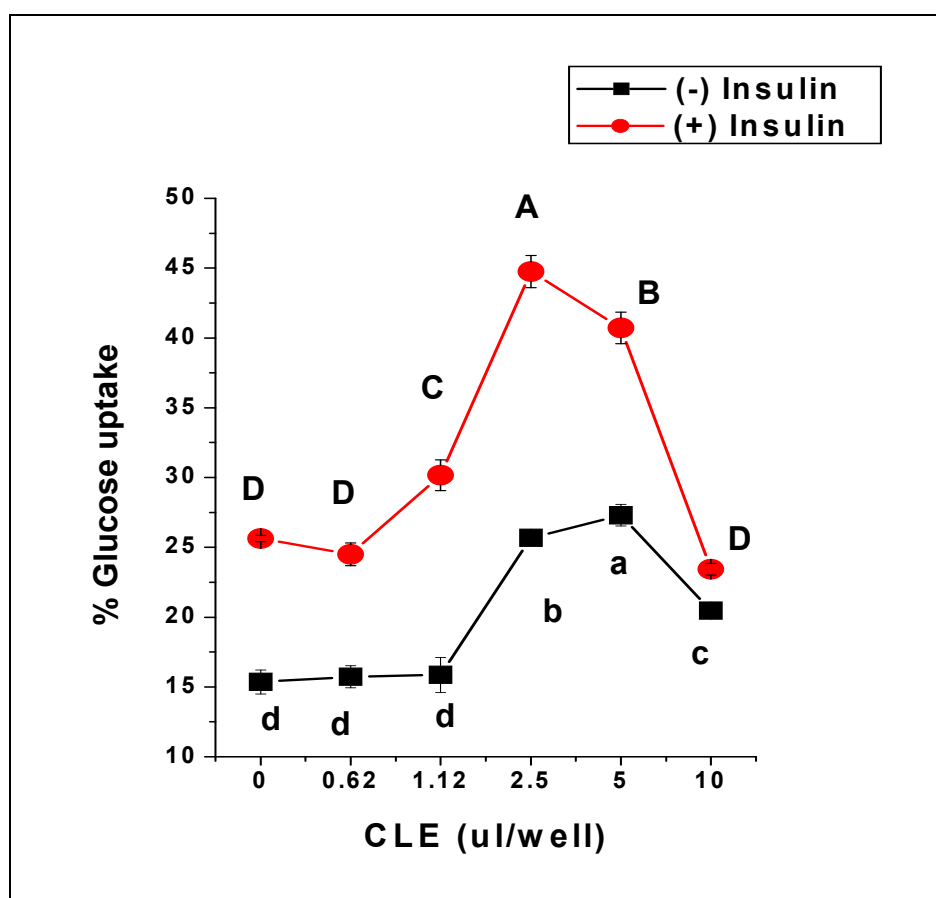


Figure 4-4 Effect of CLE (0-10 µl) on *in vitro* glucose uptake by mouse abdomen muscle tissues under hyperglycemic culture condition over chronic incubation (24 hours)
 (-■-): without added insulin. (-●-): with insulin (100 nM). Values are expressed as mean ± S.E Means indicated without a common letter are significantly different (p<0.05).

CLE induced a dose-dependent stimulatory effect on glucose uptake from abdomen muscle tissues at hyperglycemic condition in both the presence and the absence of insulin. The peak activities at a dose of 5 µl (- insulin; $27.3 \pm 0.77 \%$) and 2.5 µl (+ insulin; $44.76 \pm 1.16 \%$) were significantly different from the controls in the absence ($15.3 \pm 0.86 \%$) and the presence ($25.63 \pm 0.23 \%$) of insulin respectively, which indicates that the presence of CLE significantly potentiated the effect of insulin.

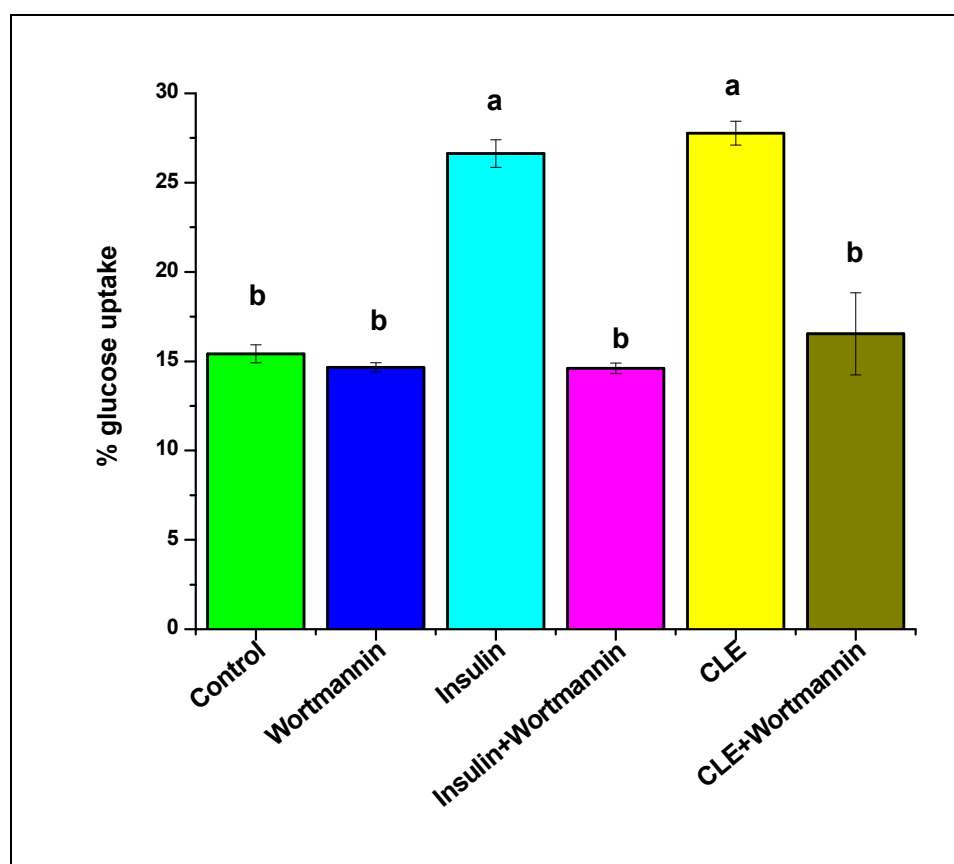


Figure 4-5 Effect of CLE (5 μ l), insulin (100 nM), wortmannin (100 nM), CLE combined with wortmannin and insulin with wortmannin on *in vitro* glucose uptake by mouse abdomen muscle tissues under hyperglycemic culture condition over chronic incubation (24 hours). Values are expressed as mean $\pm$ S.E Means without a common letter are significantly different ($p < 0.05$).

Figure 4-5 shows, the effect of wortmannin (100 nM) on peak glucose uptake activity of CLE (5 μ l) and insulin (100 nM). Under hyperglycemic culture conditions where tissues were incubated for 24 hours, glucose uptake activity increased with CLE (27.77 ± 0.67 %) and insulin (26.63 ± 0.77 %) alone ($p > 0.05$), whereas when these treatments were combined with wortmannin, the resultant % glucose uptake was not different to the control or wortmannin by itself ($p > 0.05$).

4.3.2.2 Effect of PME on muscle glucose uptake

Muscle glucose uptake in response to treatment with varying doses of PME in the presence or absence of insulin (100 nM) under hyperglycemic culture conditions were measured and compared with basal glucose uptake (Figure 4-6).

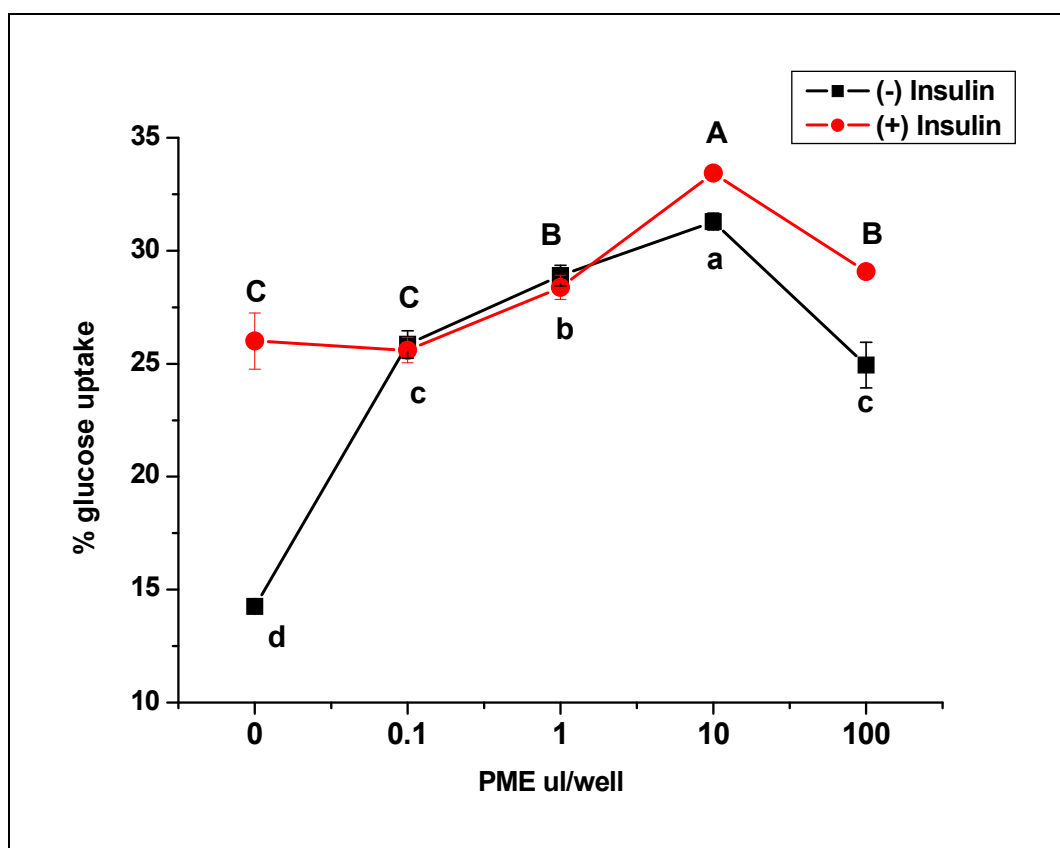


Figure 4-6 Effect of PME (0-100 µl) on glucose uptake by abdomen muscle tissues under hyperglycemic culture condition over chronic incubation (24 hours) *in vitro* (-■-): without added insulin. (-●-): with insulin (100 nM). Values are expressed as mean $\pm$ S.E Means indicated without a common letter are significantly different ($p < 0.05$).

PME exhibited a dose-dependent stimulatory effect on glucose uptake from abdomen muscle tissues under hyperglycemic conditions in both the presence and absence of insulin. The peak activity of PME at a dose of 10 μ l was significantly different from the control in both the absence (31.29 ± 0.37 %) and presence (33.43 ± 0.12 %) of insulin ($p < 0.05$). The presence of insulin significantly potentiated the glucose uptake activity in the higher doses of PME (10 and 100 μ l) but, the lower doses of PME (0.1 and 1 μ l) did not significantly alter the stimulatory effect of insulin. The effect of added insulin was greater in the control wells (11.76 %) than in the wells treated with PME.

A dose of PME (10 μ l), the volume with peak glucose uptake activity, was tested by spiking with wortmannin, and compared with treatments such as insulin (100 nM), wortmannin (100 nM), and insulin spiked with wortmannin in order to study the specificity of the insulin signaling pathways. Data are shown in Figure 4-7.

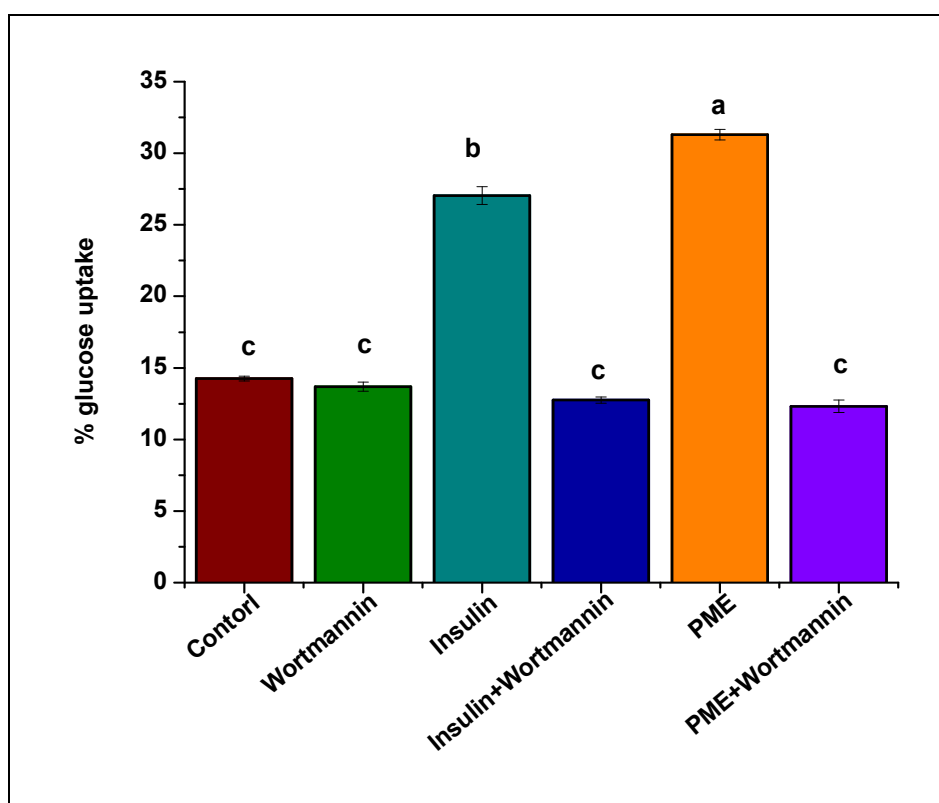


Figure 4-7 Effect of PME (10 μ l), insulin (100 nM), wortmannin (100 nM), PME combined with wortmannin and insulin with wortmannin on *in vitro* glucose uptake by mouse abdomen muscle tissues under hyperglycemic culture conditions over chronic incubation (24 hours) Values are expressed as mean $\pm$ S.E Means without a common letter are significantly different ($p < 0.05$).

The stimulatory effect of PME on mouse glucose uptake at a dose of 10 μ l (31.29 ± 0.37 %) was significantly more potent than the effect of insulin (27.04 ± 0.62 %; $p < 0.05$) and the control (14.24 ± 0.17 %; $p < 0.05$). However, the stimulatory effects of PME and insulin were cancelled in tissues when these treatments were spiked with wortmannin (12.75 ± 0.22 ; 12.31 ± 0.44 %). Wortmannin treatment alone had no significant effect on glucose uptake activity (13.68 ± 0.32 %; $p > 0.05$).

4.4 Discussion

Despite numerous studies on CL and PM and their active principles in the treatment and management of diabetes, no study to date has demonstrated the effect of the aqueous extracts of CL and PM specifically on the tissues such as pancreas and skeletal muscle tissues which are involved in glucose homeostasis. The present study reports that the water soluble phyto-ingredients of CL and PM stimulate insulin secretion from mouse pancreatic tissues and increase glucose uptake by mouse muscle tissues.

Chronic incubations of pancreatic tissues with CLE (10 μ l) showed a significant insulin releasing effect without showing any toxicity to the tissues under hyperglycemic culture conditions. The lower dose (0.1 μ l) of CLE had no effect on the insulin secretion compared to the control over 24 hours and the higher dose of CLE (100 μ l) appeared to be inhibitory or toxic with an inhibited insulin secretion. The peak activity at a dose of 10 μ l of CLE over chronic incubation (24 hours) but not in acute incubation (15 and 30 min) suggests its possible involvement in the metabolic process of insulin secretion and also the potent stimulatory effect of CLE in response to the elevated glucose concentration suggests that it may act via protective pathways rather than a stimulatory pathway.

Similarly, chronic incubation of pancreatic tissues with PME showed a significant dose-dependent insulin-releasing effect under hyperglycemic culture condition. However,

PME treatments under basal (normoglycemic) culture condition or following acute incubations (15 and 30 min) have no significant effect on insulin secretion. Enhancement of insulin secretion by PME treatment at a dose of 10 μ l after chronic incubation, but not following acute incubation suggests its potential role in the metabolic process of insulin secretion within culture conditions. Moreover, the potent stimulatory effect of PME in response to the elevated glucose concentration suggests that β -cell metabolism is able to augment the insulintropic effect.

The stimulation of insulin secretion by both CLE and PME indicates that these treatments may have active constituents which can augment or protect or regenerate β -cell metabolism. Hypoglycemia induced by excessive insulin secretion is a major complication of current pharmacological treatments of T2D (Henquin, 2004). The enhancement of insulin secretion only at hyperglycemic but not in normoglycemic condition suggests that these treatments do not provoke hypoglycemia under basal physiological condition.

Impairments in the insulin signaling cascades, glucose transports and GLUT4 translocation are the major defects in insulin resistance and T2D (Kellerer *et al.*, 1999). Experimental findings on other aqueous plant extracts such as elder, agrimony, coriander, and mushroom have shown an insulin secretion stimulatory effect coupled with insulin-like effect on peripheral glucose uptake *in vitro* (Gray & Flatt, 1998a, 1998b, 1999; Gray *et al.*, 2000). Current advancement of diabetes treatment and management focuses both

insulin secretion and action. Thus the current study examined the effect of CLE and PME (0-100 μ l) on abdominal skeletal muscle tissue.

Skeletal muscle is the major site for primary glucose disposal and utilization. In the skeletal muscles, glucose uptake is stimulated by the activation of the translocation of GLUT4 vesicles by the presence of insulin and also involving many downstream pathways (Shepherd & Kahn, 1999). CLE and PME treatment of skeletal muscle tissues significantly enhanced the glucose uptake in absence or presence of insulin. Although glucose uptake activity was observed in the absence of insulin, it does not preclude the involvement of residual insulin receptor binding within the muscle preparation.

The potential enhancement of glucose uptake activity of CLE and insulin in combination and also the declining response with respect to increasing concentrations of CLE suggests that these extracts possibly act via an insulin-mediated enhanced peripheral glucose uptake pathway, which may be very similar to the effect of the established antihyperglycemic drug metformin (Bailey & Puah, 1986; Prager *et al.*, 1986).

In contrast to CLE, PME showed less potentiation of glucose uptake activity in the presence of a saturating dose of insulin. The combined actions of PME and insulin were not additive, indicating that the active constituents of PME may act via pathways (at least terminally) that are utilized by insulin. Interestingly, the effect of PME on glucose uptake differs significantly from metformin.

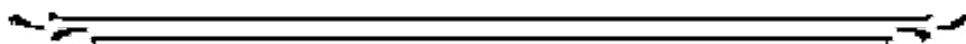
The pharmacological inhibitor, wortmannin inhibits insulin-mediated glucose uptake by inhibiting PI3-kinase (Hausdorf *et al.*, 1999). The inhibitory response of CLE and PME with wortmannin further suggests that these extracts act via the classical PI3-kinase pathway. Curcumin the active principle of CL was reported to have an inhibitory effect on insulin-induced GLUT4 translocation and glucose transport in 3T3-L1 adipocytes (Ikononov *et al.*, 2002) and cyclic-AMP protein kinase (Hasmeda & Polya, 1996). The curcuminoid content of commercially available CL powder was reported to be 2.7- 5.18 g/100 g (Hiserodt *et al.*, 1996). Thus, the declining glucose uptake effect of CLE (>5 µl) may be attributed to the presence of curcumin. The significant glucose uptake effect of CLE at 5 µl dose without insulin and at 2.5 µl dose with insulin suggests the presence of insulin sensitizing constituents in CL. In contrast to CLE, the lack of significant potentiation of glucose uptake activity by PME and insulin (saturating dose) in combination and also the suppression of activity when combined with wortmannin reveals that the constituents of PME may have insulin-like or mimicking active constituents.

The inhibitory response for the highest concentration (100 µl) of both CLE and PME on tissue involved in glucose homeostasis is possibly due to the presence of *in vitro* assay interfering or inhibitory or synergetic compounds such as polyphenols, saponins and sugars in the aqueous extract of plant materials (Soumyanath & Srijoyanta, 2006). However, the lower doses were found to be non-toxic in both normal and hyperglycemic conditions. Moreover, the potentiation of activity only under hyperglycemic conditions

further confirms this effect was not a mere consequence by *in vitro* assay interfering compounds.

Overall, CL and PM have water-soluble principles capable of stimulating insulin secretion and which also sensitise or mimic insulin action on the peripheral tissues involved in glucose homeostasis.

5 Bioassay-guided purification of PME



5.1 Introduction

A number of Ayurvedic traditional medicinal plants are known to have preventive and therapeutic effects in diabetes and obesity, but their active components have not yet been characterised, except in few cases. As reviewed in Section 1.4.4, an aqueous extract of heartwood of *Pterocarpus marsupium* Roxb (PM) has been used in the Ayurvedic system of medicine for the treatment and management of diabetes since time immemorial (Satyavathi *et al.*, 1987; Warriar *et al.*, 1995). Although many encouraging experimental studies report that PM has antidiabetic action, to date no confirmative evidence is available which identifies the active constituent(s) responsible for the action.

Chakravarthy and colleagues (1981a) isolated (-)-epicatechin from the bark of PM and claimed it to be the hypoglycemic principle, which had protective and restorative effects on β -cells and could reduce blood glucose by increasing insulin production and secretion of alloxan-induced diabetic rats (Chakravarthy *et al.*, 1980; Chakravarthy *et al.*, 1981a; Chakravarthy *et al.*, 1981b; Chakravarthy & Gode, 1985; Ahmad *et al.*, 1991a; Ahmad *et al.*, 1991b). However, subsequent attempts to replicate these studies by Kolb and coworkers (1982) failed to confirm the blood glucose lowering effects of (-)-epicatechin. A study by Sheehan and colleague (1983), confirmed that (-)-epicatechin does not effectively stimulate islet β -cell regeneration or decrease hyperglycemia once the diabetic state has been firmly established. Therefore, these conflicting results warrant further

robust investigation before (-)-epicatechin can be considered as a viable hypoglycemic agent in clinical trials (Sheehan *et al.*, 1983).

Ayurveda advocates the heartwood rather than the bark of PM to treat diabetes (Chopra *et al.*, 1958; Jain, 1968; Satyavathi *et al.*, 1987) although it has been reported that (-)-epicatechin is not present in the heartwood (Manickam *et al.*, 1997; Mallavadhani & Gayatri, 2003). In this context, Manickam and colleagues (1997) investigated the aqueous extract of heartwood and isolated three main isoflavones from the ethyl acetate soluble fraction of the aqueous extract of the heartwood of PM, and reported marsupsin and pterostilbene were both effective antidiabetic agents. However, all these findings have failed to ascertain the precise mechanistic pathways involved in the glucose lowering properties of PME. The significant antidiabetic effects of aqueous extract of heartwood of PM in newly diagnosed T2D patients and also their restrictions to mild diabetics warrant more scientific investigations to identify the active constituent of PM (ICMR, 1998). Moreover, our preliminary studies (see Chapter 3 and 4) strongly suggest that the aqueous extract of heartwood of PM has potent hypoglycemic constituent(s), which stimulate both insulin secretion and action.

Identification and isolation of the pure, biologically potent constituent(s) from natural sources remains a long and tedious process (Hostettmann, 1999). A major focus for phytochemical analysis is the characterisation of an active agent responsible for some beneficial or toxic effect exhibited by a crude plant extract when tested against a living organism. Chemical screening can be performed to allow the localization and targeted

isolation of such constituent(s) with potential activity. Bioassays are therefore essential for monitoring each stage of extraction and separation procedures in order to follow the active material as it is purified (Harbourne, 1973).

Despite many experimental findings suggesting that heartwood of PM has antidiabetic action in both experimental animals and humans and also some reports on its possible hypoglycemic constituent(s), there are currently no studies available, which have identified the hypoglycemic constituent(s) of aqueous extract of heartwood of PM.

The present study is aimed to identify and isolate the potent hypoglycemic constituent(s) from an aqueous extract of heartwood PM (PME) using *in vitro* bioassay-guided fractionation techniques.

5.2 Material and methods

5.2.1 General methods

Extract preparation, animal ethics approval, statistical analysis, *in vitro* bioassay and sample analysis for glucose and insulin have been described in Chapter 2.

The effect of treatments on insulin secretion from mouse pancreatic tissues and on glucose uptake by mouse abdomen muscle tissues were used as a bioassay guide for identifying and isolating antidiabetic constituent(s) from PME. To determine a peak effective concentration of PME, a series of 10 fold dilutions (0-100 μ l) from its original volume were examined for its potential effects on insulin secretion and glucose uptake under hyperglycemic conditions *in vitro*. The dose that showed peak activity (10 μ l) was used in subsequent experiments (see Figures 4-3 and 4-6). All samples from subsequent purification stages were consistently reconstituted to the original volume of PME that showed a peak activity, unless otherwise specified.

5.2.2 Data analysis

All the experiments were repeated thrice (n=3) and the data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as mean $\pm$ S.E., unless otherwise specified.

5.2.3 Preliminary purification studies

5.2.3.1 *Aqueous extraction*

Aqueous extraction of PM was carried out as described in Section 2.2. A pool of extract was divided into 10 ml aliquots and stored at -20°C. An aliquot of PME was freeze dried under reduced pressure at -50°C and further used for subsequent purification (FPME).

To determine the effect of heat on the nature of hypoglycemic constituent(s) of PME, 100 µl of PME was boiled for an hour. The extract thus obtained (BPME) was allowed to cool down to room temperature and then filtered through syringe filters (0.2 µm). BPME was stored at -20°C until analysed for *in vitro* antidiabetic activity.

5.2.3.2 *Organic solvent extraction*

Freeze dried extract of PM (500 mg; FPME) was dissolved in 50 ml of distilled water and subsequently extracted with equal volumes of ethyl acetate and butyl alcohol (see Figure 5-1). After separation the fractions were dried overnight under vacuum at room temperature, and stored at -20°C until analysed.

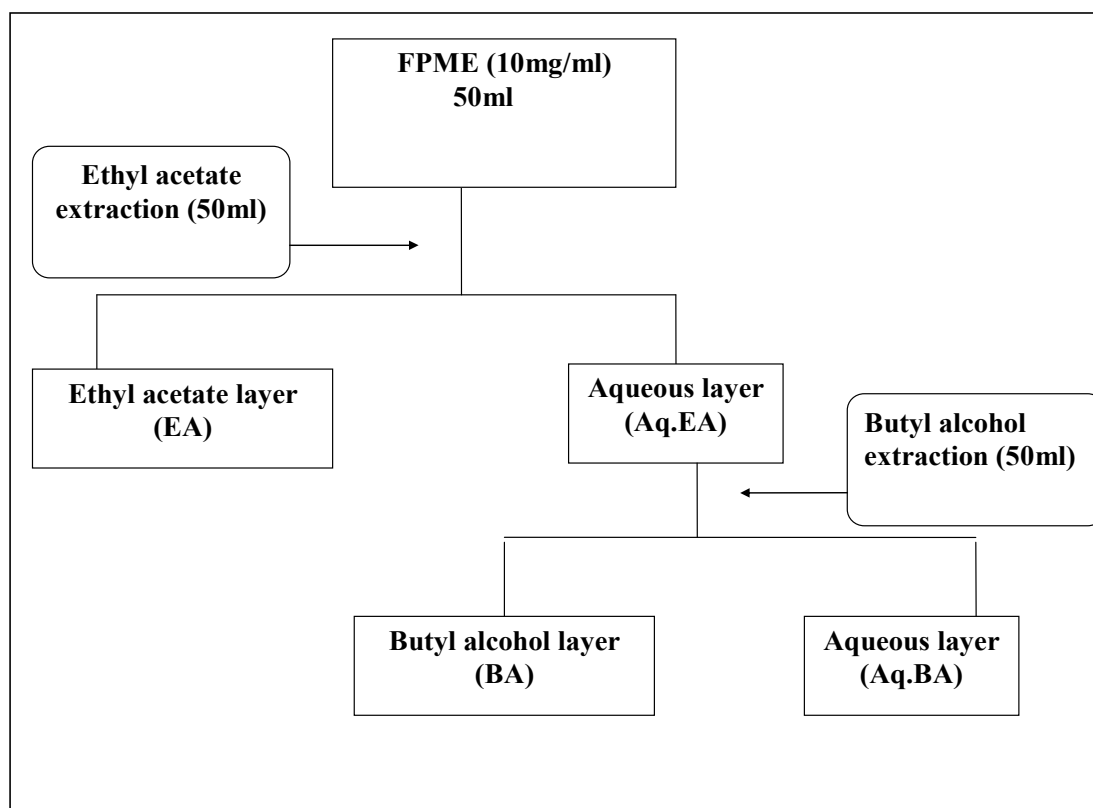


Figure 5-1 Organic solvent extraction of FPME with ethyl acetate and butyl alcohol

5.2.3.3 Ultrafiltration

Ultrafiltration of FPME through 10 and 30 kDa molecular weight cut-off membrane was used to determine the molecular weight range of the hypoglycemic constituent(s) of FPME (Figure 5-2). 400 mg of FPME was dissolved in 100 ml distilled water and concentrated through 10 kDa cut-off (YM10) ultrafiltration membrane (DIAFLO, Amicon Scientific Australia). The extract that was retained by the membrane (10KR) was

washed with equal volumes of distilled water at least 3 times and further concentrated to a final volume of 10 ml.

In a further experiment, 2 ml of 10KR material was then concentrated to approximately 200 μ l with a centrifugal filter device with 30 kDa molecular-mass cut-off (YM-30 Centricon, Millipore, MA, USA) at 5000 x g for 30 minutes. The concentrated extract retained by the 30 kDa cut-off membrane (30KR) and the extract that passed through the membrane (30KE) were examined for their *in vitro* activity.

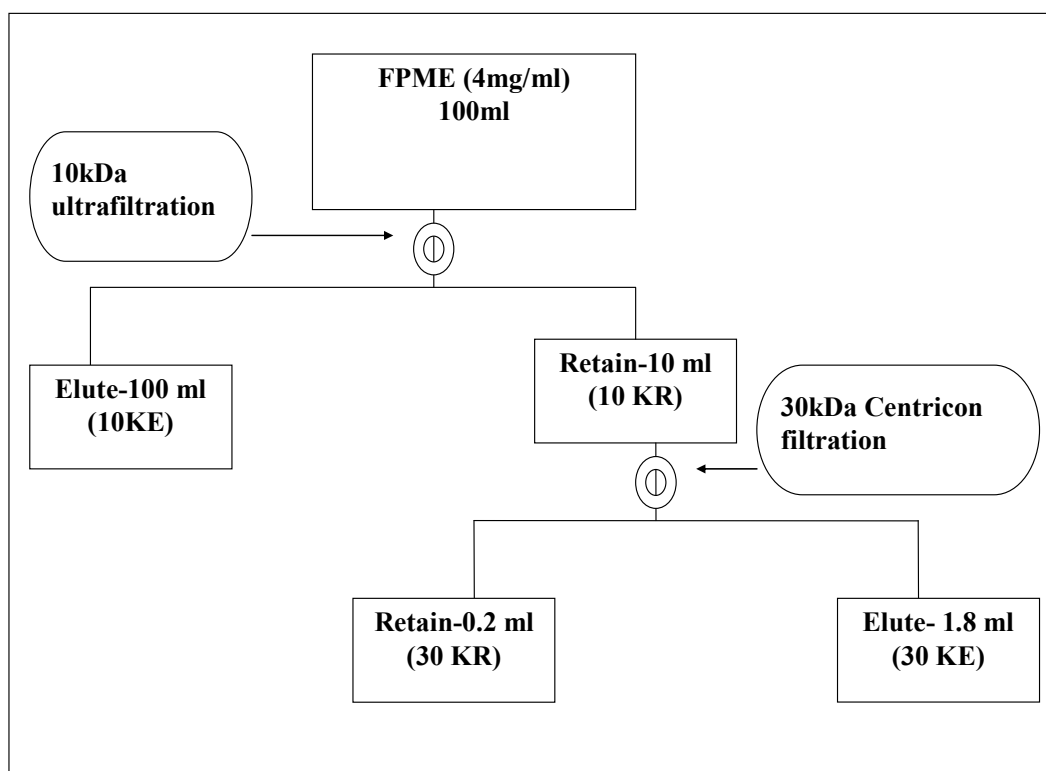


Figure 5-2 Ultrafiltration of FPME through 10 and 30k molecular mass cut-off membrane

5.2.3.4 *Sephadex G-25 column chromatography*

A pre-packed column containing Sephadex G-25 medium (PD-10; Amersham Biosciences, Piscataway, NJ, USA) was used in the preliminary fractionation of FPME (Figure 5-3). The column was washed and equilibrated with approximately 25 ml of 0.05 M phosphate buffer (pH 7.4) immediately prior to loading sample. 400mg of FPME was dissolved in 10 ml of 0.05M phosphate buffer (pH 7.4) and centrifuged at 13,000 rpm for 30 min to remove any fine particles. The clear supernatant obtained (10 ml) was used in subsequent purification. 1 ml of the sample was loaded on a Sephadex G-25 column and eluted with 0.05 M phosphate buffer (pH 7.4). Fractions were collected in 1ml volumes. The initial two fractions were discarded to nullify the exclusion volume. After collecting 10 fractions, the column was washed with 25 ml of 0.05M phosphate buffer (pH 7.4) before reloading further samples. This method of fractionation was repeated until the entire sample was loaded and eluted through the column. Each individual fraction (1-10) from the entire run was then pooled respectively and examined for its *in vitro* activity.

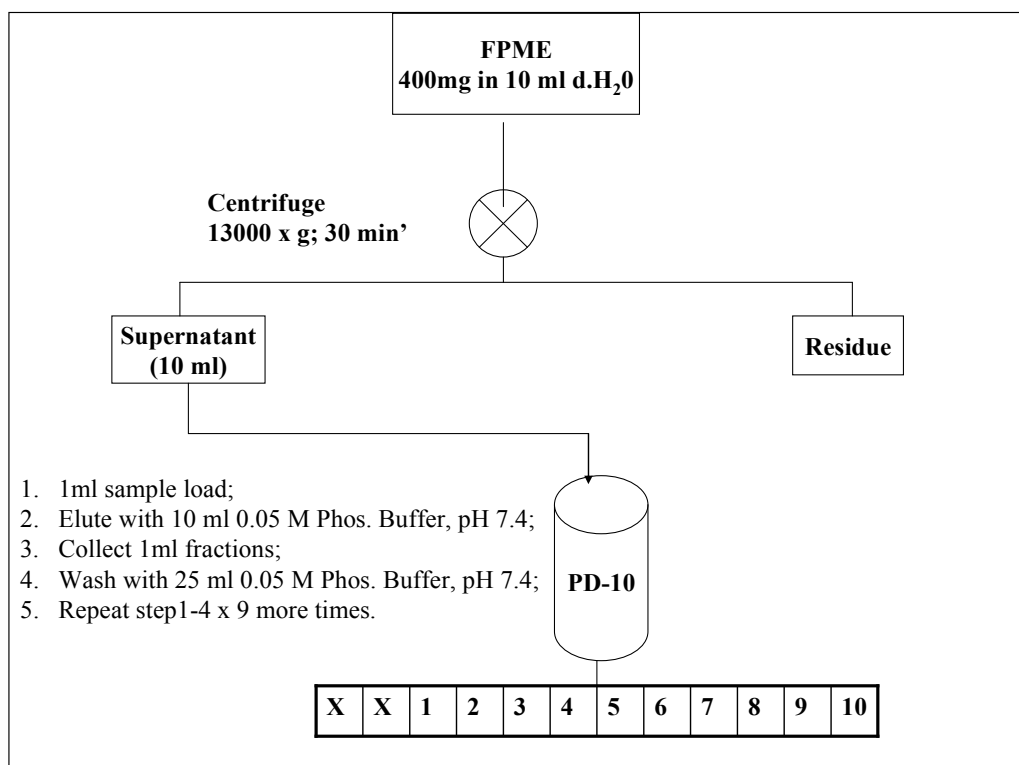


Figure 5-3 Sephadex G-25 column chromatography fractionation of FPME

5.2.3.5 Trypsin digestion

To investigate the nature of hypoglycemic constituent(s) of PME, 100 µl of PME was digested with trypsin and assayed for antidiabetic activity. Briefly, 100 µl of trypsin (1 mg/ml) in 0.1M sodium bicarbonate binding buffer, pH- 9.6 was adsorbed into 96 well ELISA plates and incubated for 1 hour, washed and followed by site blocking with 1 % skim milk (200 µl/well) for 1 hour. The wells were then washed 5 times with Tris wash buffer. 100 µl of PME was then added to each well and incubated overnight at room

temperature. A similar plate (with no trypsin coating) was constructed and treated as trypsin uncoated. The samples from both trypsin digested (+ Trypsin) and undigested plates (- Trypsin) were examined for *in vitro* antidiabetic activity.

5.2.3.6 *Ion exchange chromatography*

The selection of ion exchange resins was determined from multiple preliminary studies conducted using anion or cation exchange resins. The anion resin (Q Sepharose) that showed a maximal binding capacity and compatibility with FPME was selected (Data not shown).

Anion exchange resin chromatography was carried out to purify the FPME (Figure 5-4), Briefly, 400 mg of FPME was dissolved in 100 ml distilled water and concentrated through 10k cut-off ultrafiltration membrane. The retentate (10KR) was washed with an equal volume of 20 mM Tris/HCl buffer, pH 8.0. The 10KR fraction was then loaded on to a Q Sepharose ion exchange (Amersham Biosciences, Piscataway, NJ, USA) resin column with an internal diameter of 2.5 cm and a bed height of 5 cm. The column had been equilibrated with 20 mM Tris/HCl buffer, pH 8.0, and the flow rate was maintained at 1 ml/min throughout the run. The sample loaded on the column was then eluted with an equal volume of stepwise gradient of NaCl (0, 0.25, 0.5, 0.75, 1 M NaCl) in 20 mM Tris/HCl buffer, pH 8.0. The fractions were assayed for antidiabetic activity.

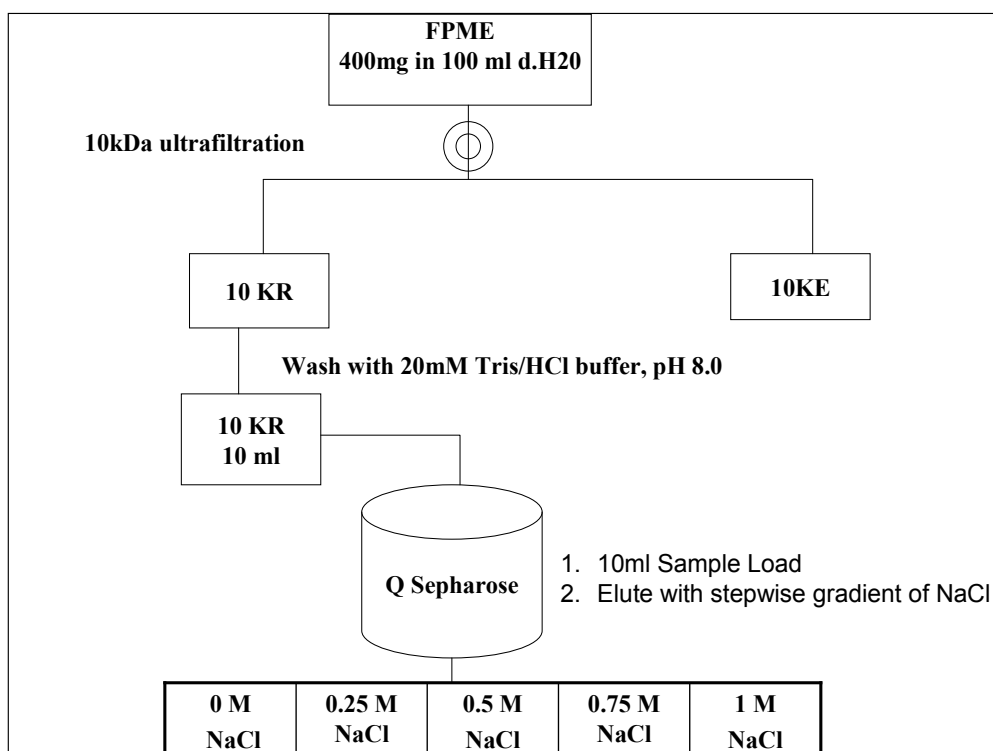


Figure 5-4 Anion exchange chromatography fractionation of FPME

5.2.4 Bioassay-guided purification of PME

Based on the above preliminary studies, the purification and isolation of the antidiabetic compounds from PME was carried as described in Figure 5- 5. Briefly, 30 litres of PME was eluted through Q Sepharose anionic ion exchange resin column (500 ml) that had been pre-equilibrated with 20 mM Tris/HCl buffer, pH 8.0, the column had an internal diameter of 4.5 cm and a bed height of 15 cm and the flow rate was maintained at 10 ml/min throughout the run. The material that eluted through the column (QSE) was collected and stored at -20°C for further investigations. The material retained on the

column was then eluted using 1 litre of 1M NaCl in 20 mM Tris/HCl buffer, pH 8. The salt eluted material (QSR) from the column was then concentrated through 10 kDa cut-off ultrafiltration membrane to a final volume of 10 ml and washed with distilled water. The material that diffused through 10 kDa cut-off membrane (QSR/10KE) was collected and stored at -20°C for further investigations. The material that was retained by the membrane (QSR/10KR) was further fractionated using a Sephadex G25 column as described in Section 5.2.3.4. This fractionation process yielded 10 fractions (Sx 1-10); each consisting of 10 ml. Based on our preliminary results, the fraction no 2 (Sx 2) was selected for further purifications, structural elucidation and pharmacological examinations. This fraction was identified as #SK/PME/07. 100 µl aliquots of #SK/PME/07 were prepared and stored at -20°C.

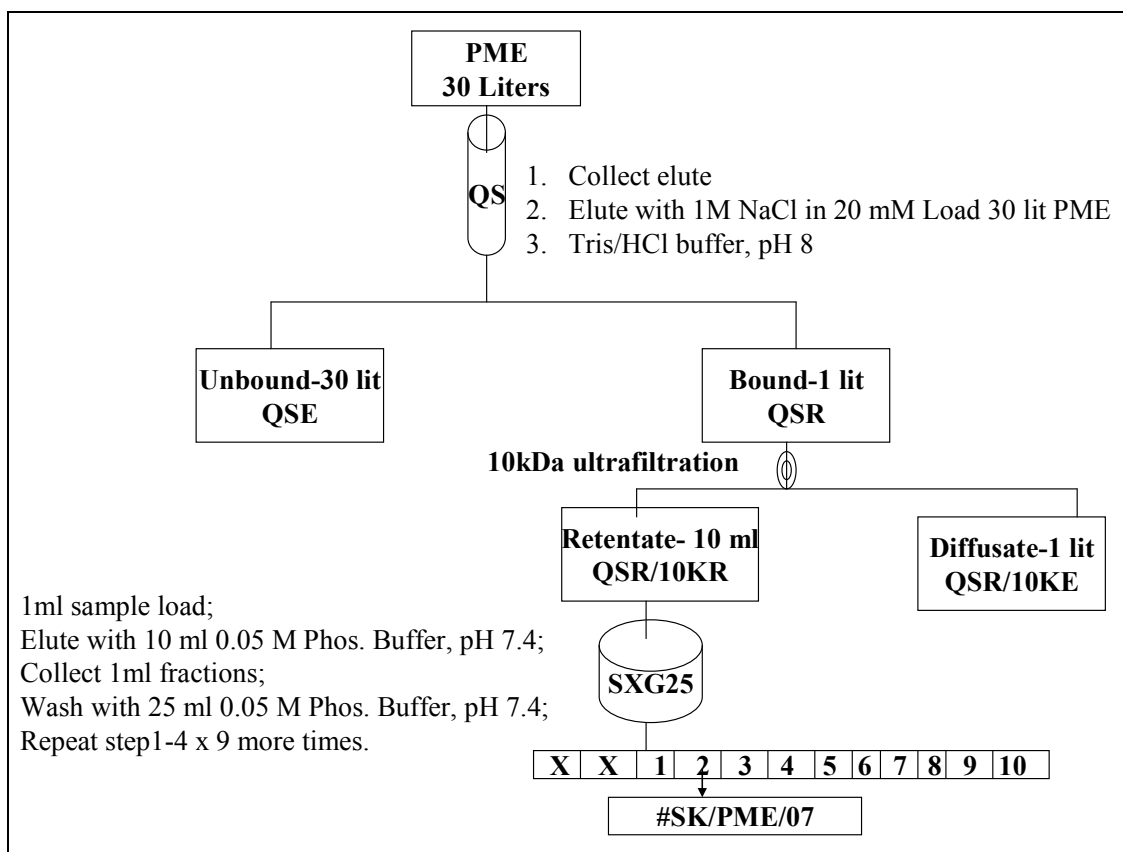


Figure 5-5 Bioassay-guided fractionation of PME

5.2.5 Molecular weight determination

Molecular weight of the constituent(s) of #SK/PME/07 was determined by SDS-PAGE as described in Section 2.8. Gels were stained for both proteins and glycoproteins.

5.2.6 Peroxidase activity

Peroxidase activity of #SK/PME/07 was determined to investigate the presence of any peroxidase molecule in the sample. Briefly, 10 μ l of #SK/PME/07 or horse radish

peroxidase (STD) was added to 250 µl of TMB substrate (Appendix) in separate tube and incubated for 60 minutes. The appearance and intensity of blue colour in the sample was compared with the standard.

5.2.7 Purification of #SK/PME/07

5.2.7.1 *Affinity Chromatography*

Concanavalin A coupled to Sepharose 4B, (Con A Sepharose™, Amersham Biosciences, Piscataway, NJ, USA) was used to study the nature of constituent(s) of #SK/PME/07. Con A binds with molecules containing α -D-mannopyranosyl, α -D-glucopyranosyl and sterically related residues. Briefly, a Con A column was washed with 10 ml of 1 M glucose solution followed by 15 ml of starting buffer (20mM Tris-HCl buffer containing 0.5M NaCl, pH- 7.4). 1 ml of #SK/PME/07 was run through the Con A column which was rinsed with starting buffer. These fractions were pooled and identified as Con A unbound (CAUB). 1 M glucose solution was used to elute the Con A bound protein until no further protein was detected by Bradford's reagent in the fractions. These fractions were then pooled and identified as Con A bound (CAB). Both CAB and CAUB fractions were concentrated and examined for their *in vitro* antidiabetic activity.

5.2.7.2 *PVPP treatment*

Insoluble polyvinylpolypyrrolidone (PVPP) was used to remove tannins from #SK/PME/07. Briefly, 100 µl of #SK/PME/07 was diluted with distilled water (1 ml) and

solute added to 25 mg of PVPP and placed on a shaking rack for 30 minutes. After shaking, the sample was centrifuged and the supernatant was filtered through a syringe filter (0.2 μ m) to remove the PVPP. These entire PVPP treated fractions (PVPPT) were then examined for antidiabetic activity.

5.2.7.3 *Isoelectric focusing*

Isoelectric focusing was carried out using a MicroRotofor™ Liquid Phase IEF cell (BIO-RAD) to separate the molecules which differ in their charge characteristics and also to assess the complexity and purity of #SK/PME/07. Briefly, 150 μ l of ampholyte (BioLyte 3-10) was added to 2 ml of #SK/PME/07 and the volume adjusted to 3 ml with distilled water. The sample was loaded on the focusing assembly and the run was performed as per manufacture's instructions. The pH of the fractions (1-10) obtained were readjusted immediately to neutral pH and examined for their *in vitro* antidiabetic effects. Further the molecular weights of these fractions were examined by performing SDS-PAGE.

5.2.7.4 *Chloroform –Methanol precipitation*

Concentration of proteins in the sample was carried out as described by Wessel and Flugge (1984). In brief, 0.1 ml of #SK/PME/07 was added to 0.4 ml of methanol and vortexed for 10 seconds. To this solution, 0.1 ml of chloroform was added and mixed, and it was then centrifuged at 13000 rpm for 10 seconds. After this, 0.3 ml of water was added, mixed vigorously and centrifuged at 13000 rpm for 1 min. The upper phase was removed. 0.3 ml of methanol was added to the lower phase and interphase which contains

precipitated proteins. This solution was mixed and centrifuged at 13000 rpm for 2 min to pelletise the proteins. A portion of pellet was dissolved in 2x non-reducing sample buffer for SDS-PAGE and the remaining portion was examined for antidiabetic activity.

5.2.8 Dose response curve

The dose responses of various concentrations of #SK/PME/07 and PME, produced by serial 10 fold dilutions of the original fractions, on glucose uptake by mouse skeletal muscle tissues *in vitro* were determined as described in Section 2.4.

5.2.9 N-terminal Sequencing

Electrophoresis of #SK/PME/07 was performed as described in Section 2.8. The gels were stained and de-stained as described in Section 2.8.1. After de-staining, the gel was washed with distilled water overnight. Two major protein bands $\leq 27\text{kDa}$ and $\leq 20\text{kDa}$ (see Figure 5-13) was cut and transferred to extraction buffer (Sodium acetate 100mM; SDS 0.1%; DTT 10mM in distilled water). The gel bands were homogenized in extraction buffer for 60 minutes and centrifuged at 10000 rpm for 15 minutes. The supernatant was collected and concentrated to 100 μl volume and submitted for N-terminal sequencing.

5.3 Results

5.3.1 Preliminary purification studies

5.3.1.1 Aqueous extraction

The aqueous extraction of PM yielded a dark brown colour with bluish green fluorescence in the extract. The concentration of PME after freeze drying was determined to be 0.4 mg/ml. The effect of heat on the nature of hypoglycemic constituent(s) of PME was investigated by boiling the PME (BPME) (Figure 5-6).

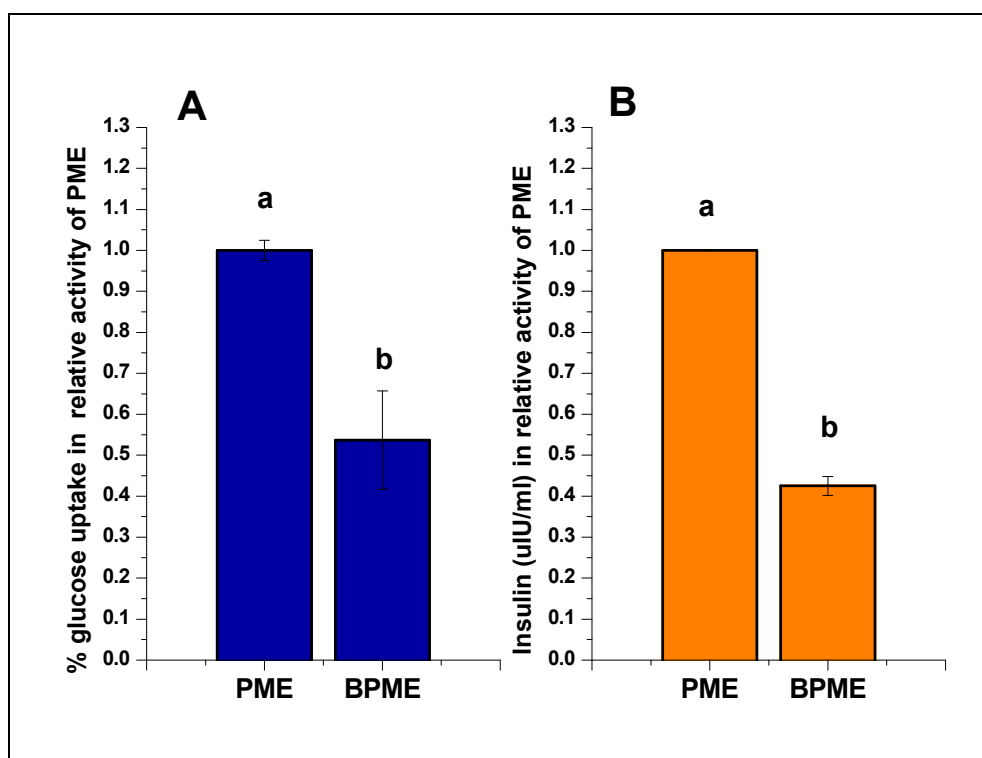


Figure 5-6 Effect of PME and BPME on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. Means without a common letter are significantly different ($p < 0.05$). BPME- Boiled PME.

The effect of heating significantly inhibited both glucose uptake (0.53 ± 0.12 % glucose uptake in relative activity of PME) and insulin secretion (0.42 ± 0.02 insulin (μ IU/ml) in relative activity of PME) activity of PME, but it has no significant influence on basal glucose uptake.

5.3.1.2 *Organic solvent extraction*

Sequential extraction of PME with ethyl acetate and butyl alcohol yielded four different fractions: ethyl acetate soluble (EA), aqueous layer after ethyl acetate extraction (Aq.EA), butyl alcohol soluble (BA), aqueous layer after butyl alcohol extraction (Aq.BA). All these fractions were examined for both *in vitro* glucose uptake and insulin secretion activity (Figure 5-7). Some fractions (EA, and Aq.BA) exhibited enhanced glucose uptake activity (1.56 ± 0.15 ; 1.61 ± 0.12 ; % glucose uptake in relative activity of PME; respectively) but, Aq.EA (1.05 ± 0.05 % glucose uptake in relative activity of PME) had no significant effect on the glucose uptake activity of PME (Figure 5-7A). The effect on insulin secreting activity (Insulin (μ IU/ml) in relative activity of PME) in decreasing order 1.45 ± 0.21 ; 1.42 ± 0.13 ; 0.81 ± 0.10 ; 0.63 ± 0.19 of fractions: Aq.BA, Aq.EA, EA and BA respectively; were significantly different from the PME ($p < 0.05$) (Figure 5-7B). Aq.BA and Aq.EA fractions showed enhanced insulin secreting activity and fractions: EA and BA showed an inhibited insulin secretion when compared with the insulin secreting activity of PME.

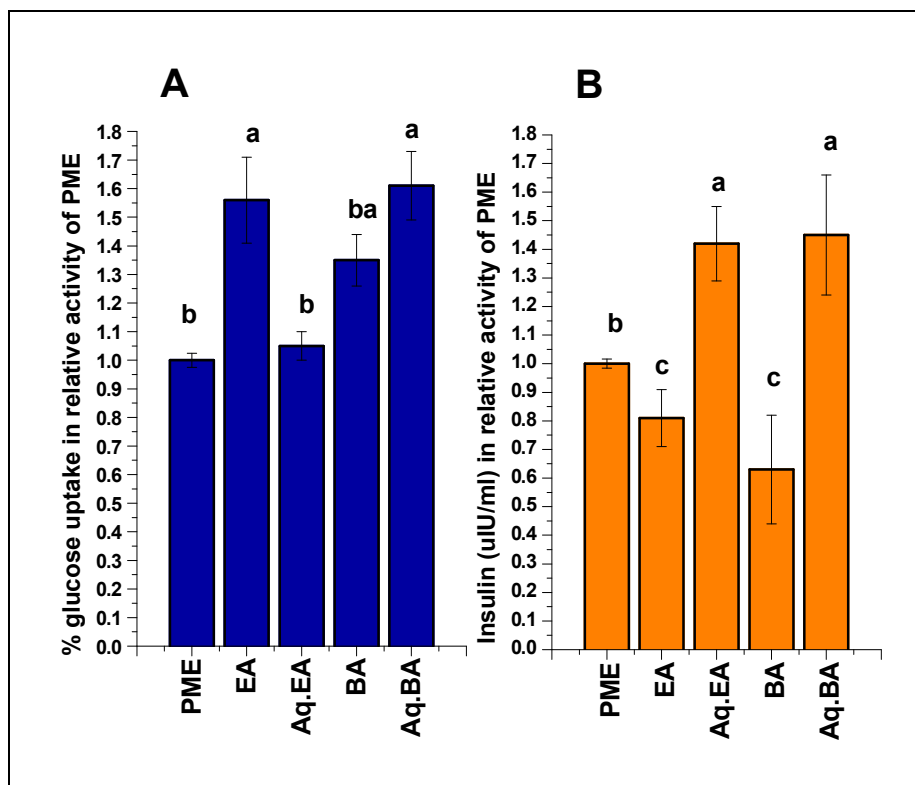


Figure 5-7 Effect of PME, EA, Aq.EA, BA and Aq.BA fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. Means without a common letter are significantly different ($p < 0.05$). EA- Ethyl Acetate soluble; Aq.EA- Aqueous layer after Ethyl Acetate extraction; BA- Butyl Alcohol soluble; Aq.BA- Aqueous layer after Butyl Alcohol extraction.

5.3.1.3 Ultrafiltration

Molecular weight range of hypoglycemic constituent(s) of PME was determined by concentrating PME through 10 kDa cut-off ultrafiltration membrane and 30 kDa cut-off centrifugal filters. The fractions obtained: 10KR, 10KE, 30KR and 30KE were investigated for their effects on glucose uptake activity and insulin secretion *in vitro*

(Figure 5-8). The fractions 10KR and 30KE showed enhanced glucose uptake activity (1.75 ± 0.16 ; 1.88 ± 0.1 % glucose uptake in relative activity of PME) and increased insulin secretion (1.81 ± 0.2 ; 1.72 ± 0.13 insulin ($\mu\text{IU/ml}$) in relative activity of PME); both significantly greater than the PME values ($p < 0.05$).

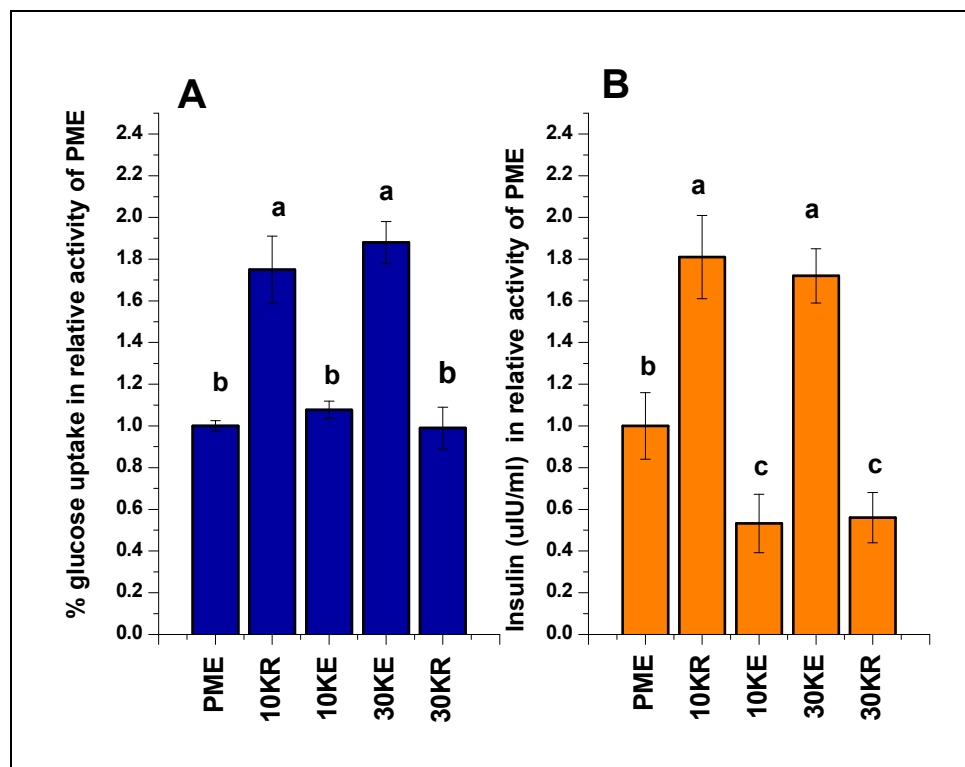


Figure 5-8 Effect of 10KR, 10KE, 30KE and 30 KR fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. Means without a common letter are significantly different ($p < 0.05$). 10KR- 10 kDa cut-off Retain; 10KE- 10 kDa cut-off Elute; 30KE- 30 kDa cut-off Elute; 30KR-30 kDa cut-off Retain.

The percentage of glucose uptake activity of 10KE and 30 KR (1.07 ± 0.04 ; 0.99 ± 0.1 % glucose uptake in relative activity of PME) were not significantly different from PME. However these fractions showed inhibited insulin secretion (0.532 ± 0.14 ; 0.56 ± 0.12 insulin ($\mu\text{IU/ml}$) in relative activity of PME) and it was significantly less than the insulin

secreting activity of PME ($p < 0.05$). This suggests that molecules greater than 10 kDa and less than 30 kDa are the insulin secretion stimulating and glucose uptake activity.

5.3.1.4 *Sephadex G-25 column chromatography*

Preliminary fractionation of PME was carried out using Sephadex G-25. The fractions were known as Sx1, Sx2.....Sx10. All these fractions were examined for their effect on both glucose uptake and insulin secretion activity *in vitro* (Figure 5-9). Of these fractions, Sx2 and Sx3 showed peak activity in both glucose uptake (1.87 ± 0.39 ; 1.96 ± 0.47 % glucose uptake in relative activity of PME) and insulin secretion (2.46 ± 0.15 ; 1.84 ± 0.49 insulin ($\mu\text{IU/ml}$) in relative activity of PME) which was significantly greater than that of PME ($p < 0.05$).

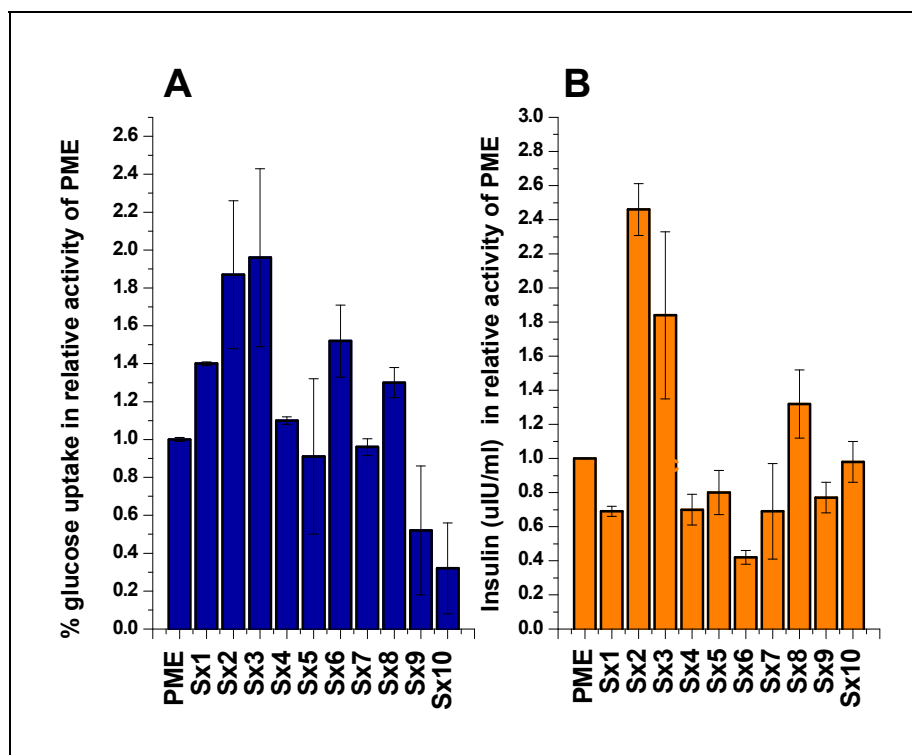


Figure 5-9 Sephadex G-25 fractionation of PME Sx1, Sx2, ...Sx10 fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. Sx1, Sx2.....Sx10- Sephadex-G25 fractions.

5.3.1.5 *Trypsin digestion*

The preliminary data suggested that the active ingredient was a large molecular weight molecule(s). These are likely to be carbohydrates, tannins, proteins and/or glycoproteins. Trypsin digestion of PME was carried out to investigate the nature of hypoglycemic constituent(s) of PME. The samples obtained from +Trypsin and –Trypsin were examined for their effect on tissues involved in glucose homeostasis (Figure 5-10).

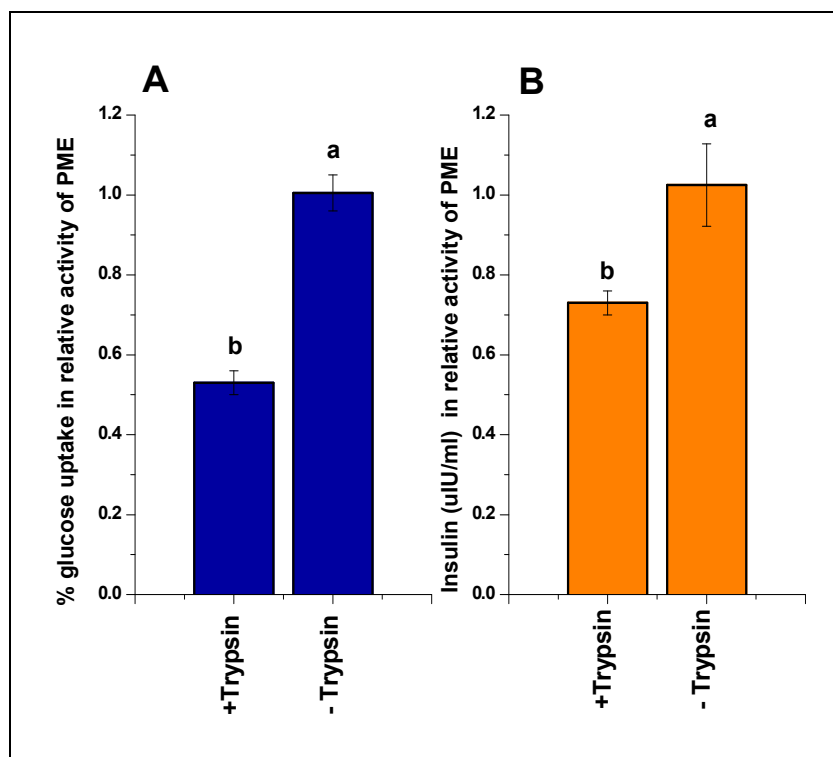


Figure 5-10 Trypsin digestion of PME; +Trypsin and -Trypsin samples on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. Means without a common letter are significantly different ($p < 0.05$). +Trypsin- PME incubated with trypsin; -Trypsin- PME incubated without trypsin.

The sample incubated with trypsin (+Trypsin) showed inhibition of both glucose uptake (0.53 ± 0.03 % glucose uptake in relative activity of PME) and insulin secretion (0.73 ± 0.03 insulin (μ IU/ml) in relative activity of PME) and both activities were significantly lower than that of PME incubated without trypsin (-Trypsin) ($p < 0.05$).

5.3.1.6 Ion exchange chromatography

Anion exchange chromatography was carried out to fractionate hypoglycemic constituent(s) of PME. The fractions were designated as 0M, 0.25M, 0.5M, 0.75M and

1M on the basis of concentrations of NaCl used for elution. *In vitro* examinations of these fractions on glucose uptake and insulin secretion are presented in Figure 5-11. All fractions showed an enhanced glucose uptake and insulin secretion activity. However, the peak activity was detected in the 0.5M fraction in terms of stimulating both glucose uptake (2.472 ± 0.11 % glucose uptake in relative activity of PME) and insulin secretion (4.09 ± 0.16 insulin (μ IU/ml) in relative activity of PME), and it was significantly more potent than PME alone ($p < 0.05$). The 0.75M and 1M fractions also showed an enhanced activity compared to PME ($p < 0.05$).

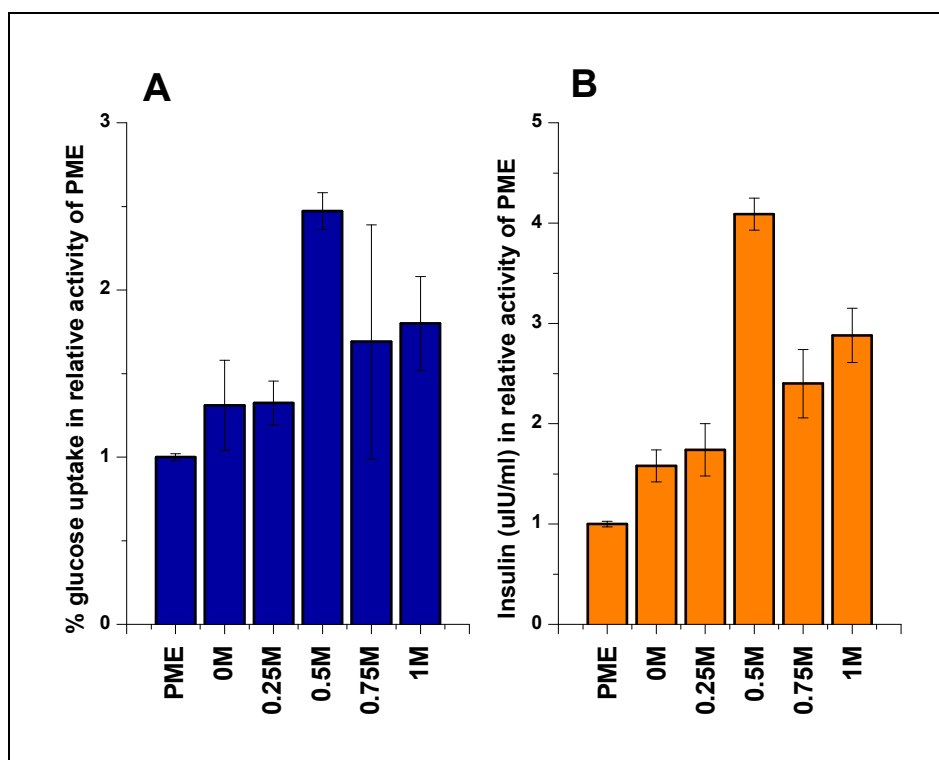


Figure 5-11 Anion exchange chromatography of PME; eluted with various concentrations of NaCl (as indicated) A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

5.3.2 Bioassay-guided purification of PME

Based on the above preliminary results (Section 5.2.3), a bioassay-guided purification was performed on the PME in order to isolate the bioactive compound(s) responsible for antidiabetic activity. After every fractionation process, the fractions were tested for their effects on glucose uptake by mouse muscle tissue and insulin secretion from mouse pancreas tissues *in vitro*. The results obtained from both glucose uptake and insulin secretion *in vitro* bioassay, were converted in relative activity of PME and presented in Figure 5-12.

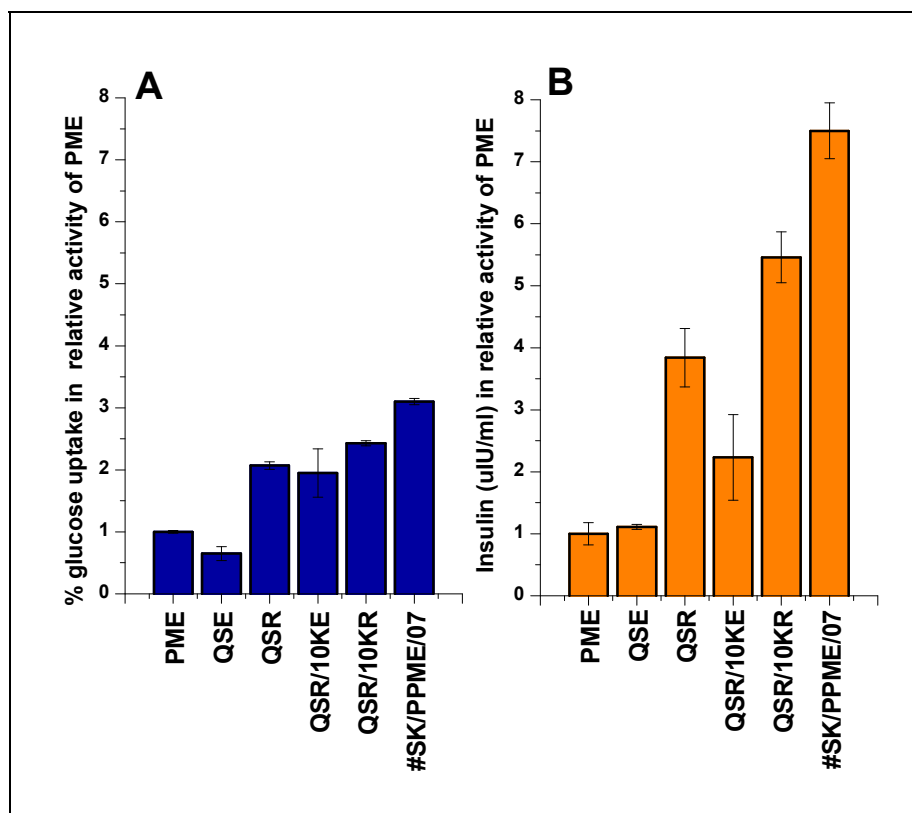


Figure 5-12 Bioassay-guided fractionation of PME; QSE, QSR, QSR/10KE, QSR/10KR and #SK/PME/07 fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. QSE- Q Sepharose Elute; QSR- Q Sepharose Retain; QSR/10KE- Q Sepharose Retain/10 kDa cut-off Elute; QSR/10KR-Q Sepharose Retain/10kDa cut-off Retain; #SK/PPME/07- Q Sepharose Retain/10 kDa cut-off Retain/Sephadex G25 fraction no-2.

The first step of bioassay guided purification of PME was performed through Q Sepharose ion exchange chromatography, yielded two fractions QSR and QSE, of these two fractions QSR showed a significantly enhanced activity ($p < 0.05$) on both glucose uptake (2.07 ± 0.06 % glucose uptake in relative activity of PME) and insulin secretion (3.84 ± 0.47 insulin ($\mu\text{IU/ml}$) in relative activity of PME). However, the effects of QSE under similar conditions on both glucose uptake and insulin secretion were not significantly different from PME ($p > 0.05$). Based on these results, the active fraction QSR was considered for the next step of purification. In the second step, concentration of QSR through 10 kDa molecular weight cut-off membrane yielded two fractions, QSR/10KR and QSR/10KE. The *in vitro* antidiabetic effect of QSR/10KR was found to be significantly greater than the activity of PME ($p < 0.05$). The insulin secretion (5.46 ± 0.41 insulin ($\mu\text{IU/ml}$) in relative activity of PME) was elevated in the QSR/10KR treated groups and it was significantly different from QSR treated groups ($p < 0.05$), but there was no significant difference in glucose uptake between the QSR/10KR (2.43 ± 0.04 % glucose uptake in relative activity of PME) and QSR treated groups ($p > 0.05$). The effect of QSR/10KE on glucose uptake activity (1.952 ± 0.39 % glucose uptake in relative activity of PME) had no significant difference from QSR and its effect on insulin secretion (2.23 ± 0.69 insulin ($\mu\text{IU/ml}$) in relative activity of PME) was significantly lower than that of QSR. The active fraction from the second step, QSR/10KR was further fractionated using Sephadex G25 column chromatography, with this fractionation step yielding 10 fractions, each consisting of 10 mL. Of these fractions, fraction no.2 (Sx-2) showed strong activity on both glucose uptake (3.10 ± 0.05 % glucose uptake in relative activity of PME) by mouse muscle tissue and insulin secretion (7.5 ± 0.45 insulin

(μ IU/ml) in relative activity of PME) from mouse pancreas tissue and it was significantly more potent than all other earlier fractions ($p < 0.05$). This fraction was brown in color and its concentration was found to be 0.83 mg/ml. The purified potent fraction, Sx-2 from the bioassay-guided purification process was named as #SK/PME/07 and used for subsequent purification and structural elucidation.

5.3.3 Molecular weight determination

To investigate the molecular weight of the constituent(s) present in the #SK/PME/07, SDS-PAGE was performed and stained for both proteins and glycoprotein, as described in Section 2.8. Coomassie R250 stained gel showed two major protein bands at ≤ 27 kDa and ≤ 20 kDa (Figure 5-13).

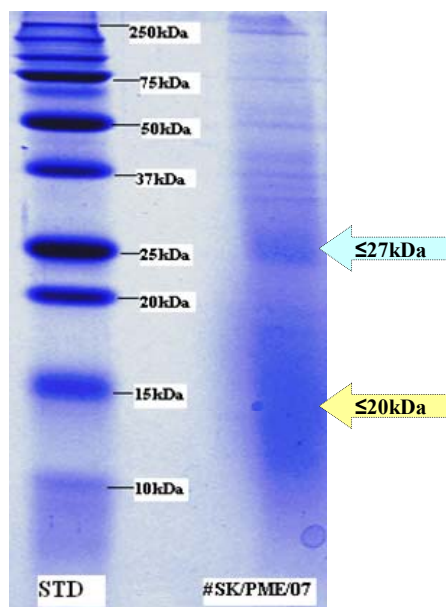


Figure 5-13 Coomassie R250 stained SDS-PAGE of marker proteins (STD) and #SK/PME/07. The arrow indicates the two major protein bands of #SK/PME/07

Glycoprotein staining was carried out as per standard staining protocol and guidelines described by GYCO-PRO kit. The purple colour development after staining in both the horseradish peroxidase standard (HRP) and #SK/PME/07 indicates the presence of glycoproteins (Figure 5-14).



Figure 5-14 Glycoprotein stained SDS-PAGE of horseradish peroxidase and #SK/PME/07.

5.3.4 Peroxidase activity

Peroxidase activity of #SK/PME/07 was determined to investigate the presence of any peroxidase molecule in the sample. Incubating horseradish peroxidase (HRP) with TMB substrate developed an intense blue colour. However, under similar conditions, #SK/PME/07 remains unchanged, indicating the absence of peroxidase molecules in the #SK/PME/07.

5.3.5 Purification of #SK/PME/07

5.3.5.1 *Affinity Chromatography*

Con A column chromatography was performed to purify hypoglycemic constituent(s) of #SK/PME/07 and the obtained fractions were examined for their *in vitro* antidiabetic activity (Figure 5-15). The effect of Con A unbound (CAUB) on both glucose uptake (0.96 ± 0.06 % glucose uptake in relative activity of PME) and insulin secretion (0.99 ± 0.04 Insulin (μ IU/ml) in relative activity of PME) was significantly less than the effect of both CAB and #SK/PME/07 ($p < 0.05$). The Con A bound (CAB) material showed a potent activity with both glucose uptake (3.58 ± 0.10 % glucose uptake in relative activity of PME) and insulin secretion (3.21 ± 0.24 insulin (μ IU/ml) in relative activity of PME) and there were significantly different from the effects of PME. However, the antidiabetic effects of CAB were significantly lower than the effect of #SK/PME/07 ($p < 0.05$).

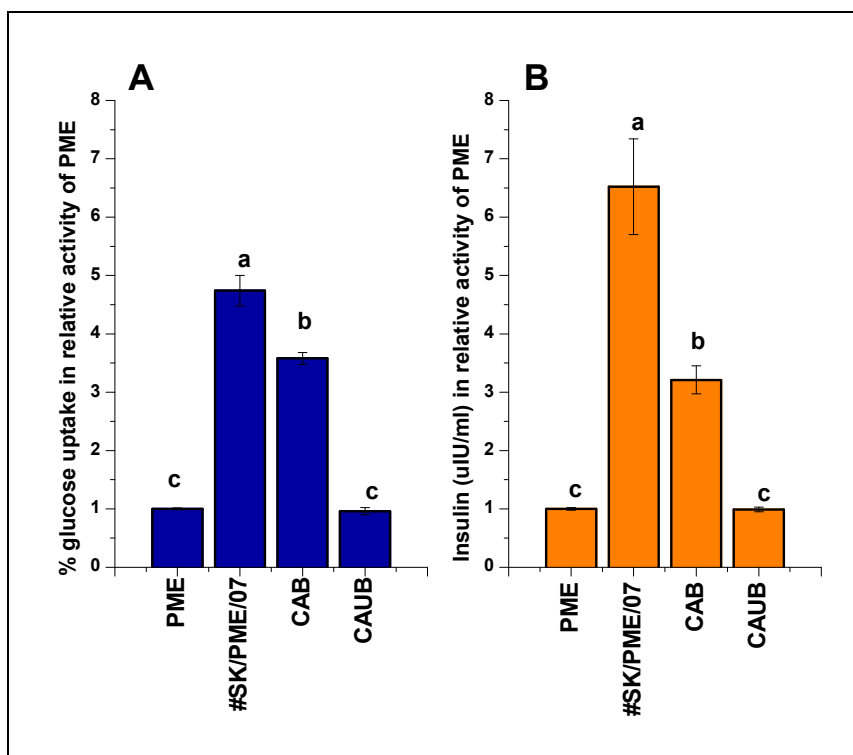


Figure 5-15 Purification of #SK/PME/07; PME, #SK/PME/07, CAB and CAUB fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. Means without a common letter are significantly different ($p < 0.05$). #SK/PME/07- A potent fraction of PME; CAB- Con A Bound material; CAUB- Con A UnBound material.

5.3.5.2 PVPP treatment

Removal of tannins and other high molecular weight polyphenolic impurities from #SK/PME/07 was carried out by treating with PVPP. The PVPP unabsorbed material that obtained after PVPP treatment showed no significant difference from #SK/PME/07 on either glucose uptake or insulin secretion ($p > 0.05$) (data not shown). This indicates tannins are not part of the active molecule.

5.3.5.3 Isoelectric focusing

Isoelectric focusing was carried out to separate and concentrate the proteins present in the #SK/PME/07 based on their charge characteristics. The fractions (ISF 1-10) obtained were investigated for their effects on glucose uptake and insulin secretion (Figure 5-16). The pH range of each fraction has been calculated from the pH range of ampholyte (Bio-Lyte pH 3-10) used in the experiment. The 7 pH unit range was separated across 10 fractions and the estimated pH range for each fraction was ~0.7 pH units.

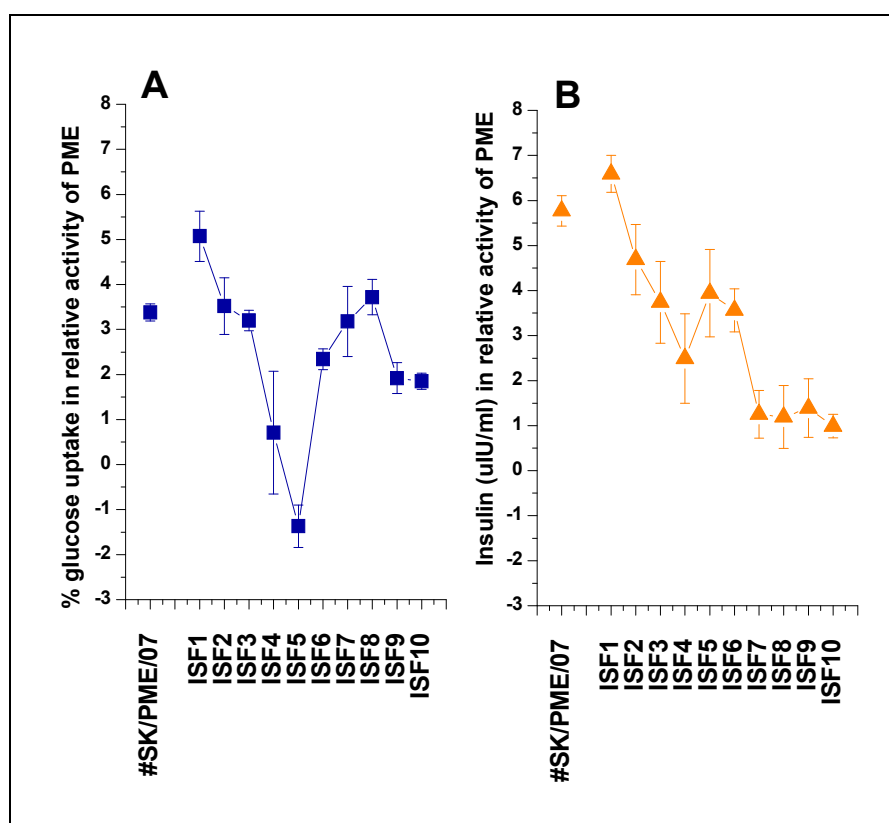


Figure 5-16 #SK/PME/07 and ISF fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. ISF (1-10) – Isoelectric focusing fractions

The effect of ISF (1-10) fractions on glucose uptake showed a peak activity in fraction no.1 and the activity tends to gradually decline until fraction no.5 and then increased until fraction no.8 and again declined with respect to increase in the pH units of fractions. Of these fractions, ISF1 (pH ~ 3) showed a peak glucose uptake and it was greater than the effect of #SK/PME/07 and all other ISF fractions. The effects of fractions ISF2, ISF3, ISF7 and ISF8 on glucose uptake were similar to the effects of #SK/PME/07. All other fractions (ISF4, ISF5, ISF6, ISF9 and ISF10) showed an inhibited glucose uptake and were lower than the effect of #SK/PME/07 ($p < 0.05$). Similarly, the effect of ISF (1-10) fractions on insulin secretion also showed a peak activity in fraction no.1 (ISF1) and the activity tends to gradually decline until fraction no.4, increases in the activity in fraction no.5, and then declined with respect to increase in pH units of fractions. With insulin secretions all ISF fractions except ISF1, were lower than the effect of #SK/PME/07.

SDS-PAGE of these fractions showed streaking of bands in all fractions (gel not shown), suggests the active protein bands probably masked by the presence of ampholyte.

5.3.5.4 Chloroform-Methanol precipitation

Precipitation of proteins of #SK/PME/07 was performed to concentrate the proteins present in the #SK/PME/07 and also to determine its effect on mouse glucose uptake and insulin secretion (Figure 5-17). The precipitate (PPT) that was obtained after chloroform-methanol precipitation showed an enhanced glucose uptake activity and insulin secretion. The glucose uptake activity (5.42 ± 0.19 % glucose uptake in relative activity of PME) of

PPT was not significantly different from the effect of #SK/PME/07 ($p>0.05$). However, the insulin secretion activity (7.90 ± 0.24 Insulin ($\mu\text{IU/ml}$) in relative activity of PME) of PPT was significantly greater than the effect #SK/PME/07. The effects of supernatant (SN) on both insulin secretion (1.86 ± 0.14 Insulin ($\mu\text{IU/ml}$) in relative activity of PME) and glucose uptake (0.64 ± 0.16 % glucose uptake in relative activity of PME) were significantly inhibited ($p<0.05$). SDS-PAGE of the PPT dissolved in 2x non-reducing sample buffer (Figure 5-18) showed a major band at $\leq 27\text{kDa}$ and some $\leq 20\text{kDa}$.

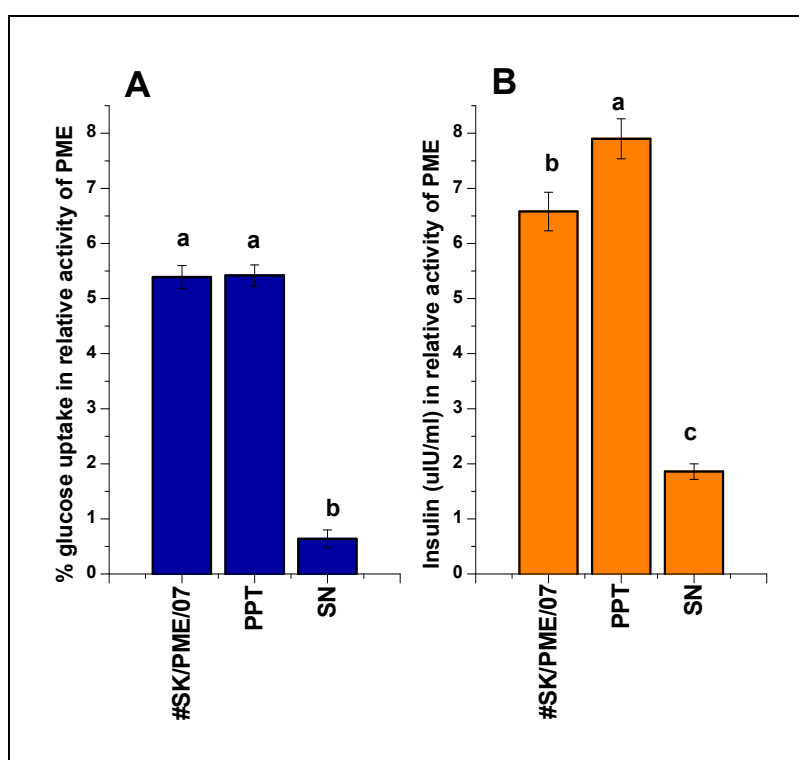


Figure 5-17 #SK/PME/07, PPT and SN fractions on A- glucose uptake by mouse muscle tissues and B- insulin secretion from mouse pancreatic tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) obtained were converted as relative activity of PME and presented as Mean $\pm$ S.E. Means without a common letter are significantly different ($p < 0.05$). PPT- Precipitate; SN- Supernatant.

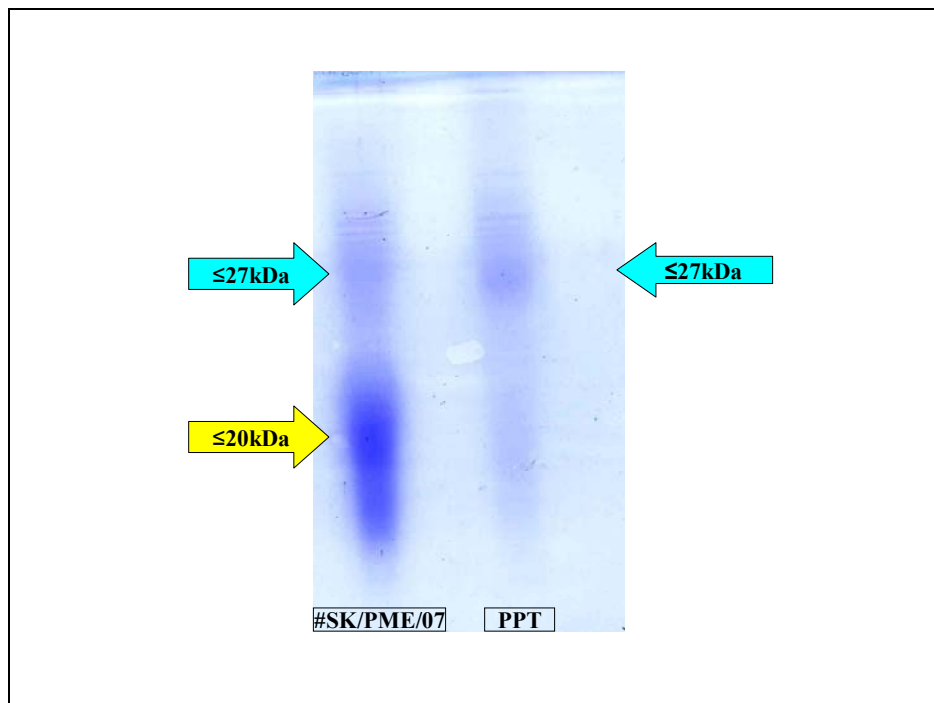


Figure 5-18 Coomassie R250 stained SDS-PAGE of #SK/PME/07 and PPT. The arrow indicates the major protein bands of #SK.PME/07 and PPT.

5.3.6 Dose response curve

The effect of 10 fold diluted doses of #SK/PME/07 and PME on mouse muscle glucose uptake activity and pancreas insulin secretion *in vitro* is shown in Figure 5-19. Chronic incubation of pancreatic or muscle tissues with the PME (0-100 μ l) or #SK/PME/07 (0-1 μ l) of increasing final concentration under hyperglycemic culture condition exerted a dose-dependent stimulatory effect.

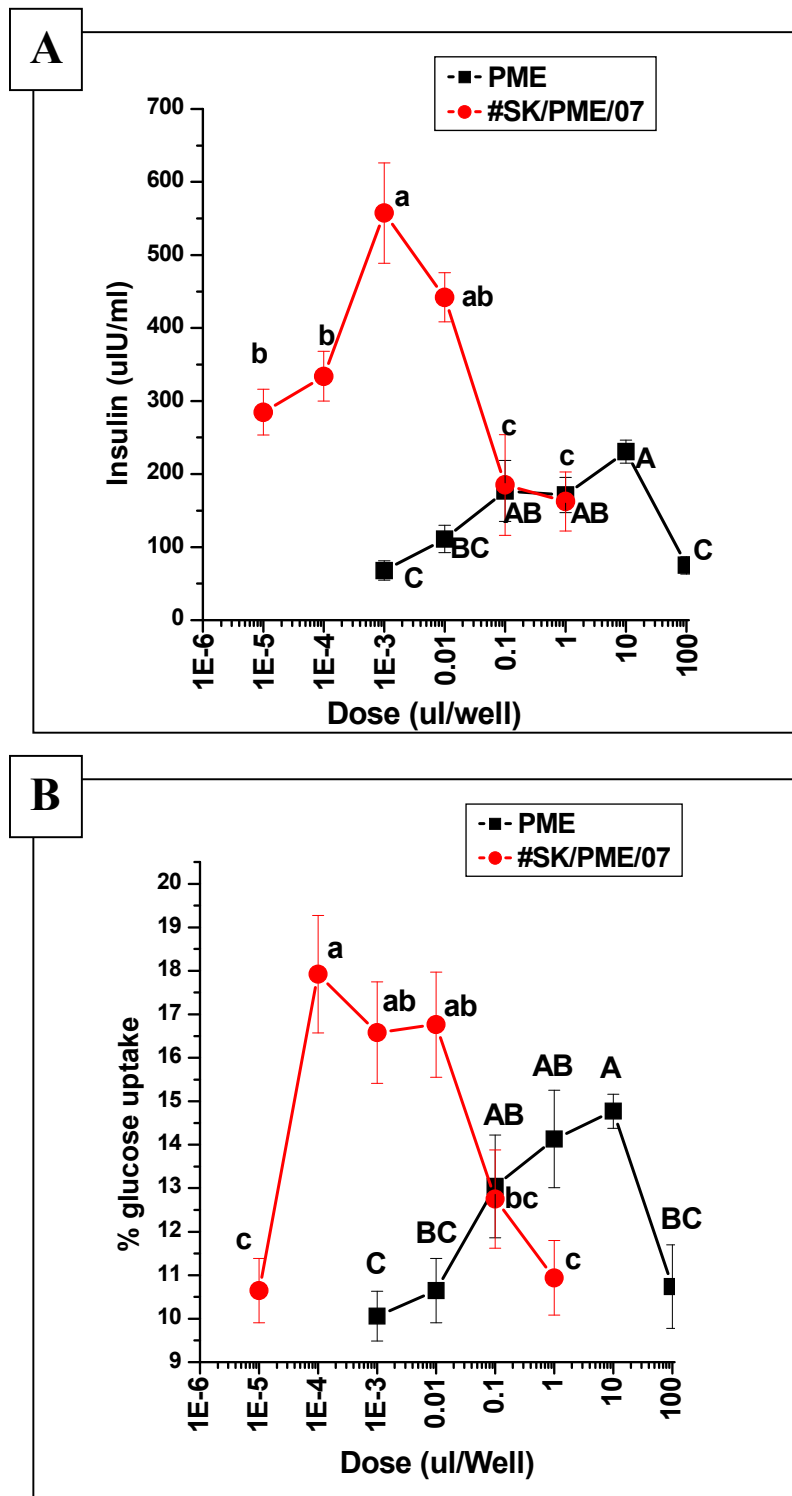


Figure 5-19 Dose response curve of PME and #SK/PME/07 A) on insulin secretion from mouse pancreas tissues and B) on glucose uptake by mouse skeletal muscle tissues over chronic incubation under hyperglycemic culture conditions.

Data (glucose uptake and insulin) are presented as Mean $\pm$ S.E. Means without a common letter are significantly different ($p < 0.05$).

5.3.7 N-terminal sequencing

Repeated attempts tried to sequence the #SK/PME/07 were unsuccessful, because the volume of the final sample was small.

5.4 Discussion

Pterocarpus marsupium (PM), an Ayurvedic traditional medicine, is known to have preventive and therapeutic effects in T2D. The hypoglycemic activity of PM has been confirmed by many modern scientific investigations. However, studies directed to the isolation, purification and identification of substances responsible for the hypoglycemic activity have been lacking. The present study for the first time reports the identification and isolation of more potent antidiabetic constituent(s) of PM using a bioassay-guided purification technique.

The Ayurvedic system of medicine strongly recommends water stored in a tumbler made from heartwood of PM for effective diabetes treatment (Chopra *et al.*, 1958; Jain, 1968; Satyavathi *et al.*, 1987) and it has been shown to have some therapeutic benefits in humans (Sepaha & Bose, 1956; Pandey & Sharma, 1975; Rajasekharan & Tuli, 1976; Kedar & Chakrabarti, 1981; ICMR, 1998). The present study prepared the aqueous extract of heartwood of PM by soaking the heartwood in water at room temperature. Aqueous extracts of PM (PME) yielded a dark brown color extract and its concentration was found to be 0.4 mg/ml. The yield from the raw material was 10.2 %. This compares favorably with the percentage yield of aqueous decoction of heartwood of PM in the

earlier literature, which range from 8-10 % (ICMR, 1998). The effects of PME on both glucose uptake by mouse muscle tissues and insulin secretion from mouse pancreas tissues have been already discussed in Chapter 3 and 4.

Manickam and colleagues (1997) isolated three main isoflavones, marsupsin, pterosupin and pterostilbene, from the ethyl acetate soluble fraction of a hot aqueous decoction of the heartwood of PM and reported marsupsin and pterostilbene to be effective antidiabetic agents. Similarly, Maurya and colleagues (2004) isolated five new flavanoids C-glucosides from the butyl alcohol soluble portion of a hot aqueous decoction of the heartwood of PM. In our study boiling the PME (BPME) destroyed both glucose uptake and insulin secretion activity of PME, indicating that the hypoglycemic constituent(s) present in the PME is heat sensitive. Our study also examined the ethyl acetate and butyl alcohol soluble fraction of PME and found that the most activity towards both glucose uptake and insulin secretion remains in the aqueous portion after ethyl acetate and butyl alcohol extraction. These differing results from the previous reports are possibly due to the difference in the experimental model used to determine the antidiabetic activity. Nevertheless, in terms of stimulating insulin release and glucose uptake under hyperglycemic conditions, the aqueous extract of PM remains potent even after ethyl acetate and butyl alcohol extraction. This prompted the detailed investigation and purification of PME.

The ultrafiltration of PME through 10 and 30 kDa molecular mass cut-off membranes indicated that the molecular weight ranges of hypoglycemic constituents of PME is in the

range of 10 kDa to 30 kDa. The PME fractionated on Sephadex G25 yielded 10 fractions. Of these fractions, fractions no 2 & 3 (Sx 2 and 3) showed peak glucose uptake and insulin secretion activity. The peak activity at fraction 2 and 3 further confirmed that the hypoglycemic constituent of PME is/are high molecular weight molecule(s) and also fractionation through Sephadex G-25 probably eliminates all low molecular weight inhibitory or inactive substances from PME.

High molecular weight polyphenols are abundant in plants and includes other subclasses besides flavanoids such as phenolic acids, stilbenes, lignans, oxidized polyphenols and tannins. Polyphenols have various biological properties and they have been mainly used as antioxidants in the prevention of several chronic diseases involving oxidative stress (Rice-Evans, 1995). PM is a rich source of polyphenols (Seshadri, 1972; Adinarayana & Syamasundar, 1982; Maurya *et al.*, 1982; Mathew & Rao, 1983; Maurya *et al.*, 1984; Bezuidenhoudt *et al.*, 1987; Maurya *et al.*, 2004). In this study, trypsin digestion destroyed the activity of PME, suggesting that the hypoglycemic constituent(s) present in the PME is/are protein(s) or perhaps that the association of proteins is necessary for the antidiabetic activity. Based on these results, anion exchange chromatography was performed to purify the hypoglycemic constituent(s) of PME. The result of ion exchange chromatography indicated that the 0.5M salt eluted material showed the most potent activity.

Based on these studies, the bioassay-guided purification of PME was carried out. and yielded a more potent antidiabetic fraction identified as #SK/PME/07. The concentration

of #SK/PME/07 was 0.83 mg/ml. Electrophoresis of #SK/PME/07 on SDS-PAGE showed many proteins bands. However, based on our earlier findings that the potent activity was evident in the molecular weight ranges between the >10 kDa and <30 kDa, the two major bands at ≤ 27 kDa and ≤ 20 kDa were considered for the further investigations. Similarly a gel stained for glycoproteins was positive. Poor resolution and fuzzy appearance of bands suggests that these proteins are highly glycosylated, and this was confirmed by glycoprotein staining. Hence, as #SK/PME/07 was brown in color, and contained highly glycosylated proteins, presence of peroxidase molecules was investigated, as the brown colour of heartwood is primarily related to the oxidation of phenolic compounds. Browning reactions are generally catalysed by polyphenoloxidases (Lee & Whitaker, 1995) or peroxidases (Higuchi, 1997) and result in the formation of quinines which subsequently polymerize to varying degrees leading to brown pigments (Rouet-Mayer *et al.*, 1990). Peroxidases are a super family of enzymes that are ubiquitous in plants, fungi, and vertebrates. The structurally diverse secretory plant peroxidases are haem-containing enzymes which oxidize several substrates in the presence of H_2O_2 (Penel *et al.*, 1992). However, results from our peroxidase activity assay indicate that #SK/PME/07 had no peroxidase molecules.

Con A coupled to Sepharose resin is routinely used for separation and purification of glycoproteins, polysaccharides and glycolipids. In our present study, Con A chromatography of #SK/PME/07 failed to enhance or recover the full activity of #SK/PME/07, indicating the possible interaction of proteins present in the #SK/PME/07 with the Con A. This was further confirmed by running SDS-PAGE. The Coomassie

stained gel of these fractions showed the presence of concanavalin proteins in the fractions and this exchange of #SK/PME/07 with the Con A suggested that the glycoprotein(s) of #SK/PME/07 had high affinity with Con A and due to this, this method of purification was abandoned.

Tannins, a group of high molecular weight polyphenolic compounds form complexes with proteins through peptide or amide linkages, or irreversibly by covalent condensations (Loomis & Battaile, 1966; Stern *et al.*, 1996). Removal of tannins from the solution can be achieved by adding a substance that contains groups similar to peptide bonds, such as using nylon or insoluble polyvinylpyrrolidone (PVPP) (Toth & Pavia, 2001). The effect of PVPP treated #SK/PME/07 (PVPPT) on glucose uptake and insulin secretion showed no difference to the effect of #SK/PME/07. This strongly suggests that #SK/PME/07 either contains no tannins or that there is no association of tannins with the active protein molecules of #SK/PME/07.

As it has been confirmed that the hypoglycemic constituent(s) of #SK/PME/07 is/are heavily glycosylated proteins showing two major fuzzy bands at $\leq 27\text{kDa}$ and $\leq 20\text{kDa}$, further purification experiments were performed to purify and also to identify the band(s) responsible for the antidiabetic activity, using, isoelectric focusing (ISF) to separate the protein based on their charge characteristics. The distribution of both positive and negative activity of ISF fractions suggested that, #SK/PME/07 may contain two or more isoforms of glycosylated proteins. SDS-PAGE of ISF fractions showed streaking of bands all over the fractions possibly due to the presence of ampholytes. Hence this

observation recommends further experiment on the removal of ampholytes from the ISF fractions before running SDS-PAGE to provide a clear molecular profile of these isoforms of ISF fractions. Nonetheless, the presence of peak activity at acidic pH region, suggesting that the negatively charged protein(s) present in the #SK/PME/07 is/are possibly the highly potent antidiabetic molecule.

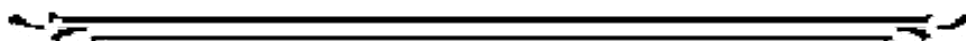
Following this, the precipitate (PPT) that was obtained after chloroform methanol precipitation of #SK/PME/07 showed an enhanced insulin secretion and similar glucose uptake activity, while the supernatant had little activity. The results from the chloroform-methanol precipitation experiment again confirmed that the hypoglycemic constituent(s) of #SK/PME/07 is/are likely to be proteins.

Although many possible techniques can be performed to separate these isoforms, the results from our current study are encouraging and therefore further experiments on identification and purification of antidiabetic glycoproteins from #SK/PME/07 using isoelectric focusing method are recommended. The putative roles of the sugar chains in glycoproteins such as their effect on solubility, oligomeric assembly, recognition by receptors, and biological activity are often conflicting. Hence the results from the present study indicate further investigations on the glyco-moiety of the protein(s) present in the #SK//PME/07 as well.

In summary, the present study for the first time identified and isolated the more potent hypoglycemic constituent from an aqueous extract of heartwood of PM. This study

reports that the hypoglycemic constituent(s) of PM are two or more isoforms of glycosylated proteins. However, these promising *in vitro* results must be ascertained *in vivo* using animal models of insulin resistance and diabetes. Hence, further pharmacological study in both *in vitro* and *in vivo* experimental models would be required prior to their being considered for clinical application. Nevertheless, the findings from this study may provide a rationale for the further development of a novel antidiabetic molecule

6 Antidiabetic properties of #SK/PME/07



6.1 Introduction

In Chapter 5, a glycoprotein(s) (#SK/PME/07) was extracted and purified from an aqueous extract of heartwood of *Pterocarpus marsupium* (PM). This glycoprotein exhibits *in vitro* both an insulintropic and an insulin sensitising activity (Chapter 5). Interestingly, due to its potent insulin-releasing and sensitising effect, demonstrated both in isolated mouse pancreas and muscle tissues, #SK/PME/07 may be considered as a novel secretagogue and sensitiser with the potential to be developed into a treatment option of T2D.

In general, T2D occurs when insulin secretion no longer compensates for insulin resistance which is seen associated with obesity, aging and illness. Many new pharmacological agents have been added to our armamentarium of treatments for T2D in the last few decades. The goal of all treatments is the same irrespective of the cause of the T2D: namely, to normalise blood glucose. For normal glucose metabolism insulin must be released from the pancreas in an exquisitely exact amount, at the correct time and in a correct pattern. The normal pancreas also senses fasting and fed states as well as the energy content of the meals eaten. So far, no pharmacological agent can take over or restore this exquisite sensing capacity when it is diseased, and no agent can restore the

exact pattern of insulin kinetics. The presence of so many compounds to treat T2D is a testament to the complexity involved in normalising blood glucose.

Under normal physiological conditions, glucose homeostasis is predominantly maintained by a balance between insulin secretion and insulin action, because of the robustness within the system in non-diabetic subjects, alteration in one of these will lead to compensation by the other. Before the onset of diabetes, a pre-diabetic state exists, in that the pancreas is secreting an increasing amount of insulin, in the face of increasing insulin resistance, to maintain non-diabetic levels of blood glucose. At that point in the maintenance of glucose homeostasis the balance is fragile. Moreover, in the presence of T2D, the introduction of any agent that has a negative impact on glucose homeostasis could increase blood glucose and require adjustments to the diabetes treatment regimen. Therefore, knowledge of possible or even theoretical interactions of pharmacological agents that have an impact on glucose homeostasis would be beneficial in treating and managing T2D.

In this context, and extending the *in vitro* bioassay results, described previously in this thesis, the present work was designed to investigate firstly, the precise pharmacological effects of #SK/PME/07 on tissues involved in glucose homeostasis such as on isolated mouse pancreas and muscle tissues *in vitro*, and second, the insulintropic effect of #SK/PME/07 as well as its consequence on glucose clearance in two different age groups, in normoglycemic, non-diabetic sheep *in vivo*. This would likely suggest possible

physiological mechanisms involved and responsible for the antidiabetic properties of #SK/PME/07.

6.2 Material and methods

6.2.1 General methods

Animal ethics approval, statistical analysis, *in vitro* and *in vivo* studies and sample analysis for glucose and insulin have been described in Chapter-2.

6.2.2 *In vitro* studies

6.2.2.1 *Insulin secretion in vitro*

Insulin secretion was evaluated using mouse pancreas tissues as described in Section 2.4. In brief, 5 pancreatic tissues per well were added to tissue culture wells containing culture media. Following incubation, media samples were removed from each well and stored at –20°C until analyzed for insulin content as described in Section.2.7.1.

In the first experiment tissues were incubated for 24 hours in 5 mM glucose (normoglycemic) and 12 mM glucose (hyperglycemic) media in the presence or absence of #SK/PME/07 and tolbutamide.

The second experiment was an investigation of the effects of test agents such as tolbutamide (0.1 mM; a first generation K⁺-ATP channel blocker), diazoxide (0.5 mM; an established opener of K⁺-ATP channels), and tolbutamide and diazoxide combined, arginine (10 mM; a cationic amino acid that depolarizes β -cells because of its entry as a positively charged form) and potassium chloride (KCl; 25 mM; a membrane depolarizer) on the insulin secreting activity of #SK/PME/07. Pancreatic tissues were incubated for 24 hours at 12 mM glucose with these test reagents in the absence or presence of #SK/PME/07.

To investigate the effect of #SK/PME/07 on insulin synthesis within culture conditions, mouse pancreatic tissues were incubated at 12 mM glucose for 24 hours in the presence or absence of #SK/PME/07; the samples were withdrawn for analysis at 12 and 24 hours. After this period, the tissues were allowed to recover in culture media containing 5 mM glucose for 24 hours. The tissues from the wells were then transferred and homogenized in tubes containing 1 ml RIA buffer. The tissue and media samples from each well were then stored at -20°C until analyzed for insulin content as described in Section 2.7.1.

6.2.2.2 *Glucose uptake in vitro*

To determine the glucose uptake, square strips of mouse abdomen muscle were used as described in Section 2.4. Briefly, muscle tissues were added to the wells containing culture media. Following incubation, media samples were removed from each well and stored at -20°C until analyzed for glucose content as described in Section.2.6.

To investigate the effect of #SK/PME/07 on glucose uptake activity by muscle tissues at both normoglycemic (5 mM glucose) and hyperglycemic (12 mM glucose) culture conditions, muscle tissues were incubated for 24 hours at 5 mM glucose and 12 mM glucose in the presence or absence of #SK/PME/07 and metformin (10 mM; an oral hypoglycemic agent that increases insulin sensitivity).

The following experiment investigated the effects of test agents such as insulin (100 nM), metformin, wortmannin (100 nM; a pharmacological inhibitor of PI3-kinase), insulin and wortmannin combined, insulin and metformin combined and metformin and wortmannin combined on glucose uptake activity of #SK/PME/07. The muscle tissues were incubated for 24 hours at 12 mM glucose with the test agents in the absence or presence of #SK/PME/07 and test agents.

6.2.2.3 In vitro data analysis

All the experiments were repeated thrice unless otherwise specified (n=3). Basal glucose uptake was calculated based on the difference in glucose concentration between control wells with tissue and the control wells without tissue. Glucose uptake in response to various treatments was calculated as a percent variation from basal levels. Insulin (μ IU/ml) and glucose uptake (%) data were statistically analyzed as mentioned in Section 2.9 and expressed as means $\pm$ SE unless otherwise mentioned.

6.2.3 *In vivo* studies

6.2.3.1 *Experiment 1*

A preliminary experiment was carried out to investigate the effects of aqueous extract of *Pterocarpus marsupium* (PME) and an intermediate fraction (PPME) obtained during the bioassay-guided fractionation of PME, on glucose clearance in normoglycemic sheep. In the first set of experiments, 12 Merino wethers of 2 to 3 years of age were weighed and randomly divided into three groups, control, PME and PPME. The sheep were acclimatised for 3 days, followed by 10 days experimental protocol. On the day prior to beginning of the experiment (day -1), jugular catheters were inserted and the animals remained untreated. The following morning (day 0), blood samples were collected via catheter to evaluate basal glucose and insulin levels. After this collection, intravenous glucose was administered (2.5 mg per kg body mass) via catheter as a bolus over 1 min to initiate the glucose tolerance test (GTT). Subsequent blood samples were taken at 15, 30, 45, 60, 90, 120 and 180 minutes after the glucose bolus. On this day (0) immediately after the completion of GTT and again on the next two days (days 1 & 2), the treatment groups PME and PPME and the control group, received a daily intravenous injections of 50 ml PME, 0.5 ml of PPME and 50 ml of saline (0.9% NaCl), respectively. On day 3, an intravenous GTT (IVGTT) was performed again as mentioned previously. The blood samples were immediately centrifuged at 3000 rpm for 20 minutes, the plasma separated and stored at -20°C until analysed. Glucose and insulin concentration were then assayed for each sample as outlined in Section 2.6 and 2.7.2

6.2.3.2 Experiment 2

This experiment was carried out to investigate the effects of administration of three consecutive intravenous doses of #SK/PME/07 (5 µl) on glucose clearance and on insulin secretion in young (2 years of age) normoglycemic sheep over three weeks. From the, dose response effects of #SK/PME/07 on tissues involved in glucose homeostasis *in vitro* experiments, a dose that showed a peak effects on both insulin secretion and action and also a dose that is equivalent to original starting volume and mass was selected for the *in vivo* experiments (see Figure 5-19)

Four Merino wethers of 2 years of age were weighed and randomly divided into two groups, control and #SK/PME/07. The sheep were acclimatised for 3 days in individual pens. On the day prior to beginning experiment (day -4), jugular catheters were inserted and the animals remained untreated. The following morning (day -3) and also over the next two days (day -2 and -1), intravenous glucose was administrated (2.5 mg per kg body mass) via catheter as a bolus over 1 min to stabilise the normal glucose clearance. On day 0, blood samples were collected via catheter to evaluate the basal glucose and insulin levels (0 min). After this, an IVGTT was performed. 10 ml blood samples were taken at 15, 30, 45, 60, 90, 120 and 180 minutes after the glucose bolus. Following the day 0 IVGTT and over the next two days (days 1 & 2), the treatment group #SK/PME/07 and the control group, received a daily intravenous injections of 5 µl of #SK/PME/07 (in 10 ml saline) and 10 ml of saline (0.9% NaCl) respectively. Additionally on these days, both these groups also received an intravenous glucose (2.5 mg per kg body mass) bolus via catheter over 1 min. On day 3, an IVGTT was performed. After the day 3 IVGTT,

catheters were removed and the animals were moved to an open paddock. On day 20, again all these animals were brought back to the pens and catheters were reinserted. The following morning (day 21), an IVGTT was performed. Over the next two days both these groups received an intravenous glucose (2.5 mg per kg body mass) via catheter as a bolus over 1 min. On day 25 IVGTT was performed. All blood samples were centrifuged immediately after bleeding at 3000 rpm for 20 minutes and the plasmas collected were stored at -20°C until analyzed. Glucose and insulin concentration were then assayed for each sample as outlined in Section 2.6 and 2.7.2.

6.2.3.3 *Experiment 3*

To investigate the effects of three treatments of #SK/PME/07 at two different dose levels (5 and 15 μl) on glucose clearance and insulin secretion in adult (5-6 years), normoglycemic sheep, over three weeks, 12 Merino wethers of 5-6 years of age were weighed and randomly divided into three groups, saline (control), #SK/PME/07 (5 μl) and #SK/PME/07 (15 μl). The sheep were acclimatised for 3 days. On the day prior to beginning of the experiment (day -4), jugular catheters were inserted and the animals remained untreated. The following morning (day -3) and also over the next two days (day -2 and -1), intravenous glucose (2.5 mg per kg body mass) via catheter was administered as a bolus over 1 min to stabilise the normal glucose clearance. On day 0, blood samples were collected via catheter to evaluate the basal glucose and insulin levels (0 min). After this, an IVGTT was performed. The blood samples were taken at 15, 30, 45, 60, 90, 120 and 180 minutes after the glucose bolus. On this day (day 0) immediately after a GTT and over the next two days (day 1 & 2), the treatment groups #SK/PME/07 (5 and 15 μl)

and the control group, received a daily intravenous injections of 5 and 15 μ l of #SK/PME/07 (in 10 ml saline) and 10 ml of saline (0.9% NaCl), respectively. On these days, these groups also received an intravenous glucose (2.5 mg per kg body mass) via catheter as a bolus over 1 min. On day 3, an IVGTT was again performed as previously detailed. All blood samples were immediately centrifuged at 3000 rpm for 20 minutes and the plasma collected stored at -20°C until analysed. Glucose and insulin concentration were then assayed for each sample as outlined in Section 2.6 and 2.7.2

6.2.3.4 In vivo data analysis

Plasma glucose and insulin values presented in the results are the mean ($\pm$ S.E) of duplicate replicates. From the IVGTT data, a glucose and insulin curve was plotted. From this plot of the rapid increase and gradual decay of glucose, and subsequently insulin, a value for area under the curve was derived using Origin™ software (Version 7 – Microcal Software Inc., Northampton, MA, USA). Variables derived were, 1) glucose intolerance measured as the area above basal plasma glucose and under the plasma glucose curve ($\text{Glu}_{\text{AUC (0-180 min)}}$; min·mmol/L), 2) absolute insulin secretion measured as the area above basal plasma insulin curve ($\text{Ins}_{\text{AUC (0-60 min)}}$; min· μ IU/ml). Data were statistically analyzed as mentioned in Section 2.9 and expressed as means $\pm$ S.E, unless otherwise mentioned.

6.3 Results

6.3.1 *In vitro* studies

6.3.1.1 *Insulin secretion in vitro*

To understand the possible pharmacological mechanisms responsible for the insulin releasing activity of #SK/PME/07, the effect of some known pharmacological substances on insulin secreting activity of #SK/PME/07 from mouse pancreas tissues *in vitro* were investigated and the results obtained are shown in Figure 6-1, 2 & 3.

The insulin secreting activity of #SK/PME/07 at basal glucose (5 mM) over chronic incubation (24 hours) was not significantly different from that of the control ($p > 0.05$). However under similar culture conditions, the insulin releasing effect of tolbutamide ($138.89 \pm 11.83 \mu\text{IU/ml}$) was significantly greater than the effect of both control and #SK/PME/07 ($p < 0.05$). The presence of high glucose (12 mM) potentiated the insulin secreting activity of both #SK/PME/07 ($334 \pm 28.31 \mu\text{IU/ml}$) and tolbutamide ($293.56 \pm 6.91 \mu\text{IU/ml}$) which were significantly greater than the hyperglycemic control ($69.04 \pm 6.06 \mu\text{IU/ml}$). However, #SK/PME/07 and tolbutamide treatment were not significantly different ($p > 0.05$) from each other (Figure 6-1).

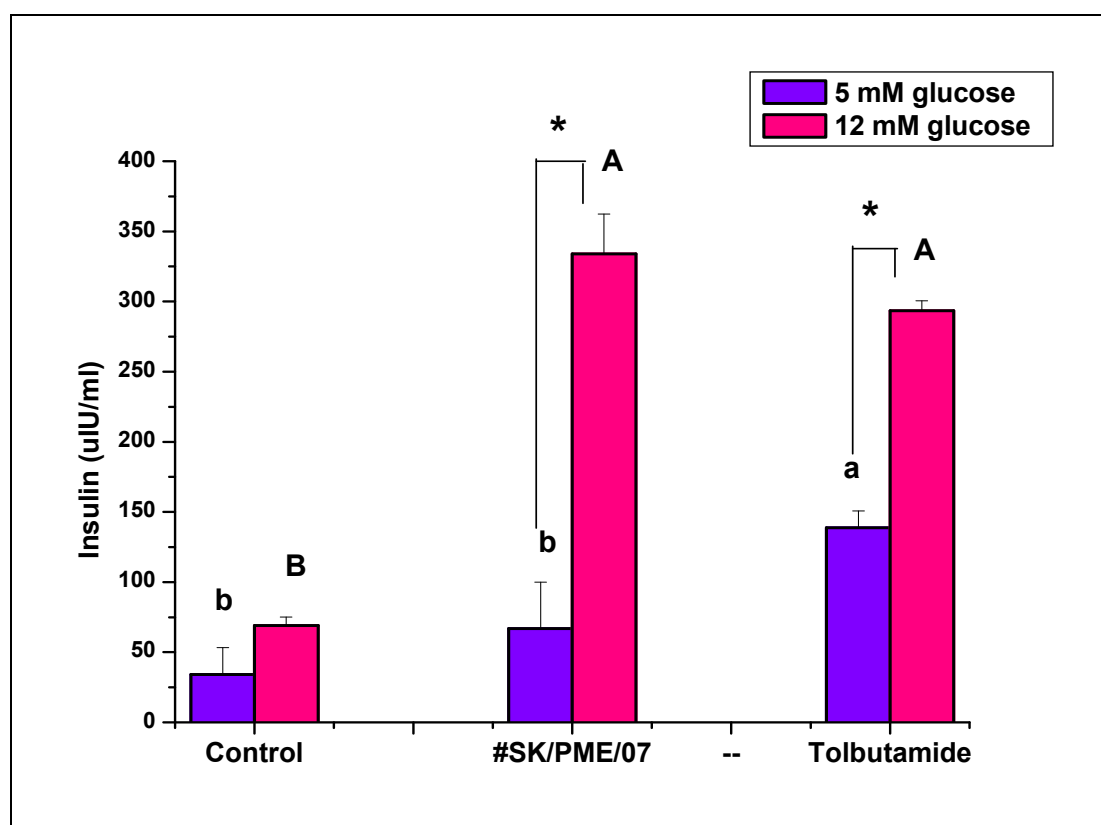


Figure 6-1 Effect of glucose (5 and 12 mM) on insulin secreting activity of #SK/PME/07 and tolbutamide (0.1 mM) from mouse pancreatic tissues *in vitro*. Values are expressed as mean $\pm$ S.E. Means indicated without a common letter are significantly different relative to the control group ($p < 0.05$) and means indicated with an asterisk (*) are significantly different relative to the treatment group ($p < 0.05$).

Figure 6-2 shows that under hyperglycemic conditions, the effect of tolbutamide in the presence ($298.66 \pm 28.58 \mu\text{IU/ml}$) or absence ($293.56 \pm 6.91 \mu\text{IU/ml}$) of #SK/PME/07 was not significantly different, indicating the presence of tolbutamide has no significant influence on insulin secreting activity of #SK/PME/07. The presence of diazoxide (0.5 mM) significantly inhibited the insulin releasing effect of #SK/PME/07 ($140.31 \pm 28.58 \mu\text{IU/ml}$) and tolbutamide in either absence ($83.82 \pm 18.98 \mu\text{IU/ml}$) or presence ($100.73 \pm 22.28 \mu\text{IU/ml}$) of #SK/PME/07 ($p < 0.05$). However, the insulin releasing activity of

diazoxide in the absence of both #SK/PME/07 and tolbutamide was significantly higher than the control ($p < 0.05$).

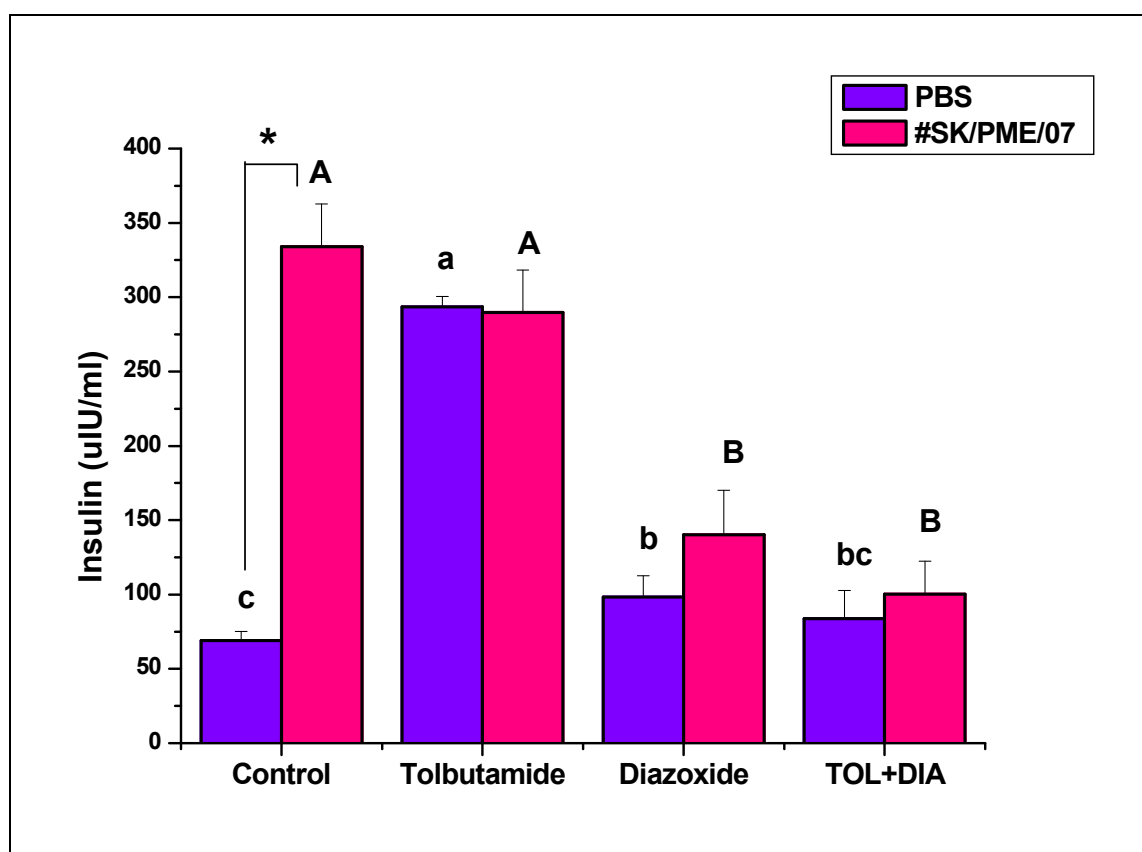


Figure 6-2 Effect of control (PBS), tolbutamide, diazoxide and tolbutamide + diazoxide (TOL+DIA) on insulin secretion from mouse pancreas tissues under hyperglycemic culture condition (12 mM glucose) in absence or presence of #SK/PME/07

Values are expressed as mean $\pm$ S.E Means indicated without a common letter are significantly different relative to the control group ($p < 0.05$) and means indicated with an asterisk (*) are significantly different relative to the treatment group ($p < 0.05$)

The effect of arginine (10 mM) on mouse pancreas tissues in hyperglycemic conditions showed a significant activation ($p<0.05$) of insulin secreting activity (246.8 ± 7.31 μ IU/ml) (Figure 6-3). However, the arginine treatment in the presence of #SK/PME/07 did not augment insulin secretion (254.03 ± 31.93 μ IU/ml) under hyperglycemic culture conditions.

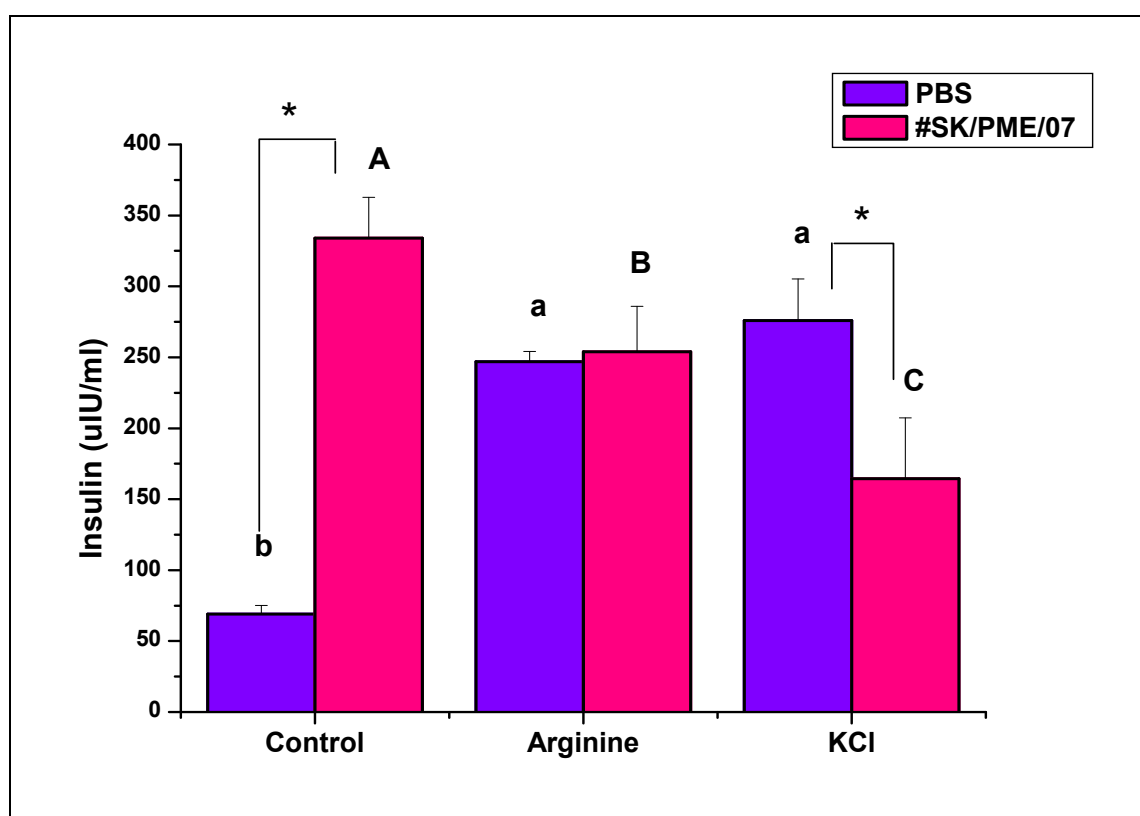


Figure 6-3 Effect of control (PBS), arginine and KCl on insulin secretion from mouse pancreas under hyperglycemic culture conditions (12 mM glucose) in absence or presence of #SK/PME/07. Values are expressed as mean $\pm$ S.E. Means indicated without a common letter are significantly different relative to the control group ($p<0.05$) and means indicated with an asterisk (*) are significantly different relative to the treatment group ($p<0.05$).

Under similar culture conditions, the treatment of potassium chloride (KCl) on pancreatic tissues showed a significantly ($p<0.05$) potent insulin secreting activity (275.87 ± 7.31 μ IU/ml). Interestingly, the presence of KCl significantly ($p<0.05$) inhibited the insulin secreting activity of #SK/PME/07 (164 ± 42.98 μ IU/ml).

The results of the potential effect of #SK/PME/07 on insulin synthesis by pancreatic tissues are presented in Figure 6-4. The media insulin concentrations of #SK/PME/07 treated groups at both 12 hours (287.3 ± 12.83 μ IU/ml) and 24 hours (380.57 ± 2.43 μ IU/ml) incubation, under hyperglycemic culture (12 mM) conditions and followed by a 24 hours (141.68 ± 14.27 μ IU/ml) incubation under normoglycemic culture (5 mM) conditions were significantly greater than the control group ($p<0.05$). Interestingly, the insulin content in the tissues treated with of #SK/PME/07 (715 ± 11.23 μ IU/ml) was significantly higher than the control tissues ($p<0.05$), indicating that #SK/PME/07 stimulates both insulin secretion and synthesis.

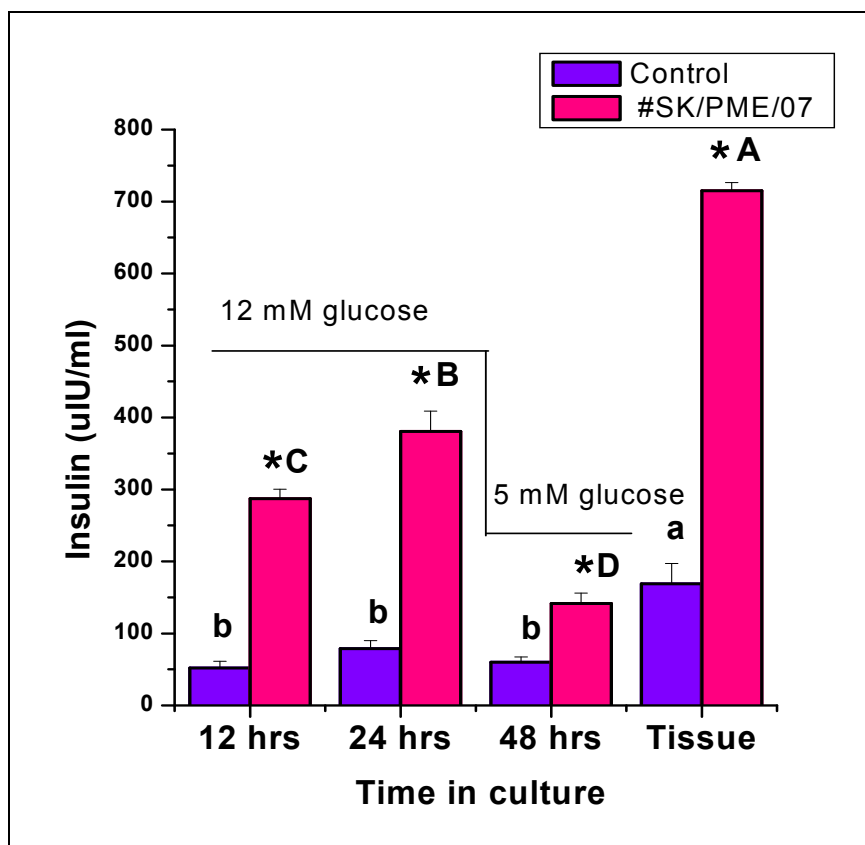


Figure 6-4 Effect of #SK/PME/07 on insulin secretion and synthesis by pancreatic tissues over 24 hours under hyperglycemic glucose (12 mM) and followed by 24 hours under basal glucose (5 mM). Media samples were withdrawn at 12, 24 and 48 hours. Tissues from respective treatments were homogenized and analyzed.

Values are expressed as mean $\pm$ S.E Means indicated without a common letter are significantly different relative to the control group ($p < 0.05$) and means indicated with an asterisk (*) are significantly different relative to the treatment group ($p < 0.05$)

6.3.1.2 Glucose uptake *in vitro*

The possible pharmacological mechanisms responsible for the glucose uptake activity of #SK/PME/07 were examined. The results of the effect of some known pharmacological substances on glucose uptake activity of #SK/PME/07 by mouse muscle tissues *in vitro* are presented in Figures 6-5 & 6-6.

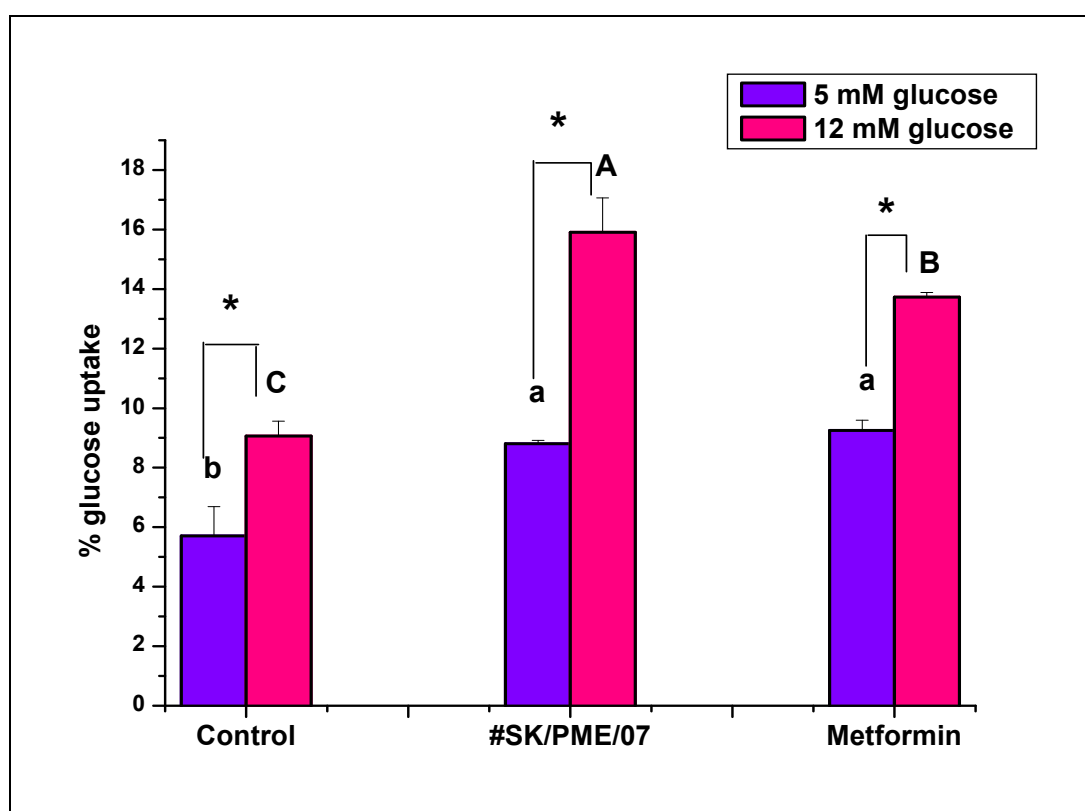


Figure 6-5 Effects of glucose (5 and 12 mM) on glucose uptake activity of #SK/PME/07 and metformin (10 mM) by mouse muscle tissues *in vitro*

Values are expressed as mean $\pm$ S.E Means indicated without a common letter are significantly different relative to the control group ($p < 0.05$) and means indicated with an asterisk (*) are significantly different relative to the treatment group ($p < 0.05$)

The glucose uptake activity of #SK/PME/07 (8.8 ± 0.12 and 15.91 ± 1.5 %, respectively) and metformin (9.25 ± 0.34 and 13.73 ± 0.16 %, respectively) were significantly greater ($p < 0.05$) than the control (5.7 ± 0.99 and 9.06 ± 0.5 %, respectively) in both basal and hyperglycemic culture conditions (Figure 6-5). This potent stimulation of glucose uptake by #SK/PME/07 in hyperglycemic culture condition was significantly greater than the effects of metformin.

The treatment with insulin (100 nM) significantly potentiated ($p < 0.05$) the glucose uptake in either absence (15.83 ± 0.91 %) or presence (21.09 ± 1.62 %) of #SK/PME/07 (Figure 6-6). Similarly, metformin (10 mM) also significantly potentiated ($p < 0.05$) the glucose uptake in either absence (13.73 ± 0.81 %) or presence (19.47 ± 2.8) of #SK/PME/07, which suggests insulin and metformin have significant additive effects on glucose uptake activity of #SK/PME/07

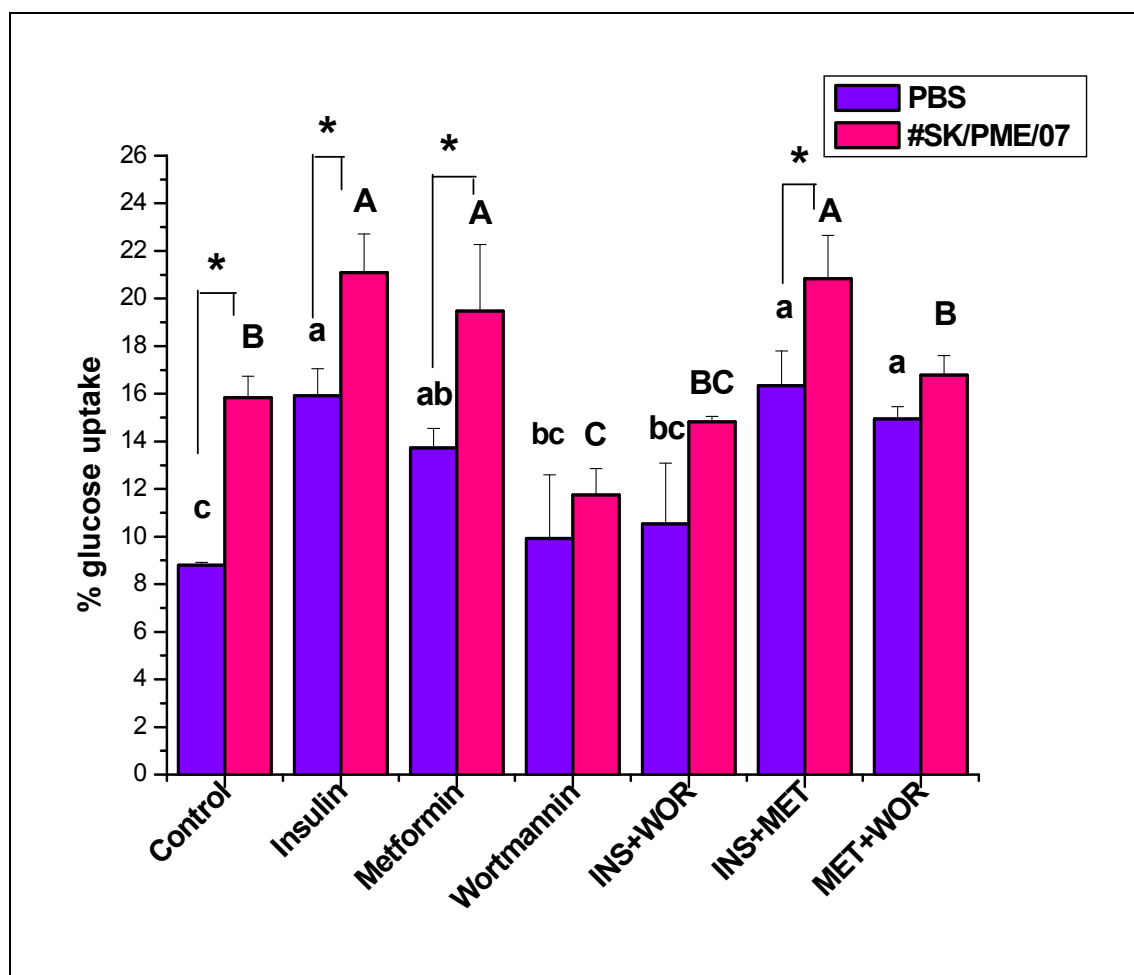


Figure 6-6 Effect of control (PBS), insulin (100 nM), metformin (10 mM), wortmannin (100 nM) insulin +wortmannin (INS+WOR), insulin + metformin (INS+MET) and metformin +wortmannin (MET+WOR) on glucose uptake by mouse skeletal muscle tissues incubated for 24 hours in hyperglycemic condition (12 mM glucose) in absence or presence of #SK/PME/07

Values are expressed as mean $\pm$ S.E Means indicated without a common letter are significantly different relative to the control group ($p < 0.05$) and means indicated with an asterisk (*) are significantly different relative to the treatment group ($p < 0.05$)

Under similar conditions, the presence of wortmannin (100 nM) significantly inhibited ($p < 0.05$) the glucose uptake activity of #SK/PME/07 (11.75 ± 1.11 %) and insulin (10.53

± 2.55 %) but failed to inhibit the activity of metformin (14.95 ± 0.51 %), insulin and #SK/PME/07 combined (14.82 ± 0.23 %) and metformin and #SK/PM/07 combined (16.78 ± 0.82 %). Interestingly, wortmannin (9.92 ± 2.67 %) showed no inhibitory effects on basal glucose uptake. The effect of insulin and metformin in combination showed no significant ($p < 0.05$) additive effect on glucose uptake activity in the absence of #SK/PME/07 (16.34 ± 1.46 %) and also had no significant ($p < 0.05$) additive effects in the presence of #SK/PME/07 (20.82 ± 1.83 %) suggesting that #SK/PME/07 may exhibit insulin and/or metformin like properties on mouse skeletal muscles within culture conditions.

6.3.2 *In vivo* studies

6.3.2.1 *Experiment 1*

A preliminary study in normal sheep, investigated the effects of three daily intravenous administrations of PME and PPME on intravenous glucose tolerance (IVGTT). The profiles of plasma glucose on day 0, when untreated and on day 3, when treated during IVGTT are shown in Figure 6-7. After the intravenous glucose bolus, plasma glucose concentration was significantly increased and then progressively decreased (6-7A & B). Basal plasma glucose at 0 min, immediately before GTT, of animals treated with PME and PPME had no significant difference from the control group ($p>0.05$) (Figure 6-7C). Interestingly, the plasma glucose at 60 minutes during GTT of PME (4.62 ± 0.66 mmol/L) and PPME (3.62 ± 0.63 mmol/L) treated groups were significantly lower ($p<0.05$) than the control group (7.05 ± 0.41 mmol/L). After 120 minutes of GTT, both control (3.42 ± 0.29 mmol/L) and PME (3.62 ± 0.63 mmol/L) treated groups were not significantly different ($p>0.05$), but the plasma glucose of PPME (2.82 ± 0.22) treated group was significantly lower ($p<0.05$) at that time point than both control and PME treated groups. In addition, glucose tolerance: as defined by the area above basal plasma glucose under plasma glucose curve ($\text{Glu}_{\text{AUC (0-180 min)}}$; min·mmol/L) has been calculated (Figure 6-7D). The effect of PME (147.17 ± 56.15 min·mmol/L; -50.43 % decrease from control) and PPME (65.92 ± 13.91 min·mmol/L; -79.6 % decrease from control) on

glucose AUC (0-180 min) during IVGTT were significantly lower ($p<0.05$) than the control group (322.31 ± 26.11 min·mmol/L).

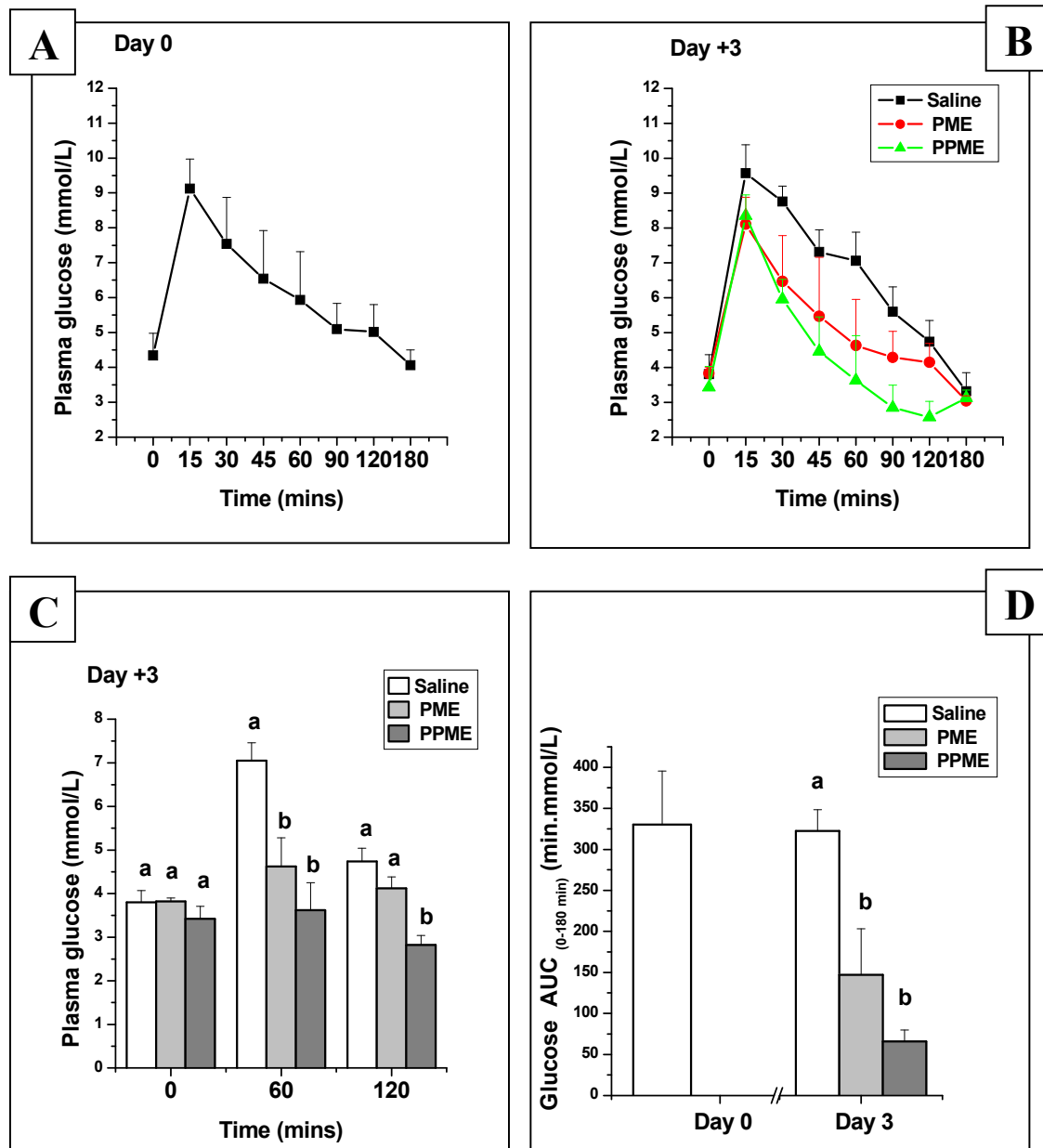


Figure 6-7 Effect of three daily intravenous administrations of PME (50 ml) and PPME (0.5 ml) on plasma glucose clearance in normal sheep. Mean ($\pm$ S.E.) glucose clearance in sheep on day 0 (A) when untreated, and on day 3 (B) when treated. (C) Plasma glucose (mean $\pm$ S.E.) at 0, 60 and 120 minutes after an IVGTT of saline, PME and PPME treated groups. (D) Mean ($\pm$ S.E.) glucose AUC_(0-180 min) (min·mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3) treating with saline, PME and PPME. Means indicated by different letters are significantly different relative to the control group ($P<0.05$).

The profiles of plasma insulin on day 0, when untreated and on day 3, when treated during IVGTT are shown in Figure 6-8. As expected, the rise in plasma glucose was associated with a simultaneous increase in insulin secretion.

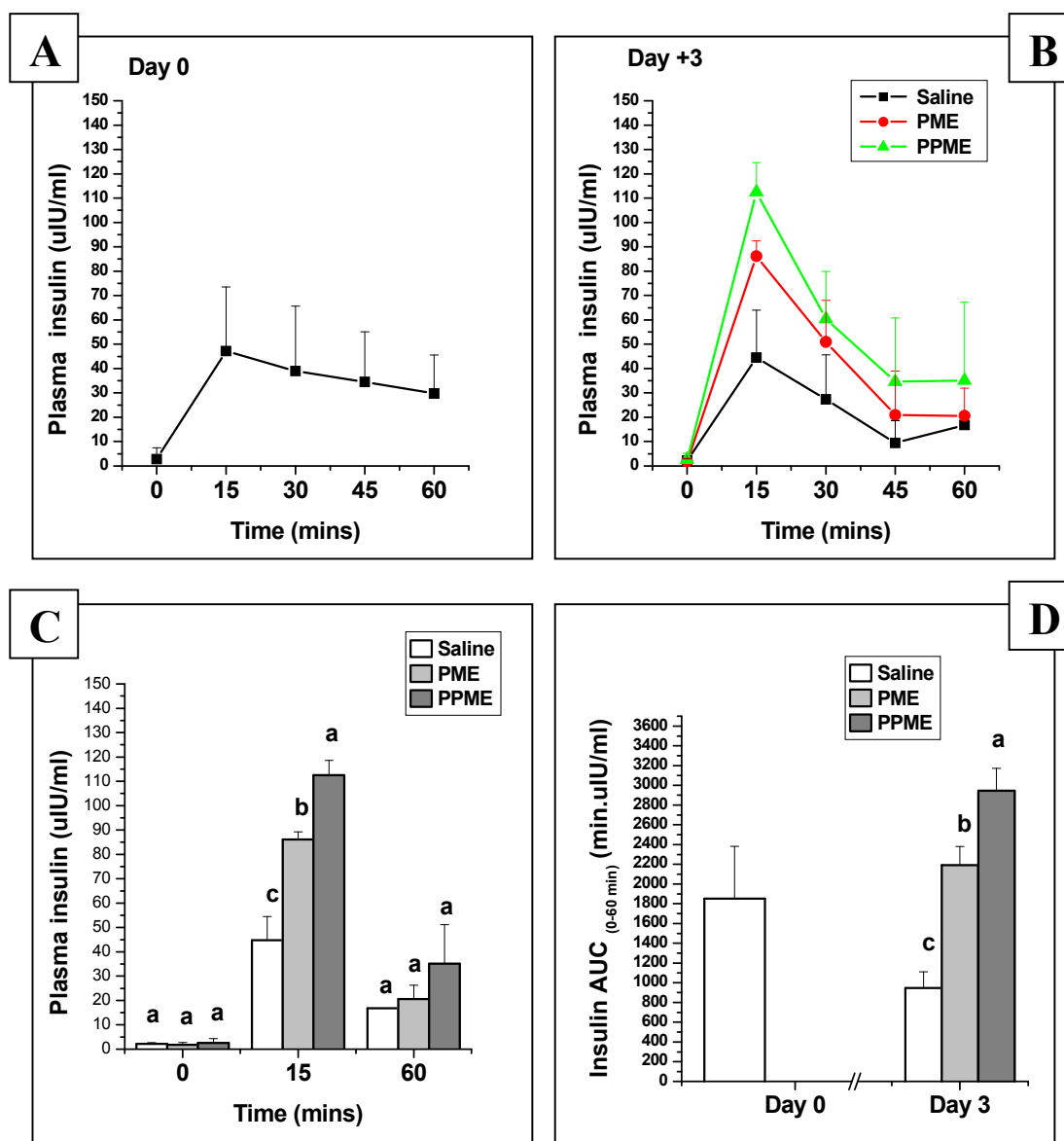


Figure 6-8 Effect of three daily intravenous administrations of PME (50 ml) and PPME (0.5 ml) on plasma insulin in normal sheep

Mean ($\pm$ S.E.) plasma insulin levels in sheep on day 0 (A) when untreated, and on day 3 (B) when treated. (C) Plasma insulin (mean $\pm$ S.E.) at 0, 15 and 30 minutes after an IVGTT of saline, PME and PPME treated groups. (D) Mean ($\pm$ S.E.) insulin AUC (0-60 min) (min·uIU/ml) in sheep, after an IVGTT performed before (day 0) and after (day 3) treating with saline, PME and PPME. Means indicated by different letters are significantly different relative to the control group ($P < 0.05$).

Plasma insulin of animals treated with PME and PPME on day 3 were not significantly different ($p>0.05$) at 0 and 60 minutes to the control during GTT (Figure 6-8C). However, the plasma insulin at 15 minutes of GTT of PME ($86.12 \pm 3.12 \mu\text{IU/ml}$) and PPME ($112.52 \pm 6.04 \mu\text{IU/ml}$) treated groups were significantly higher ($p<0.05$) than the control group ($44.75 \pm 9.75 \mu\text{IU/ml}$). Absolute insulin secretion ($\text{Ins}_{\text{AUC (0-60 min)}}$; $\text{min} \cdot \mu\text{IU/ml}$) of PME, PPME and control groups were calculated, and are shown in Figure 6-8D. The effect of PME ($2191.25 \pm 188.66 \text{ min} \cdot \mu\text{IU/ml}$; +132 % increase from control) and PPME ($2944.11 \pm 229.48 \text{ min} \cdot \mu\text{IU/ml}$; + 210 % increase from control) on insulin AUC (0-60 min) during GTT were significantly higher ($p<0.05$) than the control group ($946.87 \pm 164.68 \text{ min} \cdot \mu\text{IU/ml}$) and also significantly different from each other ($p<0.05$).

6.3.2.2 Experiment 2

The effect of three daily intravenous administrations of #SK/PME/07 (5 μl) on glucose clearance and insulin secretion in young (2 years of age) normal sheep over three weeks were investigated. The profiles of plasma glucose on day 0, when untreated and on days 3, 21 and 25 when treated with #SK/PME/07 during IVGTT are shown in Figure 6-9. After the intravenous glucose bolus, plasma glucose concentration was significantly increased and then progressively decreased. In this set of experiments, in young animals, the plasma glucose of the #SK/PME/07 treated group on days 21 and 25 at 0, 60 and 120 minutes during GTT have no significant difference ($p>0.05$) from the control group although there are decreased plasma glucose levels in the #SK/PME/07 treated group at 60 and 120 minutes at day 3 (Figure 6-10A). The glucose AUC (glucose tolerance; $\text{Glu}_{\text{AUC (0-180 min)}}$; $\text{min} \cdot \text{mmol/L}$) of #SK/PME/07 ($396.22 \pm 40.72 \text{ min} \cdot \text{mmol/L}$; -40.29 %

decrease from control) treated group on day 3 was significantly lower ($p < 0.05$) than the control ($663.32 \pm 46.47 \text{ min} \cdot \text{mmol/L}$) (Figure 6-10B). However, the glucose AUC of the #SK/PME/07 treated group on day 21 and 25 was not significantly different ($p > 0.05$) from that of the control group.

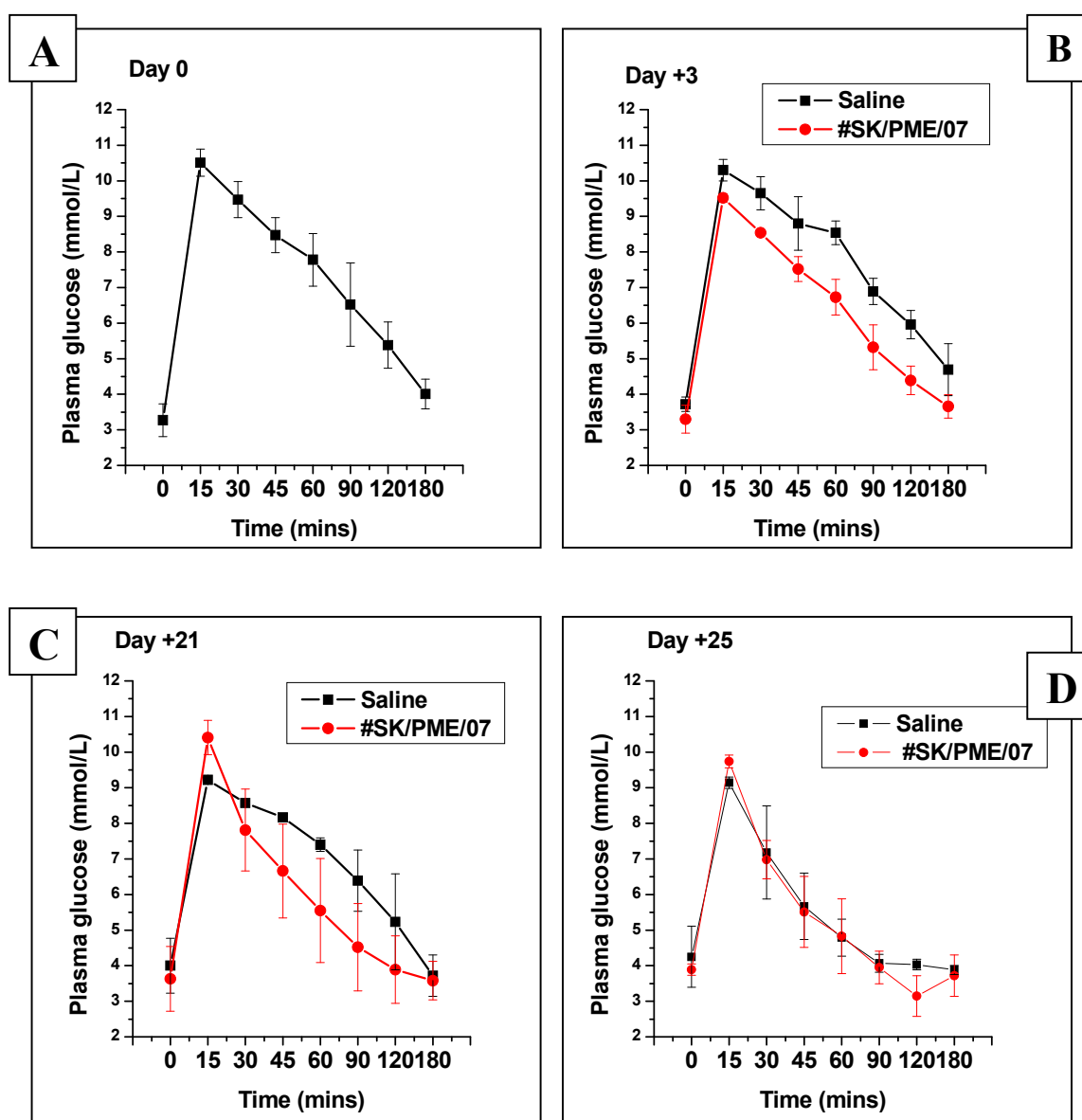


Figure 6-9 Plasma glucose clearance after an IVGTT performed in saline and #SK/PME/07 (5 µl) treated young sheep at 2 yr of age. Mean ($\pm$ S.E) plasma glucose clearance in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated.

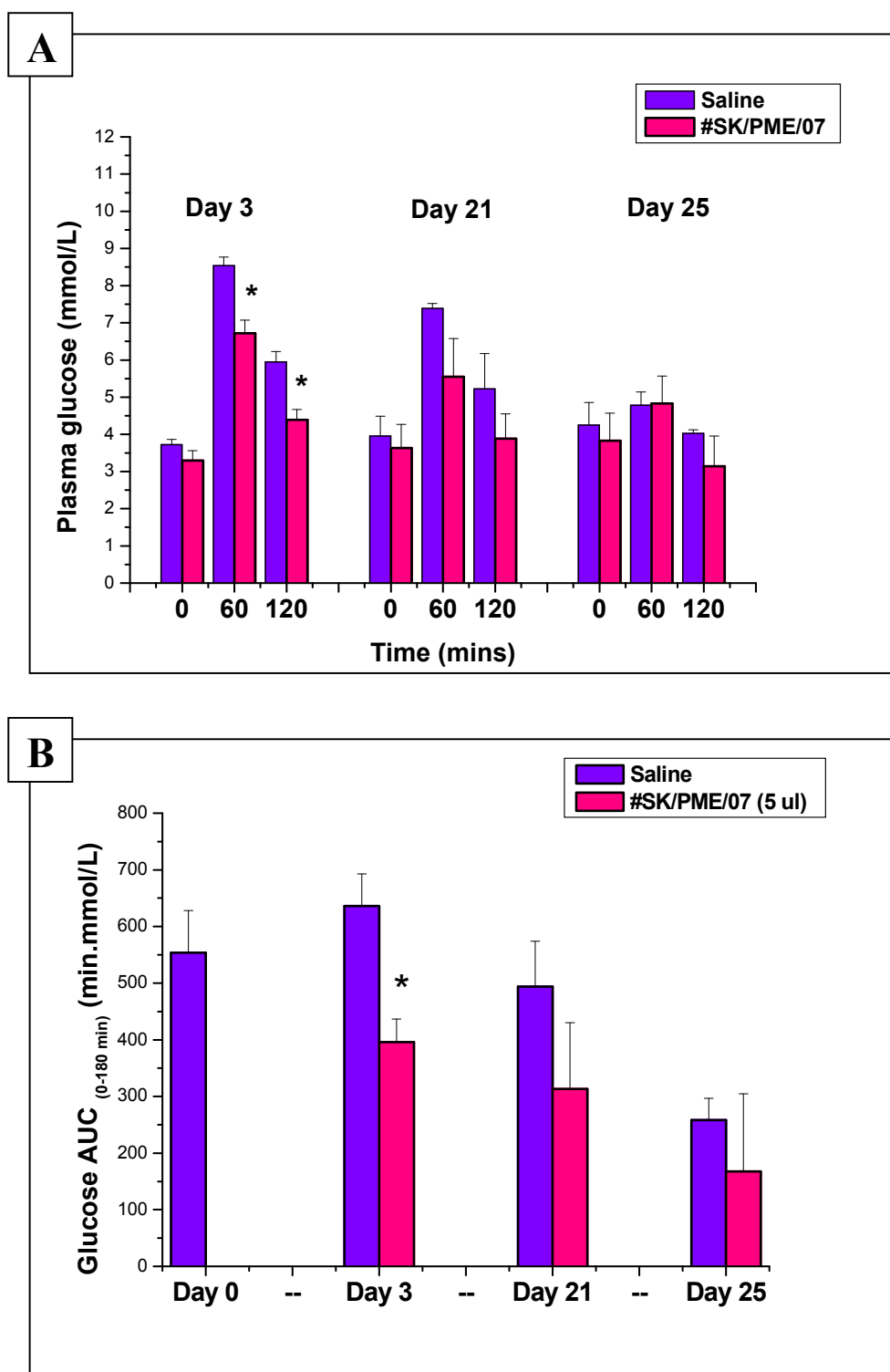


Figure 6-10 Plasma glucose (mean $\pm$ S.E) at 0, 60 and 120 minutes after an IVGTT of saline, and #SK/PME/07 treated groups (A). Mean ($\pm$ S.E.) glucose AUC_(0-180 min) (min·mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3, 21 and 25) treating with saline, and #SK/PME/07 (B).

Means indicated by an asterisk (*) are significantly different relative to the control group ($P < 0.05$).

The profiles of plasma insulin on day 0, when untreated and on day 3, 21 and 25, when treated with #SK/PME.07 during IVGTT are shown in Figure 6-11.

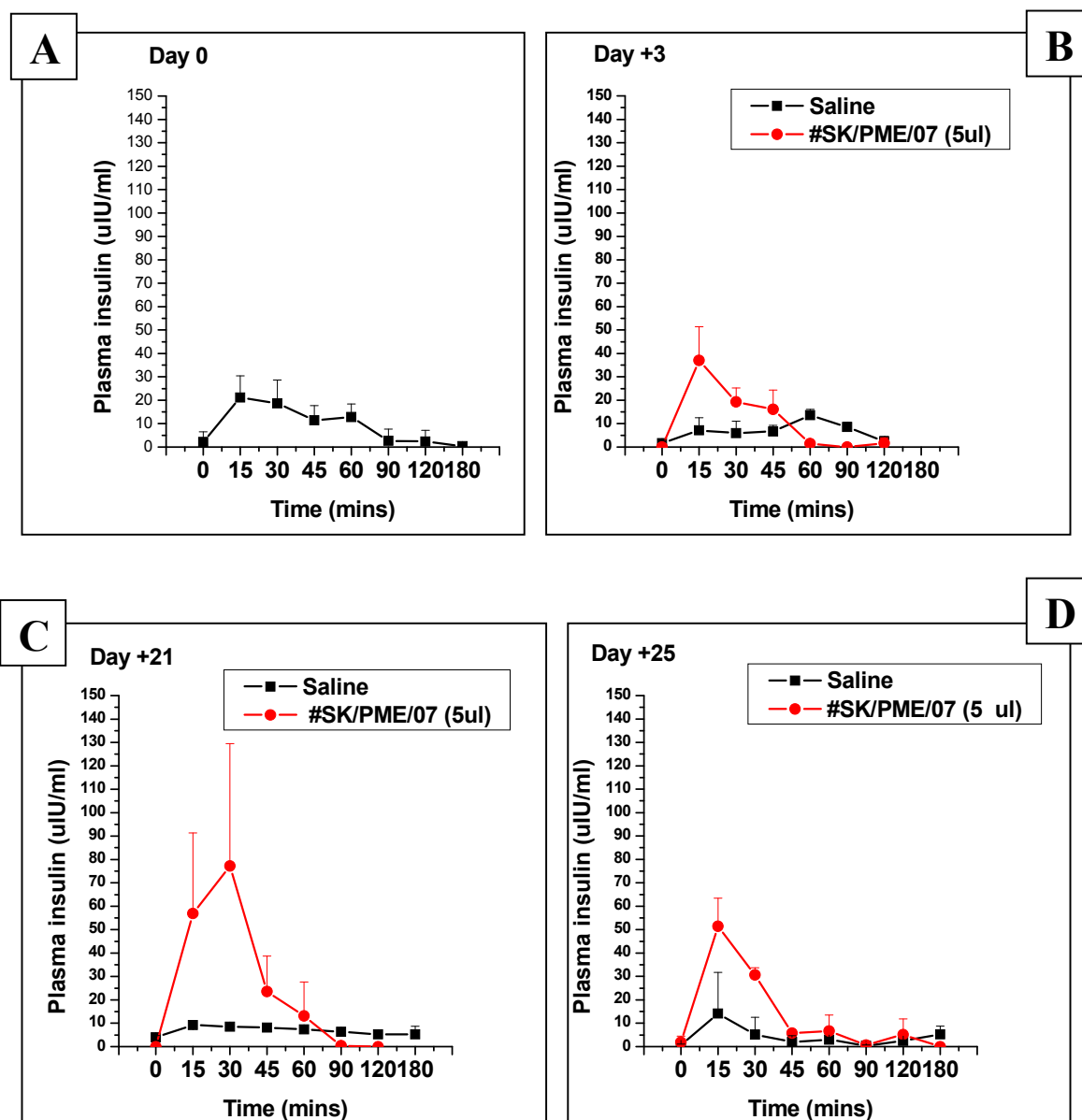


Figure 6-11 Plasma insulin levels after an IVGTT performed in saline and #SK/PME/07 (5 μ l) treated young sheep (2 yr of age). Mean ($\pm$ S.E) plasma insulin in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated.

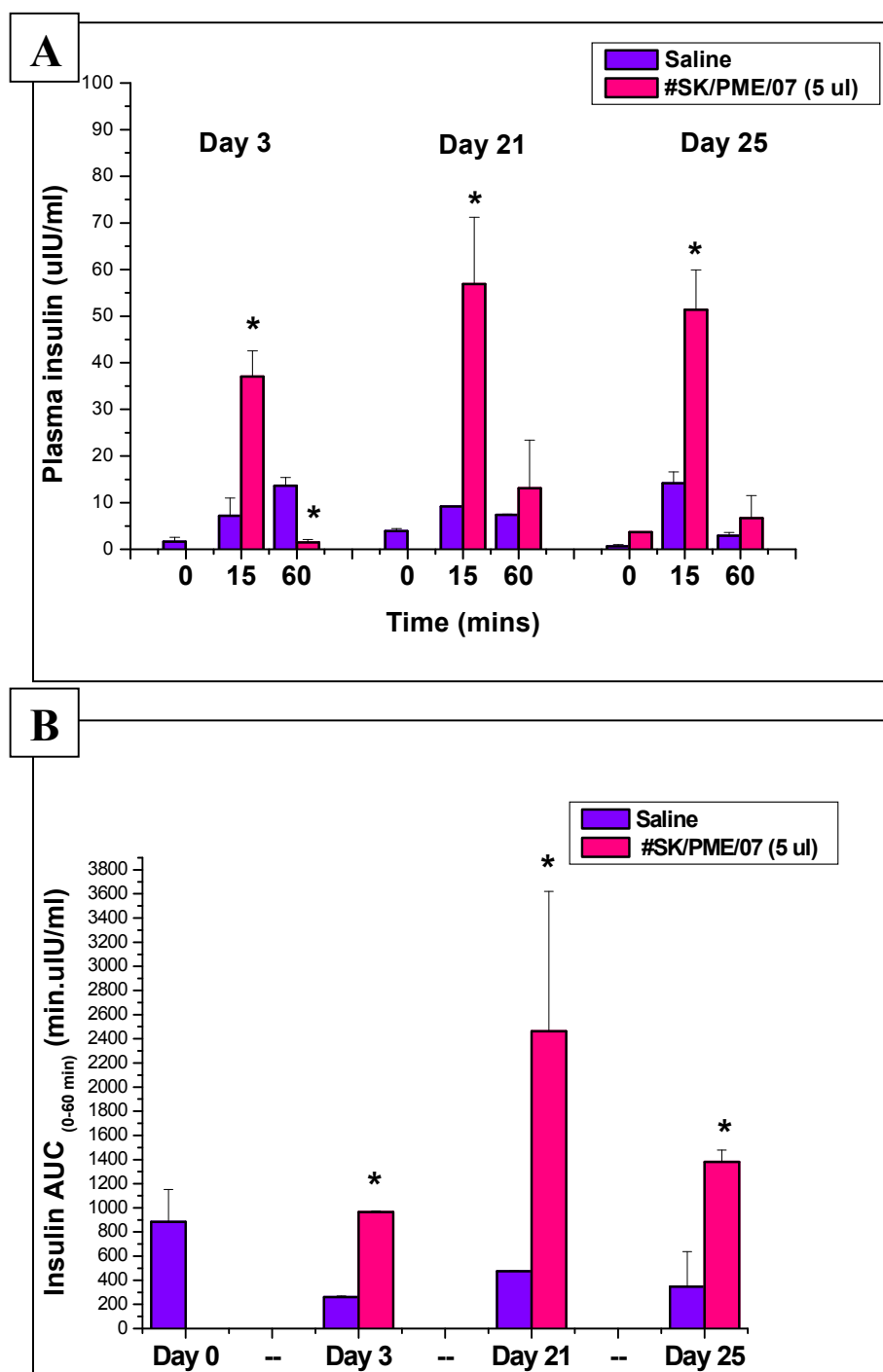


Figure 6-12 Plasma insulin (mean $\pm$ S.E.) at 0, 15 and 30 minutes after an IVGTT of saline, and #SK/PME/07 treated groups (A). Mean ($\pm$ S.E) insulin AUC_(0-60 min) (min $\cdot$ mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3, 21 and 25) treating with saline and #SK/PME/0. Means indicated by an asterisk (*) are significantly different relative to the control group ($P < 0.05$).

The comparison of insulin levels on days 3, 21 and 25 at 0, 15 and 60 minutes of the control and #SK/PME/07 treatment are shown in Figure (6-12A). No significant difference ($p>0.05$) in insulin level on days 21 and 25 at time 0 and 60 minutes were observed for either group, although on day 3 insulin was significantly decreased at 60 minutes in the #SK/PME/07 group. However, at 15 minutes on days 3, 21 and 25, the plasma insulin level of the #SK/PME/07 treated group (37.05 ± 5.5 , 56.85 ± 14.35 and 51.4 ± 8.5 $\mu\text{IU/ml}$, respectively) was significantly higher ($p<0.05$) than the control group (37.05 ± 5.5 , 56.85 ± 14.35 and 51.4 ± 8.5 $\mu\text{IU/ml}$, respectively). In addition, the insulin AUC (absolute insulin secretion; $\text{Ins}_{\text{AUC (0-60 min)}}$; $\text{min} \cdot \mu\text{IU/ml}$) of the #SK/PME/07 treated group on days 3, 21 and 25 (965 ± 8.25 , 2462 ± 1159 and 1380 ± 98.5 $\text{min} \cdot \mu\text{IU/ml}$; + 298, 419 and 298 % increase from control, respectively) was significantly higher than the control group (260.62 ± 9.37 , 474.67 ± 4.35 and 346 ± 291.75 $\text{min} \cdot \mu\text{IU/ml}$, respectively) (Figure 6-12B).

6.3.2.3 *Experiment 3*

The effects of three daily intravenous administrations of #SK/PME/07 at two different dose levels (5 and 15 μl) on glucose clearance and insulin secretion in adult (5-6 years) normoglycemic sheep over three weeks were studied. The profiles of plasma glucose on day 0, when untreated and on day 3, 21 and 25 when treated with #SK/PME/07 (5 and 15 μl) during IVGTT are shown in Figure 6-13.

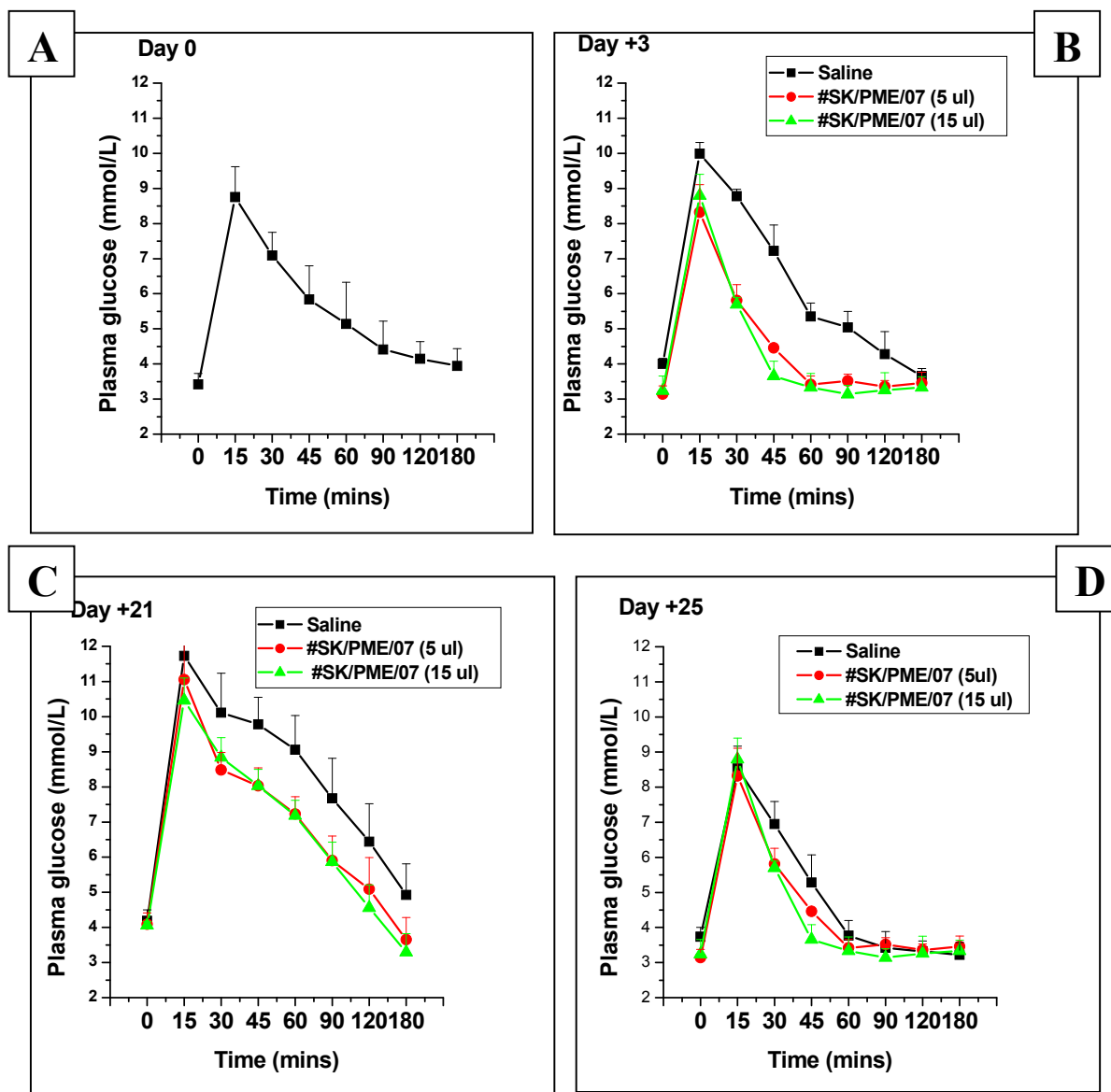


Figure 6-13 Plasma glucose clearance after an IVGTT performed in saline and #SK/PME/07 (5 and 15 μ l) treated adult sheep (5-6 yr of age). Mean ($\pm$ S.E) plasma glucose clearance in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated.

The plasma glucose at 0, 60 and 120 minutes on day 3, 21 and 25 of groups treated with #SK/PME/07 (5 and 15 μ l) and control were compared, and are presented in Figure 6-14A. Interestingly, in the adult animals, the basal plasma glucose (0 min) of treatments #SK/PME/07 (5 μ l) and #SK/PME/07 (15 μ l) on day 3 were significantly lower ($p<0.05$) than the control group. Similarly, at 60 minutes, #SK/PME/07 (5 μ l) and #SK/PME/07 (15 μ l) treatments on days 3 and 21 were significantly lower than the control. However, on day 25 no significant difference in the plasma glucose at 0, 60 and 120 minutes were observed in any group ($p>0.05$).

The glucose AUC (glucose tolerance; $\text{Glu}_{\text{AUC (0-180 min)}}$; $\text{min}\cdot\text{mmol/L}$) of 5 and 15 μ l of #SK/PME/07 (109.6 ± 18.51 and 81.99 ± 12.29 $\text{min}\cdot\text{mmol/L}$; -69.85 and - 77.44 % decrease from control, respectively) treated group on day 3 and (423.4 ± 73.54 and 454.31 ± 26.61 $\text{min}\cdot\text{mmol/L}$; -43.5 and - 39.18 % decrease from control, respectively) on day 21 were significantly lower ($p<0.05$) than the control (Figure 6-14B). However, the glucose AUC of #SK/PME/07 (5 and 15 μ l) treated groups on day 25 showed no significant difference ($p>0.05$) from the control group.

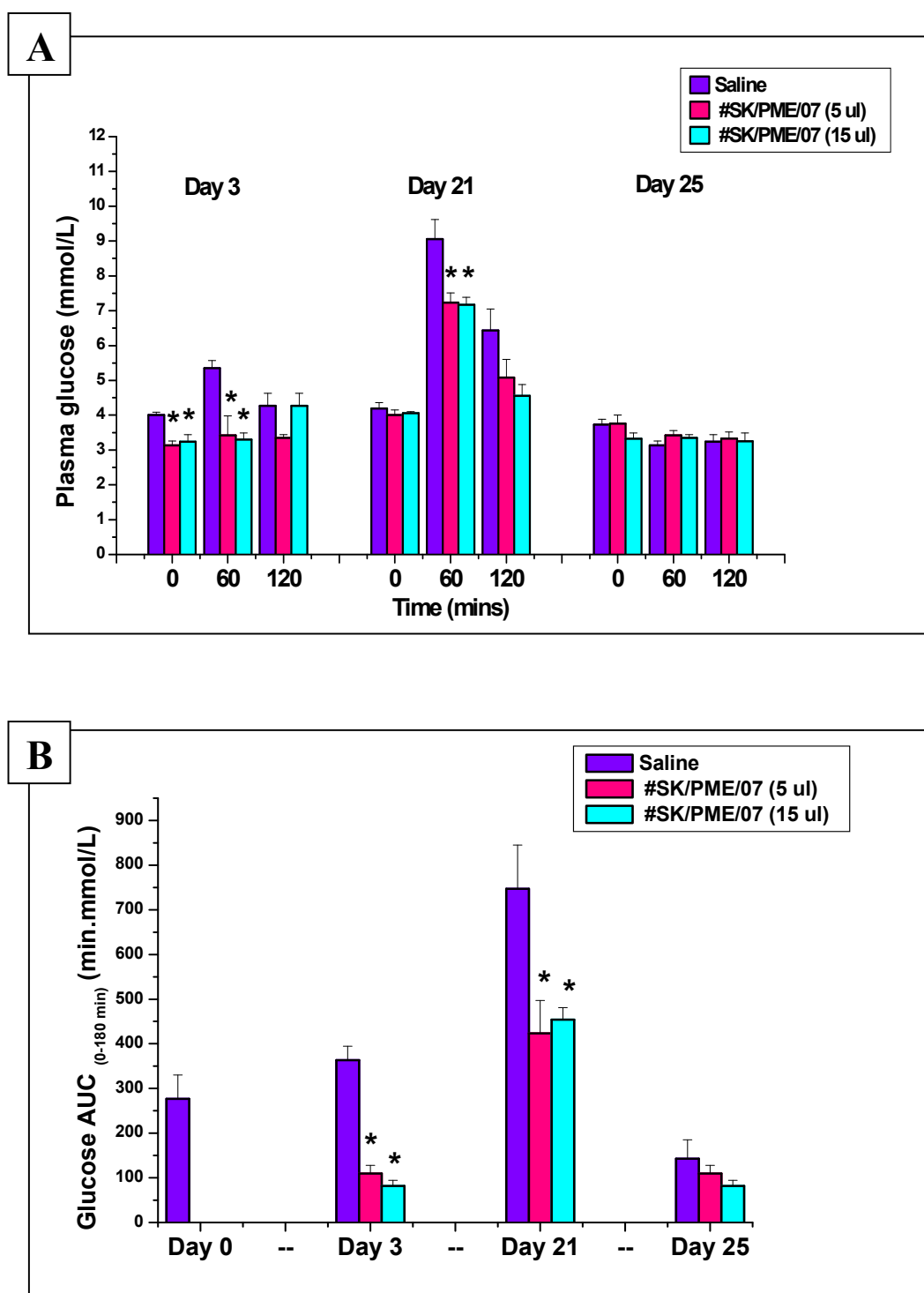


Figure 6-14 Plasma glucose (mean $\pm$ S.E.) at 0, 60 and 120 minutes after an IVGTT of saline, and #SK/PME/07 (5 and 15 μ l) treated groups (A). Mean ($\pm$ S.E.) glucose AUC (0-180 min) (min·mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3, 21 and 25) treating with saline, and #SK/PME/07 (5 and 15 μ l) (B). Means indicated by an asterisk (*) are significantly different relative to the control group ($P < 0.05$)

The profiles of plasma insulin on day 0, when untreated and on day 3, 21 and 25, when treated with #SK/PME.07 (5 and 15 μ l) during IVGTT are shown here in Figure 6-15.

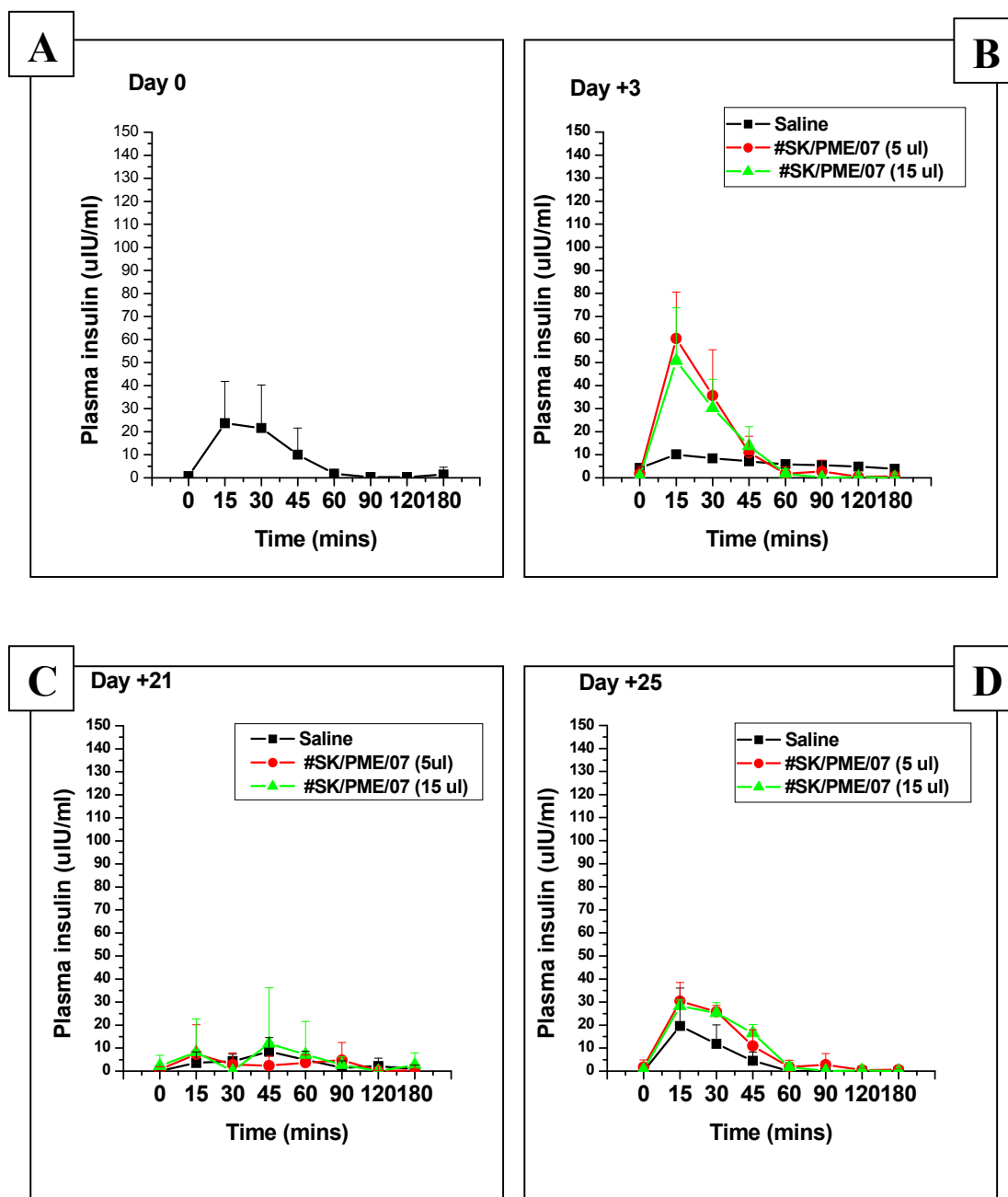


Figure 6-15 Plasma insulin levels after an IVGTT performed in saline and #SK/PME/07 (5 and 15 μ l) treated adult sheep (5-6 yr of age). Mean ($\pm$ S.E) plasma insulin in sheep on day 0 (A) when untreated, and on day 3, (B) day 21, (C) and day 25 (D) when treated.

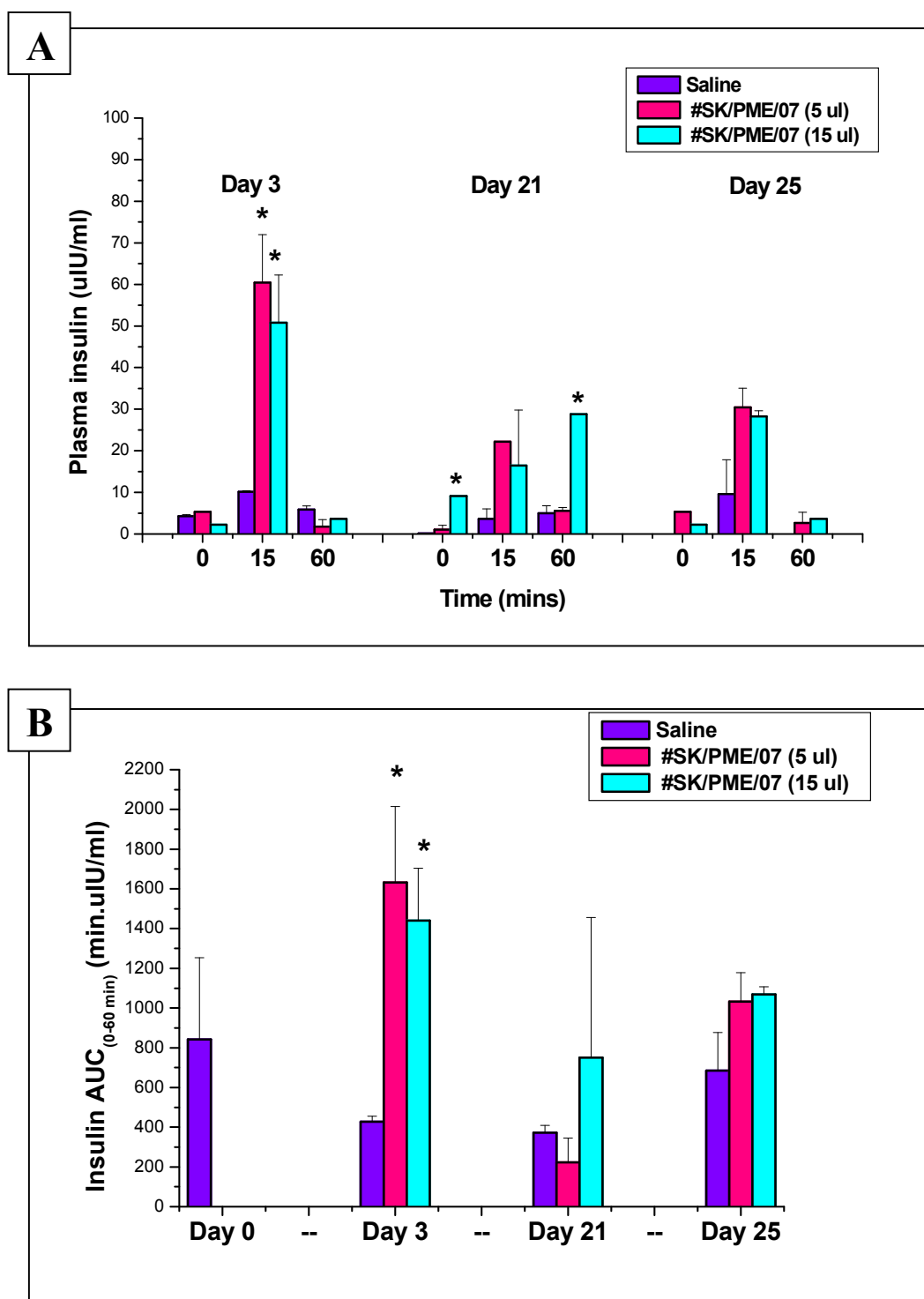


Figure 6-16 Plasma insulin (mean $\pm$ S.E.) at 0, 15 and 30 minutes after an IVGTT of saline, and #SK/PME/07 (5 and 15 μ l) treated groups (A). Mean ($\pm$ S.E) insulin AUC_(0-60 min) (min.mmol/L) in sheep, after an IVGTT performed before (day 0) and after (day 3, 21 and 25) treating with saline and #SK/PME/07 (5 and 15 μ l).

Means indicated by an asterisk (*) are significantly different relative to the control group ($P < 0.05$).

The comparison of insulin levels on days 3, 21 and 25 at 0, 15 and 60 minutes between control and #SK/PME/07 (5 and 15 μ l) is shown in Figure (6-16A). No significant difference ($p>0.05$) in insulin level on day 3, and 25 at time 0 and 60 minutes was observed for either group. However, on day 21 the plasma insulin level of #SK/PME/07 (15 μ l) at 0 and 60 minutes was significantly higher ($p<0.05$) than the control and #SK/PME/07 (5 μ l). On day 3 the plasma insulin level of both #SK/PME/07 (5 and 15 μ l) was significantly greater than the control group at 15 minutes. In addition, the insulin AUC (absolute insulin secretion; $\text{Ins}_{\text{AUC (0-60 min)}}$; $\text{min}\cdot\mu\text{IU/ml}$) of both #SK/PME/07 (5 and 15 μ l) treated group (1632 ± 382.43 and 1441.13 ± 262.95 $\text{min}\cdot\mu\text{IU/ml}$; +282 and +237 % increase from control, respectively) on day 3 were significantly higher ($p<0.05$) than the control group (427.5 ± 27.7 $\text{min}\cdot\mu\text{IU/ml}$) (Figure 6-16B).

6.4 Discussion

The present study investigated the pharmacological/physiological mechanisms responsible for the antidiabetic properties of #SK/PME/07 on both tissues involved in glucose homeostasis *in vitro* and on glucose clearance in normoglycemic, non-diabetic sheep *in vivo*.

6.4.1 *In vitro* studies

The present *in vitro* studies both on insulin secretion from mouse pancreas tissue and on glucose uptake by mouse skeletal muscle tissues, reports the possible pharmacological mechanisms involved in the glucose lowering properties of the bioassay guided-purified fraction of PME (#SK/PME/07).

Insulin secretion by pancreatic β -cells in response to glycemic control is maintained by glucose, other nutrients, neurotransmitters and other hormones (Wollheim *et al.*, 1996). However, glucose is the primary regulator of insulin synthesis and secretion (Goodman, 2001). Pharmacological agents may alter insulin secretion by influencing the myriad regulated physiologic molecular processes in the β -cell or modifying insulin secretion by cytolytic or cytotoxic means (Doyle & Egan, 2003).

Experiments using mouse pancreas tissues showed that chronic incubation of pancreatic tissues with the bioassay-guided purified fraction of PME (#SK/PME/07) showed a significant glucose dependent insulin-releasing effect under hyperglycemic culture conditions (12 mM glucose) *in vitro*. Moreover, this insulin releasing effect of #SK/PME/07 was comparable with the similar effects of tolbutamide (0.1 mM). The potent insulin secretion stimulatory effect of #SK/PME/07 in response to elevated glucose concentration suggests that β -cell metabolism is able to augment the insulin release stimulus.

Unlike tolbutamide, the effect of #SK/PME/07 treatment under normoglycemic (5 mM glucose) conditions on mouse pancreatic tissues has shown no significant effect on basal insulin secretion. The absence of a significant effect on basal insulin secretion by #SK/PME/07 supports the earlier findings that the insulin secretion activity of #SK/PME/07 is not a mere consequence of cytotoxic or cytolytic events and therefore is regulated by physiologic molecular processes in the β -cell. Moreover, hypoglycemia induced by excessive insulin secretion is a major complication of current pharmacological treatments of T2D (Henquin, 2004). The enhancement of insulin secretion only at hyperglycemic but not in normoglycemic conditions suggests that this substance would not provoke hypoglycemia under basal physiological condition.

Studies to evaluate the possible pharmacological mechanisms underlying the insulin-releasing action of #SK/PME/07 indicated a similarity to the sulphonylurea class of drug. Until recently, sulfonylureas were the only drugs used in the treatment of T2D to

stimulate insulin secretion. The sulphonylureas mimic the effect of glucose on the insulin secretory pathway. These agents act by binding to sulphonylurea receptors in the pancreatic β -cells, resulting in closure of plasma membrane $[K^{+} \text{ sub.ATP}]$ channels, membrane depolarization, opening of voltage dependent Ca^{2+} channels, elevation of intracellular Ca^{2+} and triggering insulin secretion (Proks *et al.*, 2002; Henquin, 2004; Rendell, 2004). Defective closure of $[K^{+} \text{ sub.ATP}]$ channels in diabetic β -cells may be expected to impair the production of a triggering signal by agents that depolarize by inward current. Diazoxide, a pharmacological inhibitor of sulphonylurea receptors, blocks insulin secretion by preventing closure of K^{+} -ATP channels (Henquin & Meissner, 1982; Trube *et al.*, 1986). In this study, as expected, the insulin releasing effect of tolbutamide (first generation sulphonylurea) was inhibited by diazoxide. Interestingly, diazoxide also inhibited the insulin releasing effect of #SK/PME/07, indicating that the involvement of K^{+} -ATP channels closure in the insulin secretion stimulatory action of #SK/PME/07. In the present study tolbutamide failed to augment the insulin-releasing effects of #SK/PME/07, suggesting that the effect of #SK/PME/07 together with tolbutamide on pancreas tissues may rationally use the same pathways to exhibit insulin-releasing effect that may lead to saturation of insulin releasing activity. However another possibility for this lack of synergism is that these tissues are already saturated with the peak active concentrations of tolbutamide and #SK/PME/07 when combined.

As a general concept, at the molecular level a drug may influence insulin secretion by 1) acting primarily on β -cells, and or 2) by influencing the variety of secondary messenger pathways and the secretory machinery in the β -cells. Consistent with this view, arginine,

which promotes insulin secretion by depolarizing the pancreatic β -cells, failed to affect the insulin-releasing effect of #SK/PME/07. Arginine and other cationic amino acids depolarize β -cells because of their entry as a positive charged form (Hermans *et al.*, 1987). Unlike sulphonylureas (Eliasson *et al.*, 1996), #SK/PME/07 does not stimulate insulin secretion from chemically depolarized pancreatic tissues incubated with 25 mM of KCl, indicating the absence of K^+ -ATP channels-independent effects. Furthermore, the insulin-releasing activity of #SK/PME/07 increased gradually from 12 hours to 24 hours under hyperglycemic culture conditions followed by a decrease under basal culture conditions, indicating its glucose-mediated stimulatory effects on insulin secretion. Moreover, the high insulin content in pancreatic tissues treated with #SK/PME/07 supports the possible role of #SK/PME/07 on insulin biosynthesis. Unfortunately, there are no dose response data to compare the combined effects of pharmacological test substances and #SK/PME/07, limiting suggestions as to how precisely #SK/PME/07 exhibits insulin releasing activity.

In summary, the treatment of #SK/PME/07 on mouse pancreas tissues stimulates glucose-dependent insulin secretion by possibly acting on K^+ -ATP channels, like glucose and sulphonylureas. However unlike sulphonylureas, the absence of significant effects on basal insulin secretion, absence of synergetic effects with tolbutamide and absence of potentiation of insulin secretion from chemically depolarized pancreatic tissues of #SK/PME/07, prompted us to suggest that #SK/PME/07 may also act on other downstream, secondary insulin secretory pathways such as G-protein-coupled receptor pathways. This is particularly true of potentiators such as the gut hormones that stimulate

intracellular cAMP productions, activation of protein kinase A (PKA) and protein kinase C (PKC). The resultant activation of PKA and PKC, in turn, can phosphorylate and activate the K⁺-ATP channels and cause exocytosis of insulin (Doyle & Egan, 2003). Nevertheless, these results indicate #SK/PME/07 exhibits insulin releasing effects, partially by mimicking the effects of sulphonylureas on the insulin secretory pathway and partially by having additional action on either pancreatic β -cell nutrient metabolism or second messenger pathways or proinsulin biosynthesis.

The present study also examined the possible pharmacological mechanisms responsible for the glucose uptake activity of #SK/PME/07 using an isolated mouse skeletal muscle preparation.

Skeletal muscle tissue is responsible for the majority of the glucose disposal and plays a major role in glucose homeostasis (Bonadonna *et al.*, 1993). #SK/PME/07 showed a potent glucose uptake activity at both basal and hyperglycemic culture condition. There are a number of ways in which #SK/PME/07 may be increasing uptake of glucose in skeletal muscle. In the skeletal muscles, glucose uptake is stimulated by the activation of the translocation of GLUT-4 vesicles by the presence of insulin (Shepherd & Kahn, 1999). Thus it is plausible that #SK/PME/07 is directly increasing the glucose uptake by activating the translocation of GLUT-4 vesicles present in the skeletal muscle tissues.

The potent glucose uptake effect in hyperglycemic culture condition was of a similar magnitude to that of 100 nM insulin. Although this effect was observed in the absence of

exogenously added insulin it does not preclude a possible involvement of residual insulin receptor binding within the muscle preparation. The presence of insulin significantly potentiated the glucose uptake activity of #SK/PME/07. This significant synergism is probably due to its potential effects on secondary glucose uptake pathways other than the classical insulin-dependent pathway.

Like insulin, 10 nM of metformin also potentiated the glucose uptake effects of #SK/PME/07. Metformin acts primarily by suppressing hepatic glucose production mostly by gluconeogenesis (Nosadini *et al.*, 1987; DeFronzo *et al.*, 1991; Stumvoll *et al.*, 1995; Cusi *et al.*, 1996) and to a small extent enhancing the insulin sensitivity in peripheral tissues (Inzucchi *et al.*, 1998; Johansen, 1999). In the skeletal muscle, metformin enhances the insulin-stimulated translocation of GLUT4 in muscle tissue to increase glucose uptake (Bailey & Puah, 1986; Matthaei *et al.*, 1991). Metformin also reduces hyperglycemia in the absence of insulin, through the exercise-stimulated GLUT4 translocation pathway by activating 5'AMP-activated kinase (AMPK) in muscle (Hayashi *et al.*, 2000; Zhou *et al.*, 2001). In our study, the significant potentiation of the combination of metformin and #SK/PME/07 suggests that #SK/PME/07 exerts effects on glucose transport via either peripherally enhanced insulin-mediated glucose uptake or exercise stimulated glucose uptake or both.

To determine, the exact mechanistic pathway involved in the glucose uptake activity of #SK/PME/07 and also whether or not #SK/PME/07 has an insulin mimetic effect, a pharmacological inhibitor of PI3-kinase (wortmannin) was used to block the insulin-

mediated glucose uptake pathway. Insulin-mediated activation of PI3-kinase is necessary for translocation of GLUT4 vesicles from an intracellular site to the plasma membrane to facilitate glucose uptake (Despres & Marette, 1999). Wortmannin has been shown to completely inhibit the activity of PI3-kinase and consequently, insulin-mediated glucose uptake (Hausdorf *et al.*, 1999), but has no effect on contraction-stimulated glucose uptake (Lund *et al.*, 1995). In the present study, wortmannin inhibited the glucose uptake activity of insulin and #SK/PME/07ant the combination of metformin and #SK/PME/07 but failed to inhibit the effects of metformin or the combination of insulin and #SK/PME/07. This inhibitory effect of wortmannin on glucose uptake activity of #SK/PME/07 was comparable with similar effects of insulin, suggesting that, the glucose uptake activity of #SK/PME/07 is possibly regulated by pathways that are utilised by insulin such as PI3-kinase pathway. However, the absence of inhibition by wortmannin on insulin combined with #SK/PME/07 suggests that, #SK/PME/07 also possibly has some additional effects on the other secondary insulin-mediated glucose uptake pathways. Some studies indicate that the activation of PI3-kinase alone does not result in glucose transportation in muscle and adipose tissues indicating there may be an alternative PI3-kinase independent pathway involved in the signal transduction at this level (Despres & Marette, 1999). The exact biological mechanisms which underlie the other PI3-kinase independent glucose uptake pathways such as p38 mediated glucose uptake pathways (Konrad *et al.*, 2001) and a c-Cbl/CAP signaling pathway are poorly understood (Ross *et al.*, 2004). An alternative suggestion is that #SK/PME/07 may increase basal glucose uptake by facilitating the movement of glucose through GLUT1 and GLUT3 transporters which have been found in skeletal muscle (Haspel *et al.*, 1986; Gould *et al.*, 1991). It has also

been suggested that metformin also increases the translocation of GLUT1 from intracellular compartments (Hundal *et al.*, 1992). The presence of synergism in the combined #SK/PME/07 and metformin suggests it is unlikely that the major mechanism by which #SK/PME/07 stimulates glucose uptake in skeletal muscle is via GLUT1 translocation. Alternatively, GLUT1 translocation has been shown to have a minimal impact on glucose homeostasis (Pilch *et al.*, 1993). Nevertheless the results from this study confirm that #SK/PME/07 stimulates glucose uptake by enhancing insulin-mediated glucose uptake rather than mimicking insulin action on the insulin-signaling pathways in the skeletal muscle tissues.

6.4.2 In vivo studies

The present study shows that the observed insulintropic and insulin sensitising effect of #SK/PME/07 *in vitro* is maintained *in vivo* and accounts for the improvement of glucose tolerance occurring in normoglycemic, non-diabetic sheep. More importantly, the present study for the first time demonstrated that #SK/PME/07, a glycoprotein(s) from an aqueous extract of *Pterocarpus marsupium*, is able to significantly improve glucose tolerance in normal sheep, suggesting that #SK/PME/07 has promise as a novel potential antidiabetic drug.

Physiologically, glucose tolerance involves a complex interaction among pancreatic β -cell insulin secretion, action of insulin to increase glucose disappearance and decrease endogenous glucose production (insulin sensitivity), liver clearance rate of insulin, and the ability of glucose itself (independent of insulin) to increase glucose uptake and

suppress endogenous glucose production (glucose efficiency) (Bergman, 1989). A preliminary study investigated the effects of PME (an aqueous extract of *Pterocarpus marsupium*) and PPME (an intermediate fraction from bioassay-guided purification of PME) on glucose clearance in normal sheep, and as expected, in normal, non-diabetic sheep after an intravenous glucose bolus, plasma glucose and insulin concentrations were significantly elevated and then progressively decreased. In this study three, daily intravenous administrations of PME and PPME had no effects on both basal glucose and insulin levels. This finding supports the earlier studies from our laboratory which showed that oral administration of PME had no effects on basal glucose (Verschuuren, 2001) and is also in contrast to the results of previous studies which showed that PME decreases blood glucose in normal rabbits (Shah, 1967) and also in alloxan induced diabetic rats (Ahemed *et al.*, 1991). Plasma glucose clearance rapidly increased in sheep treated with PME and PPME during GTT and this effect was clearly associated with the insulintropic effects of PME and PPME. Although both PME and PPME showed a similar plasma glucose clearance, the stimulation of insulin secretion by PPME was significantly greater than the insulintropic effect of PME. This was further confirmed by calculating glucose tolerance and absolute insulin secretion. The improved glucose tolerance (decrease in glucose AUC) of PPME >PME >control, was clearly associated with the absolute insulin secretion of PPME > PME > control, possibly due to the presence of insulin secreting and sensitising constituents in PME and PPME. The present study clearly differentiates the insulintropic effects of PPME from PME. The enrichment of insulin secreting activity in PPME has been considered as an important confirmatory measure during the bioassay-guided purification of PME. In view of these

present results, it can be assumed that both PME and PPME have a significant effect on lowering blood glucose and stimulate insulin responsiveness after a glucose challenge.

This preliminary *in vivo* experiment, lead to the investigation of the *in vitro* bioassay-guided purified fraction (#SK/PME/07) from an aqueous extract of *Pterocarpus marsupium* on insulin secretion as well as its consequence on glucose clearance in two different age groups (young and adult), in normoglycemic and non-diabetic sheep *in vivo* over three weeks. The intravenous administration of glucose (2.5 mg per kg) before and during the experiments stabilised the normal glucose clearance and insulin secretion of both age groups.

In young animals (2 years of age), treatment showed significant effects on plasma glucose clearance and tolerance on day 3 but this effect was normalised on day 21 and 25 in some individuals. This glucose intolerance on day 21 and 25 is possibly related to insulin resistance in young animals and not insulin deficiency as indicated by the increased insulin AUC during the glucose tolerance test. The impairment of glucose tolerance with aging from the young animal through puberty to the young adult is common in the sheep (Gatford *et al.*, 2004) and rat (Bracho-Romero & Reaven, 1977), in contrast to humans, where glucose tolerance is maintained across this period by compensatory changes in insulin secretion (Amiel *et al.*, 1986; Bloch *et al.*, 1987).

The acute plasma insulin secretion at 15 min in response to intravenous glucose bolus during GTT was markedly elevated on day 3, 21 and 25. Animals exhibiting high levels

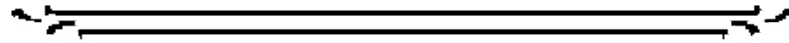
of insulin when there is minimal change in glucose levels in our studies is in parallel with a previous study by Gupta (1963), where PME was infused via stomach tube and reported that sensitization of β -cells of the islets of the pancreas in albino rats resulted in secretion of large amounts of insulin in response to glucose. Moreover, the rise in plasma insulin secretion in response to glucose reveals that the insulin releasing effect of #SK/PME/07 is glucose dependent. Interestingly, #SK/PME/07 has no significant influence on basal glucose or insulin levels over the days after treatment, indicating that the administration of #SK/PME/07 is relatively safe and would not provoke hypoglycemia unlike some other insulintropic agents.

Similarly in adult animals (5-6 years of age), treatments (5 and 15 μ l of #SK/PME/07) showed a significant prolonged effects on both insulin secretion and plasma glucose clearance. However, no marked significant differences in glucose clearance and insulin secretion between the two doses (5 and 15 μ l) of #SK/PME/07 were observed. In contrast to young animals,

The clear difference in the kinetics of plasma insulin levels on day 21 and 25, of #SK/PME/07 treatment, between young and adult sheep suggests that #SK/PME/07 exhibits its hypoglycemic activity majorily thorough insulin secretory pathway. However, these interesting finding demands further investigations to study the effect of #SK/PME/07 treatment in animals with different ages.

In summary, the present *in vivo* experimental results indicate that three daily intravenous administrations of #SK/PME/07 have prolonged effects on insulin secretion as well as on glucose clearance in normal, non-diabetic sheep. Importantly, the potent effect of #SK/PME/07 on both insulin secretion and glucose tolerance supports our *in vitro* findings that, constituent(s) of #SK/PME/07 have direct effects on tissue involved in glucose homeostasis. In view of the results, it can be assumed that the use of #SK/PME/07 to combat the adverse effects of hyperglycemia appears to be beneficial by enhancing the target area directly associated with the normal glucose regulatory processes and moreover results provide evidence that the principal effects are prolonged by many hours to days, unlike the many antidiabetic drugs which overstimulate the β -cells or pose as a risk of hypoglycemia.

7 General Discussion



The current treatment of T2D includes oral antidiabetics and/or insulin injections. Current medication options for the treatment and management of T2D are relatively limited, have non-negligible side effects and/or must often be prescribed in combination. In most cases, the current oral antidiabetics and/or exogenously administered insulin can maintain blood glucose within a normal physiological range but cannot simulate the pancreas of a healthy individual; hence diabetics within 15 to 20 years of diagnosis develop complications. Given that most T2D patients respond to exogenously administered insulin, interventions aimed at mimicking insulin's effects and augmenting insulinotropic and insulin synthesis function may prove beneficial.

Natural products historically have been a source of therapeutically useful drugs. In the particular case of diabetes, a number of plant derived secondary metabolites have been described to possess antidiabetic activity in a variety of model systems (Marles & Farnsworth, 1995; Gray & Flatt, 1997; Alarcon-Aguilara *et al.*, 1998; Yeh *et al.*, 2003). Indeed, the widely prescribed antidiabetic drug, metformin was derived from guanidine, a molecule isolated from *Galega officinalis*.L (French lilac) (Bailey & Day, 2004). Ayurveda, an ancient Indian system of medicine advocates a number of traditional medicinal plants to treat and manage T2D (Trivedi, 1963; Nagarajan *et al.*, 1982; Grover *et al.*, 2002b; Kar *et al.*, 2003). The hypoglycemic activity of Ayurvedic traditional medicinal plants has been confirmed using modern scientific investigations. This has caused an increase in the number of experimental and clinical investigations directed towards the validation of the antidiabetic properties, which are empirically attributed to

these remedies. However, studies specifically directed to the isolation, purification and identification of substances responsible for the hypoglycemic activity and also the mechanisms of hypoglycemic activity of these constituent(s) have often been lacking. In this context, the present study aimed to identify and isolate a potent antidiabetic constituent(s) from some extensively advocated Ayurvedic medicinal plants such as *Trigonella foenum-graecum* Linn, *Pterocarpus marsupium* Roxb, *Gymnema sylvestre* R.Br and *Curcuma longa* Linn.

While there have been numerous studies in these particular plants that demonstrate the antidiabetic potential in various animal models (Shah, 1967; Saifi *et al.*, 1971; Okabayashi *et al.*, 1990; Khosla *et al.*, 1995; Persaud *et al.*, 1999; Zia *et al.*, 2001; Arun & Nalini, 2002b; Kuroda *et al.*, 2005) as well as in humans (Rajasekharan & Tuli, 1976; Baskaran *et al.*, 1990; Sharma *et al.*, 1996), no experiment to date has provided convincing evidence as to which are the major sites of action of these plants or determined how these hypoglycemic effects are achieved. The present study represents a substantial step towards identifying the major mechanism through which these selected Ayurvedic plants are achieving their hypoglycemic effects. An in-house developed *in vitro* bioassay method was employed in the present study to determine the effects of treatments on insulin secretion from mouse pancreas tissues and on glucose uptake by mouse skeletal muscle tissues under both normoglycemic and hyperglycemic culture conditions (Chapter 3). The finding that all these plants enhance glucose homeostasis by increasing either insulin secretion or peripheral glucose uptake or both indicated the relative mechanistic targets by which of these plants achieve antidiabetic effects in the

experimental *in vivo* model. The findings of antidiabetic properties in the tissue culture-based bioassay support the ethnopharmacological use of these Ayurvedic plant and also indicate the possible mechanistic ways by which they achieve glucose lowering effects. Moreover, the *in vitro* model and methods described in this study could be used for the development of new antidiabetic drugs. Nonetheless, these promising *in vitro* results must be ascertained in various *in vitro* and *in vivo* animal models of insulin resistance and diabetes.

The molecular basis of T2D pathogenesis is complex and largely unknown. Although T2D has many unresolved complexities, insulin resistance and β -cell dysfunction are the major components of diabetes pathogenesis (DeFronzo *et al.*, 1992). Many currently available medications for T2D diabetes reduce hyperglycemia either by increasing insulin secretion, production and sensitivity or reducing hepatic glucose uptake and absorption of dietary carbohydrates or delaying gastric emptying or mimicking insulin action or altering renal glucose handling, but none have so far convincingly demonstrated that they can beneficially alter the natural history of progressive loss of pancreatic insulin which normally results in exogenous insulin therapy. Therefore research into the novel molecules that could preserve, protect or indeed increase β -cell mass along with other beneficial peripheral effects would be a major advance in the treatment of diabetes. Hence, the current advancement of novel antidiabetic molecule interventions focuses on the plant derived molecules which have potent effects on both insulin secretion and action (Bailey & Flatt, 1995; Oubre *et al.*, 1997). The present work was carried on plants that have potent effects on both insulin secretion and action. Of these Ayurvedic plant

treatments, the potent effects of aqueous extracts of *Curcuma longa* (CLE) and *Pterocarpus marsupium* (PME) on both insulin secretion and action indicated that these plants have single or multiple constituents that may act directly or indirectly on glucose homeostatic pathways of insulin secretion and action. Similarly, experimental findings on other aqueous plant extracts, such as elder, agrimony, coriander, and mushroom, have shown to have an insulin secretion stimulatory effect coupled with insulin-like effect on peripheral glucose uptake *in vitro* (Gray & Flatt, 1998a, 1998b, 1999; Gray *et al.*, 2000).

Although all these selected Ayurvedic plants have shown potential effects on tissues involved in glucose homeostasis, CLE and PME were selected for further studies due to their complementary effects on both insulin secretion and action. The dose dependent effects of both CLE and PME on insulin secretion occurred only at hyperglycemic culture conditions but not in normoglycemic conditions indicating that these treatments would not provoke hypoglycemia under basal physiological conditions. Moreover, the absence of glucose uptake and insulin secretion in skeletal muscle and pancreas tissues respectively following acute incubations (15 and 30 minutes) of both CLE and PME suggests that regulation may occur at the transcriptional or translational level. Alternatively, cellular uptake of the active compound may require facilitative transport and be subjected to the kinetics of such a transport mechanisms (Strobel *et al.*, 2005).

Similarly, CLE and PME treatments in the absence or presence of insulin on mouse skeletal muscle tissues *in vitro* showed a dose-dependent glucose uptake activity. The enhancement of glucose uptake activity of CLE in the presence of added insulin suggests

that these extracts possibly act via insulin-mediated enhanced peripheral glucose uptake pathway which may be very similar to the effect of well established antihyperglycemic drug, metformin (Bailey & Puah, 1986; Prager *et al.*, 1986). In contrast to CLE, the treatments of PME on mouse muscle tissues have shown no additive effects in the presence of insulin, suggesting that the active constituents of PME may act via pathways (at least terminally) that are utilized by insulin. Interestingly, wortmannin (a PI3-kinase inhibitor), inhibited the glucose uptake effects of both CLE and PME suggesting that these extracts act via the classical PI3-kinase pathway. In contrast to CLE, the lack of significant potentiation of glucose uptake activity of PME in the presence of insulin (saturating dose) and inhibition of activity in the presence of wortmannin shows that the constituents of PME may have insulin-like or mimicking active constituents. Therefore, treatment with CLE and PME may offer patients an effective alternative treatment to improve glucose homeostasis with no demonstrated side effects. However, in terms of their comparative effects on tissues involved in glucose homeostasis, PME (3-3.5 g/L) was found to be more potent than CLE (10 g/L) with respect to their original plant material to extract concentration. These results prompted us to identify the hypoglycemic constituent(s) of PME.

Based on these findings (Chapter 3 & 4), in order to isolate the bioactive compound(s) responsible for antidiabetic activity, a bioassay-guided purification was performed on the PME (Chapter 5). The results indicated that 1) boiling destroyed both glucose uptake and insulin secretion activity of PME, 2) it remains potent even after ethyl acetate and butyl

alcohol extraction, 3) the potent activity was evident in the molecular weight ranges between the >10K and <30K, and 4) trypsin digestion destroyed the activity of PME.

Based on these purification studies, the bioassay-guided purification of PME was carried out. Bioassay-guided purification yielded a potent antidiabetic fraction, #SK/PME/07. Dose response of #SK/PME/07 on insulin secretion and glucose uptake was compared with the starting material (PME). #SK/PME/07 and PME exerted a subtle antidiabetic effect on glucose-induced insulin secretion and glucose-mediated glucose uptake consisting of both an increase in maximal response as well as a leftward shift in the dose response curve (Figure 5-19).

Electrophoresis of non-reduced #SK/PME/07 on SDS-PAGE showed the two major fuzzy protein bands at ≤ 27 kDa and ≤ 20 kDa. Gels stained to detect the presence of glycoprotein showed positive and the poor resolution and fuzzy appearance of the bands in the gel, suggested that these proteins are heavily glycosylated. In addition, #SK/PME/07 showed negative test for peroxidase activity, indicating that #SK/PME/07 has no peroxidase molecules. The results of Con A chromatography of #SK/PME/07 indicated that the glycoproteins of #SK/PME/07 have high affinity towards the Con A. However, the appearances of cross linkage of #SK/PME/07 with Con A proteins resulted in abandonment of this method of purification. In another experiment, isoelectric focusing (ISF) of #SK/PME/07 indicated that #SK/PME/07 may have two or more negatively charged isoforms of glycosylated protein(s). Absence of significant potentiation of activity of #SK/PME/07 after treating with an insoluble

polyvinylpolypyrrolidone (PVPP) suggested that #SK/PME/07 contains either no tannins or that there is no association of tannins with the active protein molecules of #SK/PME/07. The protein precipitate (≤ 27 kDa) that was obtained after chloroform-methanol precipitation of #SK/PME/07 was significantly more potent than the #SK/PME/07, further supporting our previous findings that the hypoglycemic constituent(s) of #SK/PME/07 is/are proteins.

All these results suggested that the hypoglycemic constituent(s) of #SK/PME/07 is/are two or more isoforms of negatively charged, heavily glycosylated and hydrophobic protein(s). Similarly, experimental findings on other plants such as curry patta (*Murraya koenigii*) and pumpkin (*Cucurbitatmoschata duch*) also reports the isolation of glycoproteins (protein-bound polysaccharide) with potent antidiabetic activity (Yadav *et al.*, 2002; Quanhong *et al.*, 2005). These encouraging results of #SK/PME/07 on insulin-releasing and sensitising effect, as demonstrated both in isolated mouse pancreas and muscle tissues *in vitro* suggest it might be considered as a novel secretagogue and sensitiser with potential interest for the treatment of T2D.

The pharmacological mechanisms underlying the insulin releasing and insulin sensitising activity of #SK/PME/07 demonstrated a diverse class of antidiabetic molecule (Chapter 6). There are a number of ways in which #SK/PME/07 may be increasing the insulin secretion from pancreas. Pharmacology experiments indicated that, #SK/PME/07 exhibits insulin releasing effects, partially by mimicking the effects of sulphonylureas on insulin secretory pathway and partially having additional actions on either pancreatic β -cell

nutrient metabolism or second messenger pathways or proinsulin biosynthesis. In pancreas tissues, #SK/PME/07 exerted a potent glucose-induced insulin secretion, without a significant effect on basal secretion. These are highly desirable properties in an antidiabetic medication, as they minimize the potential for inducing hypoglycemia, an important concern with sulphonylureas and insulin secretagogues (Bailey, 1999). More importantly, a single treatment of #SK/PME/07 to pancreatic tissues has shown prolonged effects on glucose-stimulated insulin secretion suggesting #SK/PME/07 may have either direct or indirect effects on proinsulin synthesis or proliferation of replicating β -cells or tissue regenerative effects. In the light of existing evidence of β -cell mass plasticity in the adult pancreas (Bouwens & Rooman, 2005), such a prolonged effect could represent a valuable antidiabetic potentially capable of retarding β -cell decompensation in advanced T2D. Another interesting finding of this work is that #SK/PME/07 also possesses considerable potential effects on glucose uptake activity, as evidenced by enhancement of insulin-mediated glucose uptake rather than mimicking insulin action on the insulin-signaling pathways in skeletal muscle tissue culture-based bioassay.

In addition to increasing insulin secretion and the uptake of glucose in pancreas and muscle tissues respectively *in vitro*, #SK/PME/07 may also improve the whole body metabolism of glucose. Support for this hypothesis comes from the *in vivo* experiments described in Chapter 6. Three daily intravenous administrations of #SK/PME/07 have prolonged effects on insulin secretion as well as on glucose tolerance in normal, non-diabetic sheep, using both young (2 years old) and old (5-6 years old). The insulintropic

and insulin sensitising effect of #SK/PME/07 *in vitro* is maintained *in vivo* and accounts for the improvement of glucose tolerance.

Several constituents from PME have been tested for hypoglycemic activity in a number of species, all producing varying results. Interestingly marked elevation of insulin in #SK/PME/07 treated animals even after the end of experiment (25 days) raise the possibility it may in fact be maintained for several months. Strong evidence suggest that this prolonged effect is possibly due to the proliferation of β -cells (Chakravarthy *et al.*, 1980). It would be consistent with the majority of literature that examined the restoration of β -cell, causing enhanced synthesis of proinsulin or conversion proinsulin to insulin (Chakravarthy *et al.*, 1981a; Chakravarthy *et al.*, 1981b; Sheehan *et al.*, 1983). The regeneration would result from increased mitosis and therefore a rise in β -cell population causing and in turn causing the sequential elevation in insulin synthesis and release (Chakravarthy *et al.*, 1980; Chakravarthy *et al.*, 1981a). The proliferation of these β -cells would imply the plasma insulin would be elevated throughout the entire experiment, as was clearly obvious (Section 6.4.1). Once these changes in pancreatic function were established, insulin responsiveness was maintained at, a higher set point, even though there was minimal change in glucose levels, suggesting that this is a form of down regulation of receptors, which leads to insulin resistance. With respect to obese individuals, the fasting insulin level correlates with the degree of insulin resistance (Beard *et al.*, 1987), suggesting that resistance to insulin action leads to a compensatory increase of insulin secretion. Administration of thiazolidinediones, which reduces insulin

resistance in obese humans, also reduces the hyperinsulinaemia (Nolan *et al.*, 1994), as does weight loss and exercise.

Consequently, it is also possible that #SK/PME/07 may have beneficial effects on enzymes that regulate glucose metabolism. Support for this suggestion also come from recent experiments that have shown that plants traditionally used for their hypoglycemic activity regulate the activity of enzymes involved in glucose metabolism. For example it has been shown that some traditional plants increase the activity of enzymes such as glucose-6-phosphate, fructose-6-phosphate, glyoxalase 1 and creatinine kinase (Shibib *et al.*, 1993; Raju *et al.*, 2001; Genet *et al.*, 2002). However, evidence against this hypothesis comes from the fact that the rate-limiting step in glucose utilization is glucose uptake and not glucose metabolism (Clow *et al.*, 2004).

In most cases, as reported by many earlier workers, the glucose reductions following treatment with PME treatment is a result of whole-body effects of these plant extracts. Given the substantial impact that liver and adipose tissues have on glucose homeostasis, the hypoglycemic activity of PME and #SK/PME/07 on these tissues needs to be investigated in culture conditions. This would help to further identify other potential sites upon which #SK/PME/07 may have potential effects. Also there is an array of hormones and other metabolic products such as free fatty acids, resistin, tumor necrosis factor- α (TNF- α), adiponectin, and leptin which have been directly or indirectly implicated in the pathogenesis of insulin resistance and β -cell dysfunction as well as in the etiology of diabetes (as reviewed in Section 1.3) and therefore further research into the whole body

effects of #SK/PME/07 is required as it is possible that #SK/PME/07 may affect the production and release of any one of these hormones or interact with their sites of action. Complications secondary to chronic hyperglycemia are numerous and include damage to vascular endothelial cells, kidney podocytes, and neural tissue (Wendt *et al.*, 2003; Ristow, 2004; Rojas & Morales, 2004; Stas *et al.*, 2004). Cell death from hyperglycemia is thought to be due to oxidative stress, protein glycation and other mechanisms. The current experimental results also indicate that #SK/PME/07 has preservative tissue effects on both pancreas and skeletal muscle. These findings of *in vitro* and *in vivo* antidiabetic properties of #SK/PME/07 open the possibility that this may be used in the future as a means of protecting β -cells of the pancreas from apoptosis in T2D patients or to promote the regeneration of residual β -cells in early type-1 diabetes (Rubin, 1997). These preservative effects may also prove helpful in the treatment and prevention of other degenerative and autoimmune disease, as apoptosis is a prominent feature in nervous system development and disease. This finding is an important discovery, because at present, there is no conventional treatment that has protective effects against β -cell degeneration or promotes residual β -cell regeneration following damage (Saxena & Vikram, 2004). While the mechanism behind these protective effects are unclear, they may occur through a decrease in lipid peroxidation, protein oxidation and/or protein glycation (Mchugh & Mchugh, 2004). However, it may also be due to antioxidant activity of the treatment as this has been shown to have tissue preservative effects. Nevertheless, these findings possibly initiate a novel insight in the development of means to prevent diabetes. Similarly, previous experiments in a number of animal studies have demonstrated that PME exerts a protective and restorative effect against both

streptozotocin and alloxan toxicity, as well as reversing damage to the muscle, kidney and liver caused by hyperglycemia (Chakravarthy *et al.*, 1980; Chakravarthy *et al.*, 1981a; Sheehan *et al.*, 1983; Ahmad *et al.*, 1991a; Ahmad *et al.*, 1991b; Manickam *et al.*, 1997; Anandharajan *et al.*, 2005)

In summary, this investigation has provided convincing evidence as to which are the major sites of action and described how these hypoglycemic effects of Ayurvedic antidiabetic plants such as *Trigonella foenum-graecum* Linn, *Pterocarpus marsupium* Roxb, *Gymnema sylvestre* R.Br and *Curcuma longa* Linn are achieved. It demonstrated the possible mechanistic pathways attributed to the potential effects of *Curcuma longa* and *Pterocarpus marsupium* on insulin secretion from pancreas and glucose uptake by muscle *in vitro*. Importantly, the present study identified and isolated glycoproteins (#SK/PME/07) from an aqueous extract of *Pterocarpus marsupium* using tissue culture-based bioassay-guided purification methods. Finally, this study elucidated both the *in vitro* and *in vivo* antidiabetic properties and the pharmacological mechanisms involved in the glucose lowering effects of #SK/PME/07.

Despite these findings, a great deal of research is still needed to isolate and identify the active glycoprotein responsible for the hypoglycemic activity of #SK/PME/07 as well as the exact biological mechanisms responsible for the blood glucose lowering effects of #SK/PME/07. Importantly, the effects of #SK/PME/07 on different stages of pathogenesis of T2D as well as on different animal models of both insulin resistance and diabetes and also any toxic or adverse effects associated with the long term use would be

required prior to their being considered for clinical application. Nevertheless, the findings from this investigation are particularly interesting from a commercial aspect and may provide rationale for the further development of novel antidiabetic molecules and also some new potential insights into the antidiabetic potential of *Pterocarpus marsupium*.

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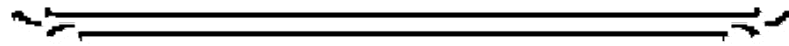
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Appendix



RIA protocol for insulin assay

Day 1

Step 1

- Label 20 tubes 'std 1-std 10'
- Label 3 tubes 'T.C. (total count)
- Label 2 tubes 'NSB & 2 'B0'
- Label sample tubes (duplicate for each sample)

Step 2

- Pipette 300 µl of charcoal stripped horse serum or DMEM into tubes 'std 2-10'.
- Pipette 100 µl of charcoal stripped horse serum into all NSB and B0 tubes
- Pipette 300 µl of Bovine Insulin standard in tube 1

Step 3

- Pipette 100 µl of std 1 into std 2
- Pipette 100 µl std 2 into std 3.... Continued until std 10

Step 4

- Make up a antibody solution containing 5 µl Antibody & 4ml of CTS
- Add antibody solution into all tubes **except NSB and TC**
- Add 100 µl of CTS to NSB tube

Step 5

- Make up tracer solution: 22 µl tracer & 8 ml of RIA buffer
- Pipette 100 µl of tracer solution into 3 TC tubes
- Take 1 of the tubes & test cpm – If around 20,000 then pipette 100 µl of solution in to all tubes. This step should be carried out on the radiation bench with gloves. Vortex briefly.
- Cover the tray of tubes with alfoil and put into the cold room overnight.

Day 2

- Add 1.2 ml of 20 % PEG to all tubes **except TC** (*Add 100 µl charcoal striped serum for the tissue culture media samples*)
- Vortex, and let stand on bench for 15-30 mins
- Place in centrifuge & spin for 30 mins @ 3,500 rpm at 5°C.

-
- When spin completed, **Remove immediately** and aspirate all **except TC**
 - Place tubes in gamma counter in radiation lab
 - ❖ Place TC, BO, NSB in first rack labeled Protocol 13
 - ❖ Then load standards in order (1,1,2,2,3,3.....)
 - ❖ Then samples
 - ❖ Make sure a stop rack is at the end of the run

Reagents required for insulin RIA

RIA buffer (0.05M Phosphate, 0.15M NaCl)

10X PBS stock	100 ml
5 mM EDTA	1.86 g
BSA	1.0 g
10 % Azide (0.02 %)	2.0 ml
Dist. H ₂ O to give 1000 ml total volume	

CTS buffer

Sodium deoxycholate	5 g
Triton-x100	10 ml
Sodium dodecyl sulphate (SDS)	0.5 g
Dist. H ₂ O to give 100 ml total volume	

22 % PEG

Polyethylene glycol 6000 (PEG)	550 g
NaCl	22.5 g
Dist. H ₂ O to give 2500 ml total volume	

10x Phosphate buffered saline (PBS, 0.5 M Phosphate, 1.5 M NaCl, pH 7.4)

Na ₂ HPO ₄ (141.96) (Na ₂ HPO ₄ .12H ₂ O (MW-358.15))	57.49 g
KH ₂ PO ₄ (136.09)	12.39 g
NaCl (58.44)	87.66 g
Adjust pH to 7.4 and make up to 1 L with distil.H ₂ O. Dilute 1:10 to prepare 0.05 M (1x)	

SDS Polyacrylamide Gel Electrophoresis

Method

1. All equipment, particularly glass and aluminum plates, must be spotlessly clean before beginning. Any dirt on plates causes gel to stick and tear.
2. Every half dozen runs spray the aluminum plates with a light coating of silicon. When dry wash off any greasy residue thoroughly.
3. Combs are also washed thoroughly and stored in ethanol when not in use.
4. Make sure casting plate is ready prior to adding final ingredients to separation gel solution.
5. Grease spacers (1.5 mm) lightly with Vaseline and place in between glass and aluminum plates. The lower edge of these must be absolutely flat to minimize leakage. Lightly grease lower corners of combined plates. Place into casting plate.
6. Add a small amount of distilled water using a syringe to check for leakages and then drain.
7. Prepare separating gel by adding lower gel stock, acrylamide and water to conical flask. Swirl gently over very low heat. This is to remove oxygen from solution which will prevent the polymerization process. Note- **Do not over heat solution as it will prevent it from setting.**
8. To conical flask add ammonium persulfate and TEMED. This solution sets rapidly.
9. Using a syringe slowly add solution to mold ensuring no bubbles.
10. Overlay gel with very small amount of n-butanol taken from the top layer of the n-butanol bottle. A distinct surface will be apparent beneath the n-butanol.
11. This gel will take approximately 30 minutes to set.
12. Mix stacking gel as described for the running gel ensuring TEMED and ammonium persulfate are not added until ready.
13. When running gel has formed, tip off the n-butanol, and wash gel surface with 3x distilled water.
14. Remove comb from ethanol in which it was stored and dry thoroughly. Insert into casting mold.
15. Add TEMED etc to stacking gel and quickly syringe onto the surface of the mold. Ensure no bubbles around comb.
16. Let stacking gel form, then remove comb carefully with slow even force.
17. Flush gel and bottom of wells with running buffer to remove any unpolymerised gel out of wells.
18. Wells may need to be straightened following washing. This is also a good time to mark where the first well is to improve visibility.
19. Dissolve samples (25 μ l) in sample buffer (25 μ l) and boil 90-100°C for 12-15 minutes. Spin in microfuge for 1-2 minutes.

20. Apply samples (10 μ l) using a pipette-ensure that samples do not contaminate other wells. When pipetting into well keep pipette tip straight to ensure wells are not shifted.
21. Place gel in electrophoresis apparatus and apply 30 mA/2 gels. After tracking dye has reached the bottom.
22. Run gel until tracking gel has reached the bottom
23. Turn off current and remove gel from the apparatus.
24. Carefully remove the spacers from between plates and remove plates. Wash gel from plate surface using distilled water wash bottle. Try to minimize gel breakage.
25. Stain overnight in Roeder's stain. De-stain until background clear.
26. TO DRY GEL: In a shallow dish add gel shrinking solution for approximately 15 minutes.
27. Take cellophane membrane and moisten in distilled water to soften.
28. Using a frame and plate place cellophane membrane then gel then the additional layer of cellophane membrane on top.
29. Take second frame and clamp to form sandwich
30. Leave set up to dry over a period of 2-3 days.

Reagents required:

4x Lower Gel Stock Solution (1.5 M Tris/HCl + 0.4 % SDS)

Tris/HCl	181.7 g
SDS	4.0 g

Bring to 900 ml with distilled water, adjust pH to 8.8 and make final volume of 1000 ml with distilled water. Filter through 0.45 μ m and store refrigerated.

4x Upper Gel Stock Solution (0.5 M Tris/HCl + 0.4 % SDS)

Tris/HCl	60.6 g
SDS	4.0 g

Bring to 900 ml with distilled water, adjust pH to 6.8 and make final volume of 1000 ml with distilled water. Filter through 0.45 μ m and store refrigerated.

Acrylamide Stock Solution (30% + 0.8% Bis)

Acrylamide	90 g
N, N'-methylene bis acrylamide	2.4 g

Bring to 300 ml with distilled water, filter through 0.45 μ m and store refrigerated in a foil covered bottle.

10 % Ammonium Persulphate (APS)

Ammonium persulphate	0.1g
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Add 1 ml dist.water.

Seperating Gel (for 2 gels) (15 % acrylamide)

4x Lower gel stock	5 ml
Acrylamide stock	6.66 ml
Water	8.35 ml
APS	50 µl
TEMED	5 µl

Stacking Gel (for 2 gels) (4.06 % acrylamide)

4x Upper gel stock	1.4 ml
Acrylamide stock	0.75 ml
Water	3.45 ml
APS	30 µl
TEMED	10 µl

2x Non-Reducing Sample buffer

4x Upper gel stock	5 ml
Glycerol	13 ml
SDS	0.9 g
Nonident P-40 detergent (NP-40)	225 µl
Bromophenol blue (small amount to color samples)	
Bring to 15 ml with dist.water.	

10x Running Buffer

Tris	45.5 g
Glycine	216 g
SDS	15 g
Make up to 1.5 L with water and store at room temperature	

Roeder's stain

Coomassie R-250	125 mg
Isopropanol	125 ml
Acetic Acid	50 ml
Combine these ingredients and make final volume to 500 ml with distilled water.	

Destaining Solution

Acetic acid	175 ml
Methanol	125 ml
Make up to 2500 ml with water.	

Peroxidase activity

Method

- Label tubes 'std' and 'sample'
- Add 250 µl TMB-substrate to both tubes
- Add 10 µl sample to 'sample' tube and horseradish peroxidase (STD) to 'std' tube.
- Compare the development of blue colour after substrate addition (15, 30, 60 and 120 min).

Reagent required

TMB Stock

3,3',5,5' tetramethylbenzidine	100 mg
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Dissolve in 1 ml methanol.

TMB-Substrate

- Add 10 µl of TMB stock into 1 ml of 0.1 M Citric acid
- Add this to 9 ml 0.1 M Sodium acetate, pH 6
- Add 10 µl of 30 H₂O₂ just before use.

Miscellaneous Buffers

Binding Buffer pH 9.6

Na ₂ CO ₃ (alkaline)	1.59 g
NaHCO ₃ (acid)	2.93 g
NaN ₃ (Sodium azide)	2 ml of 2 % solution
Distilled water to	1000 ml
Adjust pH by adding Na ₂ CO ₃ or NaHCO ₃ (in solution) and store at 4°C	

Tris Wash Buffer

1 M Tris (pH-8.0)	100 ml
NaCl	90 g
Distilled water to	10 L