Introduction

Diabetes and its chronic complications lead to extensive quality of life and economic burdens that are shared across the world (Wild et al., 2004; Ettaro et al., 2004). It is indicative of inadequate or resistance of insulin for glucose metabolism. Insulin signalling regulates a large number of cellular processes (Shepherd et al., 1998). Sustained periods of hyperglycaemia are considered a contributing factor in the development of diabetic complications, including retinopathy, nephropathy and neuropathy. Diabetic patients are also at increased risk for developing central nervous system (CNS) dysfunction (Biessels et al., 1999; Ryan et al., 2003; Allen et al., 2004), including impaired central motor conduction (Tchen et al., 1992; Abbruzzese et al., 1993) and, on rare occasions, hemichorea-hemiballismus associated with nonketotic hyperglycaemia (Lee et al., 2002). Diabetes associated complications are major reason for morbidity and mortality associated with this disease and is a major factor causing ramifications in life of diabetic patients. Even though insulin secretion is mainly regulated by changes in circulating concentrations of glucose and other metabolic fuels, stimuli such as neurotransmitters and gastrointestinal hormones make an important contribution to the overall regulation of pancreatic β cell function.

The CNS neurotransmitters play an important role in the regulation of glucose homeostasis. These neurotransmitters mediate rapid intracellular communications not only within the CNS but also in the peripheral tissues. They exert their function through receptors present in both neuronal and non-neuronal cell surface that trigger second messenger signaling pathways (Julius et al., 1989). Neurotransmitters have been reported to show significant alterations during hyperglycaemia resulting in altered functions causing neuronal degeneration (Bhardwaj et al., 1999). Chronic hyperglycaemia during diabetes mellitus is a major initiator of diabetic micro-vascular complications like retinopathy, neuropathy and nephropathy (Sheetz & King, 2002;
Monnier et al., 2009). The autonomic nervous system plays a prominent role in the regulation of insulin secretion. It has been proposed that neuronal afferent signals delivered to the pancreatic β cell through the vagus are responsible for the cephalic phase of insulin secretion. In pancreatic β-cells, IP₃ mobilizes Ca²⁺ from intracellular stores, resulting in an elevation of the intracellular concentration of Ca²⁺ and allowing activation of Ca²⁺/calmodulin. DAG on the other hand, activates PKC (Nishizuka, 1995; Renstrom et al., 1996). PKC, like Ca²⁺/calmodulin, accelerates exocytosis of insulin granules (Nakano et al., 2002). Chronic hyperglycaemia is strongly implicated in the development of vascular complications of diabetes, including gradual damage to the CNS (Brands et al., 2004).

Approaches to the control and prevention of hyperglycaemia are central to the management of diabetes (Herman & Crofford, 1997). The development of new dietary adjuncts and novel anti-diabetic agents, which reinstate a normal metabolic environment, thereby reducing the long term complications associated with diabetes. Such agents would both ideally stimulate the secretion and improve the action of insulin (Bailey & Flatt, 1995; Bashan et al., 2009). It has been widely accepted that peripherally synthesized insulin can be transported into the brain via the cerebrospinal fluid. Recent molecular biological evidence suggests that it can also be synthesized de novo by neurons, because the presence of preproinsulin I and II mRNA or insulin receptor mRNA was observed in cultured neurons. Moreover, insulin immunoreactivity occurs in the endoplasmic reticulum and Golgi apparatus in vivo (Schechter et al., 1998; Zhao et al., 1999).

In the CNS, insulin seems to play an important role, particularly in the complications caused by diabetes, involving the regulation of brain metabolism (Shah et al., 1993; Santos et al., 1999), neuronal growth and differentiation (Schechter et al., 1998; Gerozissis et al., 2003), or neuromodulation (Gerozissis et al., 2003; Rhoads et al., 1984). Insulin also protects against brain damage, induced by stress conditions,
such as oxidative stress or ischemia (Santos et al., 1999; Duarte et al., 2003). Brain glucose utilization and metabolism are essential to cognitive functions, and a disturbance in both or in the desensitization of brain insulin receptors are involved in the intellectual decline in Alzheimer’s disease and related neurodegenerative disorders (Biessels et al., 2002; Hoyer, 2002), in which excitotoxicity and oxidative stress have been shown to occur (Bohr, 2002).

Traditional medicinal plants treatment for diabetes exists and therein lays a hidden wealth of potentially useful natural products for diabetes control (Bailey & Day, 1989; Gray & Flatt, 1997; Swanston-Flatt et al., 1991). Despite this, few anti-diabetic medicinal plants have received scientific or medical scrutiny and the World Health Organization (1980) recommended that this area warrants further attention. Medicinal plants provide a potential source of anti-hyperglycaemic drugs because many plants and plant derived compounds have been used in the treatment of diabetes. There are probably several contributing factors, including changes in the epidemiology of diabetes that attributes to its control. Gray & Flatt (1987) gathered scientific validation for the use of certain traditional anti-diabetic plants and this has encouraged botanical exploration in the quest for new anti-diabetic drugs. Additionally there is the wider appeal of ‘natural’ dietary adjuncts as functional foods through which patients can gain added benefits to the management of their disease (Swanston-Flatt et al. 1991). Many traditional anti-diabetic plants probably act at least in part through their fibre, vitamin or mineral content. Mineral deficiencies are common in diabetes and can exacerbate insulin resistance. Several of these minerals are co-factors for signalling intermediaries of insulin action and key enzymes of glucose metabolism. Several medicinal plants have anti-hyperglycaemic agents in the Indian system of medicines, including Ayurveda. Many Indian plants have been investigated for their beneficial use in different types of diabetes and reports occur in numerous scientific journals (Mukherjee et al., 2006).
Aegle marmelose Corr. (Rutaceae) commonly called as ‘Koovalam’ in Malayalam and ‘Bael’ in Hindi is indigenous to India. Preliminary reports indicate Aegle marmelose leaf extract exhibits anti-diabetic action in glucose-induced hyperglycaemic rats (Sachdewa et al., 2001) and in alloxan induced diabetic rats (Ponnachan et al., 1993). Aegle marmelose extract, which is being used in the traditional medicine to reduce the serum glucose level, has significant antioxidant activity in vitro (Sabu & Kuttan, 2000). Diabetes has been shown to damage islet cells of pancreas by the liberation of oxygen radicals (Halliwell & Gutteridge, 1985). Aegle marmelose leaf extract is found to reduce blood sugar levels and markers of oxidative stress i.e. lipid peroxidation, conjugated diene and hydroperoxide levels in serum and catalase, glutathione and superoxide dismutase in blood and liver of rats (Maxwell et al., 1997; Wen-Chi et al., 2009). Natural antioxidants strengthen the endogenous antioxidant defences from reactive oxygen species (ROS) and restore the optimal balance by neutralizing the reactive species. They are gaining immense importance by virtue of their critical role in disease prevention. In this context, A. marmelose is rightly mentioned as a medicinal plant of varied properties (Halliwell & Gutteridge, 1985).

Pyridoxine (Vitamin B₆) a water-soluble vitamin that exists in three major chemical forms: pyridoxine, pyridoxal and pyridoxamine (Leklem, 1999; Bender, 1989). It performs a wide variety of functions in your body and is essential for good health. Vitamin B₆ is needed for more than 100 enzymes involved in protein and red blood cell metabolism. Body needs vitamin B₆ to make hemoglobin which carries oxygen from red blood cells to tissues (Bender, 1994). Vitamin B₆ also helps increase the amount of oxygen carried by hemoglobin. Vitamin B₆ deficiency can result in iron deficiency anaemia (Allen & Kollas, 1997). Through its involvement in protein metabolism and cellular growth, is important to the immune system. It helps maintain
the health of lymphoid organs (thymus, spleen and lymph nodes) that make your white blood cells (Gerster, 1996). Animal studies show that a vitamin B6 deficiency can decrease your antibody production and suppress immune response (Chandra & Sudhakaran, 1990). Vitamin B6 helps maintain your blood glucose within a normal range. Beaton & Goodwin (1954) found that in vitamin B6 deficient rats there was a significant decrease in fasting blood sugar, pyruvic acid, lactic acid and glycogen levels. Improper diet leads to deficient vitamin B6 to help convert stored carbohydrate or other nutrients to glucose to maintain normal blood glucose levels (Huang & Wang, 1999).

Pyridoxine has been implicated for many years in the metabolism of proteins, amino acids and fat. Pyridoxine is used for relieving headaches and depression associated with low dose oral contraceptives (Villegas et al., 1997). The nervous and immune systems need vitamin B6 to function efficiently and it is also needed for the conversion of tryptophan (an amino acid) to niacin (a vitamin). Beaton et al., (1956) found that pyridoxine deficient rats given daily injections of insulin were able to maintain an amount of body fat similar to their respective controls. Huber et al., (1902) reported that insulin-like activity in the serum and pancreas of pyridoxine deficient rats was significantly lowered, compared to control rats. Pyridoxine functions in decarboxylation system as a coenzyme of amino acid decarboxylase. Snell (1958) reviewed and indicated that all amino acid decarboxylases in animal tissues and in bacteria were pyridoxal phosphate dependent. Neurotransmitters are the products of metabolic process. Pyridoxal phosphate (PLP), the major co-enzyme form of pyridoxine, inhibits GDH through Schiff’s base formation with an amino group of a lysine residue (Anderson et al., 1996; Cho et al., 1996). The ratio of pyridoxal phosphate to pyridoxal in the plasma was decreased by streptozotocin (STZ) injection (Okada et al., 1997). Pyridoxine deficient rat is showed a significant increase in glutamate concentration (Nayeemunnisa et al., 1977).
Serotonergic control is suggested to exert different effects on insulin secretion according to the activation of different receptors subclasses (Pontiroli et al., 1975, Schilman et al., 2010). Our previous studies reported increased monoamine content in the plasma and platelet of diabetic patients (Jackson et al., 1997). In addition to this mechanism, the secretion of insulin is dependent on the turnover ratio of endogenous 5-hydroxy tryptophan (5-HTP) to 5-HT in the pancreatic islets (Jance et al., 1980). CNS is the pathway triggered by decrease in the brain 5-HT content brought about by a decrease in transport of tryptophan across the blood-brain-barrier (BBB). This transport of tryptophan across the BBB depends on the circulating tryptophan and decreased uptake of it into the brain leading to decreased brain 5-HT synthesis (Fernstorm & Fernstorm, 1995; Fernstorm, 1991; Fernstorm, 1979; Biggio et al., 1974). This decreased brain 5-HT stimulates the over expression of 5-HT2A receptors in the brain regions, which lead to sympathetic stimulation that inhibits insulin release (Jackson et al., 2004). Once the circulating insulin content is reduced, it leads to an increase in large neutral amino acids, which compete with tryptophan for uptake into brain. Diabetes is a peculiar case because it is influenced by glutamate receptors present outside of the central nervous system and it also influences glutamate receptors in the central nervous system. Diabetes an endocrine disorder, induces cognitive impairment and defects of long-term potential in the hippocampus, interfering with synaptic plasticity. Defects of long-term potential in the hippocampus are due to abnormal glutamate receptors, specifically the malfunctioning NMDA glutamate receptors during early stages of the disease (Trudeau et al., 2004).

Research is being done to address the possibility of using hyperglycaemia and insulin treatment to regulate these receptors and restore cognitive functions. Pancreatic islets regulating insulin and glucagon levels also express glutamate receptors (Weaver et al., 1996). Metabotropic glutamate receptors (mGluRs)
indirectly activate ion-channels on the plasma membrane through a signaling cascade that involves G proteins. Ionotropic receptors tend to be quicker in relaying information but metabotropic are associated with a more prolonged stimulus. The increase in GDH activity in the diabetic group may be the cause for the increase in glutamate content (Nayeemunisa et al., 1977). Treatment using pyridoxine and insulin reversed the enzyme activity to control level (Aswathy et al., 1998). Glutamate binding to the extracellular region of an mGluR causes G proteins bound to the intracellular region to be phosphorylated, affecting multiple biochemical pathways and ion channels in the cell (Platt, 2007). Because of this, mGluRs can both increase or decrease the exitability of the post synaptic cell, thereby causing a wide range of physiological effects. Over stimulation of glutamate receptors causes neuronal degradation and death through a process called excitotoxicity.

Excessive glutamate, or excitotoxins acting on the same glutamate receptors, overactivates glutamate receptors, causing high levels of calcium ions (Ca\(^{2+}\)) to influx into the postsynaptic cell (Dubinsky, 1993). High Ca\(^{2+}\) concentrations activate a cascade of cell degradation processes involving proteases, lipases, nitric oxide synthase and a number of enzymes that damage cell structures often to the point of cell death (Manev et al., 1989). Ingestion or exposure to excitotoxins that act on glutamate receptors induce excitotoxicity and cause toxic effects on the CNS (Meldrum, 1993).

Peripheral control of insulin secretion occurs directly within the pancreatic β-cells. During diabetes the amount of 5-HT within the pancreatic islets increases. This excess 5-HT binds and down regulates nuclear receptors and directly alter the transcription of insulin gene from the β-cells. The present work is to understand the alterations of serotonin, subtype 5-HT\(_{2A}\) receptors in brain regions and pancreatic islets of streptozotocin induced diabetic rats. Pharmacologically, Ayurveda, Aegle marmelose has a combination of constituents that are beneficial in the management of
diabetic stress. Glucose consumption in the brain is required to meet the energy demands of brain cells for metabolic and physiologic processes (Sokoloff et al., 1977). Due to pyridoxine deficiency there is an impairment leading to brain cognitive functional activities in diabetes (Wei et al., 1999). The work focuses on the evaluation of the anti-hyperglycaemic activity of pyridoxine alone and in combination with insulin and Aegle marmelose leaf extract and the changes in the Serotonin, subtype 5-HT$_{2A}$, glutamate receptors kinetics, gene expression and immunohistochemical studies during diabetes and regulation of insulin secretion. This will help to elucidate the role of serotonin, subtype 5-HT$_{2A}$ and glutamate receptors in diabetes and the regulatory activity of this treatment on insulin secretion.
OBJECTIVES OF THE PRESENT STUDY

1. To study the anti-hyperglycaemic activity of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extracts in streptozotocin induced diabetic animal model.

2. To measure the circulating insulin level, T3 concentration of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats.

3. To quantify serotonin in the brain regions - cerebral cortex (CC), brain stem (BS), cerebellum (CB) and Hippocampus (Hippo) of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats.

4. To study the serotonin, 5-HT$_{2A}$ subtype receptor binding parameters in brain regions and pancreas of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats.
5. To quantify glutamate content in the brain regions – brain regions and pancreas of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats.

6. To study the Glutamate receptor binding parameters in brain regions and pancreas of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats.

7. To study the expression of 5-HT$_{2A}$, 5-HT transporter, mGluR5 glutamate receptor subtype and GLAST glutamate transporter gene expression in the brain regions and pancreas of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats using Real Time PCR.

8. To study the expression of insulin receptors and status of antioxidants-superoxide dismutase and glutathione peroxidase gene expression in the brain regions and pancreas of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats using Real Time PCR.
9. To study the localization of 5-HT$_{2A}$, 5-HT transporter, mGluR5 glutamate receptor subtypes using confocal microscope by immunofluorescent receptor specific antibodies in the brain slices of CC, BS, CB, Hippo and pancreatic islets of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats.

10. To study the intracellular calcium release in pancreatic islets of pyridoxine alone and in combination with insulin and *Aegle marmelose* leaf extract treated streptozotocin induced diabetic rats.

11. To study the behavioural changes in Control and Experimental rats using Rotarod test, Elevated Plus-Maze and Beam walk test.