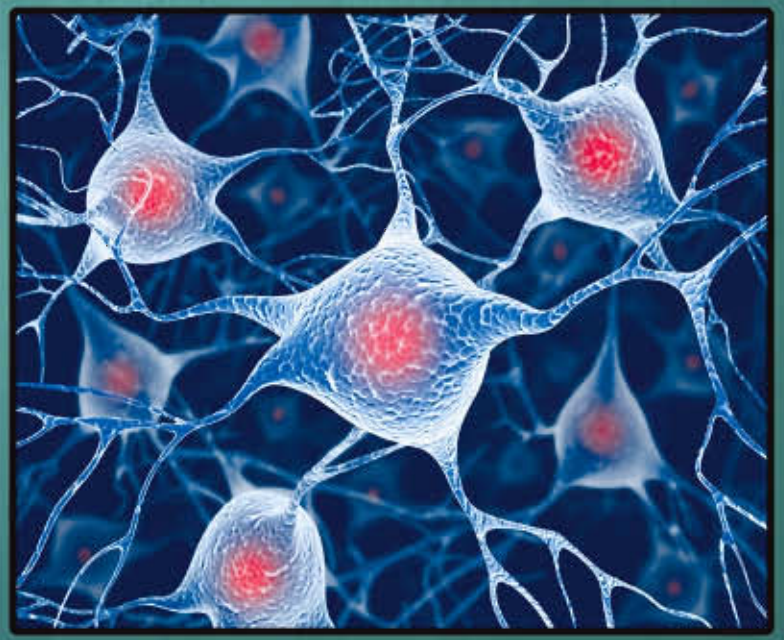
ANITHA M

Ph.D. THESIS

JULY 2011

Neuronal degeneration in streptozotocin induced diabetic rats: Effect of Aegle marmelose and pyridoxine in pancreatic β cell proliferation and neuronal survival

Ph.D. Thesis



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COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

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BY

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CERTIFICATE

This is to certify that the thesis entitled "***Neuronal degeneration in streptozotocin induced diabetic rats: Effect of Aegle marmelose and pyridoxine in pancreatic β cell proliferation and neuronal survival***" is a bonafide record of the research work carried out by **Mrs. Anitha M** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

Cochin – 682 022

July 11, 2012

(C. S. Paulose)

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DECLARATION

I hereby declare that the thesis entitled "***Neuronal degeneration in streptozotocin induced diabetic rats: Effect of Aegle marmelose and pyridoxine in pancreatic β cell proliferation and neuronal survival***" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Prof. C. S. Paulose, Director, Centre for Neuroscience, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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Anitha Malat

Dedicated to my family. .

ABBREVIATIONS

5-HTP	5-hydroxytryptophan
BBB	blood-brain barrier
BDNF	Brain derived neurotrophic factor
bHLH	Basic helix–loop–helix
BrdU	Bromodeoxyuridine
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
CARD	Caspase-recruiting domain
Caspases	Cysteine-dependent aspartate-specific proteases
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CREB	Cyclic AMP binding protein
DAG	Diacylglycerol
DARPP-32	dopamine- and cAMP-regulated phosphoprotein
DED	Death-effector domain
EDTA	Ethylene diamine tetra acetic acid
ER	endoplasmic reticulum
FADD	Fas-associated death domain protein
FKHRL1	Forkhead transcription factor like 1
GABA	γ-Aminobutyric acid
GDNF	Glial cell derived neurotrophic factor
GLUT	Glucose transporter

GPx	Glutathione peroxidase
IGF-I	Insulin like groth factor-1
IP_3	Inositol trisphosphate
IP3R	Inositol trisphosphate receptor
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	NH_2-terminal Jun kinases
L-DOPA	L-dihydroxy-phenylalanine
LTD	Long-term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MSN	Medim spiny neuron
NE	Nor epinephrine
NeuroD1	Neurogenic differentiation 1
NFκB	Nuclear factor-κB
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
OGTT	Oral glucose tolerance test
PDK1	Phosphoinositide-dependent protein kinase-1
PDX-1	Pancreatic duodenal homeobox-1
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate

PKA	Cyclic AMP protein kinase A
PNS	Peripheral nervous system
ROS	Reactive oxygen species
SN*pc*	Substantia nigra *pars compacta*
SN*pr*	Substantia nigra *pars reticulate*
SNr	Substantia nigra pars reticulate
SOD	Superoxide dismutase
STN	Sub thalamic nucleus
STZ	Streptozotocin
TH	Tyrosine hydroxylase
TNFα	Tumor necrosis factor- α
Trk	Tropomyosin-related kinase
VTA	Ventral tegmental area

CONTENTS

CORPUS STRIATUM

LIVER

Introduction

Diabetes is a severe health problem and its incidence is increasing at alarming proportions. Recent estimates indicate that around 200 million people suffer from diabetes mellitus, making it the most common serious metabolic disorder worldwide. Diabetes is a major cause of mortality. Type 2 diabetes results from the interaction between a genetic predisposition, behavioural and environmental risk factors (Neel, 1962). Although the genetic basis of type 2 diabetes has yet to be identified, there is strong evidence that modifiable risk factors such as obesity and physical inactivity are the main nongenetic determinants of the disease (Manson *et al*., 1991; Hamman, 1992). Altered dietary pattern also participates in the increased incidence of diabetes.

Diabetes is characterized by hyperglycemia with slowly progressive end-organ damage in the eyes, kidneys, blood vessels, heart, peripheral nerves and brain (Gispen & Biessels, 2000; Northam *et al*., 2006). In poorly controlled diabetic animals or humans increased cell death occurs in different tissues and organs and it is involved in secondary complications of diabetes (Barber *et al*., 1998; Cai *et al.,* 2002; Li *et al.,* 2002; Pesce *et al.,* 2002; Arroba *et al*., 2003; García-Cáceres *et al.,* 2008). Chronic hyperglycemia during diabetes mellitus is a major initiator of diabetic micro-vascular complications like retinopathy, neuropathy and nephropathy (Sheetz & King, 2002).

Pancreatic islets of Langerhans, which play a crucial role in glucose homeostasis, are mainly formed by two types of exocytotic cells (α, β). Insulin and glucagon are key hormones regulating glucose homeostasis. Insulin is secreted by pancreatic β-cells when plasma glucose concentrations are elevated, leading to the insulin-dependent glucose uptake by peripheral tissues. α-cells represent 15 to 20% of islet cells and secrete glucagon in response to decreased blood glucose levels and stimulate glucose production by the liver. Impaired glucagon secretion has been associated to hyperglycaemic periods in diabetic patients. Both cell types respond oppositely to changes in blood glucose concentration: while hypoglycaemic

conditions induce α-cell secretion, β-cells release insulin when glucose levels increase (Nadal *et al*., 1999; Quesada *et al*., 2006). Insulin acts mainly on muscle, liver and adipose tissue with an anabolic effect, inducing the incorporation of glucose into these tissues and its accumulation as glycogen and fat. By contrast, glucagon induces a catabolic effect, mainly by activating liver glycogenolysis and gluconeogenesis, which results in the release of glucose to the bloodstream. Defective insulin secretion and insulin resistance is an obligatory step in the development of type 2 diabetes, is the result of decrease in β cell mass and β cell malfunction. The pathogenesis of type 2 diabetes involves a combination of factors resulting destruction of β cells that reduces insulin secretion, hindering the body's ability to maintain normoglycemia.

Hyperglycemia is a connector between diabetes with diabetic complications (Brownlee, 2001; Rolo & Palmeira, 2006). Oxidative stress plays an important role in the complications of diabetes. The central nervous system (CNS) which is both functionally and structurally connected to the peripheral nervous system (PNS) has been largely ignored in diabetic condition. In recent years it has become evident that diabetes causes significant CNS complications, resulting in important functional impairments (Mijnhout *et al*., 2006). The CNS is affected by both metabolic and vascular consequences of diabetes. Emerging evidence suggests impairment of neuronal function or loss of neurons in diabetes. Epidemiologic studies have reported that diabetes is an independent risk factor for cognitive impairment (Stewart & Liolitsa, 1999; Allen *et al*., 2004). These impairments are characterized by moderate deficits in learning and memory, psychomotor slowing and reduced mental flexibility. Furthermore, diabetic patients have increased probability of developing Alzheimer's disease and other dementias (Arvanitakis *et al*., 2004; Biessels *et al*., 2006).

Hyperglycemia results in enhanced formation of oxygen free radicals in tissues (Baydas *et al*., 2002). Oxidative stress seems to play a central role in neuronal damage (Bonnefont-Rousselot, 2002). Diabetic patients display increased retinal apoptosis compared to non-diabetic patients (Barber *et al*., 1998). The increased

oxidative stress in diabetes produces oxidative damage in many regions of brain. Brain injury appears to result from a number of processes. Many cytokines have been suggested to participate in neurodegeneration and neurotoxicity. Tumor necrosis factor (TNF) is a major mediator of apoptosis and inflammation (Chen & Goeddel, 2003; Wajant *et al*., 2003). Increased expression of tumor necrosis factor-α have been observed before neuronal death (Little & O'Callagha, 2001). NFκB (nuclear transcription factor kappa B) regulates expression of a large number of genes involved in the regulation of apoptosis. NFκB is activated in response to certain stimuli and its activation lead to the induction of apoptosis (Kuhnel *et al*., 2000). The final pathway that leads to execution of the death signal is the activation of a series of proteases termed caspases. The caspases that have been well described are caspases- 3, 6, 7, 8, and 9.

CNS neurones are supported by several neurotrophic and transcription factors. Brain-derived neurotrophic factor (BDNF) is a potent trophic factor supports striatal cells and promotes survival and/or differentiation of GABAergic neurons *in vitro* (Mizuno *et al*., 1994; Ventimiglia *et al*., 1995). The cAMP response element-binding protein (CREB) is a transcription factor, play a pivotal role in neuroplasticity. It binds to certain DNA sequences called cAMP response elements (CRE) and thereby, increases or decreases the transcription of the downstream genes (Spaulding, 1993). Protein kinase B(PKB) is another important regulator involved in neuroprotective effect against ischemic brain damage in genetically modified mice expressing an active form of PKB (known as Akt) in neuronal cells (Ohba *et al*., 2004). Many growth factors (e.g. insulin like growth factor (IGF), BDNF, nerve growth factor (NGF) promote neuronal survival and protect cells from neurotoxic insults (Kaplan & Miller, 2000; Brunet *et al*., 2001). All these factors regulate different aspects of neuronal and synaptic function.

Diabetes mellitus is defined as inappropriate glucose metabolism leading to impaired removal of glucose from the circulation. While insulin mediates the

clearance of glucose from blood by activating glucose transport into the cytosol, absolute or relative lack of insulin and/or impaired insulin action at its receptor causes delayed or almost absent metabolism of circulating glucose (Ashcroft *et al*., 1994; Kahn, 1994; Butler *et al*., 2003; Ashcroft & Rorsman, 2004). Type 2 diabetes results from insulin resistance of the target tissues (adipose tissue, skeletal muscle and liver) and decreased insulin secretion by the pancreatic β-cells. It is however still unclear, which event is the primary defect in the development of type 2 diabetes (Chang-Chen *et al*., 2008).

Multiple abnormalities have been shown to disturb the delicate balance between islet neogenesis and apoptosis in diabetic condition. Transcription factors provide the genetic instructions that drive pancreatic development and enable mature β cells to function properly. Pancreas duodenum homeobox -1 (PDX-1) and Neurogenic differentiation-1 (NeuroD1) are important transcription factors that activate pancreatic development. PDX-1 is considered to be the master regulator of pancreatic development and β-cell differentiation. PDX-1 expression is required in mature β-cells to maintain hormone production, glucose transporter-2 (GLUT-2) expression and euglycemia (Ahlgren *et al*., 1998). NeuroD1 is a transcription factor expressed in all endocrine cells (Naya *et al*., 1995) and regulates the transcription of the insulin gene. Insulin-secreting pancreatic β cells proliferate in response to increasing demand for insulin and also in response to certain stimuli. This notion raises the possibility of enhancing baseline replication of β cells as a therapeutic approach for the treatment of diabetes.

The various neurotransmitter systems, including, serotonergic, cholinergic, glutamatergic and GABAergic, undergo a significant change in diabetes (Gireesh *et al*., 2008; Antony *et al*., 2010; Anu *et al*., 2010; Kumar *et al*., 2010). Dopamine is a catecholamine neurotransmitter both in the periphery and in the CNS which plays a key role in various physiologic and behavioural homeostatic mechanisms (Kuchel & Kuchel, 1991). Dopamine is involved in various physiological functions such as cell

proliferation, gastrointestinal protection and inhibition of prolactin secretion (Freeman *et al*., 2000; Hoglinger *et al*., 2004). Dopamine action is mediated through 5 distinct G protein-coupled receptors grouped into two families, dopamine D_1-like (D_1, D_5) and dopamine D_2-like (D_2, D_3, D_4) receptors (Kebabian & Calne, 1979; Jackson & Westlind- Danielsson, 1994; Missale *et al*., 1998). Activation of dopamine receptor dopamine D_1 family stimulates adenylyl cyclase and dopamine D_2 family inhibits adenylyl cyclase (Nestler, 1994). Dopamine plays an important role in food intake. Moreover dopaminergic neurotransmission profoundly affects glucose and lipid metabolism (Meier & Cincotta, 1996). Several studies suggest that alterations in dopaminergic neurotransmission are involved in the pathogenesis of type 2 diabetes. Hyperglycemia during diabetes is reported to damage dopaminergic functions, as shown by changes in dopamine metabolism in the human brain and the brains of diabetic animals (Trulson & Himmel, 1983; Lacković *et al*., 1990). In brains of diabetic patients and type 2 diabetic animals, increased dopamine levels are measured (Lackovic *et al*., 1990; Yang & Meguid; 1995; Barber *et al*., 2003). Dopamine in the PNS modulates insulin secretion from the pancreatic islets (Nogueira *et al*., 1990). Its role is implicated in insulin and glucose metabolism.

Antioxidant treatments have been proposed to be prospective in the treatment of diabetes. Antioxidants have shown to decrease oxidative stress and diabetic complications (Rogers & Mohan, 1994). Vitamin B_6 (Pyridoxine) is water-soluble B-complex vitamins found in a variety of animal and vegetable products. Pyridoxine acts as coenzyme in biosynthesis of neurotransmitters GABA, dopamine and serotonin, important for development and function of the CNS (Ernahrungswiss, 1996). Pyridoxine is essential for proper functioning of the mechanisms that control neurotoxicity, edema formation free radical production and mediate cognitive dysfunction (Cabrini *et al*., 1998; Bender, 1999; Culskelly *et al*., 2001; Kelly *et al*., 2003; Friso *et al*., 2004). Studies show antioxidant properties of pyridoxine (Jain & Lim, 2001; Stocker *et al*., 2003). Pyridoxine show anti-hyperglycemic activity in

diabetic animals (Nair *et al*., 1998). Clearly, an effective neuroprotective strategy would be valuable for diabetic treatment to minimize neurological complications due to oxidative stress. Medicinal plants provide a potential source of anti-hyperglycaemic compounds and have been used in the treatment of diabetes. *Aegle marmelose* Corr. (Rutaceae) commonly called as 'Koovalam' in Malayalam and 'Bael' in Hindi is indigenous to India. *Aegle marmelose* extract, which is being used in the traditional medicine for the treatment of diabetes. The plant is rich in alkaloids, among which aegline, marmesin, marmin and marmelose are the major ones. *Aegle marmelose* modulates the expression of various molecular targets, such as transcription factors, enzymes, cytokines, cell cycle proteins, receptors and adhesion molecules (Narendhirakannan *et al.,* 2010). *Aegle marmelose* fruit extract exhibit antihyperlipidemic effect in streptozotocin (STZ) induced diabetic rats. *Aegle marmelose* leaf extract reduce oxidative stress by significant antioxidant activity in alloxan induced diabetes (Kamalakkannan et al., 2005; Sabu & Kuttan, 2000).

Diabetes mellitus is associated with various neurological complications. The effect of hyperglycemia on neuronal plasticity and expression of neuronal survival factors has not received much attention. The present study was designed to investigate the neuroprotective role of pyridoxine and *Aegle marmelose* on neuronal viability through the expression of neuronal survival factors- BDNF, Akt-1, CREB and factors regulating apoptosis- TNF-α, NFκB, caspase-8 in the brain regions of streptozotocin induced diabetic rats. Pancreatic regeneration is studied through Bromodeoxyuridine (BrdU) administration experiments and expression of dopamine receptors, insulin receptor, IGF-1 and GLUTs. An understanding of the critical factors and mechanisms involved in the proliferation and maintenance of large β cell mass may be of great value for developing more effective therapies to improve metabolic control in diabetes. Anti-oxidative agents that stimulate β cell proliferation and differentiation will certainly enlighten novel therapeutic possibilities for diabetes treatment.

OBJECTIVES OF THE PRESENT STUDY

1. To study the mechanism of action of pyridoxine and *Aegle marmelose* in glucose regulation of diabetic rats.

2. To determine the role of dopaminergic system in insulin secretion and pancreatic beta cell function.

3. To understand the role of dopaminergic system in the regulation of metabolic factors- insulin receptor, GLUT-2, GLUT-3, IGF-1 expressions in experimental animals.

4. To study neuronal vulnerability through Flouro jade –C staining in striatum experimental animals.

5. To study the neuroprotective role of pyridoxine and *Aegle marmelose* through the gene expression profile of apoptotic factors and neuronal survival factors in brain regions of STZ induced diabetic rats.

6. To study dopamine signaling through the expression of transcription factors and second messengers- cAMP, cGMP, IP_3 in STZ induced diabetic rats.

7. To study the expression of pancreatic regeneration markers- PDX-1, NeuroD1 in experimental group animals.

8. To study the role of pyridoxine and *Aegle marmelose* in pancreatic beta cell proliferation through BrdU administration experiments and immuno-histochemical analysis of BrdU in STZ induced diabetic rats.

Literature Review

Diabetes mellitus is one of the most common metabolic diseases. It is a chronic disease characterized by relative or absolute deficiency of insulin, resulting in glucose intolerance. Diabetes mellitus is a major global health problem that affects more than 185 million people around the world (Zimmet *et al*., 2001). It is characterized by disturbances in carbohydrate, lipid and protein metabolism and insulin resistance concomitantly with deficits of β cell mass and insulin secretion relative to metabolic demands. Type 2 diabetes is often associated with obesity and hypertension. The concomitant occurrence of these disorders is commonly referred to as 'metabolic syndrome'.

There are two main forms of diabetes (Zimmet *et al*., 2001). Type 1 diabetes is due primarily to autoimmune mediated destruction of pancreatic β-cells, resulting in dramatic insulin deficiency. Its frequency (~10%) is low relative to type 2 diabetes, which accounts for over 90% of cases. Type 2 diabetes is characterized by abnormal insulin secretion, associated with varying degrees of insulin resistance. The diabetes epidemic relates particularly to type 2 diabetes and is taking place both in developed and developing nations (Zimmet *et al*., 2001)

Pancreas

The pancreas is a mixed gland with a large exocrine and a much smaller endocrine gland. The endocrine cells are arranged into small islands of cells called the islets of Langerhans. Islets are generally oval in shape and their core consists primarily of β cells, while non-β- cells are located mostly in the surrounding mantle. They represent approximately 2% of the total pancreatic mass. The interactive function of both the exocrine and the endocrine parts are particularly important for the normal functioning of the body. The pancreas serves two major functions: (i) the production of digestive enzymes which are secreted by exocrine acinar cells and

routed to the intestine by a branched ductal network and (ii) the regulation of blood sugar which is achieved by endocrine cells of the islets of Langerhans. Like all endocrine glands, islets secrete their hormones into the bloodstream and are highly vascularised. Because of its obvious medical importance, the pancreas has been subject to decades of close study. The pancreatic islets of Langerhans are made by the aggregation of 4 major secretory cell types. Each islet is composed of 2000-4000 cells, ~60%-80% of which secrete insulin and are referred to as β cells. Glucagon secreting α cells account for ~15-20% of the islet cells, while somatostatin-secreting delta-cells and pancreatic polypeptide-secreting cells (PP) account ~ 5-10% and < 2% of the total islet cell number respectively.

Glucagon

Glucagon is the hormone secreted by pancreatic α-cells. Glucagon also plays a central role in the regulation of glucose homeostasis (Baron *et al.,* 1987; Cherrington, 1990). Glucagon stimulates glycogenolysis in the liver to increase blood glucose levels. Under postabsorptive conditions, approximately half of total hepatic glucose output is dependent on the maintenance of normal basal glucagon levels and inhibition of basal glucagon secretion with somatostatin causes a reduction in hepatic glucose production and plasma glucose concentration. After a glucose-containing meal, glucagon secretion is inhibited by hyperinsulinemia and the resultant hypoglucagonemia contributes to the suppression of hepatic glucose production and maintenance of normal postprandial glucose tolerance. It has been shown that glucagon has a striking stimulatory effect on insulin release in the absence of glucose (Sevi, 1966). Glucagon stimulates glycogenolysis in the liver to increase blood glucose levels. The presence of specific glucagon receptors on isolated rat pancreatic β -cells as well as a subpopulation of α- and δ-cells shows the relevance of glucagon on regulation of insulin secretion. Intra-islet glucagon appears to be a paracrine regulator of cAMP *in vitro* (Schuit, 1996). Glucagon stimulates insulin release by

elevating cAMP. cAMP through activation of protein kinase A, increases Ca^{2+} influx through voltage dependent L-type Ca^{2+} channels, thereby elevating Ca^{2+} and accelerating exocytosis (Carina, 1993). Protein phosphorylation by Ca^{2+}/Calmodulin and cAMP dependent protein kinase play a positive role in insulin granule movement which results in potentiation of insulin release from the pancreatic β-cell (Hisatomi, 1996).

Insulin

The biological effects of insulin in classical insulin target tissues, such as skeletal muscle, fat and liver are glucose uptake, regulation of cell proliferation, gene expression and the suppression of hepatic glucose production. Insulin decreases hepatic glucose production and increases glucose entry into muscle and fat cells. Following glucose ingestion, the increase in plasma glucose concentration stimulates insulin release and the combination of hyperinsulinemia and hyperglycemia (1) stimulates glucose uptake by splanchnic (liver and gut) and peripheral (primarily muscle) tissues and (2) suppresses endogenous (primarily hepatic) glucose production (DeFronzo, 1997, 1988; DeFronzo *et al.,* 1981; DeFronzo & Ferrannini, 1987; Mari *et al.,* 1994; Mandarino *et al.,* 2001; Mitrakou *et al.,* 1990; Cherrington, 1990). Insulin is a potent antilipolytic hormone and even small increments in the plasma insulin concentration markedly inhibit lipolysis, leading to a decline in the plasma level of free fatty acid (Groop *et al.,* 1989).

In the postabsorptive state the majority of total body glucose disposal takes place in insulin independent tissues. Approximately 50% of all glucose use occurs in the brain, which is insulin-independent (Grill, 1990). Another 25% of glucose disposal occurs in the splanchnic area (liver plus gastrointestinal tissues) which is also insulin-independent. The remaining 25% of glucose use in the postabsorptive state takes place in insulin-dependent tissues, primarily muscle and to a lesser extent adipose tissue. Approximately 85% of endogenous glucose production is derived from the liver and

the remaining 15% is produced by the kidney. Glycogenolysis and gluconeogenesis contribute equally to the basal rate of hepatic glucose production. During glucose ingestion, insulin is secreted into the portal vein where it is taken up by the liver and suppresses hepatic glucose output. If the liver does not perceive this insulin signal and continues to produce glucose, there will be two inputs of glucose into the body, one from the liver and a second from the gastrointestinal tract and marked hyperglycemia will ensue.

The actions of insulin are mediated via the insulin receptor (IR) which belongs to the family of tyrosine kinase receptors. Binding by insulin leads to rapid autophosphorylation of the receptor, followed by tyrosine phosphorylation of IRS proteins, which induce the activation of downstream pathways such as the PI_3K and the mitogen-activated protein kinase (MAPK) cascades. IR is a glycoprotein that consists of two α-subunits and two β-subunits linked by disulfide bonds (DeFronzo, 1988; Pessin & Saltiel, 2000; Whitehead *et al.,* 2000; Saltiel & Kahn, 2001). The two α subunits of the IR are entirely extracellular and contain the insulin-binding domain. The β-subunits have an extracellular domain, a transmembrane domain and an intracellular domain that expresses insulin-stimulated kinase activity directed toward its own tyrosine residues. Phosphorylation of the β-subunit, with subsequent activation of IR tyrosine kinase, represents the first step in the action of insulin on glucose metabolism. Mutagenesis of any of the three major phosphorylation sites (at residues 1158, 1163, and 1162) impairs IR kinase activity, leading to a decrease in the metabolic and growth-promoting effects of insulin (Ellis *et al.,* 1986; Chou *et al.,* 1987). Therefore IR activation and subsequent stimulation of IR second messenger cascades, including the translocation of insulin sensitive GLUTs participate in enhancing properties of insulin. Activation of the insulin signal transduction system in insulin target tissues stimulates glucose transport through a mechanism that involves translocation of a large intracellular pool of glucose transporters (associated with low-

density microsomes) to the plasma membrane and their subsequent activation after insertion into the cell membrane.

Type 2 diabetes is characterized by a progressive decline in pancreatic β cell function and chronic insulin resistance (DeFronzo, 1987; Kudva & Butler, 1997). Pancreatic β cell dysfunction is central to the development of diabetes, possibly due to a combination of decreased β-cell mass and insulin secretion defects. The number of β cells within the pancreas is an important determinant of the amount of insulin that is secreted. Most studies have demonstrated a modest reduction (20%–40%) in β cell mass in patients with long-standing type 2 diabetes (Gepts & Lecompte, 1981; Kloppel *et al.,* 1985; Clark *et al.,* 1988; Butler *et al.,* 2003). A reduction in pancreatic β-cell function and mass leads to hyperglycemia (elevated blood sugar) in both type 1 and type 2 diabetes. In type 1diabetes, autoimmune destruction of the β-cell itself severely reduces β-cell mass, resulting in marked hypoinsulinemia and potentially life threatening ketoacidosis. In contrast, during the progression to type 2 diabetes, impaired β-cell compensation in the setting of insulin resistance (impaired insulin action) eventually leads to β-cell failure and a modest but significant reduction in β-cell mass (Butler *et al.,* 2003; Yoon *et al.,* 2003). Regulation of the β-cell mass appears to involve a balance of β-cell replication and apoptosis as well as development of new islets from exocrine pancreatic ducts (Finegood *et al*., 1995; Bonner-Weir, 2000). Disruption of any of these pathways of β-cell formation or increased rates of β-cell death could cause a decrease in β-cell mass. Diabetes results when insulin production by the pancreatic islet β-cell is unable to meet the metabolic demand of peripheral tissues such as liver, fat and muscle.

The therapy for people with β cell loss is insulin administration. This exogenous insulin is deposited subcutaneously, regardless of whether it is injected or infused and has different kinetics from endogenous insulin released from β cells. Normally, insulin is released from a β cell within a minute of the cell being exposed to

glucose, with equally rapid turn-off once the blood glucose level has returned to normal. This fine-tuning is not possible with subcutaneously administered insulin. As a result blood glucose levels in those with insulin-dependent diabetes often fluctuate widely. Aggressive attempts to prevent hyperglycemia result in an increased incidence of hypoglycemia. Hypoglycaemic episodes have detrimental effects on the brain and in addition, it is associated with impairment in learning and memory (Flood *et al.,* 1990). Being a major problem in clinical practice, hypoglycemia unawareness is associated with an increased risk of coma. By contrast, prolonged hyperglycemia will lead to an increased incidence of microvascular complications such as retinopathy and nephropathy. To avoid the variations in blood glucose levels in those with insulin-dependent diabetes and to reduce the chance of long-term complications, it would be helpful for new β cells to be produced.

Diabetes and central nervous system

Diabetes mellitus is recognized as a group of heterogeneous disorders with the common elements of hyperglycaemia and glucose intolerance due to insulin deficiency, impaired effectiveness of insulin action or both. Diabetes causes a variety of functional and structural disorders in the central and peripheral nervous systems (Biessels *et al.,* 1994). The prevalence of Type 2 diabetes mellitus increases with age and dementia also increases its incidence in later life. Recent studies have revealed that Type 2 diabetes mellitus is a risk factor for cognitive dysfunction or dementia, especially those related to Alzheimer's disease. In addition to these findings, there are electrophysiological and structural abnormalities of the brain in diabetic patients providing good reasons to believe that cognitive functions is impaired in diabetes mellitus (Gispen & Biessels, 2000). Diabetes could conceivably lead to cognitive impairment through chronic hyperglycemia (Stewart & Liolitsa, 1999). Hyperglycemia is known to potentiate neurological damage in stroke and ischemia in both human and animal studies (McCall, 1993). Longitudinal studies almost

universally reveal a higher risk of dementia or significant cognitive decline in diabetic populations (Allen *et al.,* 2004; Arvanitakis *et al.,* 2004). Retrospective analysis suggests that diabetes appears to increase the risk of dementia as much as two fold as does hyperinsulinemia (Luchsinger *et al.,* 2004; Yaffe *et al.,* 2004).

Oxidative Stress and Diabetes

Oxidative stress is one of the proposed mechanisms involved in diabetic complications (Baynes, 1991; Salahudeen *et al*., 1997; Ha & Kim, 1999). Hyperglycemia is a connector between diabetes with diabetic complications (Brownlee, 2001; Rolo & Palmeira, 2006). High glucose produces ROS as a result of glucose auto-oxidation, metabolism and the development of advanced glycosylation end products. Oxidative stress helps the progression and the development of diabetes and its complications (Ha & Lee, 2000). Oxidative damage to various brain regions constitutes into the long term complications, morphological abnormalities and memory impairments (Fukui *et al.,* 2001).

The human body is exposed to exogenous and endogenous free radicals. The cells necessitate oxygen to produce the energy. During mitochondrial respiration, the cells take in oxygen, burn it, release energy and free radicals are produced. Oxidative stress occurs when the antioxidant production is decreased or free radical production exceeds the body's ability to neutralize them. Oxidative stress is defined as a tissue injury induced by increase in reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$). The reactive oxygen intermediates produced in mitochondria, peroxisomes and the cytosol are scavenged by cellular defending systems including enzymatic (eg. superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase and catalase) and nonenzymatic antioxidants (ex. glutathione G-SH, thioredoxin, lipoic acid, ubiquinol, albumin, uric acid, flavonoids, vitamins A, C and E). Antioxidants are located in cell membranes, cytosol and in the blood plasma (Maritim *et al*., 2003).

In vivo studies have revealed that oxidative stress caused by hyperglycemia occurs before the complications of diabetes become clinically evident (Koya & King, 1998; Brownlee, 2001; Rosen *et al.,* 2001). Wolff & Dean, 1987 suggested that nonenzymatic protein glycation, a mechanism proposed early on to account for glucose cytotoxicity (Brownlee & Cerami, 1981) was dependent on ROS (superoxide and hydroxyl) formation through transition metal-catalyzed glucose autoxidation. These pathways include advanced glycation end products and receptors for advanced glycation end products, protein kinase C and the polyol pathway (Brownlee, 1995; Koya & King, 1998, Stevens *et al.,* 2000). More recently, hyperglycemia has been implicated in the activation of additional biochemical pathways, including the stress-activated signaling pathways of nuclear factor-κB (NFκB), NH_2-terminal Jun kinases/stress activated protein kinases (JNK/SAPK), p38 MAP kinase and hexosamine (Marshall *et al*., 1991; Kyriakis &Avruch, 1996; Barnes & Karin, 1997).

Diabetes & Apoptosis

The brain is mainly a glucose-dependent organ, which can be damaged by hyper- as well as by hypoglycemia (Scheen, 2010). The disturbances of neuronal glucose transport and metabolism in hyperglycemia induce neuronal damages and CNS disorders. Synaptic defects undoubtedly contribute to the memory and cognitive defects that accompany neurodegeneration. However, the overwhelming feature of most neurodegenerative disorders is excessive neuronal cell death.

Human diabetic patients display increased retinal apoptosis compared to non-diabetic patients and it is possible that loss of the protective/survival effect of insulin signalling in retinal neurons contributes directly to the high rates of eye defects in diabetic patients (Barber *et al.,* 1998). Insulin protects retinal neurons from toxin and stress induced apoptosis *via* the PI_3K-PKB pathway or the mTOR-p70S6K pathway (Diaz *et al.,* 2000; Wu *et al.,* 2004). These observations show that insulin signalling protects neurons from a variety of insults. Many growth factors (e.g. IGF, BDNF,

NGF) promote neuronal survival and protect cells from neurotoxic insults, including β-amyloid (Kaplan & Miller, 2000; Brunet *et al.,* 2001).

Cell death occurs by necrosis or apoptosis (Wyllie *et al.,* 1980; Martin, 2001; Kanduc *et al.,* 2002). These two mechanisms have distinct histologic and biochemical signatures. In necrosis, the stimulus of death (e.g., ischemia) is itself often the direct cause of the demise of the cell. In apoptosis, by contrast, the stimulus of death activates a cascade of events that orchestrate the destruction of the cell. Unlike necrosis, which is a pathologic process, apoptosis is part of normal development (physiologic apoptosis) however, it also occurs in a variety of diseases (aberrant apoptosis).

Apoptotic cell death also known as programmed cell death, can be a feature of both acute and chronic neurologic diseases (Martin, 1999; Yuan, 2000; Martin, 2001). In apoptosis, a biochemical cascade activates proteases that destroy molecules that are required for cell survival and others that mediate a program of cell suicide. The major executioners in the apoptotic program are proteases known as caspases (cysteine-dependent, aspartate-specific proteases). Caspases directly and indirectly orchestrate the morphologic changes of the cell during apoptosis. Caspases exist as latent precursors, which, when activated, initiate the death program by destroying key components of the cellular infrastructure and activating factors that mediate damage to the cells. Caspases have been categorized into upstream initiators and downstream executioners. Upstream caspases are activated by the cell-death signal, tumor necrosis factor- α (TNFα) and have a long N-terminal prodomain that regulates their activation (Hengartner, 2000; Shi, 2000).These upstream caspases activate downstream caspases, which directly mediate the events leading to the demise of the cell.

Upstream caspases are subclassified into two groups, according to the molecules modulating their activation. Procaspases 1, 2, 4, 5, 9, 11, 12, and 13 have a long N-terminal prodomain called the caspase-recruiting domain (CARD). Caspases 8 and 10 have a long N-terminal prodomain called the death-effector domain (DED). A

regulating molecule is required for specific binding to the CARD/DED domain, which results in caspase activation. These molecules are caspase specific and trigger-specific. For example, after the binding of TNFα to its receptor, the TNF receptor binds to the DED molecule that mediates caspase 8 activation. Once upstream caspases are activated in an amplifying cascade, they activate the executioner caspases downstream (Hengartner, 2000; Shi, 2000; Bouchier-Hayes & Martin, 2002).

TNF- α

TNF-α, the most widely studied cytokine, plays many roles as a signaling and as an effector molecule in both physiology and pathophysiology of the central nervous system (Munoz-Fernandez & Fresno, 1998). TNF-α is released during various inflammatory diseases of the CNS, being synthesized by microglia, astrocytes and some populations of neurons (Lieberman *et al.,* 1989; Chung *et al.,* 2005). TNFα has been considered as a possible master inflammatory regulator that can induce further cytokine production, gliosis, blood-brain-barrier damage, demyelination, inflammation, cell adhesion and immune reactivity. In the CNS microglia are the primary source of TNFα and the release of TNFα by microglia is implicated in neurotoxicity (Gregersen *et al.,* 2000; Badie *et al.,* 2000; Hanisch, 2002; Taylor *et al.,* 2005). Although TNFα has not been demonstrated to cause neuronal death in healthy brain tissue or normal neurons and normal cellular architecture is maintained in mice deficient in pro-inflammatory cytokines or receptors, one can not rule out that a localized activation could initiate neuronal death (Gendelman & Folks, 1999). It has been shown to induce the activation of glial cells and macrophages for the production of a variety of neurotoxins and to initiate the death process in oligodendrocytes and neurons (Viviani *et al.,* 2004). The pleiotropic actions of TNF-α are mediated through two distinct cell surface receptors: 55 kDa TNFR1 (p55, or CD120a) and 75 kDa TNFR2 (also called p75, and CD120b) (Wajant *et al.,* 2003). TNF-α receptors in the brain are expressed by neurons and glia (Kinouchi *et al.,* 1991; Dopp *et al.,* 1997).

TNF-α receptor distribution varies depending upon activation of either apoptosis or inflammatory regulation (Figiel & Dzwonek, 2007; Lambertsen *et al.,* 2007). These differential patterns of localization of TNF-α receptors in neuronal and glial cells, their state of activation and the down-stream effectors, all are thought to play an important role in determining whether TNF-α will exert a beneficial or harmful effect on CNS. In addition, TNF receptors mediate the activation of several transcription factors leading to enhanced gene expression (Herbein & Khan, 2008).

Caspase-8

Caspases have a pivotal role in the progression of a variety of neurologic disorders. Caspase 8 is the apical protease in the extrinsic apoptotic pathway activated at the plasma membrane by various TNF family death receptors (Ashkenazi & Dixit, 1998). Stimulation of these receptors by proapoptotic ligands causes receptor clustering followed by the recruitment of Fas-associated death domain protein (FADD) which in turn binds Death Effector Domains (DEDs) in the proform of caspase 8, a protease that then cleaves and activates downstream effector caspases responsible for apoptosis (Micheau & Tschopp, 2003). In some types of cells, caspase 8 indirectly activates downstream caspases by cleaving the BH3-only protein Bid leading to activation of the Apaf-1/caspase 9 apoptosome (Scaffidi *et al.,* 1998; Zou *et al.,* 1999). In some scenarios caspase 8 activation can also occur downstream of the mitochondrial apoptotic pathway (Stennicke *et al.,* 1998). Moreover an alternative pathway involving caspase 8 activation is associated with endoplasmic reticulum (ER) stress (Ng *et al.,* 1997; Zhang *et al.,* 2000; Gervais *et al.,* 2002; Roth *et al.,* 2003). Thus caspase 8 bridges several death mechanisms including extrinsic and intrinsic apoptosis pathways, ER stress and autophagic pathways (Vandenabeele *et al.,* 2006; Hou *et al.,* 2010). A role for caspase 8 in neuronal cell death has been suggested by evidence of proteolytic processing and increases in caspase 8 protease activity in the

settings of ischemia and seizures (Velier, 1999; Harrison *et al.,* 2001; Henshall, 2001; Li *et al.,* 2006).

Factors involved in neuronal survival

Neuronal viability is maintained through a complex interacting network of signaling pathways that can be perturbed in response to a multitude of cellular stresses. A shift in one or more of these signaling pathways can alter the fate of a neuron resulting in cell death or continued survival. The nature of the stresses affecting neurons, the duration of the stresses, the developmental stage of the neuron and a variety of other factors influence the signaling pathways that are ultimately affected. These diverse parameters also regulate the temporal response as well as the final disposition of the affected neurons.

Insulin-Neuroprotective role

Insulin produced by the pancreas is proposed to cross the blood brain barrier and activate the IR in the brain. Insulin acts as a neuronal cell survival factor during development and in adult mammals (Diaz *et al.,* 2000; Li *et al.,* 2005). There have been several reports that insulin regulates synaptic plasticity in the cortex and hippocampus, which are regions of the brain generally associated with learning and memory. It protects neurons against serum deprivation-induced apoptosis in cultured cortical neurons R28 retinal neurons and cerebellar granule neurons (Tanaka *et al.,* 1995; Ryu *et al.,* 1999; Nakamura *et al.,* 2001). It protects against toxic levels of AMPA and oxygen/ glucose deprivation in hippocampal neurons (Kim & Han, 2005; Mielke & Wang, 2005).

Peripheral insulin penetrates the blood-brain barrier (BBB) and binds to brain IRs, which leads to the triggering of their intrinsic tyrosine kinase activity and, as a result, to tyrosine phosphorylation and activation of IRS proteins (Boura-Halfon & Zick, 2009). Phosphorylated IRS proteins then activate p110/p85 heterodimeric PI_3-

kinase, protein phosphotyrosine phosphatase and adaptor Shc/GRB2 dimer complex, which triggers the intracellular signaling cascades controlling the gene expression and thus regulating growth, differentiation and the other processes in neuronal cells. The activation of PI 3-kinase leads to phosphorylation and activation of Akt kinase that regulates the metabolism and cell survival *via* numerous downstream proteins in the peripheral insulin-sensitive tissues as well as in the CNS, primarily in hypothalamic neurons (Iskandar *et al.,* 2010).

The IR is expressed in all regions of the brain in both neurons and glia with highest expression in the hypothalamus, cerebellum, olfactory bulb, hippocampus and cerebral cortex (Wozniak *et al.,* 1993). In the hypothalamus, this contributes to the regulation of food intake and energy homoeostasis. The fact that these receptors are localized in the brain accounts for the role of insulin in CNS functioning. Since the main function of insulin is to regulate glucose homeostasis, central insulin and brain IRs specifically recognizing the hormone modulate the energy, glucose and fat homeostasis in the brain, being involved, in addition, in the regulation of metabolism in the peripheral tissues. However, in the brain insulin performs some other functions specific of the CNS. Interacting with the other regulatory peptides and neurotransmitters, central insulin participates in controlling the feeding behaviour, learning and memory and is involved in the intercellular communication within brain structures, the hypothalamus and the limbic system in particular (Gerozissis, 2008).

Insulin regulates neuronal synaptic activity by altering internalization of neurotransmitter receptors. Insulin also controls the internalization of GluR2 and β-adrenergic receptors and induces translation of the dendritic synapse scaffolding protein PSD-95 (Karoor *et al.,* 1998; Huang *et al.,* 2004; Lee *et al.,* 2005). These observations clearly implicate insulin as a regulator of synaptic function and neurotransmission. Other neuronal functions attributed to insulin include promotion of neurite outgrowth in neuroblastoma cells and cultured primary neurons as well as

enhancement of axonal regeneration following injury of cultured rat sensory neurons (Recio-Pinto *et al.,* 1984; Fernyhough *et al.,* 1993; Schechter *et al.,* 1998).

IGF-1

IGF-1 is genetically related polypeptide similar to insulin with similar three-dimensional and primary structures. IGF-1 is synthesized primarily in the liver and also in the brain. Its synthesis is regulated by growth hormone, insulin and nutritional intake (Mathews *et al.,* 1988; Russell-Jones *et al.,* 1992; Thissen *et al.,* 1994). IGF-1 receptors are widely expressed in the brain and are localized preferably in neuron rich structures in many brain areas, such as the granule cell layers of the olfactory bulb, hippocampal formation and cerebral cortex. It has profound effects on the regulation of proliferation and differentiation of many cell types as well as metabolic effects, which are similar to those of insulin, including actions on glucose metabolism.

IGF-1 is involved in neuronal development, stimulates neurogenesis and synaptogenesis, facilitates oligodendrocyte development, promotes neuron and oligodendrocyte survival and stimulates myelination. All this speaks about a very important role it has in preserving the integrity of neuronal cells and in protecting the brain structures from damages and injury (D'Ercole *et al.,* 2002). The alterations of proteins, the components of brain insulin- and IGF-1-regulated signaling cascades, typical of diabetes mellitus, pre-diabetic states and are the causes of the diabetes associated neurodegenerative diseases.

Akt

Protein kinase B (PKB, also known as Akt) is an important regulator involved in several cellular functions including cell growth and apoptosis (Hanada *et al.,* 2004). Akt is a member of the serine/threonine kinase family (Alessi *et al.,* 1997). Akt is an important mediator of the physiological effects of several growth and survival factors and promotes cell survival through the inhibition of apoptosis (Downward, 1998;

Datta *et al.,* 1999). Within the nucleus, Akt controls expression of genes involved in cell survival *via* the transcription factors Forkhead, NFκB and CREB (Brunet *et al.,* 2001). In non-neuronal cells certain survival stimuli activate Akt independently of PI_3-kinase including agonists of the PKA pathway and increases cytoplasmic calcium levels (Moule *et al.,* 1997; Sable *et al.,* 1997; Yano *et al.,* 1998; Filippa *et al.,* 1999).

There are three distinct isoforms (a, b, g) that are widely expressed around the body, although PKBg (Akt3) is the major isoform expressed in neurons (Brodbeck *et al.,* 1999; Masure *et al.,* 1999). It is a member of the cAMP-dependent, cGMP-dependent and protein kinase C (AGC) family of protein kinases and is a common target of growth factor signalling pathways including insulin. Activation of Akt is required for the neuroprotective function of growth factors such as IGF1 and expression of wild type Akt protects neurons against toxin-induced death ((Dudek *et al.,* 1997; Zhou *et al.,* 1998, 2000; Weihl *et al.,* 1999).

Observations over the past decade have identified the PI_3K-Akt pathway's importance in mediating survival in PC12 cells and cultured neurons from the peripheral and central nervous systems (Yao & Cooper, 1995; Ghosh & Greenberg, 1995; Crowder & Freeman, 1998). Neurotrophic factors such as NGF, BDNF, glial cell line-derived neurotrophic factor (GDNF) and IGF-1 activate the PI_3-Akt signaling cascade through corresponding receptor tyrosine kinases such as the high affinity neurotrophin receptors (Trk's) (Segal & Greenberg, 1996). After receptor dimerization, PI_3K is recruited to the plasma membrane where its catalytic subunit generates lipid second messengers, phosphoinositide phosphates (PIP_2, PIP_3), at the inner surface of the plasma membrane. Phosphoinositide-dependent protein kinase-1 (PDK1) then acts in concert with PIP_2 and PIP_3 to phosphorylate and activate Akt (Brunet *et al.,* 2001). Alternately Trk receptors stimulate PI_3K via the Ras G-protein, IRS signaling and Gab-1, an adaptor protein which binds to Trk and directly stimulates PI_3K (Holgado-Madruga *et al.,* 1997; Kauffmann-Zeh *et al.,* 1997). Studies have demonstrated downstream signaling effects that regulate cellular survival,

proliferation and metabolism. For example, Akt phosphorylates and inactivates forkhead transcription factor like 1 (FKHRL1), a member of the family of Fork head transcriptional regulators. Inactivated FKHRL1 is unable to induce the expression of death genes in cerebellar granule neurons (Brunet *et al.,* 1999). In primary hippocampal neurons subjected to hypoxia or nitric oxide, p53 activation and p53-mediated Bax upregulation are also blocked by Akt signaling (Yamaguchi *et al.,* 2001). Akt activates the CREB and NFκB, additional transcriptional regulators that promote neuronal survival (Maggirwar *et al.,* 1998; Bonni *et al.,* 1999). In addition Akt can directly inhibit the apoptotic machinery by phosphorylation at sites both upstream (BAD) and downstream (Caspase-9) of mitochondrial cytochrome c release (Datta *et al.,* 1997; Cardone *et al.,* 1998). Finally, there is evidence to support the role of Akt in promoting neuronal survival through metabolic effects by regulating glucose metabolism in neurons (Hetman *et al.,* 2000).

Glucose transporters

The expression, regulation and activity of glucose transporters play an essential role in neuronal homeostasis, since glucose represents the primary energy source for the brain (Lund-Anderen, 1979; Pardridge, 1983). The family of facilitative glucose transporter (GLUT) proteins is responsible for the entry of glucose into cells throughout the periphery and the brain (Maher *et al.,* 1994; Vannucci *et al.,* 1997; Shepherd & Kahn, 1999). GLUT-1 is the predominant glucose transporter in the insulin-independent tissues (brain and erythrocytes) but also is found in muscle and adipocytes. GLUT-2 is expressed in several peripheral tissues, in particular the pancreas and liver, where it serves to detect plasma glucose levels. This 'glucose sensor' function of GLUT-2 is most evident in the pancreas, where glucose transport mediated by GLUT-2 serves as the signal for insulin release from pancreatic β cells. GLUT-3 is the neuron-specific glucose transporter which is responsible for neuronal glucose homeostasis (Lund-Anderen, 1979; Pardridge, 1983; Duelli & Kuschinsky,

2001). Majority of glucose utilization in the CNS appears to be mediated through GLUT-1 and GLUT-3 (Duelli & Kuschinsky, 2001).

BDNF

BDNF is important in differentiation, survival and plasticity of the CNS. BDNF has been demonstrated to be important to the regulation of energy metabolism. Recent studies also have shown significant roles of BDNF in energy metabolism regulation. Effects of BDNF on energy metabolism have also been observed in human subjects. In a clinical case report, a patient with severe obesity carries a mutation in the BDNF receptor TrkB (Yeo *et al.,* 2004). These data suggest that BDNF is important to energy metabolism regulation. Hyperphagia and obesity occur in animal models with BDNF deficiency (Lyons *et al.,* 1999; Kernie *et al.,* 2000; Rios *et al.,* 2001). Chronic ventricular or peripheral administration of BDNF decreases food intake and body weight gain and reverses the phenotype of obese and hyperphagic BDNF +/_ mice (Pelleymounter *et al.,* 1999; Sauer *et al.,* 1993; Kernie *et al.,* 2000; Ono *et al.,* 2000) . BDNF is a potent trophic factor supports striatal cells and promotes survival and/or differentiation of GABAergic neurons in vitro (Mizuno *et al.,* 1994; Ventimiglia *et al.,* 1995). BDNF is a survival gene contains cAMP response element. It is a crucial neurotrophic factor and possess pro-survival and differentiation effects on several neuronal populations and synaptic plasticity (Thoenen, 2000).

NFκB

NFκB is a redox-sensitive nuclear transcriptional factor and is an important regulator of antioxidant enzymes (Rahman & MacNee, 2000). NFκB is a dimeric transcription factor composed of five members, p50, RelA/p65, c-Rel, RelB, and p52 that can diversely combine to form the active transcriptional dimer. NFκB is ubiquitously expressed in peripheral and brain cells and regulates the expression of a wide variety of genes involved in cell survival, growth, stress responses, immune and

inflammatory processes (Baldwin, 1996; Shimada *et al.,* 2001; Weih & Caamañ, 2003). NFκB controls the expression of genes that regulate a broad range of biological processes in the CNS such as synaptic plasticity, neurogenesis and differentiation (Ghosh & Hayden, 2008). NFκB family members have been implicated in the development of the nervous system and plasticity of synapses (O'Neill and Kaltschmidt, 1997; Meffert *et al.,* 2003; O'Mahony *et al.,* 2006). NFκB has also been related to ROS while certain NFκB regulated genes play a major role in regulating the amount of ROS in the cell. ROS have various inhibitory or stimulatory roles in NFκB signaling. NFκB is persistently activated in cancer, chronic inflammation, neurodegenerative diseases, stress, stroke, trauma, heart disease, and other disease conditions (Mémet, 2006; Xiao *et al.,* 2006). NFκB is an important regulator in programmed cell death and play important roles in normal brain function and neurodegenerative disorders (Grilli & Memo, 1999; Denk *et al.,* 2000; Mattson & Meffer, 2006). In the CNS, NFκB can play an anti-apoptotic or pro-apoptotic role in cell death and regulates the genes involved in neuronal death and survival (Kaltschmidt *et al.,* 2005).

CREB

CREB is a transcription factor involved in adult neurogenesis, learning and memory. CREB is involved in many functions in the nervous system, including neurogenesis and neuronal survival, development, differentiation, neuroprotection, axonal outgrowth and regeneration, synaptic plasticity (Mioduszewska *et al.,* 2003; Persengiev & Green, 2003; Dragunow, 2004; Barco & Kandel, 2006)**.** Genes whose transcription is regulated by CREB include: c-fos, BDNF, tyrosine hydroxylase and neuropeptides such as somatostatin, enkephalin, VGF and corticotropin-releasing hormone (Lauren, 2005). BDNF is a survival gene contains cAMP response element. It is a crucial neurotrophic factor and possess pro-survival and/or differentiation effects on several neuronal populations and synaptic plasticity (Thoenen, 2000).

CREB is a downstream target of cyclic AMP signaling. Multiple lines of evidence define a role for CREB in proliferation and differentiation of certain cells and tissues (Heasley *et al.,* 1991; Spaulding, 1993; Iyengar, 1996). Disruption of CREB activity, using expression of a dominant-negative CREB slows neurite outgrowth and blocks adipocyte differentiation (Engelman *et al.,* 1998; Shimomura *et al.,* 1998).

Second Messengers

Neurons use many different second messengers as intracellular signals. These messengers differ in the mechanism by which they are produced and removed, as well as their downstream targets and effects. Second messenger systems are complexes of regulatory and catalytic proteins, which are activated by first messengers to form second messengers. Second messengers relay signals received at receptors on the cell surface to target molecules in the cytosol and/or nucleus. Three major classes of second messengers are (1) cyclic nucleotides (e.g., cAMP and cGMP), (2) inositol trisphosphate (IP_3) and diacylglycerol (DAG), (3) calcium ions (Ca^{2+}). The signal transduction in metabotrophic neurotransmitters occur through activation of second messengers, whereas ionotrophic neurotransmitters act through ligand gated ion channels. The changes in neurotransmitter level and its receptor should agree with a concomitant change in second messenger for effective signal transduction.

cAMP

cAMP is produced from ATP in response to a variety of extracellular signals such as hormones, growth factors and neurotransmitters. Cyclic AMP is produced when G-proteins activate adenylyl cyclase in the plasma membrane. This enzyme converts ATP into cAMP by removing two phosphate groups from the ATP. Elevated levels of cAMP in the cell lead to activation of different cAMP targets. It was long thought that the only target of cAMP was the cAMP-dependent protein kinase (cAPK)

which has become a model of protein kinase structure and regulation (Doskeland *et al*., 1993; Francis & Corbin, 1999; Canaves & Taylor, 2002). cAMP produced by adenylyl cyclase, activates PKA by binding to the regulatory subunits in ways that result in the release and nuclear translocation of active catalytic subunits (Meinkoth *et al.,* 1993). cAMP stimulates the proliferation of many cell types, but in some cases cAMP actually inhibit cellular proliferation (Pastan *et al.,* 1975; Bokoch, 1993; Dugan *et al.,* 1999; Hagemann & Rapp, 1999; Zwartkruis & Bos, 1999; Wang *et al.,* 2000). Dopamine and cAMP regulate a diverse array of neuronal functions ranging from ion conductance and synaptic plasticity to gene expression (Young *et al.,* 1986; Oh *et al.,* 1997; Kaupp & Seifer, 2002; Cooper, 2003; Ha˚kansson *et al.,* 2004; Fernandez *et al.,* 2006).

cGMP

An essential element of the signalling cascade leading to synaptic plasticity is the intracellular second messenger molecule guanosine 3′,5′-cyclic monophosphate (cGMP). Cyclic GMP is similarly produced from GTP by the action of guanylyl cyclase. Once the intracellular concentration of cGMP is elevated, these nucleotides bind to the targets. The most common target of cGMP is cGMP-dependent protein kinase (PKG).The nitric oxide–cyclic guanosine 3',5' mono phosphate (NO-cGMP) pathway is a key player in a range of neuronal functions including neuroprotection and neurotoxicity (Weill & Green, 1984; Thippeswamy & Morris, 1997; Lipton *et al.,* 1994; Kim *et al.,* 1999; Thippeswamy *et al.,* 2001a; Fiscus, 2002; Nakamizo *et al.,* 2003; Duchen, 2004). The physiological functions of NO are mediated by activation of soluble guanylyl cyclases (sGC) which generates cGMP (Gibb *et al.,* 2003). Under pathological conditions such as cerebral ischemia, sGC-independent mechanism appears to be involved in the neurotoxic action of NO (Nelson *et al.,* 2003). Recent evidence suggests that cGMP-mediated actions of NO are protective to both neurons and glia (Thippeswamy & Morris, 1997; Thippeswamy *et al.,* 2001b, 2002; Andoh *et*

al., 2002; Ha *et al.,* 2003; Snyder & Kim, 2004). In addition, cAMP and cGMP can bind to certain ion channels, thereby influencing neuronal signaling.

IP_3

The cellular responses elicited by the interaction of many extracellular signaling molecules with their cell surface receptors are triggered by the rapid hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2). This reaction is catalyzed by phosphoinositide-specific phospholipase C (PLC) isozymes and results in the generation of two intracellular messengers, DAG and IP_3. These messengers then promote the activation of protein kinase C and the release of Ca^{2+} from intracellular stores. IP_3 is further converted by the actions of several distinct kinases and phosphatases to a variety of inositol phosphates, some of which are also implicated in intracellular signaling. IP3 is a ubiquitous second messenger that functions by binding to receptors (IP3Rs) on the ER membrane to cause liberation of sequestered Ca^{2+} (Berridge, 1998, 2002). The resultant cytosolic Ca^{2+}transients serve numerous signaling functions in neurons, including modulation of membrane excitability synaptic plasticity and gene expression (Fujii *et al.,* 2000; Miyata *et al.,* 2000; Nishiyama *et al.,* 2000; Mellstrom & Naranjo, 2001; Yamamoto *et al.,* 2002; Stutzmann *et al.,* 2003). The neuronal intracellular calcium has an important role in the regulation of synaptic plasticity (Barbara, 2002). Moreover, disruptions in this pathway are implicated in neurodegenerative disorders (Abe, 1997; Mattson *et al.,* 2000; LaFerla, 2002). Therefore, factors that modulate or disrupt IP3-mediated Ca^{2+} signaling are expected to exert powerful physiological and possibly pathological effects on the nervous system.

Insulin secretion is largely a Ca^{2+}-dependent process and restricted increases in intracellular Ca^{2+} have been related to impairment of glucose-stimulated insulin release (Boschero *et al.,* 1990). Several lines of evidence point to IP_3 playing an important role in insulin secretion. IP_3 was shown to mobilize intracellular Ca^{2+} in

permeabilized insulin-secreting cells and IP_3 production correlated with Ca2+ mobilization in intact cells (Biden *et al.,* 1984; Gromada *et al.,* 1996). In the mouse anx7 (1/2) phenotype, where there is a profound reduction in IP_3R expression and Ca^{2+} mobilization in islets, there is also defective insulin secretion (Srivastava *et al.,* 1999). Numerous stimuli have been reported to activate the IP_3 pathway and receptor stimulation is commonly linked to phospholipase C activation, as with certain neurotransmitters, growth factors and hormones (Rana & Hokin, 1990). In addition, in islets glucose stimulation acts through a Ca^{2+}-dependent mechanism to activate phospholipase C, a phenomenon also reported for brain synaptosomes and iris smooth muscle, among other tissues (Axen *et al.,* 1983; Laychock, 1985; Rana & Hokin, 1990).

Dopamine

Dopamine is a predominant catecholamine neurotransmitter in the periphery and in the CNS (Kuchel & Kuchel, 1991; Feldman *et al.,* 1997; Cooper *et al.,* 2003). Dopamine belongs to the class of catecholamine neurotransmitters which also includes epinephrine (E) and norepinephrine (NE, also known as noradrenaline). These three neurotransmitters are synthesized from the amino acid tyrosine via a series of enzymatic steps. The first enzyme in the pathway is tyrosine hydroxylase (TH) which converts tyrosine to L-dihydroxy-phenylalanine (L-DOPA) which is the rate limiting step in dopamine and NE synthesis (Holtz, 1959). TH is present in all catecholamine-producing neurons and is commonly used as a histological marker for these cell types. L-DOPA is then converted to dopamine by a carboxylase.

Dopamine containing neurons arise mainly from dopamine cell bodies in the substantia nigra and ventral tegmental area in mid-brain region (Royh *et aI.,* 1991; Carlsson, 1993; Lookingland *et aI., 1995;* Tarazi *et aI.,* 1996; 2001; Creese *et aI.,* 1997). Dopaminergic system is organized into four major subsystems (i) the *nigrostriatal* system involving neurons projecting from the substantia nigra pars

compacta to the caudate putamen of the basal ganglia. This is the major dopamine system in the brain as it accounts for about 70% of the total dopamine in the brain, and its degeneration makes a major contribution to the pathophysiology of Parkinson's disease; (ii) the *mesolimbic* system that originates in the midbrain tegmentum and projects to the nucleus accumbens septi and lateral septal nuclei of the basal forebrain as well as the amygdala, hippocampus, and the entorhinal cortex, all of which are considered components of the limbic system and so are of particular interest for the patho-physiology of idiopathic psychiatric disorders; (iii) the *mesocortical* system, which also arises from neuronal cell bodies in the tegmentum which project their axons to the cerebral cortex, particularly the medial prefrontal regions; (iv) the *tuberinfimdibular* pathway, which is a neuroendocrinological pathway arising from the arcuate and other nuclei of the hypothalamus and ending in the median eminence of the inferior hypothalamus. Dopamine released in this system exerts regulatory effects in the anterior pituitary and inhibits the release of prolactin. Dopamine is involved in the control of both motor and emotional behavior. Despite the large number of crucial functions it performs, this chemical messenger is found in a relatively small number of brain cells.

Dopamine is released by several populations of midbrain and forebrain neurons. Important nuclei that contain dopaminergic neurons include the substantia nigra pars compacta and the ventral tegmental area (Halbach & Dermietzel, 2002). These nuclei send projections to the neostriatum, the limbic cortex and other limbic structures (Feldman *et al.,* 1997). The dopaminergic neurons in the midbrain of the rat are located in three cell groups: nucleus A8 cells in the retrorubral field, nucleus A9 cells in the substantia nigra, and nucleus A10 cells in the ventral tegmental area and related nuclei. A8 and A10 to cortical and limbic structures, while the substantia nigra projects primarily to the striatum. The A8-10 mid-brain dopaminergic cell groups have extensive projections to the striatum, cortex and limbic system. The A11-14 cell populations arise from the hypothalamus and wall of the third ventrical and send

descending projections to the autonomic areas of the brain stem and spinal cord, as well as regulate neuroendocrine function.

Dopamine is known to play an important role in many brain functions. Dopamine affects the sleep-wake cycle it is critical for goal-directed behaviours and reward, learning and modulates the control of movement *via* the basal ganglia (DeLong, 1990; Graybiel *et al.,* 1994; Schultz, 2000; Wise, 2004; Dzirasa *et al.,* 2006). Cognitive processing, such as executive function and other pre-frontal cortex activities, are known to involve dopamine (Nieoullon, 2002). Finally, dopamine contributes to synaptic plasticity in brain regions such as the striatum and the pre-frontal cortex (Arbuthnott *et al.,* 2000; Jay, 2003; Yao *et al.,* 2004).

Dopamine receptors

The dopamine receptors are a super family of heptahelical G protein-coupled receptors and are grouped into two categories D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4) receptors based on functional properties to stimulate adenylyl cyclase *via* Gs/olf and to inhibit adenylyl cyclase *via* Gi/o, thereby decreasing cAMP and PKA activity (Kebabian & Calne, 1979; Stoof & Kebabian, 1981; Jackson & Westlind- Danielsson, 1994; Missale *et al*., 1998). In addition, both D_1-type and D_2-type receptors have been shown to regulate intracellular Ca^{2+} levels. D_1-type receptors can interact with calcyon and influence Ca^{2+}-dependent signaling *via* G_q-coupled release of Ca^{2+} from intracellular stores and D_2-type receptors are coupled directly to phospholipase C and the production of IP_3 and elevate intracellular calcium levels (Nishi *et al.,* 1997). D_2 receptors have been shown to have a higher affinity for dopamine than D_1 receptors which impact the relative contribution of signaling *via* these receptors in low and high dopamine states (Missale *et al.,* 1998; Hamada *et al.,* 2004).

The dopamine D1 receptor is the most widespread dopamine receptor. It has been found in the striatum, nucleus accumbens, olfactory tubercle, limbic system, hypothalamus and thalamus (Dearry *et al.,* 1990). D_1 receptors show widespread

neocortical expression including the prefrontal cortex and striatum. The dopamine D_2 receptor is also abundant in the mammalian brain. D_2 receptors are highly expressed in the striatum where they are found both post-synaptically on MSNs and pre-synaptically as autoreceptors on dopaminergic nerve terminals. D_2 receptors are also expressed at low levels in the hippocampus, amygdala, and weakly in the prefrontal cortex. Within the striatum, post-synaptic dopamine D_1 and D_2 receptors are largely segregated to the two populations of MSNs comprising the direct striatonigral and indirect striatopallidal pathways (Gerfen *et al.,* 1990). Dopamine D_2 receptors exist as two different slice variants termed $D_{2_{short}}$ and $D_{2_{long}}$. These are differentially expressed pre-synaptically ($D_{2_{short}}$) and serves presynaptic inhibitory autoreceptor functions and post-synaptically ($D_{2_{long}}$) (Centonze *et al.,* 2004). It has been detected in many areas, including the hypothalamus (Giros *et al.,* 1989; Missale *et al.,* 1998; Usiello *et al.,* 2000).

Dopamine Receptors and Synaptic Plasticity

It is widely accepted that one of the major functions of dopamine is to modulate glutamatergic signaling. In the case of the striatum this involves modulation of excitatory corticostriatal input. Glutamatergic and dopaminergic axons terminate of the head and neck, respectively, of dendritic spines on medium spiny neurons (MSN) (Chase & Oh, 2000). Dopamine D_1 receptors have been shown to interact with NMDA-type glutamate receptors which impacts the trafficking and function of each receptor in a bi-directional manner (Lee *et al.,* 2002; Scott *et al.,* 2002). *Via* several different pathways, dopamine can either enhance or inhibit NMDA signaling based on the depolarization state of the neuron (Surmeier *et al.,* 2007). Acting through the cAMP/PKA pathway, dopamine D_1 receptor signaling has been shown to increase phosphorylation of NMDA receptors and calcium channels which can enhance glutamate-induced currents (Flores-Hernandez *et al.,* 2002). It is thought that this

mechanism may underlie the D_1 receptor activation of long term potentiation (LTP), a process which is lost by treatment with dopamine D_1 receptor antagonists, or in dopamine D1 receptor knock-out mice (Calabresi *et al.,* 2007).

Dopamine D_2 receptors, conversely, have an inhibitory effect on LTP and are known to be vital in the induction of long term depression (LTD). Dopamine D_2 receptor activation leads to dephosphorylation of the GluR1 subunit of the AMPA receptor which reduces glutamate-mediated currents and can promote trafficking of the receptor out of the membrane (Hakansson *et al.,* 2006). Stimulation of dopamine D_2 receptors can also lead to diminished pre-synaptic release of glutamate (Yin & Lovinger, 2006). As mentioned previously, dopamine D_2 receptors play an important role in striatal LTD. Dopamine D_2 receptors expressed in cholinergic interneurons regulate acetylcholine release, while D_2Rs expressed post-synaptically on MSNs are important in facilitating endocannabinoid release, both essential mediators of LTD (Calabresi *et al.,* 2007).

CORTICOSTRIATAL DOPAMINERGIC PATHWAY

The striatum is the main input center for the basal ganglia which responds to and integrates signals from a variety of brain areas and neurotransmitter systems. The striatum 90-95% is comprised mainly of MSNs (Kemp & Powell, 1971). Striatum is innervated by an abundance of neurotransmitters and neuromodulators. Sriatal neurons express a wide variety of receptors and ion channels. These include ionotropic and metabotropic glutamate receptors, GABA receptors, several classes of serotonin receptors, adenosine receptors, opiate receptors and several types of dopamine receptors. MSNs are GABAergic and receives dopaminergic and glutamatergic inputs. It sends projections *via* the direct and indirect pathways to other structures of basal gnglia. Direct/striatonigral pathway neurons express type 1 dopamine receptors. Indirect/striatopallidal pathway MSN's express type 2 dopamine receptors (Gerfen *et al.,* 1990; Hersch *et al*., 1995; Surmeier *et al*., 1996; Valjent *et*

al., 2009; Bertran-Gonzalez *et al*., 2008). In the striatonigral/direct pathway neurons dopamine D_1 receptors are coupled to Gs/olf/AC/PKA signaling and activation of PKA induces the phosphorylation of PKA substrates such as DARPP-32, a dopamine- and cAMP-regulated phosphoprotein of M*r* 32 kDa and a transcription factor, CREB, leading to alterations of neuronal functions (Greengard *et al.,* 1999; Hyman & Malenka, 2001). Dopamine plays an important role in the coordination and regulation of the two output pathways by acting in a bidirectional manner. Many electrophysiological and gene transcriptional data, obtained in vitro and in vivo, suggest that dopamine exerts stimulatory effects *via* dopamine D_1 receptors and inhibitory effects via dopamine D_2 receptors (Gerfen *et al.,* 1990, Robertson *et al.,* 1990, Chergui *et al.,* 1997, Gonon, 1997; West & Grace, 2002.)

Major characteristic of the striatum is the division of its efferents into the direct striatonigral and indirect striatopallidal pathways. The direct pathway is comprised of projections from a sub-class of GABA-ergic MSNs which express dopamine D1 receptors and directly innervate the Substantia nigra pars reticulate (SNr). Indirect pathway neurons preferentially express dopamine D_2 receptors and project to the GPi/SNr *via* two intermediate structures, the GPe and subthalamic nucleus (STN). These anatomically defined cell populations are receiving more attention as it becomes increasingly clear that they have distinct physiological and signaling properties and that they can be modulated independently to result in different behavioural outcomes. In the normal state, these output pathways are highly regulated and work synergistically to produce coordinated effects on behaviour. In the case of disruption or imbalance in signaling, there are unique maladaptations which occur in each pathway that may have important consequences for targeting therapies to this brain area (Albin *et al.,* 1989; Gerfen, 2000).

Three major anatomical divisions seen in the striatum are dorsal versus ventral striatum, patch versus matrix compartments and direct versus indirect projection pathways. Dorsal region receives sensorimotor-related information from

the cortex and dopaminergic innervation primarily from the SNc (Kitai *et al.,* 1976; Donoghue & Herkenham, 1986). The ventral striatum receives visceral and limbic related afferents and higher density of inputs from the amygdala, hippocampus and ventral tegmental area (VTA) (Voorn *et al.,* 2004). The striatum can also be divided into patch and matrix compartments which were regions originally identified histologically as rich or poor in acetycholinsterase staining (Graybiel and Ragsdale, 1978). The patch and matrix areas differ in their connectivity. The striatal matrix is also associated with largely sensorimotor information. The patch compartment receives afferents from limbic areas as well as cortical layers Vb and VI and sends efferents to the SNc which can modulate dopaminergic input into the striatum (Graybiel, 1990).

Dopaminergic System in Diabetes

Hyperglycemia during diabetes is reported to damage dopaminergic functions and show changes in dopamine metabolism in the human brain and the brains of diabetic animals (Lacković *et al*., 1990; Kirchgessner & Liu, 2001). The central dopaminergic system is involved in the regulation of whole body fuel and energy homeostasis. Dopaminergic neurotransmission profoundly affects glucose and lipid metabolism (Meier & Cincotta, 1996). Selective destruction of dopaminergic neurons in the area of the suprachiasmatic nucleus severely impairs insulin sensitivity and promotes body fat accretion in lean animals (Luo *et al.,* 1997).

In obese humans and insulin resistant animals the expression of dopamine D_2 receptors in certain brain regions are reduced (Wang *et al.,* 2002; Fetissov *et al.,* 2002; Hajnal *et al.,* 2008; Davis *et al.,* 2009). Reports suggest that modulation of glucose homeostasis is achieved by activation of dopamine D_2 receptor activity and it profoundly affects energy homeostasis in humans and animals. Dopamine D_2 receptor agonist drugs reduce body weight, increase energy expenditure and improve glycemic control in obese animals and individuals (Luo *et al*., 1999; Pijl *et al*., 2000; Kuo,

2002; Kok *et al*., 2006). Dopamine D_2 receptors are modulators of insulin secretion and mice lacking dopamine D_2 receptors display an impaired glucose metabolism (Garcia *et al*., 2010). In man, treatment with dopamine D_2 receptor antagonists induces obesity and type 2 diabetes mellitus, whereas dopamine D_2 receptor activation ameliorates the metabolic profile in obese non diabetic and diabetic humans (Pijl *et al*., 2003). Drugs that block dopamine D_2 receptors enhance appetite and induce weight gain in animals and humans (Baptista *et al.,* 1987, 2002; Newcomer, 2005; Ader *et al.,* 2005). Bromocriptine controls insulin secretion and improves insulin secretion, glucose tolerance and insulin resistance in humans and animals (Cincotta *et al*., 1993; Ling *et al*., 1998; Luo *et al*., 1998; Pijl *et al*., 2000).

Dopaminergic neurotransmission in pancreas

Dopamine is involved in various physiological functions such as cell proliferation, gastrointestinal protection and inhibition of prolactin secretion (Freeman *et al.,* 2000; Hoglinger *et al.,* 2004). Anatomical studies suggest that the vagal efferent fibers originating from the nucleus ambiguus and dorsal motor nucleus of the brain stem directly innervate the pancreas through the parahypothalamic ventricular nucleus and have a role in insulin release (Bereiter *et al.,* 1981). Dopamine in the peripheral nervous system (PNS) modulates insulin secretion from the pancreatic islets (Nogueira *et al.,* 1994). The exocrine pancreas is an important source of dopamine, proposed to be implicated in gastrointestinal mucosa protection. In the vicinity of pancreatic β cells, dopamine released from neurons innervating pancreatic islets (Freeman *et al.,* 2000). Reports suggest that pancreatic β-cells express dopamine receptors and modulate the insulin secretion through its receptors (Davis & Smith, 1985; Blanca *et al.,* 2005). Pancreatic dopamine receptors modulate insulin secretion through its receptors. High concentrations of dopamine in pancreatic islets can decrease glucose stimulated insulin secretion (Tabeuchi *et al.,* 1990; Mezey *et al.,* 1996).

Pancreatic regeneration

Pancreatic β cells are in a constant state of dynamic change, with continued regeneration of islets from ductal endothelial cells of the exocrine pancreas and simultaneous apoptosis (Bonner- Weir, 2001). Transcription factors play significant role in pancreas development- specific lineage formation, differentiation and function. Transcription factors with recently discovered or newly expanded roles in pancreatic progenitors (Ptf1, PDX-1, Sox9), islet progenitors (HNF−1 β, Ngn3, and Pax4), lineage specification (Nkx2.2, Nkx6.1) and β-cell differentiation (Maf A/B, HNF4α, Foxa2) (Oliver-Krasinski & Stoffers, 2008).

Transcription factors in pancreatic regeneration

Pancreatic duodenal homeobox-1 (PDX-1)

PDX-1 is a major player of pancreas formation and it is restricted to β cells. PDX-1 is considered to be the master regulator of pancreatic development and β-cell differentiation. PDX-1 has a dual role as an inducer of the endocrine lineage from ductal epithelial cells and in the maturation of β-cells (Hill & Duvillie, 2000). It is a major transactivator of the insulin gene and of other islet specific genes, such as GLUT2, glucokinase, IAPP and somatostatin (Leonard *et al.,* 1993; Ohlsson *et al.,* 1993; Marshak *et al.,* 1996; Waeber *et al.,* 1996; Watada *et al.,* 1996a; 1996b; Carty at al., 1997). PDX-1 first appears in the digestive tract of the mouse at embryonic day (e) 8.5, a day before the dorsal bud of the pancreas develops. PDX-1 is directly activated by the transcription factor- NeuroD1 (Sharma *et al.,* 1997). When PDX-1 is inactivated, the pancreas bud is initiated and a few glucagon cells differentiate, but the expansion of the bud is limited and it does not branch properly. In addition, the ability of PDX-1 to bind the insulin and Glut2 promoters and transactivate these genes suggests that it is required for the function of β cells. Accordingly, a conditional

knockout that allows pancreas formation, but affects late PDX-1 expression in β cells results in defective insulin secretion and a diabetic condition (Ahlgren *et al.,* 1998). PDX-1 is directly activated by the transcription factors NeuroD1, Hnf-1α and Hnf-3β (Sharma *et al.,* 1997; Ben-Shushan *et al.,* 2001). Lowered PDX-1expression or activity resulting in impaired expression of both GLUT-2 and insulin could cause hyperglycemia, which progress to Type 2 diabetes. PDX-1 expression is required in mature β-cells to maintain hormone production, GLUT-2 expression and euglycemia (Ahlgren *et al.,* 1998).

Neurogenic differentiation 1 (NeuroD1/BETA2)

Neurogenic differentiation 1 or β-cell E box transactivator 2 (NeuroD1/BETA2) is a cell-type restricted basic helix–loop–helix (bHLH) transcription factor expressed in all endocrine cells (Naya *et al.,* 1995). It is expressed in the mouse pancreatic bud at (e) 9.5. NeuroD1 heterodimerizes with ubiquitous bHLH proteins of the E2A family to regulate the transcription of the insulin gene. Mice homozygous for a targeted disruption of the neuroD1/BETA2 gene survive to birth, but die within three to five days postpartum of severe hyperglycemia. The islets of these mice are dysmorphic and have markedly diminished number of endocrine cells arranged in streaks and irregular aggregates and reduced number of β-cells (Habener *et al.,* 2005).

β cell proliferation

During development, β-cells are generated from a population of pancreatic Ngn3+ progenitor cells (Edlund, 2002; Wilson *et al.,* 2003). In the neonatal period new β cells are formed by replication of differentiated β cells, which results in a massive increase in β cell mass (Finegood *et al.,* 1995; Svenstrup *et al.,* 2002). In general, formation of new β cells can take place by two pathways: neogenesis from putative progenitor stem cells or replication of already differentiated β cells (Nielsen

et al., 1999; Dor *et al.,* 2004). There have been many attempts to generate insulin-secreting cells *in vitro*. Recent studies have shown that insulin-secreting cells can be generated from non-β-cells, including mouse and human pancreatic duct cells and putative pancreatic stem/progenitor cells (Bonner-Weir, 2000; Ramiya *et al.,* 2000; Zulewski *et al.,* 2001; Wheeler *et al.,* 2004; Suzuki *et al.,* 2004; Gao *et al.,* 2007). In addition, several studies have suggested that pancreatic acinar cells can trans differentiate into insulin-secreting cells *in vitro* (Song *et al.,* 2004; Baeyens *et al.,* 2005). Discovering ways to stimulate β cell proliferation and differentiation may lead to therapeutic implications for diabetes management. Although insulin treatment has saved countless diabetics from early death, it represents an ameliorative treatment rather than a cure. A true cure for diabetes and a triumph for the concept of 'regenerative medicine' – might be achieved by the regeneration of β-cells.

Aegle marmelose

Medicinal plants have formed the basis for Indian traditional medicine systems. The medicinal plants provide a useful source of new oral hypoglycemic compounds for development of pharmaceutical entities or as a dietary adjunct to existing therapies (Bailey & Day, 1989). Few of the plants used for the treatment of diabetes have received scientific or medical scrutiny and even the WHO expert committee on diabetes recommends that this area warrant further attention. *Aegle marmelose* Corr. (Rutaceae) commonly called as 'Koovalam' in Malayalam and 'Bael' in Hindi is indigenous to India. It is a medium sized, armed deciduous tree found in wild, especially in dry forests and is also cultivated throughout Indian subcontinent. *Aegle marmelos* is a known herbal medicine for the treatment of diabetes mellitus (Alam *et al.,* 1990; Prakash, 1992). The *Aegle marmelos* belongs to the family Rutaceae. The ripe fruit and unripe fruit, as well as the roots, leaves and branches have all been used in traditional medicine. Leaves, fruits, stem and roots of *A. marmelos* have been used in ethno medicine to exploit its medicinal properties

including astringent, antidiarrheal, antidysenteric, demulcent, antipyretic and anti-inflammatory activities (Maity *et al.,* 2009).

Previous reports indicate blood glucose lowering activity of green leaves of *Aegle marmelose* (Chakrabarti *et al.,* 1960). Oral administration of aqueous decoction of *Aegle marmelose* root bark (1 ml/100 g) showed hypoglycemic effect, which was maximum (44%) at 3 h in normal fasted rats. In addition, the same extract completely prevented peak rise of blood sugar at 1 h in OGTT (Karunanyake *et al.,* 1984). Ponnachan *et al.,* (1993a, 1993b) have observed that the alkaloid extract prepared from leaves and crude aqueous leaf extract (1 g/kg for 30 days) exhibit hypoglycemic effect in alloxa induced diabetic rats. Aqueous leaf extract reversed the increase in Km values of liver malate dehydrogenase enzyme and improved histopathological alterations in the pancreatic and kidney tissues of streptozotocin (STZ) induced diabetic rats (Seema *et al.,* 1996; Das *et al.,* 1996). The aqueous extracts of fruits have also been reported to possess hypoglycemic activity (Kamalakkannan & Prince, 2003, 2004). The antidiabetic mode of action is of multidirectional as the extract can significantly lower the levels of blood glucose and glycosylated hemoglobin and increased the plasma insulin as well as liver glycogen in diabetic rats (Kamalakkanan *et al.,* 2003). Oral, as well as intraperitoneal administrations of the aqueous extract of Bael fruit exhibited hypoglycemic effect against STZ induced diabetic rats. This antidiabetic effect is probably due to the presence of Coumarins in the fruit extract, which potentiate the insulin secretion from existing β cells of the isles of Langerhans (Kamalakkanan & Prince, 2005).

Various chemical constituents like alkaloids, coumarins and steroids have been isolated and identified from different parts of tree. Marmelosin, marmesin, imperatorin, marmin, alloimperatorin, methyl ether, xanthotoxol, scopoletin, scoparone, umbelliferone, psoralen and marmelide (Farooq, 2005). Marmenol, a 7-geranyloxycoumarin [7-(2,6-dihydroxy-7-methoxy-7-methyl-3-0ctaenyloxy) Coumarins] has also been reported in *A. marmelos* (Kokate *et al.,* 2002).

From the leaves of *A. marmelos,* an alkaloidal-amide, Aegeline-2, was isolated and found to have antihyperglycemic activity as evidenced by lowering the blood glucose levels by 12.9% and 16.9% at 5 and 24 h, respectively, in sucrose challenged STZ induced diabetic rats model at the dose of 100 mg/kg body weight (Narender *et al.,* 2007). Scopoletin (7-hydroxy-6-methoxy coumarin) isolated from the leaves of *Aegle marmelose,* administration to levo-thyroxine-induced hyperthyroid rats showed its potential to regulate hyperthyroidism, lipid peroxidation and hyperglycaemia (Panda & Kar, 2006). It is also reported that there is decrease in total muscarinic and muscarinic M1 receptors during diabetes which is up regulated by *Aegle marmelose* leaf extract treatment (Gireesh *et al.,* 2008).

Antioxidative parameters like reduced glutathione, glutathione peroxidase, glutathione reductase, super oxide dismutase and catalase have shown a dose–related increase in their level /activity and a decrease in lipid peroxidation following the treatment with *A. marmelos* leaf extract (Sabu & Kuttan, 2004). The antioxidant phytochemical such as flavonoids, alkaloids, sterols, tannins, phlobotannins and flavonoid glycosides present in the leaf extract possess this free radical scavenging activity (Rajadurai & Prince, 2005). Eugenol and Marmesinin may be responsible for such activity because these compounds have independently shown their activity against oxidative stress (Vidhya & Devaraj, 1999). Natural antioxidants strengthen the endogenous antioxidant defences against 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* can rightly be mentioned as a plant of considerable interest.

Neurobiology of Pyridoxine

Pyridoxine (Vitamin B_6) is water-soluble B-complex vitamins found in a variety of animal and vegetable products. Pyridoxine is required for the production of the monoamine neurotransmitters serotonin, dopamine, norepinephrine and

epinephrine. This enzyme is responsible for converting the precursors 5-hydroxytryptophan (5-HTP) into serotonin and L-DOPA into dopamine, noradrenaline and adrenaline. It has been implicated in the treatment of depression and anxiety (Dakshinamurti *et al.,* 1990). Pyridoxine acts as coenzyme in biosynthesis of neurotransmitters- γ-aminobutyric acid, dopamine, serotonin and it is important for development and function of the CNS (Dakshinamurti *et al.,* 1988; Ernahrungswiss, 1996). Vitamin B6 has neuronal survival promoting activities *in vitro* culture of neurons by virtue of its crucial co enzymatic actions in the biosynthesis of putative neurotransmitters (Geng *et al.,* 1995).

Imbalance between dopamine and serotonin in the hypothalamus of the pyridoxine-deficient rat leads to severe neuroendocrine consequences. The decrease in pineal serotonin leads to a deficiency of melatonin (Yehuda *et al.,* 1984). The crucial role played by pyridoxine in the nervous system is evident from the fact that the putative neurotransmitters 5-HT is the product of pyridoxal phosphate (PLP)-dependent enzymatic decarboxylation (Dakshinamurti, 1982; Dakshinamurti & Paulose, 1985). A decrease in brain 5-HT content leads to an up regulation of 5-HT receptors which in turn inhibit insulin secretion due to increased sympathetic activity (Paulose & Dakshinamurti, 1985). Spontaneous or drug induced seizure activity in the pyridoxine- deficient rat is ascribed to the neurotransmitter imbalance (Dakshinamurti *et al.,* 1984).

Pyridoxine has a role in preventing heart disease. Without enough pyridoxine, a compound called homocysteine builds up in the body. Homocysteine damages blood vessel linings, setting the stage for plaque buildup when the body tries to heal the damage. Vitamin B6 prevents this buildup, thereby reducing the risk of heart attack. Pyridoxine lowers blood pressure and blood cholesterol levels and keeps blood platelets from sticking together (Perry *et al.,* 2007).

Pyridoxine is a cofactor for enzymes involved in a variety of metabolic pathways. Pyridoxine is also involved in glucose homeostasis through glycogenolysis,

glycolysis and glucose transport through its role in insulin and glucagon hormone action. Pyridoxine has anti-hyperglycemic and neuroprotective activity in diabetic animals (Abraham *et al.,* 2010; Nair *et al.,* 1998). Pyridoxine ameliorates degenerative diseases and protect against neurotoxicity (Dakshinamurti *et al.,* 2001; Hwang *et al.,* 2007). Pyridoxine inhibits lipid peroxidation, protect against neurotoxicity and ameliorate degenerative diseases (Jain & Lim, 2001; Dakshinamurti *et al.,* 2003; Hwang *et al.,* 2007). Recent studies show antioxidant properties of pyridoxine (Jain & Lim, 2001; Stocker *et al.,* 2003).

Materials and Methods

CHEMICALS USED IN THE STUDY AND THEIR SOURCES

Biochemicals

Pyridoxine, 5-hydroxy tryptamine, ascorbic acid, pargyline, calcium chloride, sulpiride, SCH 23390, ethylene diamine tetra acetic acid - EDTA, HEPES - [n′ (2-hydroxy ethyl)] piperazine-n′-[2-ethanesulfonic acid], Streptozotocin, citric acid, Tris HCl, foetal calf serum (heat inactivated), D-glucose, calcium chloride, Bromodeoxyuridine, collagenase type XI, bovine serum albumin fraction V and RPMI-1640 medium were purchased from Sigma Chemical Co., St. Louis, MO. USA). Tissue freezing medium Jung was purchased from Leica Microsystems Nussloch GmbH, Germany. All other reagents were of analytical grade purchased locally.

Radiochemicals

[^{3}H]Dopamine (Sp. activity- 45.1Ci/mmol), [^{3}H]SCH 23390 (Sp. activity 83Ci/mmol) and [^{3}H]YM-09151-2 (*cis–N-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide* Sp. activity - 85.0Ci/mmol) were purchased from NEN Life Sciences Products, Inc. Boston, USA. The [^{3}H]IP_3, [^{3}H]cGMP and [^{3}H]cAMP were purchased from American Radiolabelled Chemicals, USA . Radioimmunoassay kit for insulin was purchased from Baba Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma chemicals Co., St. Louis, MI, USA. ABI PRISM High capacity cDNA Archive kit, primers and Taqman probes for Real-Time PCR - Dopamine D_1 (Rn_02043440) Dopamine D_2 (Rn_00561126), CREB (Rn_ 00578826), Tyrosine hydroxylase (Rn_00562500), IR (Rn_00567070), Glut-3

(Rn_00567331), Glut-2 (Rn_00563565), IGF-1 (Rn_99999087), phospholipase- C (Rn_01647142), SOD (Rn_01477289), GPX (Rn_00577994), Bax (Rn_01480160), TNF-α (Rn_99999017), Nfκb (Rn_01399583), AKT-1(Rn00583646), caspase-8 (Rn_00574069), BDNF (Rn_01484924), pdx-1(Rn_00755591), Neuro-D1 (Rn_00824571) and endogenous control (β-actin) were purchased from Applied Biosystems, Foster City, CA, USA.

Confocal Dyes

Rat primary antibody for dopamine D1 (No: NRG 01691597), D2 (No: LV 1583420) Bromo deoxyuridine (Cat. No. B8434), FITC coated secondary antibody (Cat. No. No-AP307R) and rhodamine coated secondary antibody (No- AP307R) were purchased from Sigma Aldrich and Chemicon, USA.

ANIMALS

Adult male Wistar rats of 12 weeks old and 180-240g body weight purchased from Amrita Institute of Medical Sciences, Cochin and Kerala Agriculture Unviersity, Mannuthy were used for all experiments. They were housed in separate cages under 12 hour light and 12 hour dark periods and were maintained on standard food pellets and water *ad libitum*.

Plant Material

Specimen of *Aegle marmelose* were collected from Cochin University area. The plants were taxonomically identified and authenticated by Prof. K. P. Joseph, Head of the Dept. of Botany (Retd), St. Peter's College, Kolenchery and voucher specimens are deposited at the herbarium of the Centre for Neuroscience, Dept. of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala.

PREPARTION OF PLANT EXTRACT

Fresh leaves of *Aegle marmelose* plant were air dried in shade and powdered. 10g of leaf powder was mixed with 100ml of distilled water and stirred for 2hr. It was kept overnight at 4ºC. The supernatant was collected and evaporated to dryness followed by lyophylization in Yamato, Neocool, Japan lyophilizer. This was used as the crude leaf extract to study the anti diabetic effect in streptozotocin induced diabetes.

DIABETES INDUCTION

Diabetes was induced in rats by intrafemoral injection of streptozotocin (Sigma chemicals Co., St. Louis, MO, U.S.A.) freshly dissolved in citrate buffer pH 4.5 under anaesthesia (Junod *et al.,*1969). Streptozotocin was given at a dose of 55mg/Kg body weight (Hohenegger & Rudas, 1971; Arison *et al.,* 1967).

DETERMINATION OF BODY WEIGHT

Body weight of all experimental group of rats were determined gravimetrically with animal weighing balance (Essae Teraoka, India) on 0^{th}, 7^{th} and 14^{th} day of the experiment.

DETERMINATION OF BLOOD GLUCOSE

The diabetic state of animals was assessed by measuring blood glucose concentrations at 72 hours after streptozotocin treatment. The rats with a blood sugar level above 250 mg/dl were selected as diabetic rats.

DETERMINATION OF ANTI-DIABETIC POTENTIAL OF *AEGLE MARMELOSE* LEAF EXTRACT

Male Wistar rats, weighing 200 to 250g body weight were randomly divided into following groups. Each group consisted of 4-6 animals.

a) Group 1: Control (C)
b) Group 2: Diabetic (D)
c) Group 3: Diabetic treated with insulin (D+I)
d) Group 4: Diabetic treated with *Aegle marmelose* leaf extract (D+A)
e) Group 5: Diabetic treated with pyridoxine (D+P)

The D+I groups received a daily dose (1 Unit/kg body weight) of Lente and Plain insulin (Abbott India) were given for the better control (Sasaki & Bunag, 1983). The last injection was given 24 hr before sacrificing the diabetic rats. Aqueous extract of *Aegle marmelose* was given orally to the 4^{th} group of diabetic rats in the dosage of 1g/Kg body weight (Ponnachan *et al.*, 1993) at 24 hour intervals. 100 mg/kg body weight of pyridoxine dissolved in 0.2 ml of saline injected subcutaneously to the 5^{th} group (Nair *et al.*, 1998). Blood samples were collected from the tail vein at 0 hours (Before the start of the experiment), 3^{rd} day, 6^{th} day, 10^{th} day and 14^{th} day the glucose levels were estimated. Changes in the body weight of animals were monitored 0^{th} day (before the streptozotocin injection), 7^{th} and 14^{th} day.

SACRIFICE AND TISSUE PREPARATION

The animals were then sacrificed on 15^{th} day by decapitation. The corpus striatum, cerebral cortex and brain stem were dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966) and the pancreas, liver was dissected quickly over ice. The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -80° C until assay. All

animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

DETERMINATION OF PROLIFERATING CELLS IN PANCREAS OF EXPERIMENTAL RATS

Pancreatic cell replication was evaluated based on the incorporation of BrdU (Sigma-Aldrich, St. Louis, Mo), a thymidine analog that incorporates into DNA in the S phase All experimental groups of rats were intraperitoneally injected with BrdU in a dosage of 50 mg/kg body weight, dissolved in saline (Masson *et al.*, 2009) on 3rd, 10th day after streptozotocin administration. Control rats received saline alone. They were killed on 15th day.

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{(GOD)}} \text{Gluconic acid} + H_2O_2.$$

$$H_2O_2 + \text{Phenol} \quad \text{4-aminoantipyrene} \xrightarrow{\text{(Peroxidase)}} \text{Coloured complex} + H_2O$$

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration.

The absorbance was read at 510nm in (Shimadzu UV-1700 pharma SPEC) spectrophotometer.

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [^{125}I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin were separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples.

Assay Protocol

Standards, ranging from 0 to 200 μU/ml, insulin free serum and insulin antiserum (50μl each) were added together and the volume was made up to 250μl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated overnight at 2°C. Then [^{125}I] insulin (50μl) was added and incubated at room temperature for 3 hours. The second antibody was added (50μl) along with 500μl of PEG. The tubes were then vortexd and incubated for 20 minutes and they were centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with %B/Bo on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. %B/Bo was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

DOPAMINE RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Total Dopamine receptor binding studies using [^{3}H] Dopamine

Dopamine receptor assay was done using [^{3}H]dopamine according to Madras *et al*., (1988) and Hamblin and Creese, (1982). Brain tissues, pancreas and liver were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01% ascorbic acid, 4mM $MgCl_2$, 1.5mM $CaCl_2$, pH. 7.4 and centrifuged at 38,000 x g for 30min at 4°C. The pellet was centrifuged twice at 38,000 x g for 30min at 4°C and washed twice. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25nM-1.5nM of [^{3}H]DA in 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01% ascorbic acid, 1mM $MgCl_2$, 2mM $CaCl_2$, 120mM NaCl, 5mM KCl, pH.7.4 in a total incubation volume of 250µl containing 100-200µg of protein. Specific binding was determined using 100µM unlabelled dopamine.

Tubes were incubated at 25°C for 60min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Dopamine D_1 receptor binding studies using [^{3}H]SCH 23390

Dopamine D_1 receptor binding assay using [^{3}H]SCH 23390 in the brain regions, pancreas and liver were done according to the modified procedure of Mizoguchi *et al*., (2000). The tissues were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM $MgCl_2$, 1.5mM $CaCl_2$, 5mM KCl, pH. 7.4. The homogenate was centrifuged at 40,000 x g for 30min. The pellet was washed and centrifuged with 50 volumes of the buffer at 40,000 x g for 30min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.5 - 5.0nM of [^{3}H]SCH 23390 in 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM $MgCl_2$, 1.5 mM $CaCl_2$, 5mM KCl with 12µM pargyline and 0.1% ascorbic acid in a total incubation volume of 250µl containing 100-200µg protein with 50µM unlabelled SCH 23390.

Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/B filters. The filters were washed quickly by three successive washing with 5.0ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Dopamine D_2 receptor binding studies using [^{3}H]YM-09151-2

Dopamine D_2 receptor binding assay was done according to the modified procedure of Unis *et al*., (1998) and Madras *et al*., (1988). The dissected brain tissues, pancreas and liver were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl, 5mM KCl, pH 7.4. The homogenate was centrifuged at 40,000 x g for 30min. The pellet was washed and centrifuged with 50 volumes of the buffer at 40,000 x g for 30min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.1 - 2.0nM of [^{3}H]YM-09151-2 in 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl, 5mM KCl with 10µM pargyline and 0.1% ascorbic acid in a total incubation volume of 300µl containing 100-200µg of protein. Specific binding was determined using 5.0µM unlabelled sulpiride. Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Protein Determination

Protein was measured by the method of Lowry *et al*., (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in Spectrophotometer at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS

Isolation of RNA

RNA was isolated from the brain regions, pancreas and liver of control and experimental rats using the Tri reagent from Sigma Chemicals Co., St. Louis, MO, U.S.A). 25-50 mg tissue homogenates were made in 0.5 ml Tri Reagent. The homogenate was kept in the room temperature for 5 minutes. 100 µl of chloroform was added to the homogenate, mixed vigorously for 15 seconds kept in the RT for 10-15 minutes and was centrifuged at 12,000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000xg for 10 minutes at 4°C. RNA precipitated as a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 minutes at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42µg.

REAL-TIME POLYMERASE CHAIN REACTION

cDNA synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA arhive kit in 0.2ml microfuge tubes. The reaction mixture of 20 µl contained 0.2µg total RNA, 10 X RT buffer, 25 X dNTP mixture, 10 X random primers, MultiScribe RT (50U/µl)

and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 minutes and 37 °C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express software version (3.0).

Real-time PCR assays

Real Time PCR assays were performed in 96-well plates in an ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase "polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe, designed by Applied Biosystems. All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The Real-Time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20 µl contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and PCR analyses were conducted with gene-specific primers and fluorescently labelled Taqman probes of Dopamine D_1 (Rn_02043440) Dopamine D_2 (Rn_00561126), CREB (Rn_00578826), Tyrosine hydroxylase (Rn_00562500), IR (Rn_00567070), Glut-3 (Rn00567331), Glut-2 (Rn00563565), IGF-1 (Rn_99999087), phospholipase-C (Rn_01647142), SOD (Rn_01477289), GPX (Rn_00577994), Bax (Rn_01480160), TNF-α (Rn_99999017), Nfκb (Rn01399583), AKT-1(Rn_00583646), caspase-8 (Rn_00574069), BDNF (Rn_01484924), pdx-1 (Rn_00755591), Neuro-D1 (Rn_00824571). Endogenous control (β-actin) was labeled with a reporter dye (VIC). 12.5 µl of TaqMan 2X Universal PCR Master Mix was taken and the volume was

made up with RNAse free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50ºC -- 2 minutes ---- Activation

95ºC -- 10 minutes ---- Initial Denaturation

95ºC -- 15 seconds ---- Denaturation 40 cycles

50ºC -- 30 seconds --- Annealing

60ºC -- 1 minutes --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{- actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta}C\ T$).

IP_3 CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

Brain tissues-(CS, CC, BS), pancreas and liver was homogenised in a polytron homogeniser in 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 minutes and the supernatant was transferred to fresh tubes for IP_3 assay using $[^3H]IP_3$ Biotrak Assay System kit.

Principle of the assay

The assay was based on competition between [^{3}H]IP_3 and unlabelled IP_3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP_3 was then separated from the free IP_3 by centrifugation. The free IP_3 in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP_3 in the sample to be determined.

Assay Protocol

Standards, ranging from 0.19 to 25pmoles/tube, [^{3}H]IP_3 and binding protein were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15min and they were centrifuged at 2000 x g for 10min at 4°C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10min. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with %B/Bo on the Y-axis and IP_3 concentration (pmoles/tube) on the X-axis of a semi-log graph paper. $\%B/B_o$ was calculated as:

$$\frac{\text{(Standard or sample cpm} - \text{NSB cpm)}}{\text{(}B_0\text{ cpm} - \text{NSB cpm)}} \times 100$$

NSB- non specific binding and B_0 - zero binding. IP_3 concentration in the samples was determined by interpolation from the plotted standard curve.

cGMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

Brain tissues- (CS, CC, BS), pancreas and liver was homogenised in a polytron homogeniser with cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cGMP assay using [^{3}H]cGMP Biotrak Assay System kit.

Principle of the assay

The assay is based on the competition between unlabelled cGMP and a fixed quantity of the [^{3}H]cGMP for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of [^{3}H]cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurement of the antibody bound radioactivity enables the amount of unlabelled cGMP in the sample to be calculated. Separation of the antibody bound cGMP from the unbound nucleotide was done by ammonium sulphate precipitation, followed by centrifugation. The precipitate which contains the antibody bound complex was dissolved in water and its activity was determined by liquid scintillation counting. The concentration of unlabelled cGMP in the sample was determined from a linear standard curve.

Assay Protocol

Standards, ranging from 0.5 to 4.0pmoles/tube, and [^{3}H]cGMP were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The antiserum was added to all the assay tubes and then vortexed. The tubes were incubated for 90min at 2 - 8°C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5min in ice bath. The tubes were centrifuged at 12000 x g for 2min at room temperature. The supernatant was aspirated out and the pellet was dissolved in water

and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with C_o/C_x on the Y-axis and cGMP concentration (pmoles/tube) on the X-axis of a linear graph paper. C_o- the cpm bound in the absence of unlabelled cGMP; C_x- the cpm bound in the presence of standard/unknown cGMP. cGMP concentration in the samples was determined by interpolation from the plotted standard curve.

cAMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

Brain tissues-(CS, CC, BS) pancreas and liver was homogenised in a polytron homogeniser with cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cAMP assay using [^{3}H]cAMP Biotrak Assay System kit.

Principle of the assay

cAMP assay kit was used. The assay is based on the competition between unlabelled cAMP and a fixed quantity of [^{3}H]cAMP for binding to a protein which has a high specificity and affinity for cAMP. The amount of labeled protein -cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated.

Free [^{3}H] cAMP			Bound [^{3}H] cAMP-binding protein
	+ Binding protein	=	+
cAMP			cAMP-binding protein

Separation of the protein bound cAMP from unbound nucleotide is achieved by adsorption of the free nucleotide on to a coated charcoal followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

Assay Protocol

The tubes were placed on a water bath at 0°C. The assay mixture consisted of different concentrations of standard, [^{3}H]cAMP and binding protein in case of standards; buffer, [^{3}H]cAMP and binding protein for zero blank and unknown samples, [^{3}H]cAMP and binding protein for determination of unknown samples. The mixture was incubated at 2°C for 2h. Cold charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 x g for 2min at 2°C. Aliquots of the supernatant was immediately transferred to scintillation vials and mixed with cocktail-T and counted in a liquid scintillation counter (Wallac, 1409).

C_o/C_x is plotted on the Y-axis against picomoles of inactive cAMP on the X-axis of a linear graph paper, where C_o is the counts per minute bound in the absence of unlabelled cAMP and C_x is the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the C_o/C_x value for the sample, the number of picomoles of unknown cAMP was calculated.

IMMUNOCYTOCHEMISTRY OF DOPAMINE D_1, D_2 RECEPTORS IN BRAIN REGIONS AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Anaesthetized animals with chloral hydrate were transcardially perfused with 4%paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 30 µm sections

were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.05% Triton X-100) for 20 min. Brain sections were blocked with 5% normal goat serum for 4 hours. Brain sections were then incubated overnight at 4 °C with either rat primary antibody for Dopamine D_1 (No: NRG 01691597 Millipore, 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum) and Dopamine D_2. (No: LV 1583420 Millipore, 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum). After overnight incubation brain sections washed with PBS and then incubated for 1 hour with secondary antibody conjugated with FITC (No: AB7130F, Chemicon, 1:1000 dilution dilution in a 1X PBS solution containing 5% normal goat serum) in brain regions. In pancreas secondary antibody conjugated with rhodamine (No- AP307R, 1:1000 dilution in a 1X PBS solution containing 5% normal goat serum) was used. After the incubations brain sections were washed with PBS. Tap excess PBS off, the slides and mount cover glass with Prolong Gold anti-fade mounting media.The sections were observed and photographed using confocal imaging system (Leica SP 5).

Fluoro Jade- C staining

Fluoro Jade C staining was used to reveal degenerating neurons in the striatum of diabetic rats, following the protocol of Schmued *et al.,* (2005). Rats belong to different groups, control (C), diabetic (D), insulin treated diabetic (D+I), diabetic treated with *Aegle marmelose* (D+A), diabetic treated with pyridoxine (D+P) were used for the experiments. Rats were anaesthetized with chloral hydrate and transcardially perfused with 4%paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 30 µm sections were cut using Cryostat (Leica, CM1510 S). Prior to staining, sections were mounted on gelatin coated slides. Gelatin coated slides were prepared by immersion in a 60 °C solution of 1% pig skin gelatin [Sigma; type A, 300 Bloom]

and then oven dried overnight at the same temperature. Slides bearing frozen cut tissue sections were first immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min. They were then rinsed for 2 min in 70% ethanol, for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 min. Slides were then transferred for 10 min to a 0.0001% solution of Fluoro Jade-C (Histo-Chem Inc.; Jefferson, AR) dissolved in 0.1% acetic acid vehicle. The proper dilution was accomplished by first making a 0.01% stock solution of the dye in distilled water and then adding 1 ml of the stock solution to 99 ml of 0.1% acetic acid vehicle. The slides were then rinsed through three changes of distilled water for 1 min per change. Excess water was drained onto a paper towel, and the slides were then air dried on a slide warmer at 50°C for at least 5 min. The air dried slides were then cleared in xylene for at least 1 min and then coverslipped with DPX (Fluka or Sigma) nonfluorescent mounting media. The density of Fluoro-Jade C staining was calculated within striatum in five 30µ sections from 6 animals per group. Images were taken in SP5 Confocal microscope and images were analyzed using Image-J 1.41 software (NIH, Bethesda MD, USA). In order to count equal areas in every subject, a 1024× 1024 pixel frame comprising exclusively the anatomical areas of interest was applied. Using Image-J cell counter plug in, the cells were counted. The density measurements were representative of the analyzed areas. The data are presented as the mean of cell numbers ± SEM.

IMMUNOCYTOCHEMISTRY OF BROMODEOXYURIDINE IN THE PANCREAS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Anaesthetized animals with chloral hydrate were transcardially perfused with 4%paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion the pancreas was dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 30 µm sections

were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.05% Triton X-100) for 20 min. pancreatic sections were blocked with 5% normal goat serum for 4 hours. Pancreatic sections were then incubated overnight at 4 °C with mouse primary antibody for Bromo deoxyuridine (BrdU) (Cat. No. B8434, Sigma 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum). After overnight incubation pancreatic sections washed with PBS and then incubated for 1 hour with secondary antibody conjugated with rhodamine (No- AP307R, 1:1000 dilution in a 1X PBS solution containing 5% normal goat serum). After the incubation, the pancreatic sections were washed with PBS. Tap excess PBS of, the slides and mount cover glass with Prolong Gold anti-fade mounting media. The BrdU-labeled cell quantification was performed as described. The sections were observed and photographed using confocal imaging system (Leica SP 5).

STATISTICS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). Relative Quantification Software was used for analyzing Real-Time PCR results.

Results

Body weight of control and experimental rats

The body weight was significantly decreased ($p<0.001$) in diabetic rats when compared to control group. After treatment with insulin, *Aegle marmelose* leaf extract and pyridoxine supplementation for 14 days, the body weight was significantly reversed ($p<0.001$) when compared with diabetic group (Figure-1, Table-1).

Blood glucose level of control and experimental rats

Blood glucose level of all rats before streptozotocin administration was within the normal range. Streptozotocin administration led to a significant increase ($p<0.001$) in blood glucose level of diabetic group when compared to control group. Insulin, *Aegle marmelose* and pyridoxine treatments significantly reversed ($p<0.001$) the increased blood glucose level when compared with diabetic group (Figure-2, Table-2).

Circulating insulin level in the plasma of control and experimental rats

Circulating insulin level was significantly decreased ($p<0.001$) in diabetic group when compared with control. Treatment using insulin, *Aegle marmelose* leaf extract and pyridoxine significantly increased ($p<0.001$) the plasma insulin level to near control when compared with diabetic group (Figure-3, Table-3).

CORPUS STRIATUM

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH 23390 in the corpus striatum of control and experimental rats

In diabetic group, dopamine D_1 receptors were significantly decreased ($p<0.001$) with a significant decrease in K_d ($p<0.001$) compared to control. Insulin treated group showed significant reversal in K_d ($p < 0.001$) but B_{max} was not

significantly changed compared to diabetic group. In *Aegle marmelose* and pyridoxine treated group, B_{max} ($p < 0.01$) and K_d ($p < 0.001$) significantly reversed compared to diabetic group (Figure-4, Table-4).

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 against sulpiride in the corpus striatum of control and experimental rats

Binding studies of [^{3}H]YM-09151-2 against sulpiride for dopamine D_2 receptors showed that the binding parameters- B_{max} ($p<0.001$) and K_d ($p <0.001$) significantly increased in the corpus striatum of diabetic rats compared to control. Insulin, *Aegle marmelose* and Pyridoxine treated diabetic groups showed significant reversal in B_{max} ($p<0.001$) and K_d ($p<0.001$, $p < 0.05$, $p<0.01$) compared to diabetic group (Figure-5, Table-5).

Scatchard analysis of total dopamine receptor using [^{3}H] dopamine against dopamine in the corpus striatum of control and experimental rats

In diabetic group, total dopamine receptors were significantly decreased ($p<0.001$) with a significant decrease in K_d ($p<0.001$) compared to control. Insulin and *Aegle marmelose* treated group showed a significant reversal in B_{max} ($p<0.01$, $p<0.001$) with a significant increase in K_d ($p<0.001$, $p<0.01$) compared to diabetic group. In pyridoxine treated group both B_{max} and K_d significantly reversed to near control ($p < 0.001$) (Figure-6, Table-6).

Real Time amplification of dopamine D_1 receptor mRNA in the corpus striatum of control and experimental rats

Gene expression of dopamine D_1 receptor showed significant down regulation ($p<0.001$) in the corpus striatum of diabetic rats compared to control. Treatment using insulin significantly ($p<0.01$) reversed compared to diabetic group whereas *Aegle*

marmelose and pyridoxine significantly ($p<0.001$) reversed the altered expression to near control (Figure-7, Table-7).

Real Time amplification of dopamine D_2 receptor mRNA in the corpus striatum of control and experimental rats

Gene expression of dopamine D_2 receptor showed significant up regulation ($p<0.001$) in the corpus striatum of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.01$) reversed compared to diabetic (Figure-8, Table-8).

Real Time amplification of insulin receptor mRNA in the corpus striatum of control and experimental rats

Gene expression of insulin receptor showed significant down regulation ($p<0.001$) in the corpus striatum of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed the expression compared to diabetic group (Figure-9, Table-9).

Real Time amplification of GLUT-3 mRNA in the corpus striatum of control and experimental rats

Gene expression of GLUT-3 showed significant up regulation ($p<0.001$) in the corpus striatum of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* significantly ($p<0.05$) reversed GLUT-3 expression compared to diabetic group. Pyridoxine treatment ($p<0.001$) significantly reversed compared to diabetic group (Figure-10, Table-10).

Real Time amplification of IGF-1 mRNA in the corpus striatum of control and experimental rats

Gene expression of IGF-1 showed significant down regulation ($p<0.001$) in the corpus striatum of diabetic group compared to control. Insulin treatment did not show significant reversal when compared with diabetic group. Treatment using *Aegle marmelose* significantly ($p<0.01$) reversed these changes and pyridoxine treatment significantly reversed and upregulated ($p<0.001$) when compared with diabetic group (Figure-11, Table-11).

Real Time amplification of CREB mRNA in the corpus striatum of control and experimental rats

Gene expression of CREB showed significant down regulation ($p<0.001$) in the corpus striatum of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed the expression, compared with diabetic group (Figure-12, Table-12).

Real Time amplification of phospholiase-C mRNA in the corpus striatum of control and experimental rats

Gene expression of phospholiase-C showed significant down regulation ($p<0.001$) in the corpus striatum of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.05$) reversed the expression, when compared with diabetic group (Figure-13, Table-13).

Real Time amplification of TNF- α mRNA in the corpus striatum of control and experimental rats

Gene expression of TNF- α showed significant up regulation ($p<0.001$) in the corpus striatum of diabetic group compared to control. Treatment using insulin, *Aegle*

marmelose and pyridoxine significantly ($p<0.001$) reversed these changes to near control (Figure-14, Table-14).

Real Time amplification of NFκB mRNA in the corpus striatum of control and experimental rats

Gene expression of NFκB significantly ($p<0.001$) up regulated in the corpus striatum of diabetic group compared to control. Insulin, *Aegle marmelose* ($p<0.05$) and pyridoxine ($p<0.001$) treatment significantly reversed compared to diabetic group (Figure-15, Table-15).

Real Time amplification of Akt-1 mRNA in the corpus striatum of control and experimental rats

Gene expression of Akt-1 showed significant down regulation in the corpus striatum of diabetic ($p<0.001$) group. Insulin ($p<0.001$), *Aegle marmelose* and pyridoxine treatment ($p<0.01$) showed significant reversal compared to diabetic group (Figure-16, Table-16).

Real Time amplification of caspase-8 mRNA in the corpus striatum of control and experimental rats

Gene expression of caspase-8 showed significant up regulation ($p<0.001$) in the corpus striatum of diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.05$) reversed these changes compared to diabetic group (Figure-17, Table-17).

Real Time amplification of BDNF mRNA in the corpus striatum of control and experimental rats

Gene expression of BDNF showed significant down regulation ($p<0.001$) in the corpus striatum of diabetic group compared to control. Treatment using insulin,

pyridoxine ($p<0.001$) and *Aegle marmelose* ($p<0.05$) significantly reversed these changes compared to diabetic group (Figure-18, Table-18).

cAMP content in the corpus striatum of control and experimental Rats

cAMP content significantly ($p<0.001$) increased in diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.001$) significantly reversed these changes to near control (Figure-19, Table-19).

cGMP content in the corpus striatum of control and experimental Rats

cGMP content significantly ($p<0.001$) decreased in diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.001$) significantly reversed these changes to near control (Figure-20, Table-20).

IP_3 content in the corpus striatum of control and experimental Rats

IP_3 content significantly ($p<0.001$) decreased in diabetic group compared to control. *Aegle marmelose* and pyridoxine treatment ($p<0.001$) significantly reversed these changes, when compared with diabetic group. Insulin treatment did not show significant reversal compared to diabetic group. (Figure-21, Table-21).

Confocal imaging of dopamine D_1 receptor expression in the corpus striatum of control and experimental rats

Dopamine D_1 receptor antibody staining in the corpus striatum showed significant ($p<0.001$) decrease in mean pixel value of diabetic rats compared to control. Insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.05$) reversed these changes when compared with diabetic group (Figure-22, Table-22).

Confocal imaging of dopamine D_2 receptor expression in the corpus striatum of control and experimental rats

Dopamine D_2 receptor antibody staining in the corpus striatum showed significant ($p<0.001$) increase in mean pixel value of diabetic rats compared to control. Insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed to near control (Figure-23, Table-23).

Flouro Jade-C staining in the corpus striatum of control and experimental rats

Fluoro Jade positive neurons were significantly ($p<0.001$) increased in diabetic group compared to control. The number of fluorojade positive neurons were significantly reversed in insulin ($p<0.01$), *Aegle marmelose* and pyridoxine treated groups ($p<0.001$) when compared with diabetic group.
(Figure-24, Table-24).

CEREBRAL CORTEX

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH 23390 in the cerebral cortex of control and experimental rats

In diabetic group, dopamine D_1 receptors were significantly increased ($p<0.001$) with a significant increase in K_d ($p<0.05$) compared to control. Insulin, *Aegle marmelose* and pyridoxine treated groups showed significant reversal in B_{max} ($p<0.001$). Insulin, pyridoxine treated groups did not show significant reversal in K_d whereas *Aegle marmelose* treatment showed significant reversal ($p<0.001$) and increase in K_d when compared to diabetic group (Figure-25, Table-25).

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 against sulpiride in the cerebral cortex of control and experimental rats

In diabetic group, dopamine D_2 receptors were significantly decreased ($p<0.001$) with a significant decrease in K_d ($p<0.001$) compared to control. Insulin treated group showed significant reversal ($p<0.001$) and increase in B_{max} compared to diabetic group. *Aegle marmelose* treatment did not show significant reversal in B_{max} but K_d significantly ($p<0.05$) reversed compared to diabetic group. Pyridoxine treatment showed significant reversal in B_{max} and K_d ($p<0.001$) compared to diabetic group (Figure-26, Table-26).

Scatchard analysis of total dopamine receptor using [^{3}H] dopamine against dopamine in the cerebral cortex of control and experimental rats

In diabetic group, total dopamine receptors were significantly increased ($p<0.001$) with a significant increase in K_d ($p<0.001$) compared to control. Insulin *Aegle marmelose* and pyridoxine treated group showed a significant reversal ($p<0.001$) in B_{max} compared to diabetic group. Insulin, *Aegle marmelose* ($p<0.001$)

and pyridoxine ($p<0.05$) significantly reversed K_d value compared to diabetic group (Figure-27, Table-27).

Real Time amplification of dopamine D_1 receptor mRNA in the cerebral cortex of control and experimental rats

Gene expression of dopamine D_1 receptor showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed the altered expression when compared with diabetic group (Figure-28, Table-28).

Real Time amplification of dopamine D_2 receptor mRNA in the cerebral cortex of control and experimental rats

Gene expression of dopamine D_2 receptor was significantly down regulated ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment significantly ($p<0.001$) reversed the expression to near control (Figure-29, Table-29).

Real Time amplification of GLUT-3 mRNA in the cerebral cortex of control and experimental rats

Gene expression of GLUT-3 showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed to near control (Figure-30,Table-30).

Real Time amplification of CREB mRNA in the cerebral cortex of control and experimental rats

Gene expression of CREB showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. Treatment using insulin, *Aegle*

marmelose and pyridoxine significantly ($p<0.01$) reversed the expression when compared to diabetic group (Table-31, Figure-31).

Real Time amplification of phospholiase-C mRNA in the cerebral cortex of control and experimental rats

Gene expression of phospholiase-C showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. Treatment using insulin, pyridoxine ($p<0.001$) and *Aegle marmelose* ($p<0.01$) significantly reversed the expression compared to diabetic group (Figure-32, Table-32).

Real Time amplification of TNF- α mRNA in the cerebral cortex of control and experimental rats

Gene expression of TNF- α showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic group compared to control. Treatment using insulin and pyridoxine significantly increased ($p<0.01$, $p<0.001$) further down regulated the expression when compared with diabetic group. *Aegle marmelose* treatment did not show a significant reversal when compared with diabetic group (Figure-33, Table-33).

Real Time amplification of NFκB mRNA in the cerebral cortex of control and experimental rats

Gene expression of NFκB was significantly ($p<0.001$) down regulated in the cerebral cortex of diabetic group when compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment did not show a significant reversal in NFκB expression when compared with diabetic group (Figure-34, Table-34).

Real Time amplification of Akt-1 mRNA in the cerebral cortex of control and experimental rats

Gene expression of Akt-1 showed significant down regulation in the cerebral cortex of diabetic ($p<0.001$) group when compared with control. Insulin, *Aegle marmelose* and pyridoxine treatment did not show a significant reversal in Akt-1 expression when compared with diabetic group (Figure-35, Table-35).

Real Time amplification of caspase-8 mRNA in the cerebral cortex of control and experimental rats

Gene expression of caspase-8 showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic group when compared with control. Treatment using *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed caspase-8 expression when compared with diabetic group. Insulin treated group did not show reversal when compared with diabetic group. (Figure-36; Table-36).

cAMP content in the cerebral cortex of control and experimental rats

cAMP content was significantly ($p<0.001$) increased in diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.001$) significantly reversed these changes to near control (Figure-37, Table-37).

cGMP content in the cerebral cortex of control and experimental rats

cGMP content was significantly ($p<0.001$) decreased in diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.001$) significantly reversed these changes to near control (Figure-38, Table-38).

IP_3 content in the cerebral cortex of control and experimental Rats

IP_3 content was significantly ($p<0.001$) decreased in diabetic group compared to control. *Aegle marmelose* treatment ($p<0.001$) significantly reversed these changes,

when compared with diabetic group. Insulin and pyridoxine treatment did not show significant reversal in IP_3 content when compared with diabetic group. (Figure-39, Table-39).

Confocal imaging of dopamine D_1 receptor expression in the cerebral cortex of control and experimental rats

Dopamine D_1 receptor antibody staining in the cerebral cortex showed significant ($p<0.001$) increase in mean pixel value of diabetic rats compared to control. Insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed these changes, when compared with diabetic group (Figure-40, Table-40).

Confocal imaging of dopamine D_2 receptor expression in the cerebral cortex of control and experimental rats

Dopamine D_2 receptor antibody staining in the cerebral cortex showed significant ($p<0.001$) decrease in mean pixel value of diabetic rats compared to control. Insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.05$) reversed these changes, when compared with diabetic group (Figure-41, Table-41).

BRAIN STEM

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH 23390 in the brain stem of control and experimental rats

Binding studies of [^{3}H] SCH 23390 against SCH 23390 for dopamine D_1 receptors showed that the binding parameters- B_{max} and K_d ($p < 0.001$) significantly increased in the brain stem of diabetic group compared to control. Insulin treatment did not show significant reversal in B_{max} and K_d when compared with diabetic group. *Aegle marmelose* and pyridoxine treated groups showed significant reversal in B_{max} ($p<0.001$) and K_d ($p<0.001$) compared to diabetic group (Figure-42, Table-42).

Scatchard analysis of Dopamine D_2 receptor using [^{3}H]YM-09151-2 against sulpiride in the brain stem of control and experimental rats

In diabetic group, dopamine D_2 receptors were significantly decreased with a significant decrease in B_{max} and K_d ($p<0.001$) compared to control. Insulin treated group did not show a significant reversal in B_{max} and B_{max} value further decreased but K_d was significantly reversed ($p<0.01$) when compared to diabetic group. *Aegle marmelose* and pyridoxine treated group showed significant reversal in B_{max} ($p<0.001$) when compared with diabetic group. K_d of *Aegle marmelose* treated group did not change significantly whereas K_d of pyridoxine group significantly ($p<0.001$) decreased when compared with diabetic group (Figure-43, Table-43).

Scatchard analysis of total dopamine receptor using [^{3}H] dopamine against dopamine in the brain stem of control and experimental rats

In diabetic group, total dopamine receptors were significantly decreased ($p<0.001$) with a significant decrease in K_d ($p<0.001$) compared to control. Insulin, pyridoxine ($p<0.01$) and *Aegle marmelose* ($p<0.05$) treated group showed significant reversal in B_{max} when compared with diabetic group. K_d of insulin ($p<0.001$) and

Aegle marmelose treated group ($p<0.01$) significantly reversed but K_d of pyridoxine treated group did not show a significant reversal when compared to diabetic group (Figure-44, Table-44).

Real Time amplification of dopamine D_1 receptor mRNA in the brain stem of control and experimental rats

Gene expression of dopamine D_1 receptor showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed the altered expression, when compared with diabetic group (Figure-45, Table-45).

Real Time amplification of dopamine D_2 receptor mRNA in the brain stem of control and experimental rats

Gene expression of dopamine D_2 receptor is significantly down regulated ($p<0.001$) in the brain stem of diabetic rats compared to control. Treatment using insulin, pyridoxine ($p<0.001$) and *Aegle marmelose* ($p<0.01$) significantly reversed the expression compared with diabetic group (Figure-46, Table-46).

Real Time amplification of GLUT-3 mRNA in the brain stem of control and experimental rats

Gene expression of GLUT-3 showed significant up regulation ($p<0.01$) in the brain stem of diabetic rats compared to control. Treatment using insulin, pyridoxine ($p<0.01$) and *Aegle marmelose* ($p<0.05$) significantly reversed when compared with diabetic group (Figure-47, Table-47).

Real Time amplification of CREB mRNA in the brain stem of control and experimental rats

Gene expression of CREB showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.01$) reversed when compared with diabetic group (Figure-48, Table-48).

Real Time amplification of phospholiase-C mRNA in the brain stem of control and experimental rats

Gene expression of phospholiase -C showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.05$) reversed the expression when compared with diabetic group (Figure-49, Table-49).

Real Time amplification of TNF- α mRNA in the brain stem of control and experimental rats

Gene expression of TNF- α showed significant up regulation ($p<0.001$) in the brain stem of diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed these changes to near control (Figure-50, Table-50).

Real Time amplification of Akt-1 mRNA in the brain stem of control and experimental rats

Gene expression of Akt-1 showed significant down regulation in the brain stem of diabetic ($p<0.001$) group when compared with control. Insulin ($p<0.05$), *Aegle marmelose* and pyridoxine treatment ($p<0.001$) showed significant reversal compared with diabetic group (Figure-51, Table-51).

Real Time amplification of caspase-8 mRNA in the brain stem of control and experimental rats

Gene expression of caspase-8 showed significant up regulation ($p<0.001$) in the brain stem of diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed these changes, when compared with diabetic group (Figure-52, Table-52).

cAMP content in the brain stem of control and experimental Rats

cAMP content was significantly ($p<0.001$) increased in diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.01$) significantly reversed these changes when compared with diabetic group (Figure-53, Table-53).

cGMP content in the brain stem of control and experimental Rats

cGMP content was significantly ($p<0.001$) increased in diabetic group compared to control. All the treatment groups showed significant ($p<0.001$) reversal when compared with diabetic group (Figure-54, Table-54).

IP_3 content in the brain stem of control and experimental Rats

IP_3 content significantly ($p<0.001$) decreased in diabetic group compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment ($p<0.001$) significantly reversed IP_3 content compared with diabetic group (Figure-55, Table-55).

Confocal imaging of dopamine D_1 receptor expression in brain stem of control and experimental rats

Dopamine D_1 receptor antibody staining in the brain stem showed significant ($p<0.001$) increase in mean pixel value of diabetic rats compared to control. *Aegle marmelose* and pyridoxine treatment significantly ($p<0.001$) reversed when compared

with diabetic group. Insulin treatment did not show significant reversal compared with diabetic group. (Figure-56, Table-56).

Confocal imaging of dopamine D_2 receptor expression in brain stem of control and experimental rats

Dopamine D_2 receptor antibody staining in the brain stem showed significant ($p<0.001$) decrease in mean pixel value of diabetic rats compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment significantly ($p<0.001$) reversed when compared with diabetic group (Figure-57, Table-57).

PANCREAS

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH 23390 in the pancreas of control and experimental rats

Binding studies of [^{3}H] SCH 23390 against SCH 23390 for dopamine D_1 receptors showed that the binding parameters- B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly increased in the pancreas of diabetic group compared to control. Insulin ($p<0.01$), *Aegle marmelose* and pyridoxine ($p<0.001$) treated groups when compared with diabetic group. Insulin treated group did not show significant reversal in K_d whereas *Aegle marmelose* and pyridoxine treatment showed significant ($p<0.001$) reversal in K_d when compared with diabetic group (Figure-58, Table-58).

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 against sulpiride in the pancreas of control and experimental rats

In diabetic group, dopamine D_2 receptors were significantly increased with a significant increase in K_d ($p<0.001$) compared to control. Insulin, *Aegle marmelose* and pyridoxine treated group showed significant ($p<0.001$) reversal in B_{max} when compared with diabetic group. Insulin and pyridoxine treatment did not show significant reversal in K_d but *Aegle marmelose* treatment showed significant ($p<0.001$) reversal in K_d when compared with diabetic group (Figure-59, Table-59).

Scatchard analysis of total dopamine receptor using [^{3}H] dopamine against dopamine in the pancreas of control and experimental rats

In diabetic group, total dopamine receptors were significantly increased ($p<0.01$) with a significant decrease in K_d ($p<0.001$) compared to control. Insulin and pyridoxine treatment showed a significant reversal in B_{max} ($p<0.01$, $p<0.001$) but no significant change in K_d when compared with diabetic group. *Aegle marmelose*

treatment showed no significant reversal in B_{max} and but showed a significant ($p<0.001$) reversal in K_d when compared with diabetic group (Figure-60, Table-60).

Real Time amplification of dopamine D_1 receptor mRNA in the pancreas of control and experimental rats

Gene expression of dopamine D_1 receptor showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed the expression compared with diabetic group (Figure-61, Table-61).

Real Time amplification of dopamine D_2 receptor mRNA in the pancreas of control and experimental rats

Gene expression of dopamine D_2 receptor significantly up regulated ($p<0.001$) in the pancreas of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed to near control (Table-62, Figure-62).

Real Time amplification of GLUT-2 mRNA in the pancreas of control and experimental rats

Gene expression of GLUT-2 showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed compared to diabetic group (Figure-63, Table-63).

Real Time amplification of IGF-1 mRNA in the pancreas of control and experimental rats

Gene expression of IGF-1 showed significant down regulation ($p<0.001$) in the pancreas of diabetic group compared to control. Treatment using insulin, *Aegle*

marmelose and pyridoxine significantly ($p<0.001$) reversed these changes when compared with diabetic group (Table-64, Figure-64).

Real Time amplification of CREB mRNA in the pancreas of control and experimental rats

Gene expression of CREB showed significant down regulation ($p<0.001$) in the pancreas of diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed the expression compared to diabetic group (Figure-65,Table-65).

Real Time amplification of NeuroD1 mRNA in the pancreas of control and experimental rats

Gene expression of NeuroD1 showed significant up regulation ($p<0.001$) in the pancreas of diabetic, insulin, *Aegle marmelose* and pyridoxine treated groups compared to control. *Aegle marmelose* and pyridoxine treatment significantly increased the expression ($p<0.001$) compared to diabetic group (Figure-66, Table-66).

Real Time amplification of PDX-1 mRNA in the pancreas of control and experimental rats

Gene expression of PDX-1 showed significant down regulation ($p<0.001$) in the pancreas of diabetic group compared to control. *Aegle marmelose* and pyridoxine treatment significantly ($p<0.001$) reversed when compared with diabetic group. Insulin treatment did not show significant reversal when compared with diabetic group. (Table-67, Figure-67).

cAMP content in the pancreas of control and experimental Rats

cAMP content significantly ($p<0.001$) decreased in diabetic group compared to control. Treatment using insulin, pyridoxine ($p<0.001$) and *Aegle marmelose*

($p<0.01$) significantly reversed the cAMP content to near control (Figure-68, Table-68).

cGMP content in the pancreas of control and experimental Rats

cGMP content is significantly ($p<0.05$) decreased in diabetic group compared to control. Treatment using insulin ($p<0.001$) significantly reversed to near control. *Aegle marmelose* and pyridoxine treatment significantly ($p<0.01$) reversed cGMP content when compared with diabetic group (Figure-69, Table-69).

IP_3 content in the pancreas of control and experimental Rats

IP_3 content significantly ($p<0.001$) decreased in diabetic group compared to control. *Aegle marmelose* and pyridoxine treatment significantly ($p<0.001$) reversed IP_3 content compared to diabetic group. Insulin treatment did not show any reversal when compared with diabetic group. (Figure-70, Table-70).

Confocal imaging of dopamine D_1 receptor expression in the pancreas of control and experimental rats

Dopamine D_1 receptor antibody staining in the pancreas showed significant ($p<0.001$) increase in mean pixel value of diabetic group compared to control. *Aegle marmelose* and pyridoxine treatment significantly ($p<0.001$) reversed these changes when compared with diabetic group. Insulin treatment did not show any reversal when compared with diabetic group. (Figure-71, Table-71).

Confocal imaging of dopamine D_2 receptor expression in the pancreas of control and experimental rats

Dopamine D_2 receptor antibody staining in the pancreas showed significant ($p<0.001$) increase in mean pixel value of diabetic rats compared to control. *Aegle marmelose* and pyridoxine treatment significantly ($p<0.001$) reversed these changes

when compared with diabetic group**.** Insulin treatment did not show significant reversal when compared with diabetic group. (Figure-72, Table-72).

Confocal imaging of BrdU in the pancreas of control and experimental rats

BrdU positive cells were significantly increased in diabetic group when compared to control. The number of BrdU positive cells were significantly ($p<0.001$) increased further in the pancreas of *Aegle marmelose* and pyridoxine treated rats when compared with diabetic group. Insulin treatment did not show a significant increase in the number of BrdU positive cells when compared with diabetic group (Figure-73, Table-73).

LIVER

Scatchard analysis of total dopamine receptor using [^{3}H] dopamine against dopamine in the liver of control and experimental rats

In diabetic group, total dopamine receptors were significantly increased ($p<0.001$) with a significant increase in K_d ($p<0.001$) compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment showed a significant ($p<0.001$) reversal in B_{max} when compared with diabetic group. Insulin and pyridoxine treatment showed significant reversal in K_d value whereas *Aegle marmelose* treatment showed significant ($p<0.001$) increase in K_d when compared with diabetic group (Figure-74, Table-74).

Real Time amplification of insulin receptor mRNA in the liver of control and experimental rats

Gene expression of insulin receptor was significantly ($p<0.001$) up regulated in the liver of diabetic group. Treatment using pyridoxine significantly ($p<0.001$) reversed compared with diabetic group. Insulin and *Aegle marmelose* treatment did not show significant reversal in insulin receptor expression when compared with diabetic group. (Figure-75, Table-75).

Real Time amplification of GLUT-2 mRNA in the liver of control and experimental rats

Gene expression of GLUT-2 showed significant up regulation ($p<0.001$) in the liver of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.001$) significantly reversed the expression, compared to diabetic group (Figure-76, Table-76).

Real Time amplification of IGF-1 mRNA in the liver of control and experimental rats

IGF-1 gene expression showed significant down regulation ($p<0.001$) in the liver of diabetic group compared to control. Treatment using insulin, *Aegle marmelose* ($p<0.05$) and pyridoxine ($p<0.01$) significantly reversed these changes compared to diabetic group (Figure-77, Table-77).

Real Time amplification of CREB mRNA in the liver of control and experimental rats

Gene expression of CREB showed significant down regulation ($p<0.001$) in the liver of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed the altered expression, compared to diabetic group (Figure-78, Table-78).

Real Time amplification of phospholiase-C mRNA in the liver of control and experimental rats

Phospholiase-C gene expression showed significant up regulation ($p<0.001$) in the liver of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed, compared to diabetic group (Figure-79, Table-79).

Real Time amplification of TNF- α mRNA in the liver of control and experimental rats

TNF- α gene expression showed significant up regulation ($p<0.001$) in the liver of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed compared to diabetic group (Figure-80, Table-80).

Real Time amplification of NFκB mRNA in the liver of control and experimental rats

NFκB gene expression was significantly ($p<0.001$) down regulated in diabetic group compared to control. Treatment groups showed significant ($p<0.001$) reversal towards near control (Figure-81, Table-81).

Real Time amplification of Akt-1 mRNA in the liver of control and experimental rats

Akt-1 gene expression showed significant ($p<0.001$) down regulation in diabetic group when compared with control. Insulin and *Aegle marmelose* treatment showed significant ($p<0.001$) reversal when compared with diabetic group. Pyridoxine treatment showed significant reversal ($p<0.001$) and increased Akt-1 gene expression when compared with diabetic group (Figure-82, Table-82).

Real Time amplification of Caspase-8 mRNA in the liver of control and experimental rats

Gene expression of caspase-8 was significantly ($p<0.001$) up regulated in diabetic group. Insulin and *Aegle marmelose* treatment significantly reversed ($p<0.001$) and decreased the expression of caspase-8 in liver when compared with diabetic group. Pyridoxine treatment significantly ($p<0.05$) reversed the expression when compared with diabetic group (Figure-83, Table-83).

Real Time amplification of SOD mRNA in the liver of control and experimental rats

SOD gene expression showed significant up regulation ($p<0.001$) in the liver of diabetic rats compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine significantly ($p<0.001$) reversed and decreased the expression compared to diabetic group (Figure-84, Table-84).

Real Time amplification of GPX mRNA in the liver of control and experimental rats

GPX gene expression showed significant down regulation ($p<0.001$) in the liver of diabetic group compared to control. Treatment using insulin ($p<0.05$) *Aegle marmelose* and pyridoxine ($p<0.01$) significantly reversed the expression when compared to diabetic group (Figure-85, Table-85).

Real Time amplification of BAX mRNA in the liver of control and experimental rats

Gene expression of BAX showed significant up regulation ($p<0.001$) in the liver of diabetic rats compared to control. Treatment groups showed significant ($p<0.001$) reversal and decrease when compared with diabetic group (Figure-86, Table-86).

cAMP content in the liver of control and experimental rats

cAMP content in liver was significantly ($p<0.001$) decreased in diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.001$) significantly reversed the cAMP content to near control (Figure-87, Table-87).

cGMP content in the liver of control and experimental rats

cGMP content significantly ($p<0.001$) decreased in diabetic group compared to control. Treatment using insulin, *Aegle marmelose* and pyridoxine ($p<0.001$) significantly reversed cGMP content to near control (Figure-88, Table-88).

IP_3 content in the liver of control and experimental rats

IP_3 content is significantly ($p<0.001$) decreased in diabetic group compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment ($p<0.05$) significantly reversed IP_3 content compared to diabetic group (Figure-89, Table-89).

Figure-1

Body weight (g) of control and experimental rats

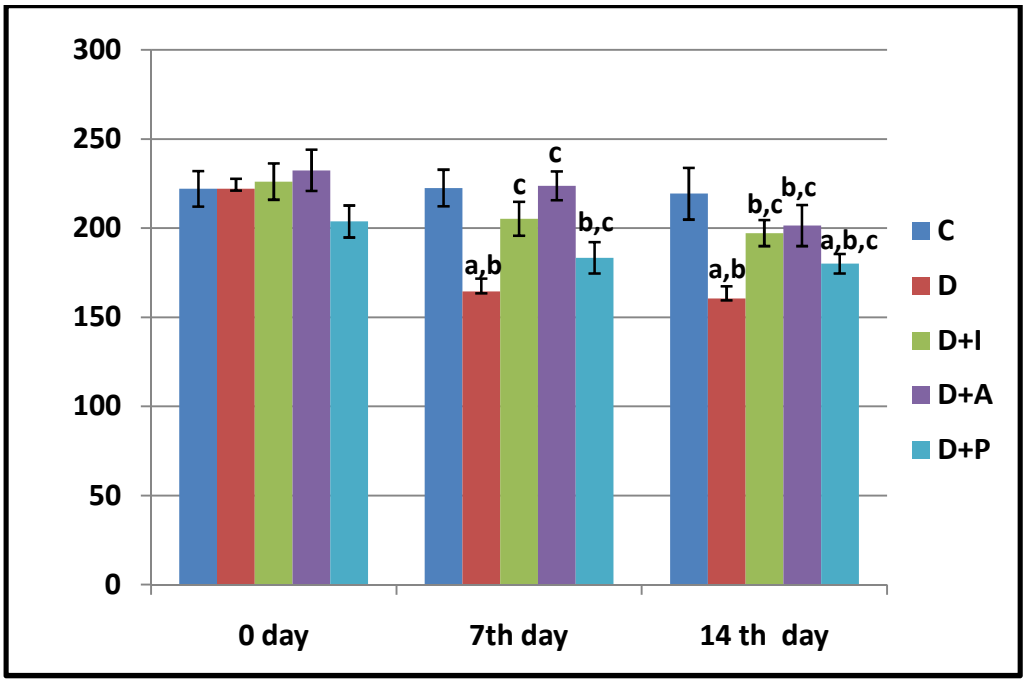


Table-1

Body weight (g) of control and experimental rats

Experimental Groups	Initial	7th day	14th day
C	220.0 ± 10.0	222.5 ± 10.3	219.3 ± 14.5
D	220.0 ± 5.7	164.4 ± 7.3 [a,b]	160.5 ± 6.9 [a,b]
D+I	226.1 ± 10.2	205.2 ± 9.5 [c]	197.2 ± 7.3 [b,c]
D+A	232.4 ± 11.6	223.7 ± 8.1 [c]	201.4 ± 11.5 [b,c]
D+P	203.7 ± 9.0	183.3 ± 8.8 [b,c]	180.0 ± 5.5 [a,b,c]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] p<0.001when compared with control;[b] p<0.001 when compared with initial weight, [c] p<0.001when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-2
Blood glucose (mg/dl) level in control and experimental rats

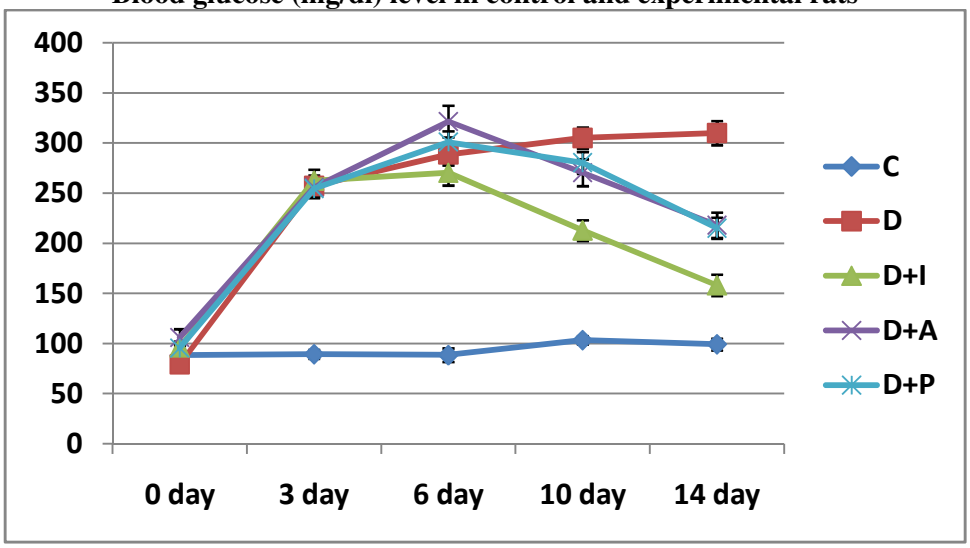


Table-2
Blood glucose (mg/dl) level in control and experimental rats

Experimental Groups	0th day (Before STZ injection)	3rd day (Initial)	6th day	10th day	14th day (Final)
C	88.25±6.1	89.33±4.5	88.41±6.8	103.26± 4.1	99.13±5.9
D	79.14±5.3	257.13±10.4[a]	288.72±10.9 [a]	305.10±10.4[a]	309.96±12.1[a]
D+I	96.09±4.5	262.36±11.1[a]	270.41±12.7 [a]	212.80±10.3[a,d]	158.0 ±10.7[a,d]
D+A	106.09±8.3	255.78±8.2 [a]	321.41±15.8[a,e]	270.40±13.4[a,e]	218.0±12.6[a,d]
D+P	95.61±6.4	254.33±9.3 [a]	300.80±10.9 [a]	280.34±10.9 [a,f]	215.0±10.5 [a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-3

Circulating Insulin level in the plasma of control and experimental rats

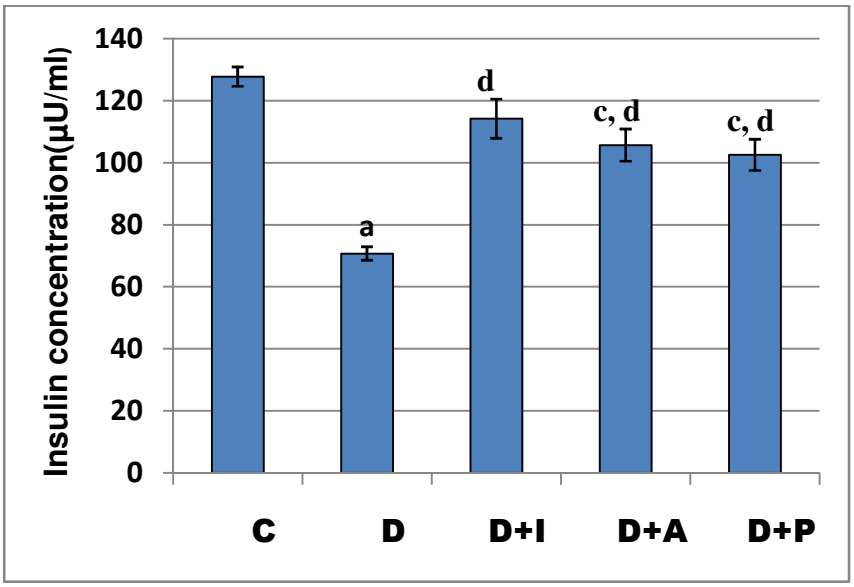


Table-3

Circulating Insulin level in the plasma of control and experimental rats

Experimental Groups	Insulin Concentration (µU/ml)
C	127.73± 3.11
D	70.70± 2.17 [a]
D+I	114.16± 4.31[d]
D+A	105.66± 4.16 [c,d]
D+P	102.50± 4.02 [c,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$ when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-4

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH23390 in the corpus striatum of control and experimental rats

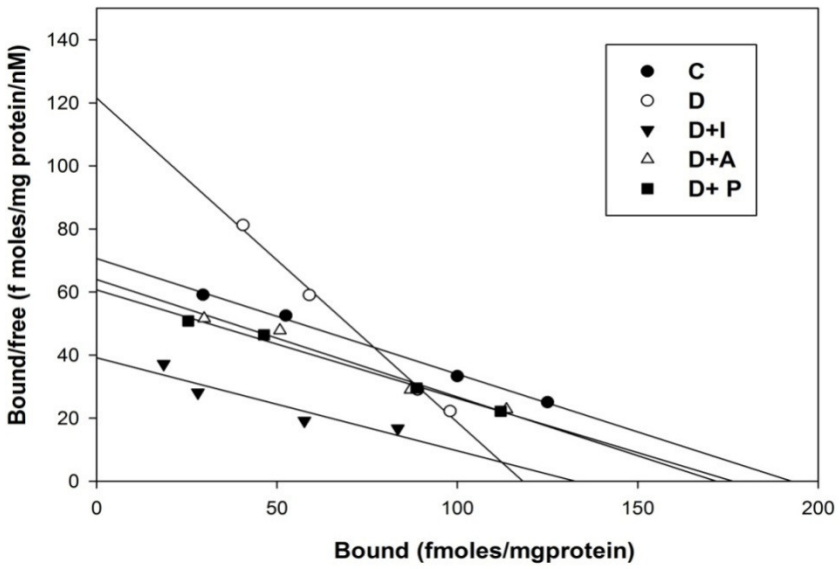


Table-4

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH23390 in the corpus striatum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	191.20 ± 14.25	2.71 ± 0.10
D	127.89 ± 10.80[a]	0.94 ± 0.01 [a]
D + I	139.29 ± 11.20 [b]	3.26 ± 0.13[c, d]
D + A	179.00 ± 13.80[c, e]	2.75 ± 0.14[d]
D + P	170.08 ± 12.80[c, e]	2.83 ± 0.14[d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared with control;[d] $p<0.001$, [e] $p<0.01$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-5

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride in corpus striatum of control and experimental rats

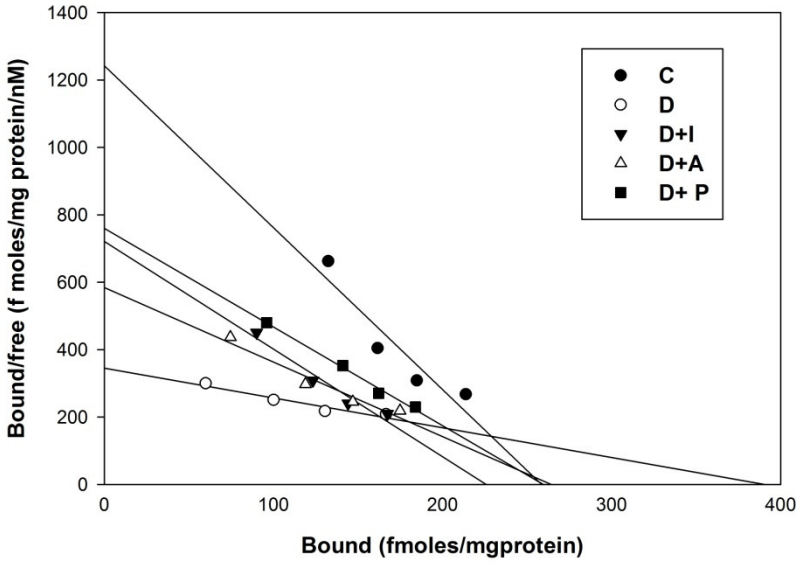


Table-5

Scatchard analysis of dopamine D_2 receptor using[^{3}H]YM-09151-2 binding against sulpiride in corpus striatum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	254.16 ± 10.28	0.19 ± 0.01
D	384.16 ± 10.83[a]	1.10 ±0.05 [a]
D + I	228.32 ± 12.27 [d]	0.30 ± 0.02 [d]
D + A	260.80 ± 10.10[d]	0.45 ± 0.05[f]
D + P	250.80 ± 9.74[d]	0.35 ± 0.04[e]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] p<0.001when compared with control;[d] p<0.001,[e] p<0.01,[f] p<0.05 when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-6
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the corpus striatum of control and experimental rats

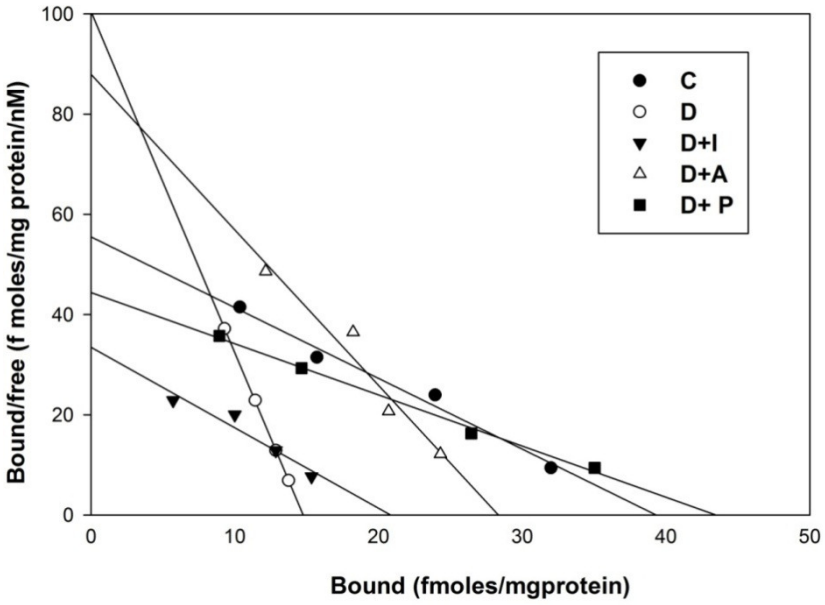


Table-6
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the corpus striatum of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	42.29 ± 3.03	0.78 ±0.03
D	14.89 ± 1.00 [a]	0.14 ± 0.01 [a]
D + I	21.98 ± 2.07[a,e]	0.67 ± 0.04[d]
D + A	28.54 ± 2.10[a,d]	0.32 ± 0.02 [e]
D + P	43.08 ± 2.80[d]	0.95 ± 0.05[d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$ when compared withcontrol;[d] $p<0.001$, [e] $p<0.01$ when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-7
Real Time amplification of dopamine D_1 receptor mRNA in the corpus striatum of control and experimental rats

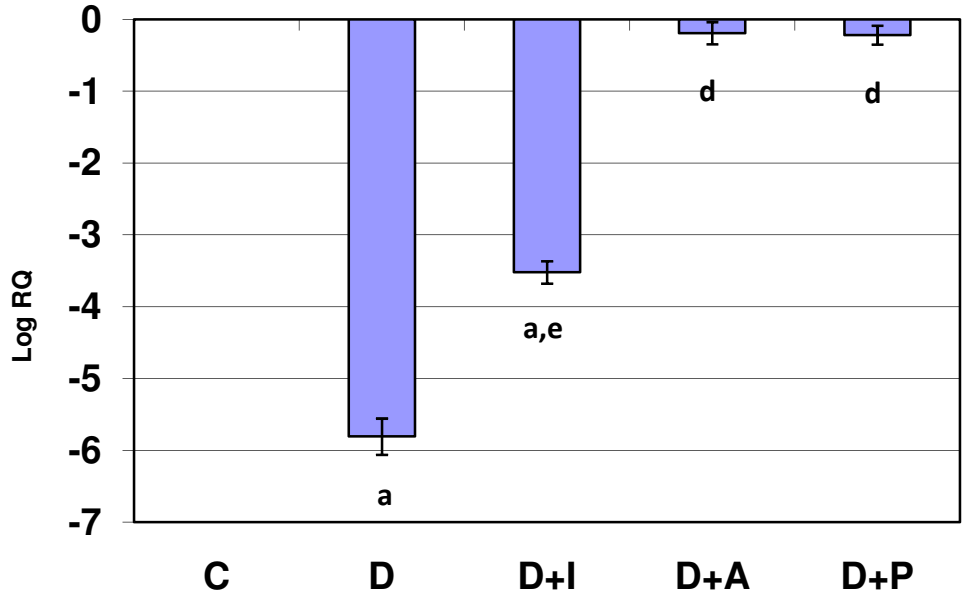


Table-7
Real Time amplification of dopamine D_1 receptor mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	-5.80 ± 0.25^{a}
D+I	$-3.52 \pm 0.05^{a,e}$
D+A	-0.19 ± 0.01^{d}
D+P	-0.20 ± 0.04^{d}

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control; [d] $p<0.001$, [e] $p<0.01$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-8
Real Time amplification of dopamine D_2 receptor mRNA in the corpus striatum of control and experimental rats

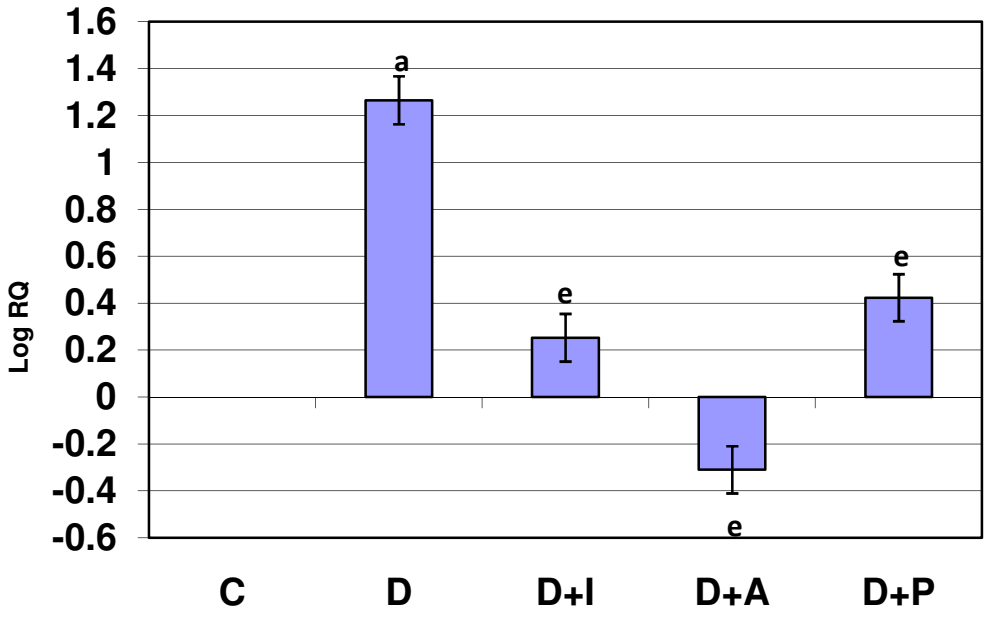


Table-8
Real Time amplification of dopamine D_2 receptor mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	1.26 ± 0.06^{a}
D+I	0.25 ± 0.02^{e}
D+A	-0.30 ± 0.02^{e}
D+P	0.43 ± 0.02^{e}

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-9
Real Time amplification of insulin receptor mRNA inthe corpus striatum of control and experimental rats

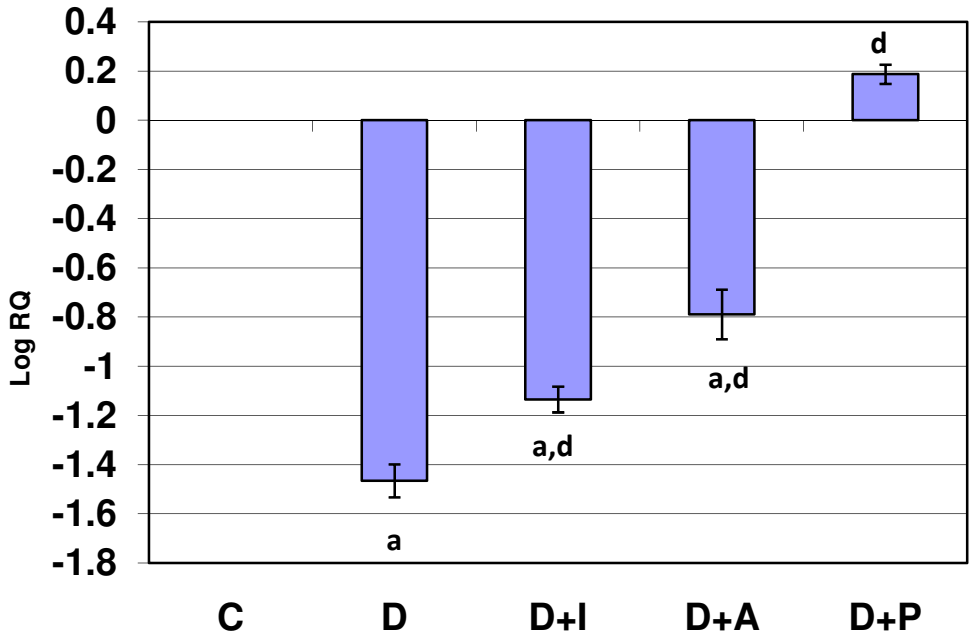


Table-9
Real Time amplification of insulin receptor mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	-1.46 ± 0.05^{a}
D+I	$-1.13 \pm 0.02^{a,d}$
D+A	$-0.78 \pm 0.01^{a,d}$
D+P	0.18 ± 0.01^{d}

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-10
Real Time amplification of GLUT-3 mRNA in the corpus striatum of control and experimental rats

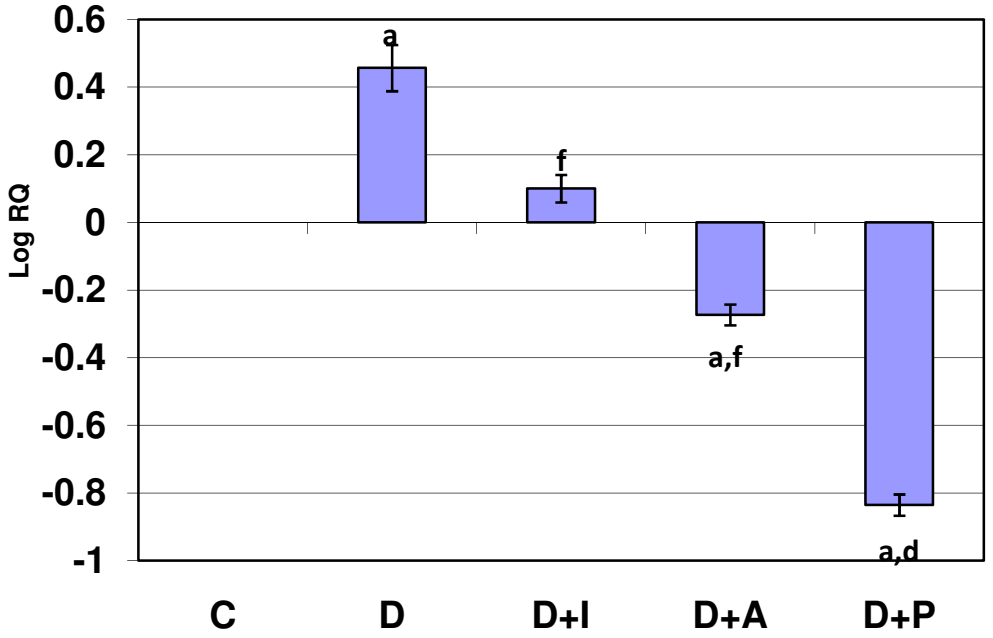


Table-10
Real Time amplification of GLUT-3 mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	0.46 ± 0.01^{a}
D+I	0.10 ± 0.01^{f}
D+A	$-0.27 \pm 0.02^{a,f}$
D+P	$-0.83 \pm 0.03^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control; [d] $p<0.001$; [f] $p<0.05$ when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-11
Real Time amplification of IGF-1 mRNA in the corpus striatum of control and experimental rats

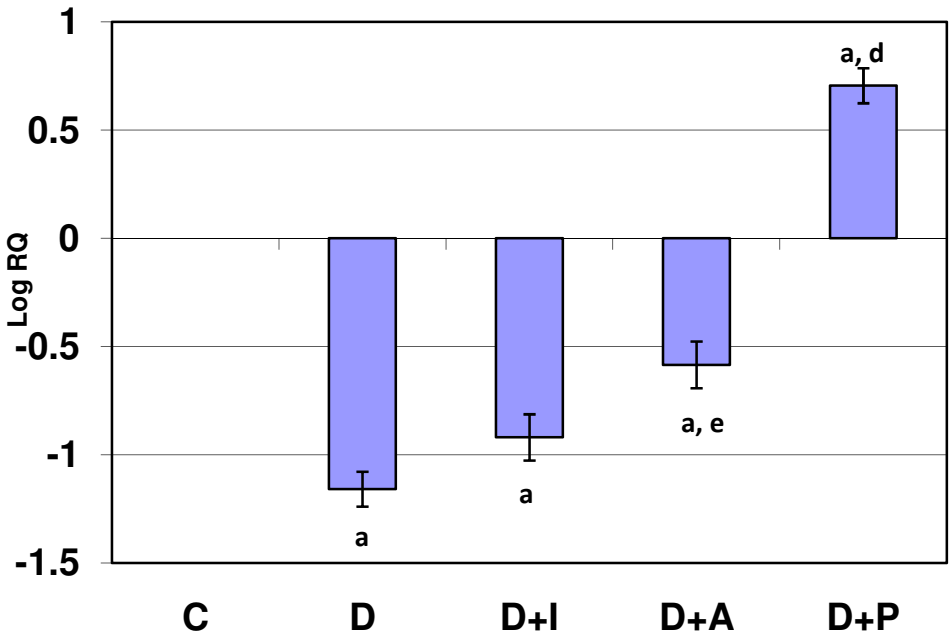


Table-11
Real Time amplification of IGF-1 mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	-1.15 ± 0.04^{a}
D+I	-0.94 ± 0.05^{a}
D+A	$-0.58 \pm 0.05^{a,e}$
D+P	$-0.66 \pm 0.05^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$, [e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-12
Real Time amplification of CREB mRNA in the corpus striatum of control and experimental rats

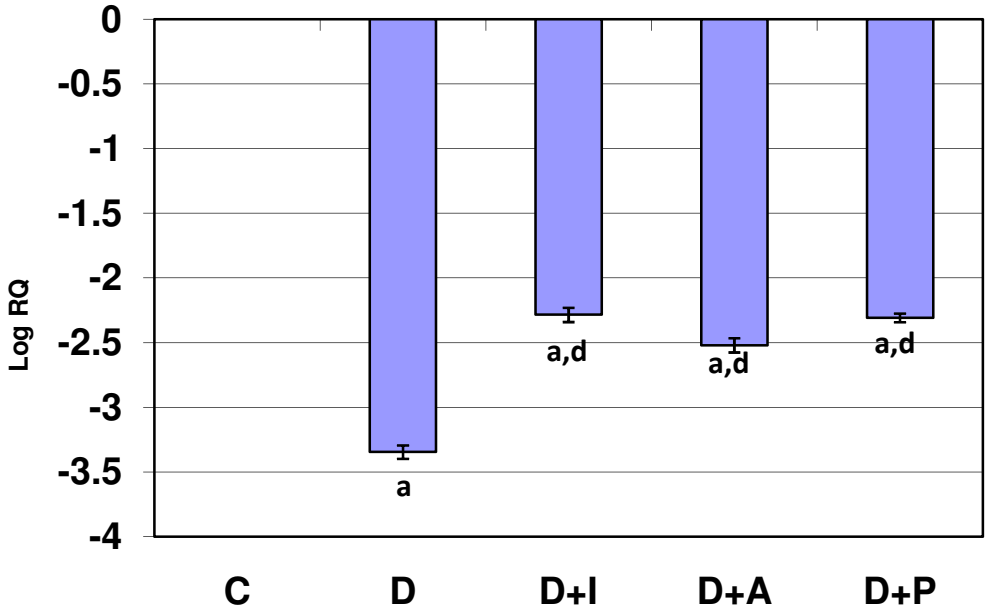


Table-12
Real Time amplification of CREB mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	-3.34 ± 0.04^{a}
D+I	$-2.28 \pm 0.03^{a,d}$
D+A	$-2.51 \pm 0.05^{a,d}$
D+P	$-2.30 \pm 0.05^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-13
Real Time amplification of phospholiase-C mRNA in the corpus striatum of control and experimental rats

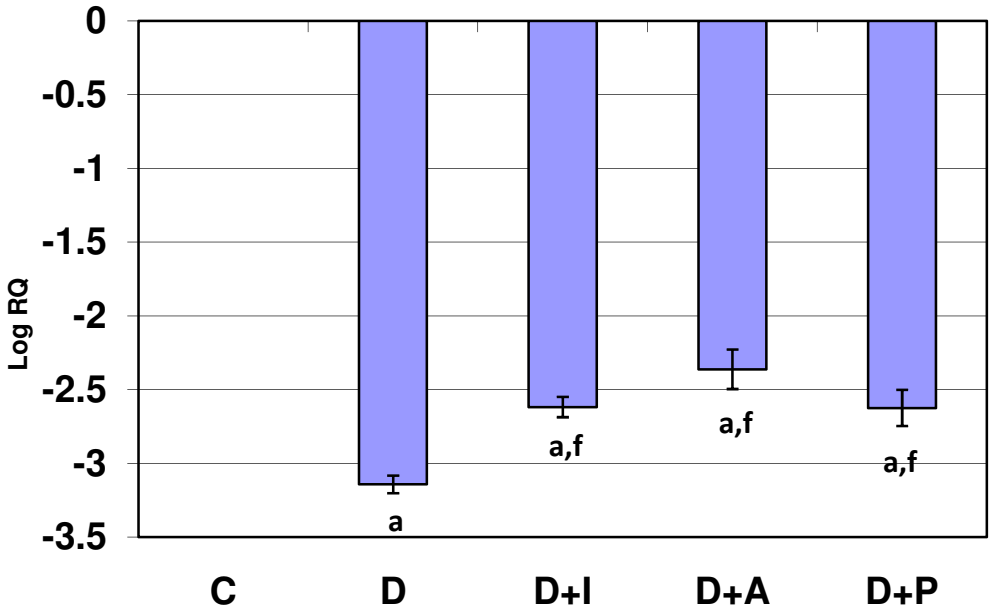


Table-13
Real Time amplification of phospholiase-C mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	-3.14 ± 0.09^{a}
D+I	$-2.61 \pm 0.02^{a,f}$
D+A	$-2.36 \pm 0.05^{a,f}$
D+P	$-2.62 \pm 0.05^{a,f}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-14
Real Time amplification of TNF-α mRNA in the corpus striatum of control and experimental rats

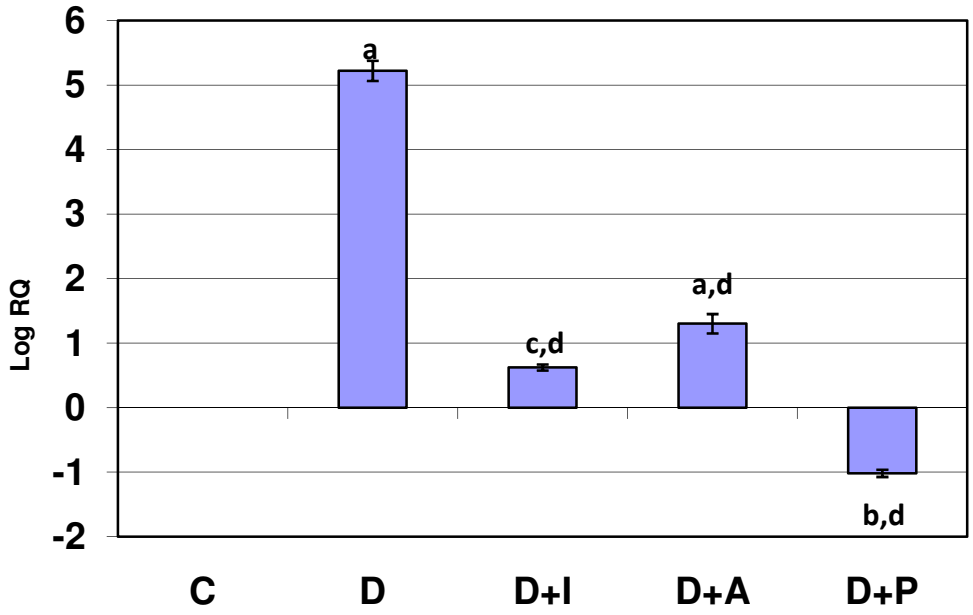


Table-14
Real Time amplification of TNF-α mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	5.22 ± 0.15^{a}
D+I	$0.66 \pm 0.04^{c,d}$
D+A	$1.36 \pm 0.07^{a,d}$
D+P	$-1.01 \pm 0.04^{b,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$,[b] $p<0.01$,[c] $p<0.05$ when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-15
Real Time amplification of NFκB mRNA in the corpus striatum of control and experimental rats

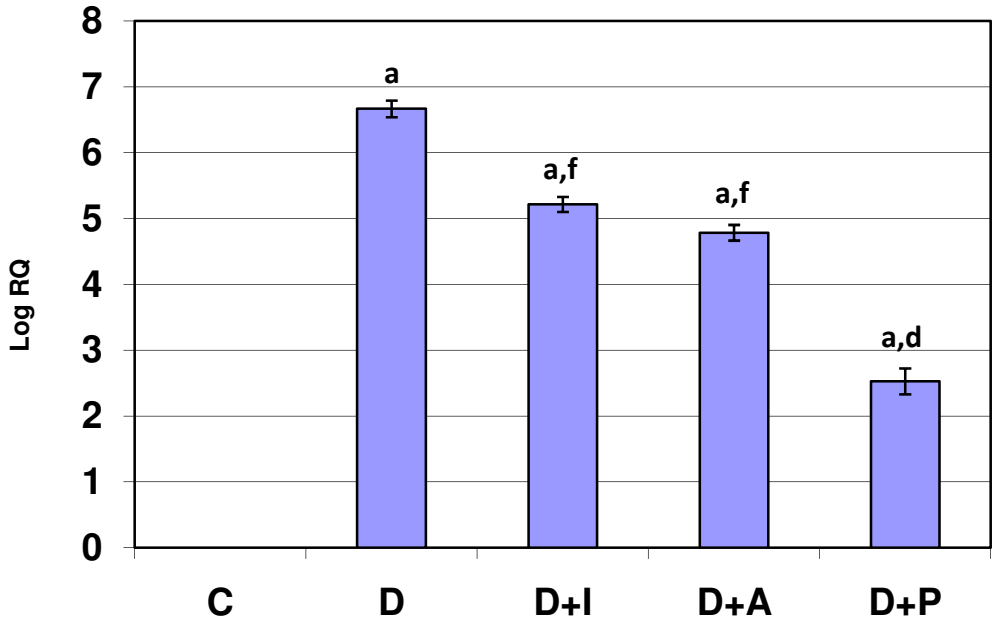


Table-15
Real Time amplification of NFκB mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	6.76 ± 0.11^{a}
D+I	$5.27 \pm 0.05^{a,f}$
D+A	$4.78 \pm 0.09^{a,f}$
D+P	$2.53 \pm 0.13^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$.[f] $p<0.05$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-16
Real Time amplification of Akt -1 mRNA in the corpus striatum of control and experimental rats

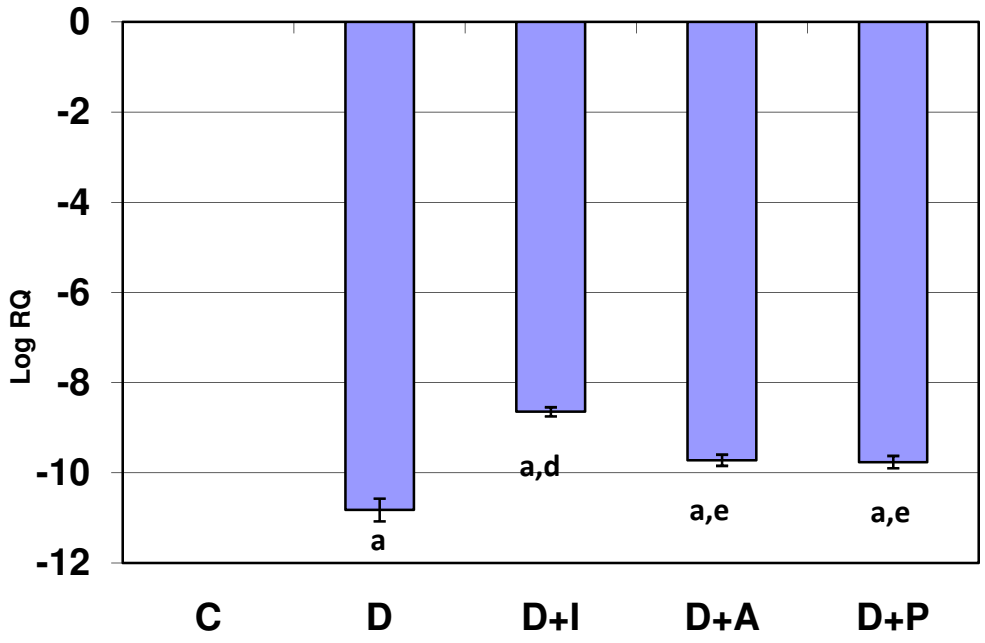


Table-16
Real Time amplification of Akt – 1 mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	-10.82 ± 0.17^{a}
D+I	$-8.41 \pm 0.12^{a,d}$
D+A	$-9.74 \pm 0.13^{a,e}$
D+P	$-9.70 \pm 0.12^{a,e}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$, [e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-17
Real Time amplification of caspase-8 mRNA in the corpus striatum of control and experimental rats

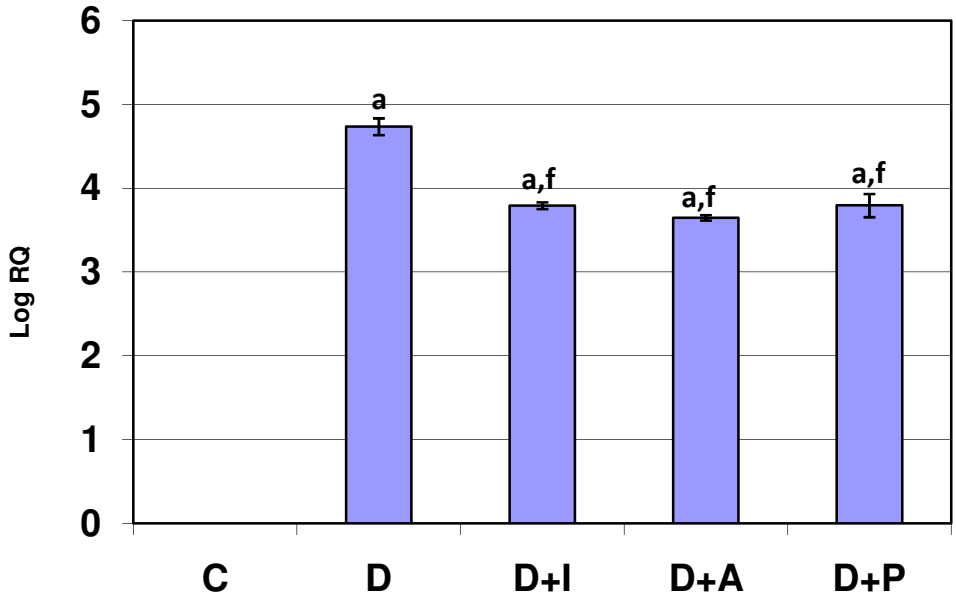


Table-17
Real Time amplification of caspase-8 mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	4.73 ± 0.13^{a}
D+I	$3.81 \pm 0.10^{a,f}$
D+A	$3.64 \pm 0.10^{a,f}$
D+P	$3.79 \pm 0.16^{a,f}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[f] $p<0.05$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-18
Real Time amplification of BDNF mRNA in the corpus striatum of control and experimental rats

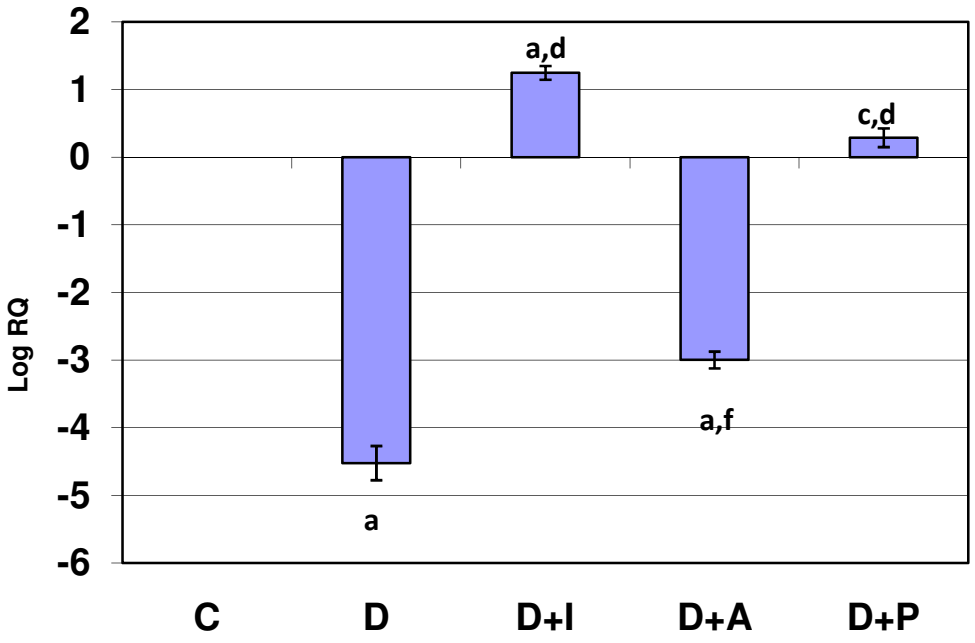


Table-18
Real Time amplification of BDNF mRNA in the corpus striatum of control and experimental rats

Experimental groups	Log RQ
C	0
D	-4.52 ± 0.10^{a}
D+I	$1.24 \pm 0.08^{a,d}$
D+A	$-2.99 \pm 0.08^{a,f}$
D+P	$0.28 \pm 0.01^{c,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$,[c] $p<0.05$when compared with control;[d] $p<0.001$; [f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-19
cAMP content in the corpus striatum of control and experimental rats

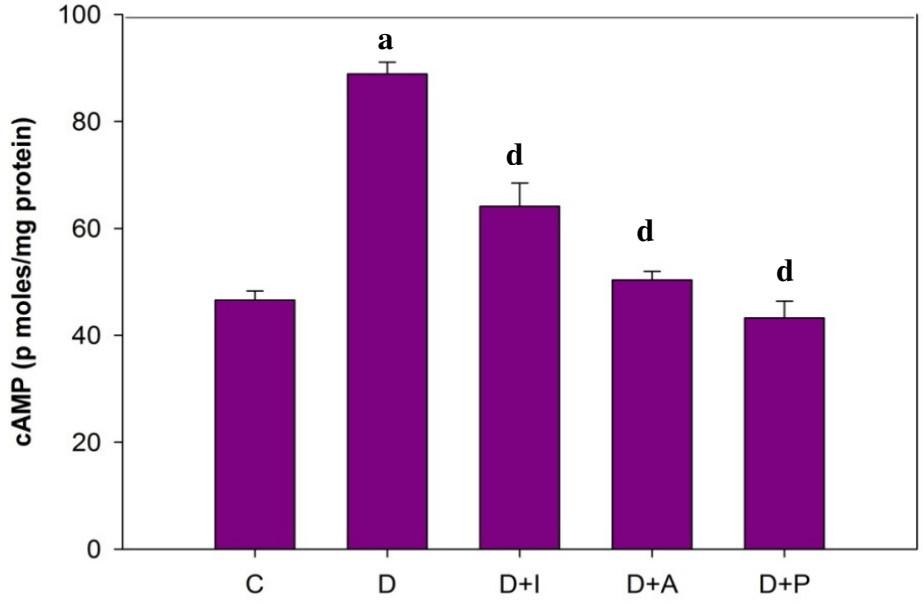


Table-19
cAMP content in the corpus striatum of control and experimental rats

Experimental groups	cAMP (pmoles/mg protein)
C	46.60 ± 1.67
D	88.88 ± 2.18 [a]
D+I	64.11 ± 4.37 [d]
D+A	50.36 ± 1.60 [d]
D+P	43.21 ± 3.19 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-20
cGMP content in the corpus striatum of control and experimental rats

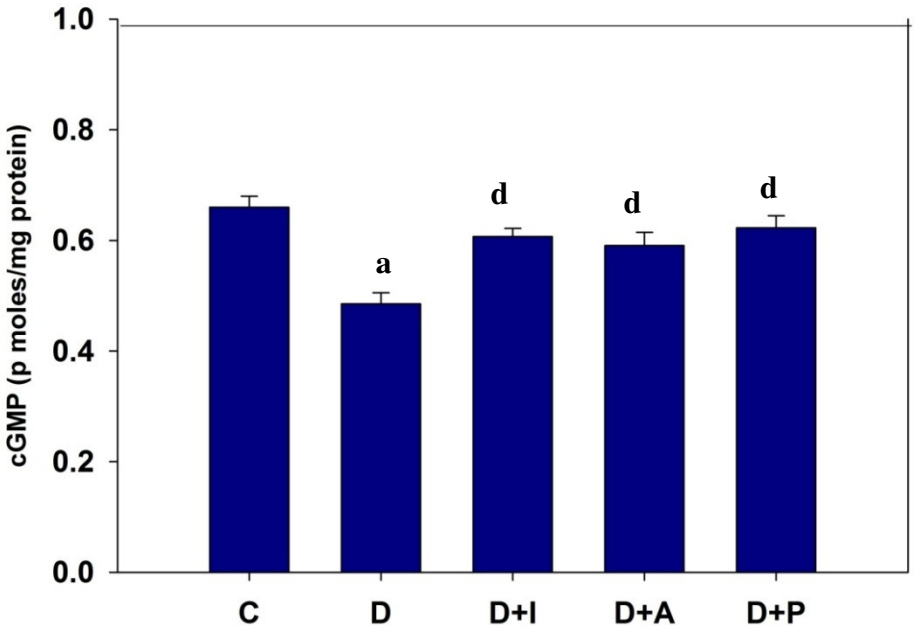


Table-20
cGMP content in the corpus striatum of control and experimental rats

Experimental groups	cGMP (pmoles/mg protein)
C	0.66± 0.02
D	0.48± 0.02[a]
D+I	0.60± 0.01[d]
D+A	0.59± 0.02[d]
D+P	0.62± 0.02[d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-21

IP_3 content in the corpus striatum of control and experimental rats

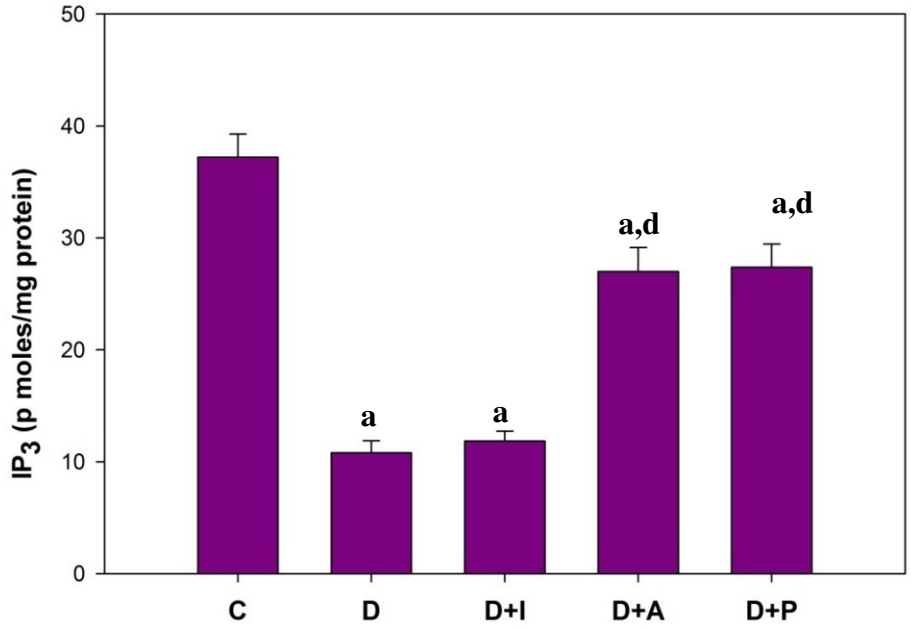


Table-21

IP_3 content in the corpus striatum of control and experimental rats

Experimental groups	IP_3(pmoles/mg protein)
C	37.20± 2.06
D	10.80± 1.08[a]
D+I	11.84± 0.90[a]
D+A	27.00± 2.14[a,d]
D+P	27.36± 2.08 [a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] p<0.001when compared with control;[d] p<0.001when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-22
Confocal imaging of dopamine D_1 receptor in the corpus striatum of control and experimental rats

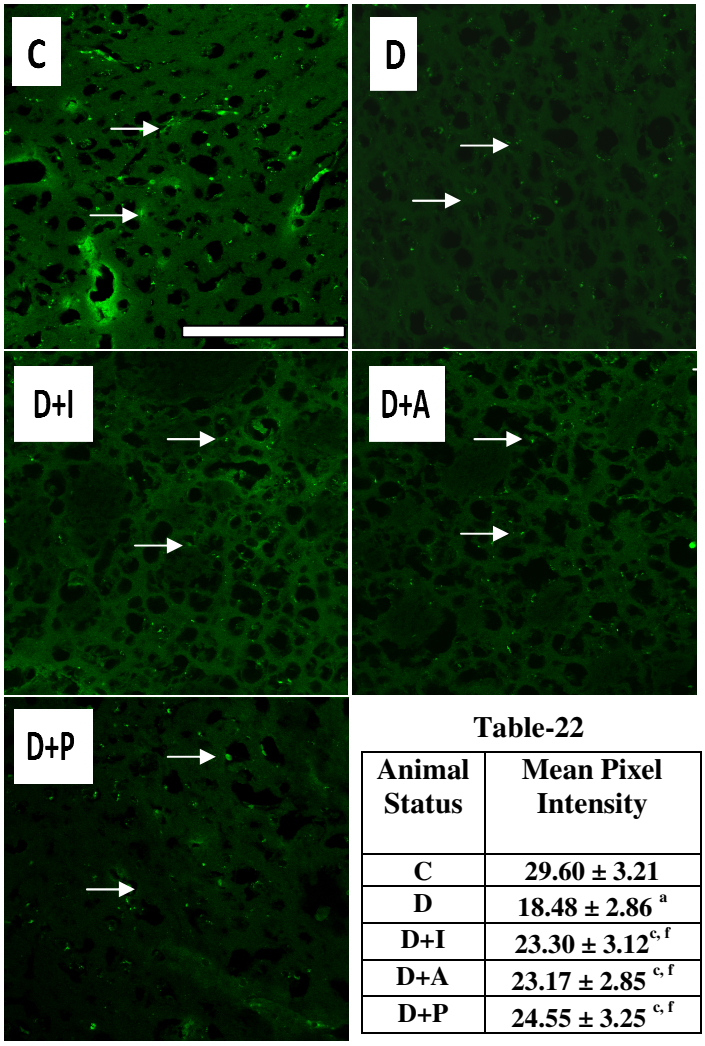


Table-22

Animal Status	Mean Pixel Intensity
C	29.60 ± 3.21
D	18.48 ± 2.86 [a]
D+I	23.30 ± 3.12 [c, f]
D+A	23.17 ± 2.85 [c, f]
D+P	24.55 ± 3.25 [c, f]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$when compared with control;[f] $p<0.05$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.Scale bar represent 200 µm
→ in white shows dopamine D_1 receptor

Figure-23
Confocal imaging of dopamine D_2 receptor expression in the corpus striatum of control and experimental rats

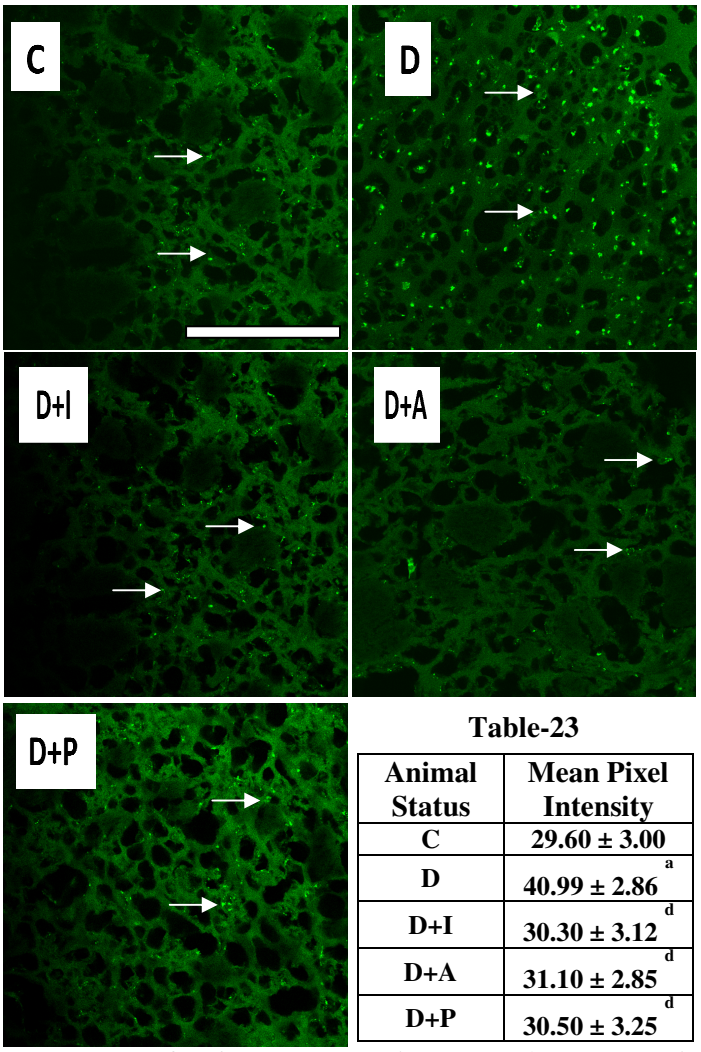


Table-23

Animal Status	Mean Pixel Intensity
C	29.60 ± 3.00
D	40.99 ± 2.86 [a]
D+I	30.30 ± 3.12 [d]
D+A	31.10 ± 2.85 [d]
D+P	30.50 ± 3.25 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control; [d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats. Scale bar - 200 µm. 150µm
→ in white shows Flouro Jade positive neurons

Figure-24
Flouro Jade-C staining in the corpus striatum of control and experimental rats

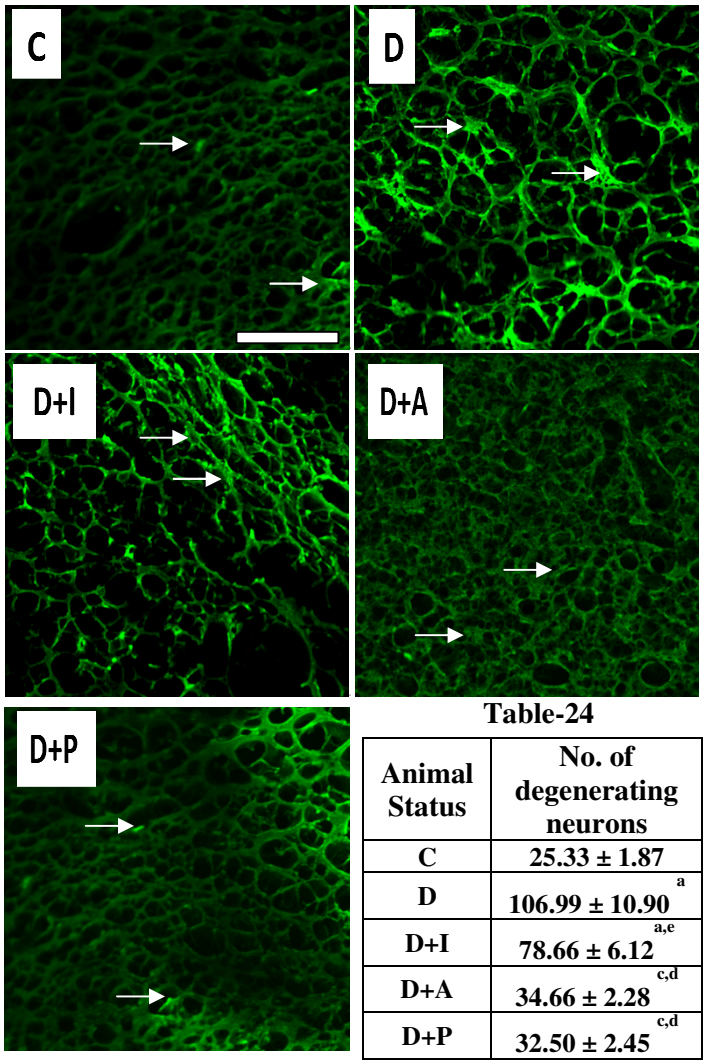


Table-24

Animal Status	No. of degenerating neurons
C	25.33 ± 1.87
D	106.99 ± 10.90 [a]
D+I	78.66 ± 6.12 [a,e]
D+A	34.66 ± 2.28 [c,d]
D+P	32.50 ± 2.45 [c,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$,[c] $p<0.05$when compared with control;[d] $p<0.001$;[e] $p<0.01$ when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats. Scale bar- 150µm

→ in white shows Flouro Jade positive neurons

Figure-25

Scatchard analysis of dopamine D_1 receptor using[^{3}H]SCH 23390 binding against SCH23390 in the cerebral cortex of control and experimental rats

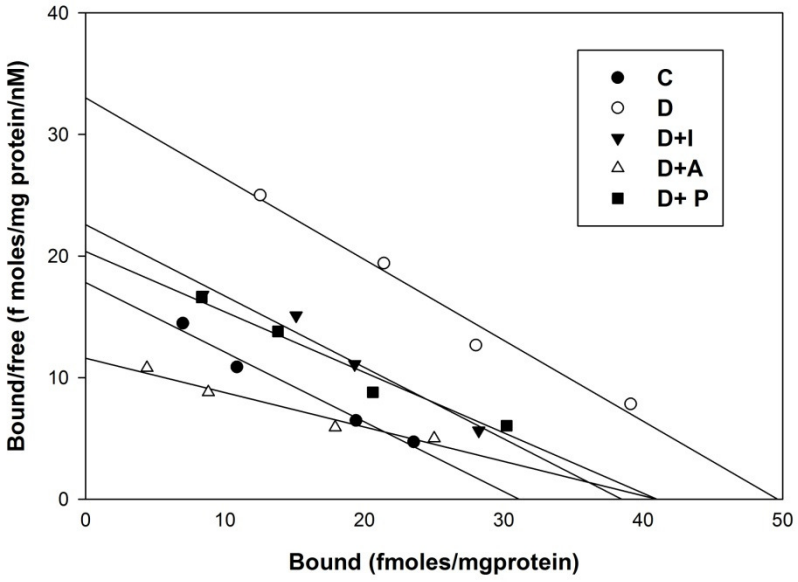


Table-25

Scatchard analysis of dopamine D_1 receptor using[^{3}H]SCH 23390 binding against SCH23390 in the cerebral cortex of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	31.83 ± 3.00	1.46 ± 0.05
D	51.58±3.03[a]	1.70 ± 0.02[c]
D + I	38.33 ± 2.57 [a, d]	1.70 ± 0.03 [c]
D + A	40.65 ± 2.06 [a, d]	3.30 ± 0.06 [a,d]
D + P	40.89 ± 2.72 [a, d]	1.76 ± 0.02[c]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-26

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride in cerebral cortex of control and experimental rats

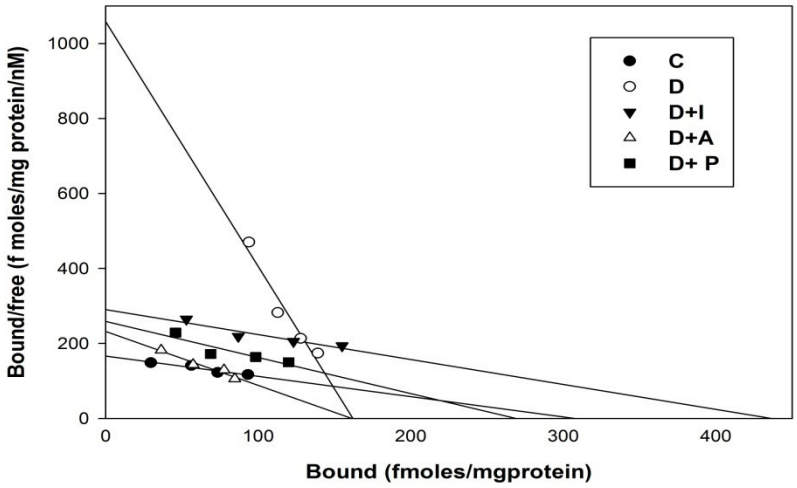


Table-26

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride in cerebral cortex of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	309.8±17.034	1.87±0.01
D	162.0±11.014 [a]	0.15±0.01 [a]
D + I	430.8±16.63 [a,d]	1.44±0.08 [d]
D + A	157.5±10.56[a]	0.62±0.05[a,f]
D + P	265.32± 16.56 [a,d]	0.97 ± 0.06 [a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$, [f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-27

Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the cerebral cortex of control and experimental rats

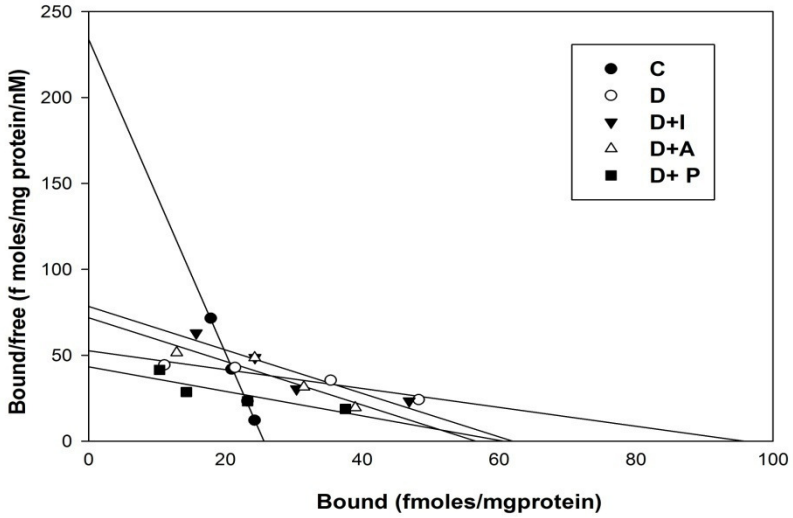


Table-27

Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the cerebral cortex of control and experimental rats

Experimental Groups	B_{max} (fmoles/ mg protein)	K_d (nM)
C	25.42 ±1.03	0.11±0.01
D	95.20 ±1.01 [a]	1.77 ±0.01[a]
D+I	61.24±1.16 [b, d]	0.78±0.02[b, d]
D+A	56.46± 2.56 [b, d]	0.78± 0.05[b, d]
D+P	60.70 ± 2.56 [b, d]	1.38 ± 0.01[a, f]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$,[b] $p<0.01$when compared with control; [d]$p<0.001$,[f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-28
Real Time amplification of dopamine D_1 receptor mRNA in the cerebral cortexof control and experimental rats

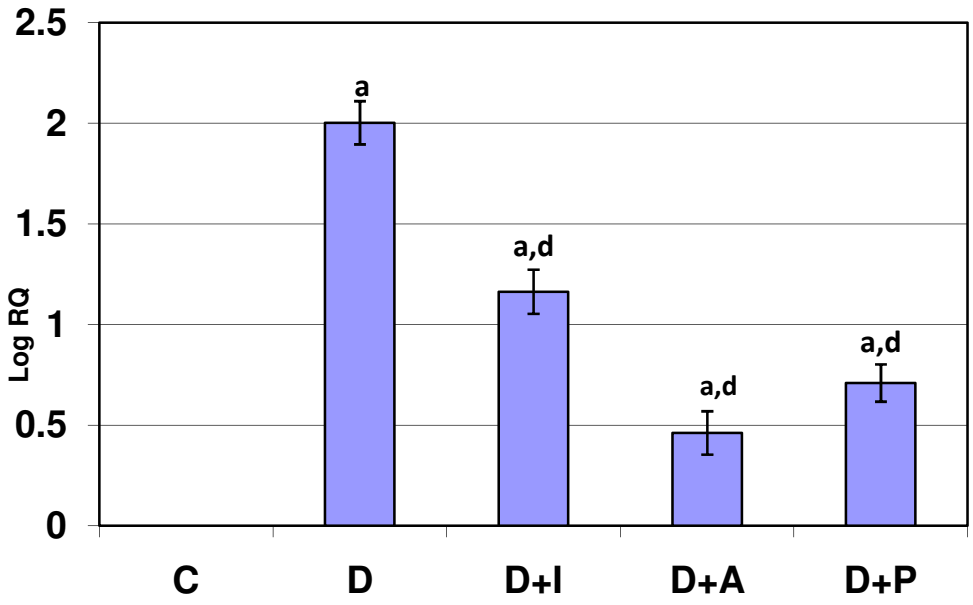


Table-28
Real Time amplification of dopamine D_1 receptor mRNA in the cerebral cortexof control and experimental rats

Experimental groups	Log RQ
C	0
D	2.00 ± 0.06^{a}
D+I	$1.11 \pm 0.03^{a,d}$
D+A	$0.46 \pm 0.05^{a,d}$
D+P	$0.69 \pm 0.05^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group.
C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-29
Real Time amplification of dopamine D_2 receptor mRNA in the cerebral cortex of control and experimental rats

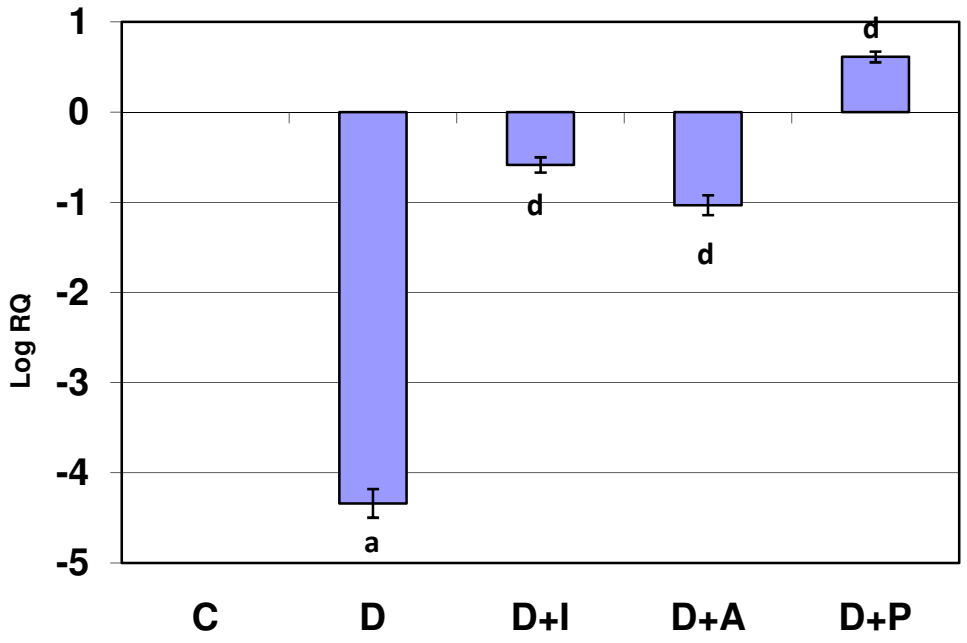


Table-29
Real Time amplification of dopamine D_2 receptor mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
C	0
D	-4.33± 0.06 [a]
D+I	0.58 ± 0.04 [d]
D+A	-1.03 ± 0.05 [d]
D+P	0.61 ± 0.01 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of4-6 rats. [a] p<0.001when compared with control; [d] p<0.001when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-30
Real Time amplification of GLUT-3 mRNA in the cerebral cortex of control and experimental rats

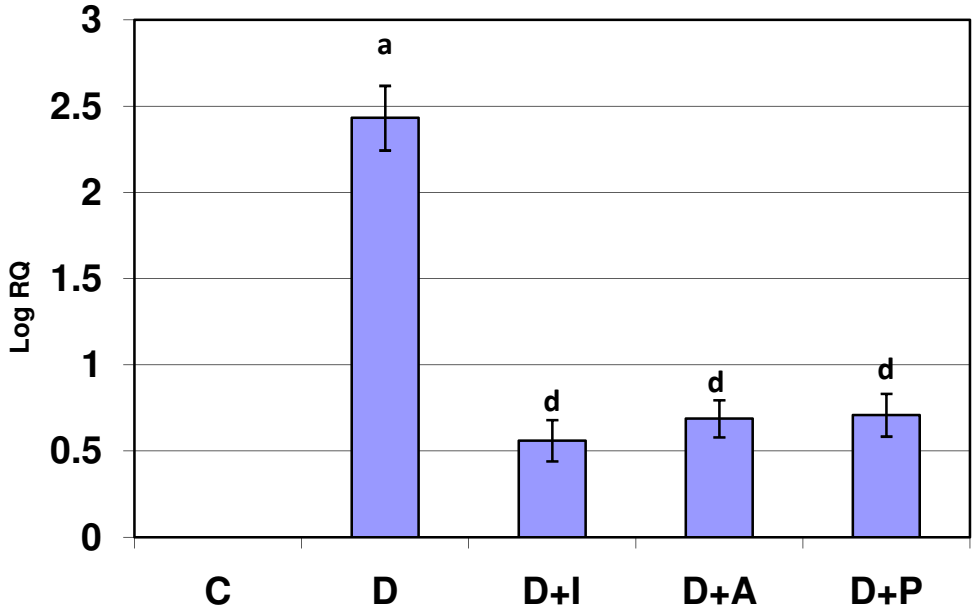


Table-30
Real Time amplification of GLUT-3 mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
C	0
D	2.46 ± 0.08^{a}
D+I	0.59 ± 0.03^{d}
D+A	0.68 ± 0.03^{d}
D+P	0.70 ± 0.03^{d}

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-31
Real Time amplification of CREB mRNA in the cerebral cortex of control and experimental rats

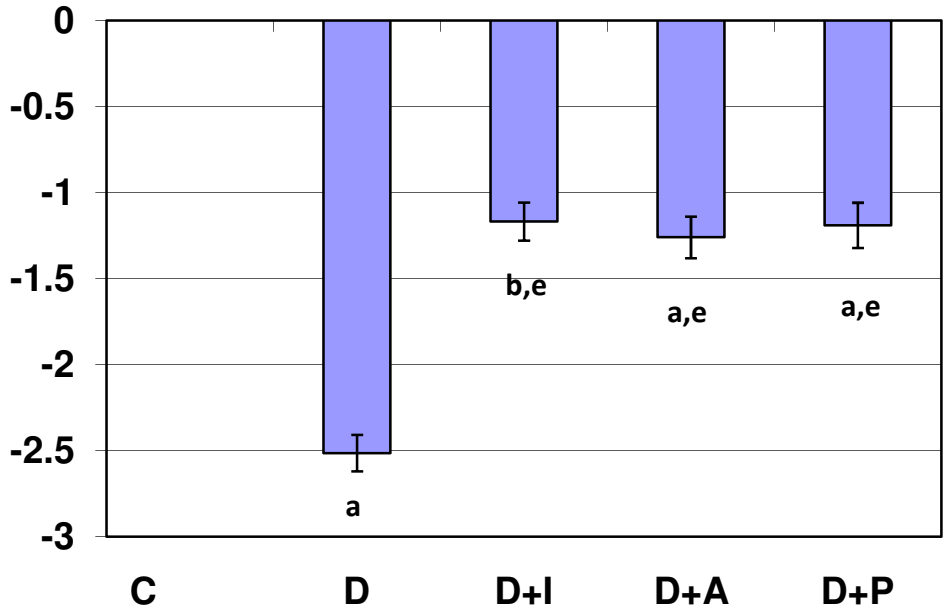


Table-31
Real Time amplification of CREB mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
C	0
D	-2.51 ± 0.02^{a}
D+I	$-1.16 \pm 0.01^{b,e}$
D+A	$-1.26 \pm 0.03^{a,e}$
D+P	$-1.19 \pm 0.03^{a,e}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [b] $p<0.01$when compared with control;[e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-32
Real Time amplification of phospholiase-C mRNA in the cerebral cortex of control and experimental rats

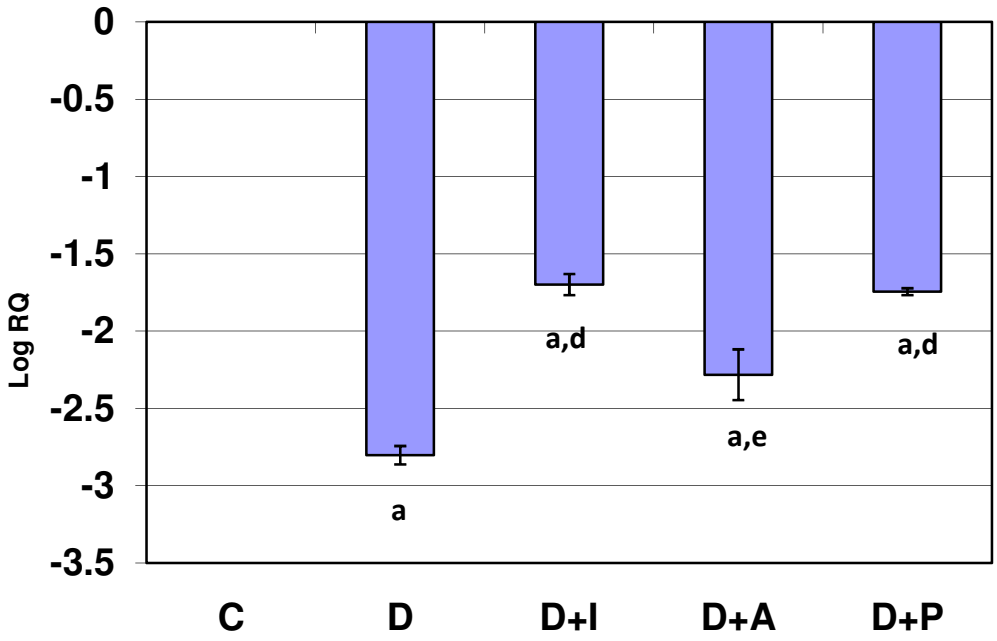


Table-32
Real Time amplification of phospholiase-C mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
C	0
D	-2.80 ± 0.04^{a}
D+I	$-1.61 \pm 0.04^{a,d}$
D+A	$-2.24 \pm 0.06^{a,e}$
D+P	$-1.74 \pm 0.02^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d]$p<0.001$, [e]$p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-33
Real Time amplification of TNF-α mRNA in the cerebral cortex of control and experimental rats

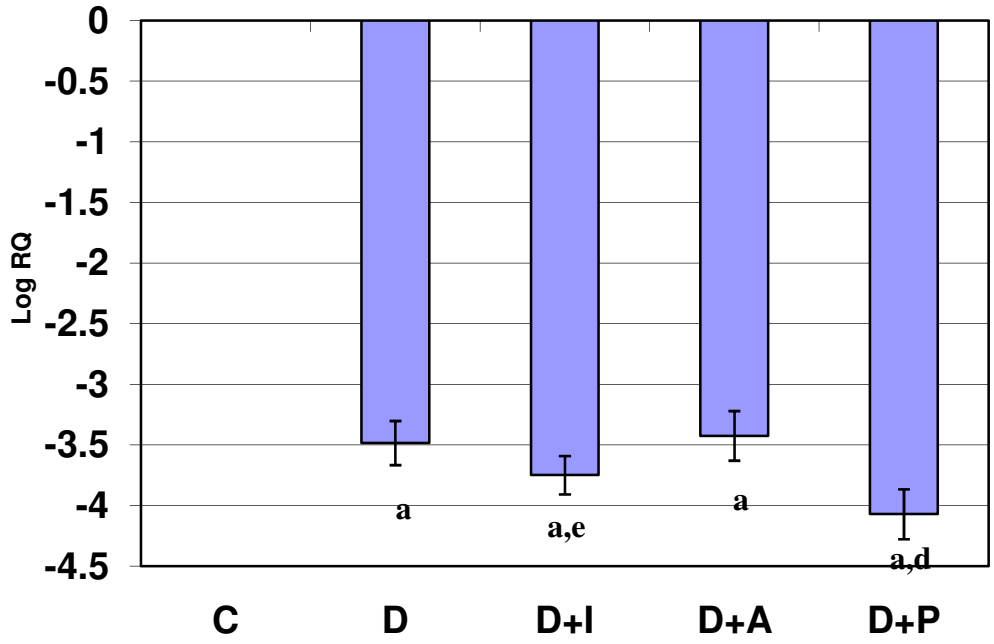


Table-33
Real Time amplification of TNF-α mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
C	0
D	-3.48 ± 0.18^{a}
D+I	$-3.74 \pm 0.15^{a,e}$
D+A	-3.42 ± 0.20^{a}
D+P	$-4.07 \pm 0.20^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$,[e] $p<0.01$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-34
Real Time amplification of NFκB mRNA in the cerebral cortex of control and experimental rats

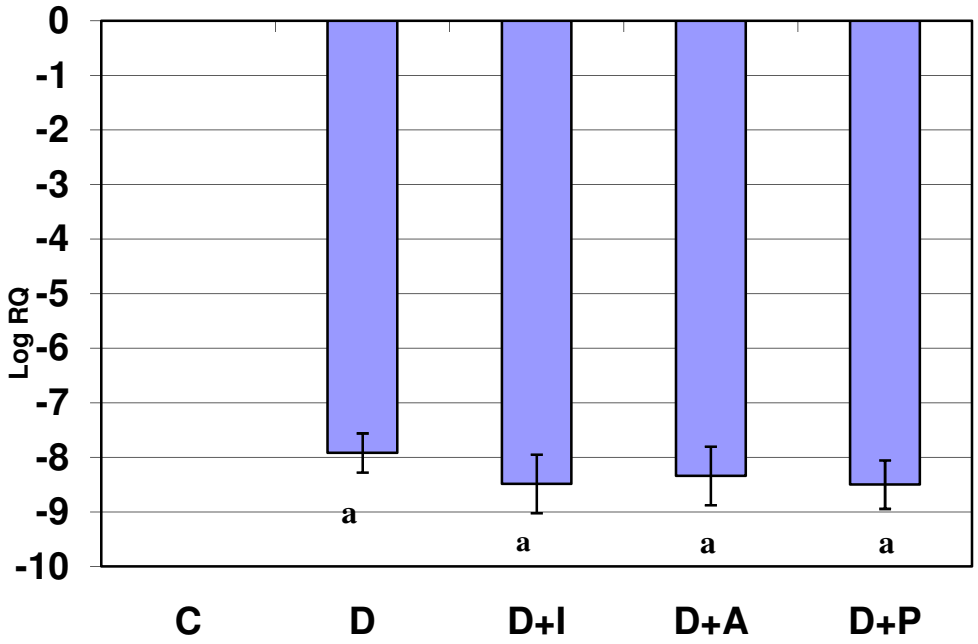


Table-34
Real Time amplification of NFκB mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
C	0
D	-7.91 ± 0.35[a]
D+I	-8.48 ± 0.53[a]
D+A	-8.33 ± 0.53[a]
D+P	-8.50± 0.44[a]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-35
Real Time amplification of Akt -1 mRNA in the cerebral cortex of control and experimental rats

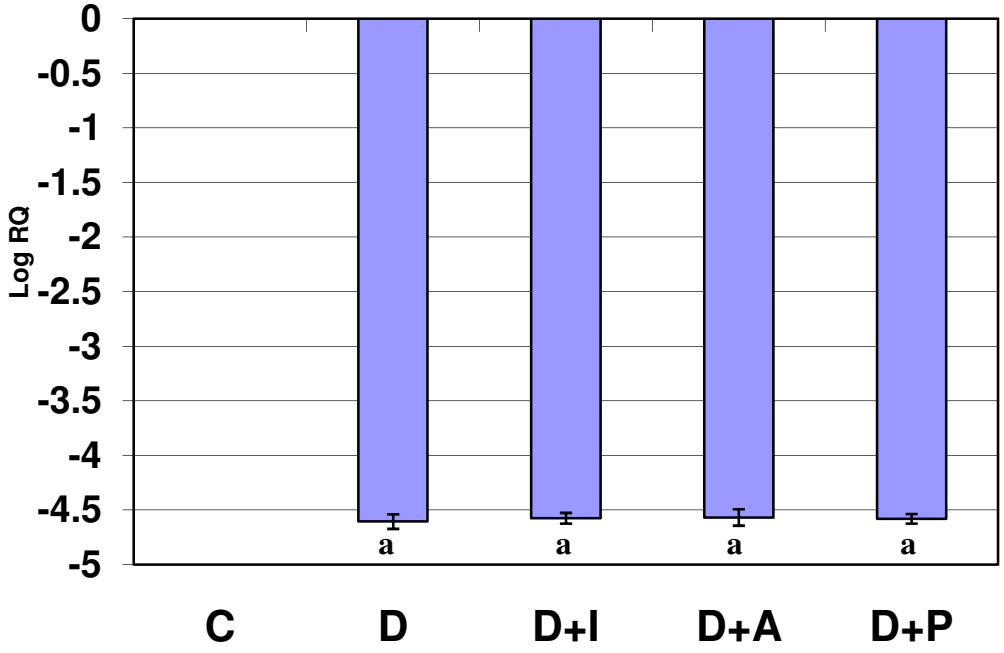


Table-35
Real Time amplification of Akt - 1 mRNA in the cerebral cortexof control and experimental rats

Experimental groups	Log RQ
C	0
D	-4.60 ± 0.06[a]
D+I	-4.57 ± 0.04[a]
D+A	-4.56 ± 0.07[a]
D+P	-4.58 ± 0.04[a]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-36
Real Time amplification of caspase-8 mRNA in the cerebral cortex of control and experimental rats

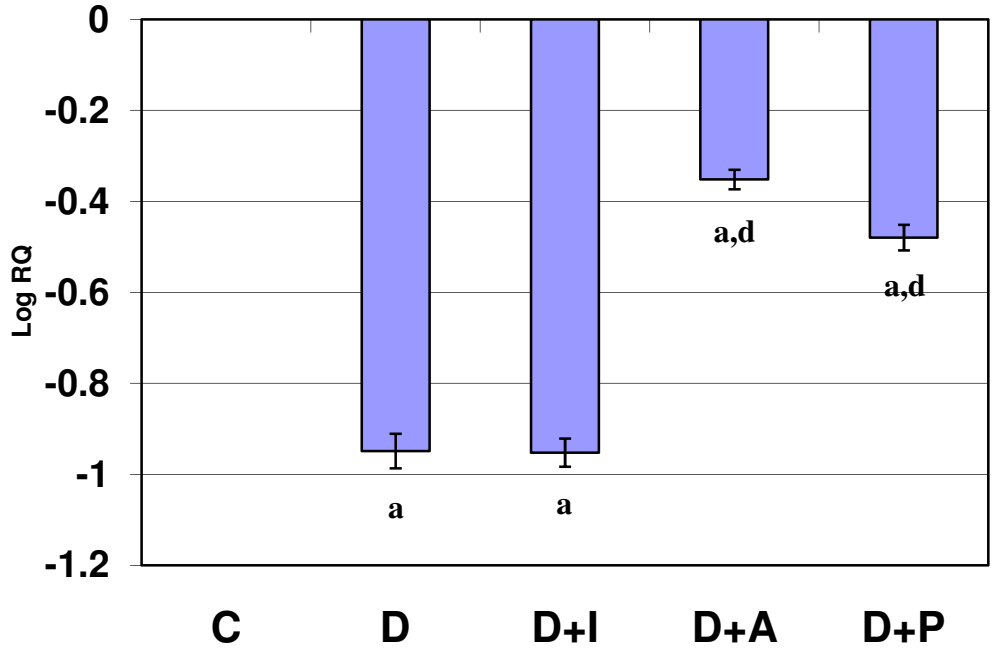


Table-36
Real Time amplification of caspase-8 mRNA in the cerebral cortex of control and experimental rats

Experimental groups	Log RQ
C	0
D	-0.94 ± 0.03^{a}
D+I	-0.95 ± 0.03^{a}
D+A	$-0.35 \pm 0.02^{a,d}$
D+P	$-0.47 \pm 0.02^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-37
cAMP content in the cerebral cortex of control and experimental rats

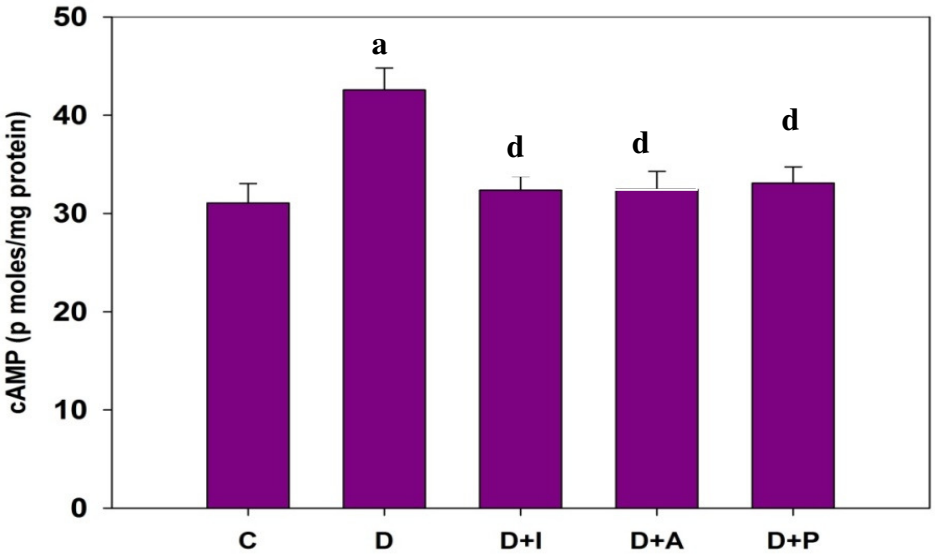


Table-37
cAMP content in the cerebral cortex of control and experimental rats

Experimental groups	cAMP (pmoles/mg protein)
C	31.07± 1.97
D	42.56± 2.24[a]
D+I	32.37 ± 1.43 [d]
D+A	32.44 ± 1.84 [d]
D+P	33.09 ± 1.65 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-38
cGMP content in the cerebral cortex of control and experimental rats

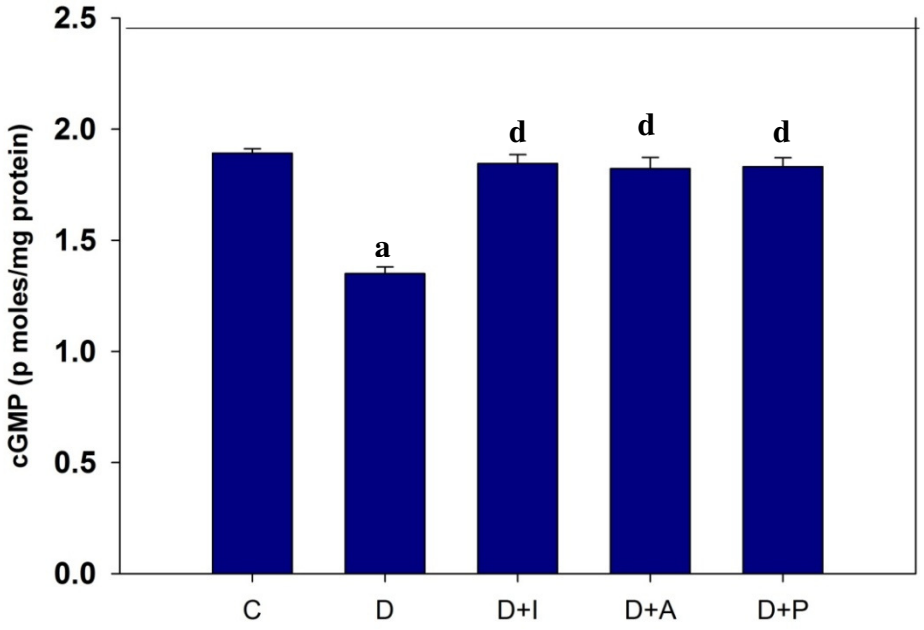


Table-38
cGMP content in the cerebral cortex of control and experimental rats

Experimental groups	cGMP (pmoles/mg protein)
C	1.89± 0.02
D	1.35± 0.03[a]
D+I	1.84± 0.04[d]
D+A	1.82± 0.05[d]
D+P	1.83± 0.04[d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] p<0.001when compared with control;[d] p<0.001when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-39

IP_3 content in the cerebral cortex of control and experimental rats

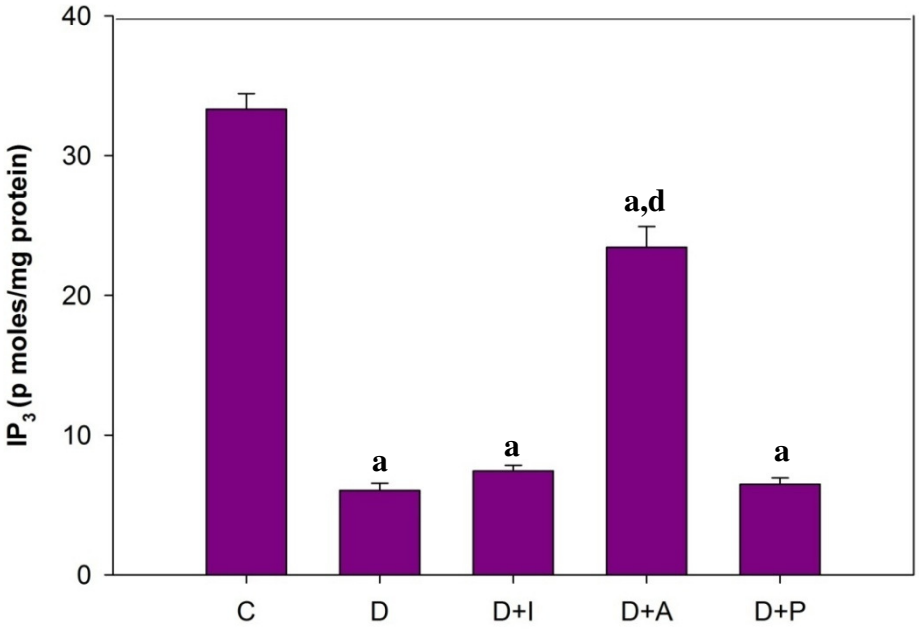


Table-39

IP_3 content in the cerebral cortex of control and experimental rats

Experimental groups	IP_3(pmoles/mg protein)
C	33.32 ± 1.10
D	6.40± 0.51[a]
D+I	7.44± 0.39 [a]
D+A	23.44± 1.48[a,d]
D+P	6.48± 0.45[a]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] p<0.001when compared with control;[d] p<0.001 when compared with diabetic group.
C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-40
Confocal imaging of dopamine D_1 receptor expression in the cerebral cortex of control and experimental rats

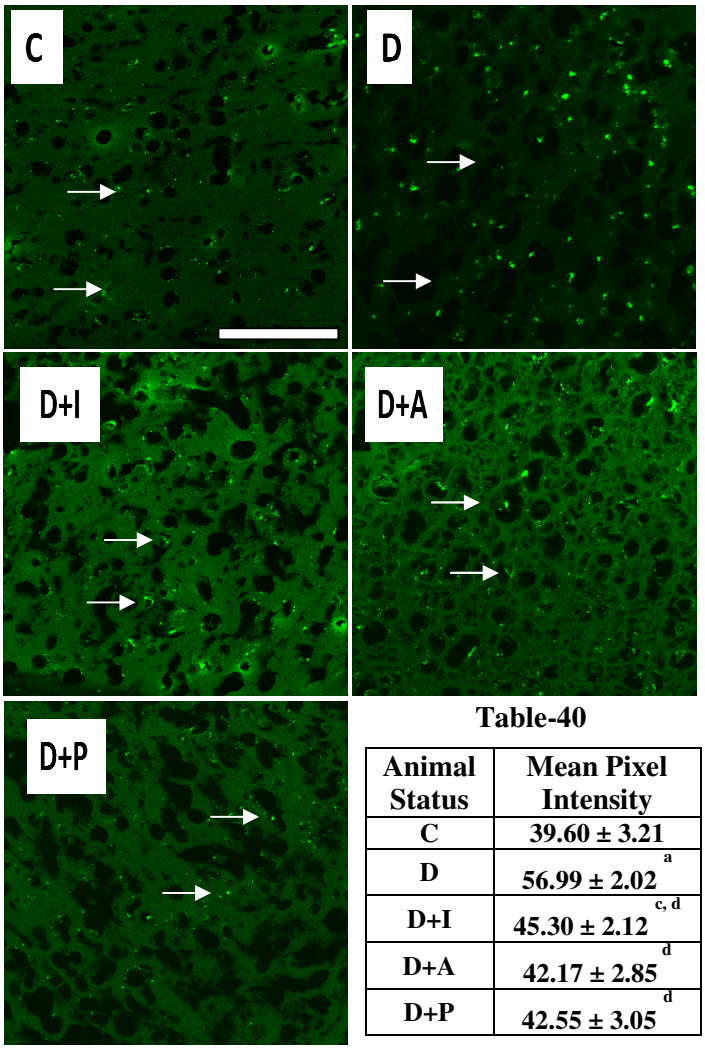


Table-40

Animal Status	Mean Pixel Intensity
C	39.60 ± 3.21
D	56.99 ± 2.02 [a]
D+I	45.30 ± 2.12 [c, d]
D+A	42.17 ± 2.85 [d]
D+P	42.55 ± 3.05 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$, [c] $p<0.01$when compared with control;[d] $p<0.001$when compared with diabetic group.
C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.Scale bars represent 200 µm
→ in white shows dopamine D_1 receptor

Figure-41
Confocal imaging of dopamine D_2 receptor expression in the cerebral cortex of control and experimental rats

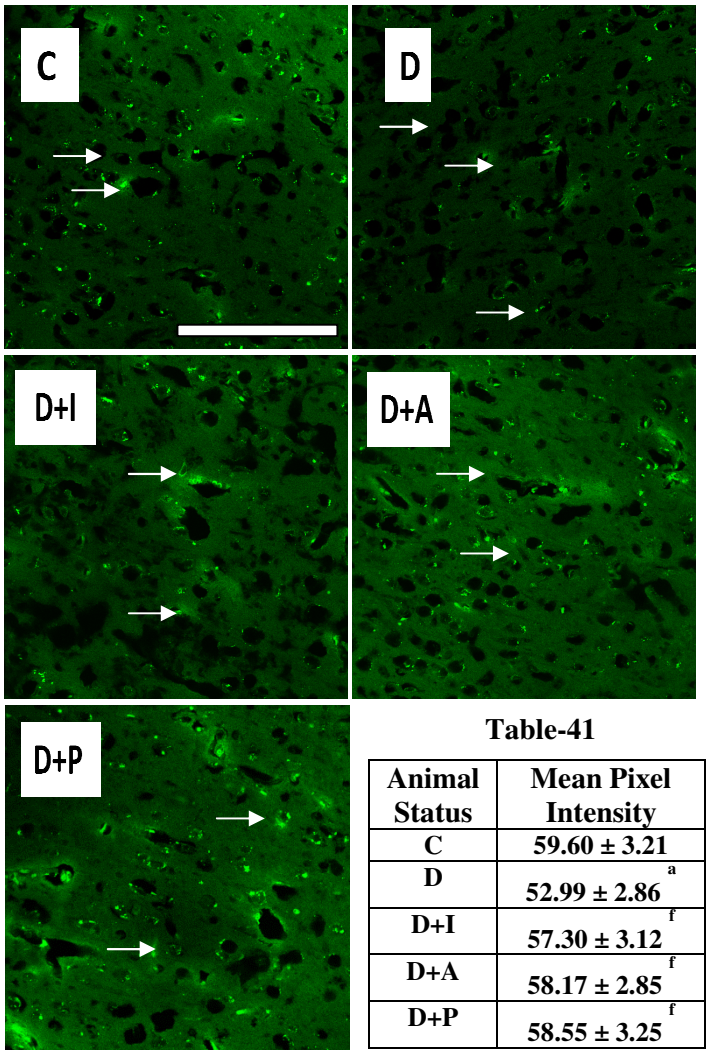


Table-41

Animal Status	Mean Pixel Intensity
C	59.60 ± 3.21
D	52.99 ± 2.86 [a]
D+I	57.30 ± 3.12 [f]
D+A	58.17 ± 2.85 [f]
D+P	58.55 ± 3.25 [f]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control; [f] $p<0.05$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.Scale bars represent 200 µm
→ in white shows dopamine D_2 receptor

Figure-42

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH23390 in the brain stem of control and experimental rats

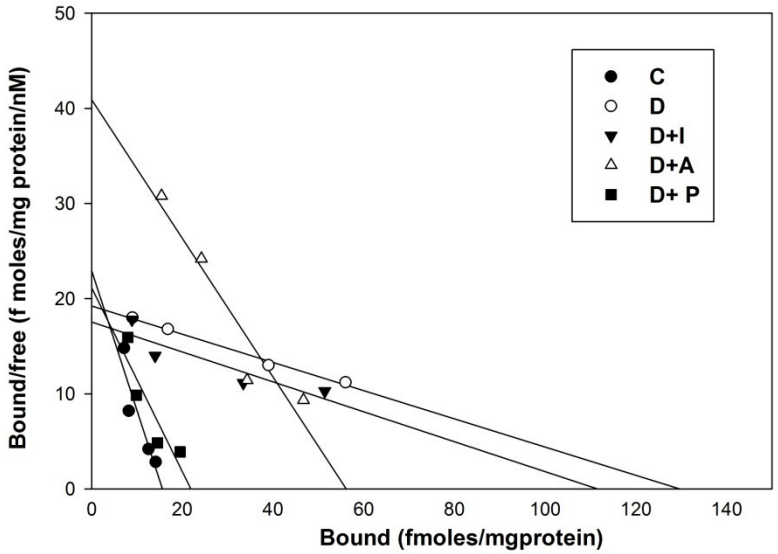


Table-42

Scatchard analysis of Dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH23390 in the brain stem of control and experimental rats

Experimental Groups	B_{max}(fmoles/ mg protein)	K_d (nM)
C	17.62 ± 1.03	0.70 ± 0.04
D	128.10 ± 20 .41[a]	6.67 ± 0.02[a]
D+I	109.23 ± 2.15 [a]	6.23 ± 0.03 [a]
D+A	56.55 ± 7.56 [a, d]	1.38 ± 0.01[d]
D+P	23.03 ± 2.16 [d]	1.04 ± 0.01[d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. B_{max} – Maximal binding; K_d – Dissociation constant, [a] $p<0.001$when compared with control; [d] $p<0.001$when compared with diabetic.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-43

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride in brain stem of control and experimental rats

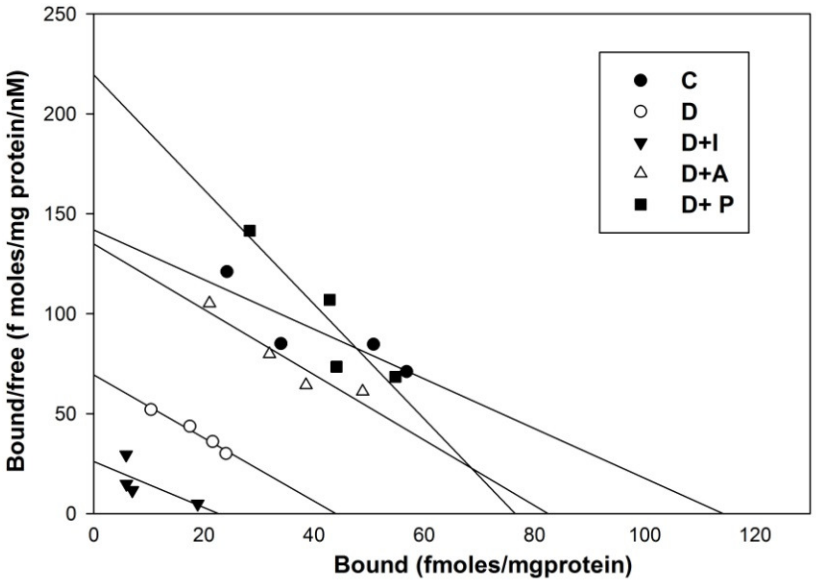


Table-43

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride in brain stem of control and experimental rats

Experimental Groups	B_{max}(fmoles/ mg protein)	K_d (nM)
C	114.0± 6.06	0.80±0.02
D	43.8± 2.16[a]	0.63± 0.01[a]
D+I	22.2±1.04[d]	0.86± 0.03[e]
D+A	82.0± 5.42[c, d]	0.60± 0.02[a]
D+P	75.81± 4.68[c, d]	0.34± 0.01[a]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$ when compared with control;[d] $p<0.001$, [e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-44
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the brain stem of control and experimental rats

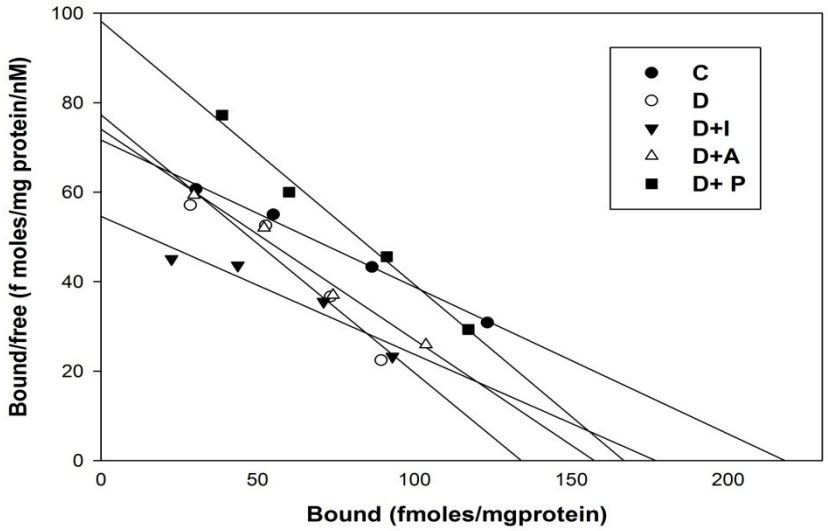


Table-44
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the brain stem of control and experimental rats

Experimental Groups	B_{max}(fmoles/ mg protein)	K_d (nM)
C	217.53±14.034	3.05±0.02
D	133.67±10.14 [a]	1.73±0.12[a]
D+I	175.85±10.16[a,e]	3.19±0.20[d]
D+A	156.67± 9.56[a,f]	2.41± 0.21[c,e]
D+P	167.86 ±8.12[a,e]	1.70 ± 0.16 [a]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant [a] $p<0.001$,[c] $p<0.05$when compared with control;[d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-45
Real Time amplification of dopamine D_1 receptor mRNA in the brain stem of control and experimental rats

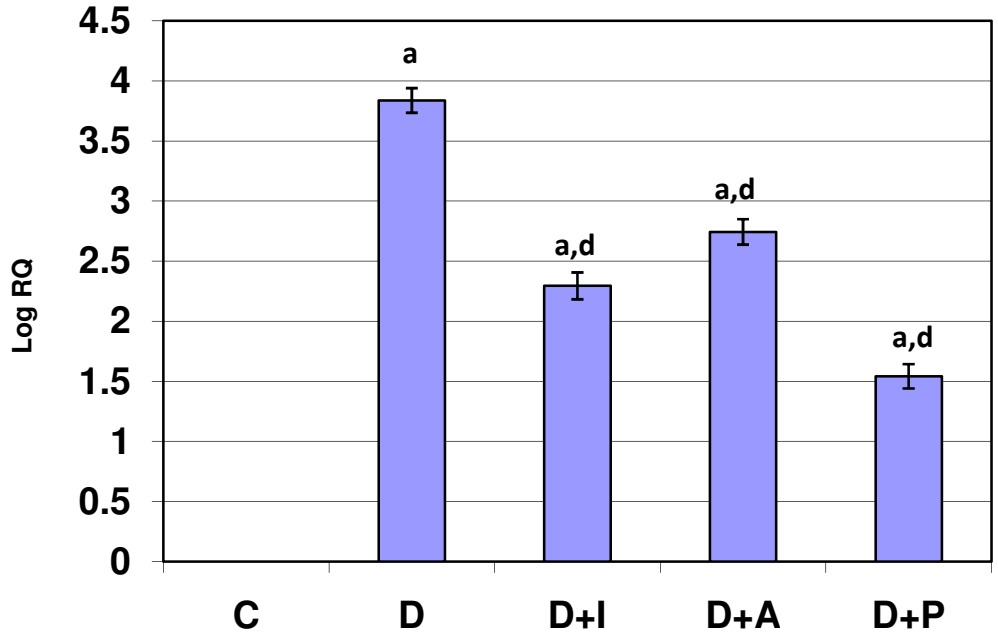


Table-45
Real Time amplification of dopamine D_1 receptor mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	3.83± 0.04[a]
D+I	2.92 ± 0.02[a,d]
D+A	2.74± 0.02[a,d]
D+P	1.54± 0.01[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-46
Real Time amplification of dopamine D_2 receptor mRNA inthe brain stem of control and experimental rats

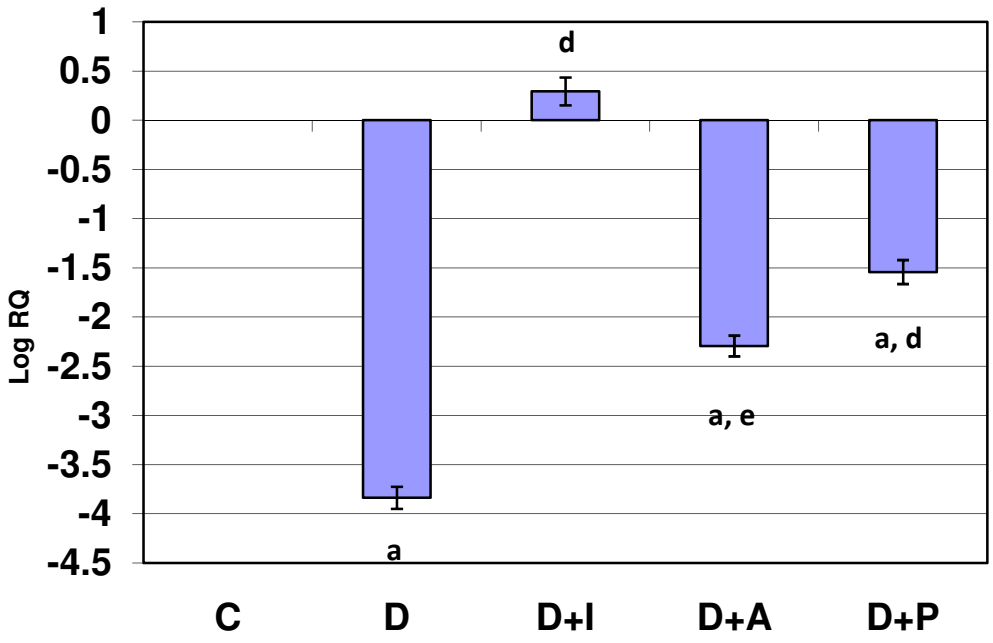


Table-46
Real Time amplification of dopamine D_2 receptor mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	-3.81 ± 0.04^{a}
D+I	-0.29 ± 0.05^{d}
D+A	$-2.29 \pm 0.04^{a,e}$
D+P	$-1.54 \pm 0.02^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$, [e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-47
Real Time amplification of GLUT-3 mRNA in the brain stem of control and experimental rats

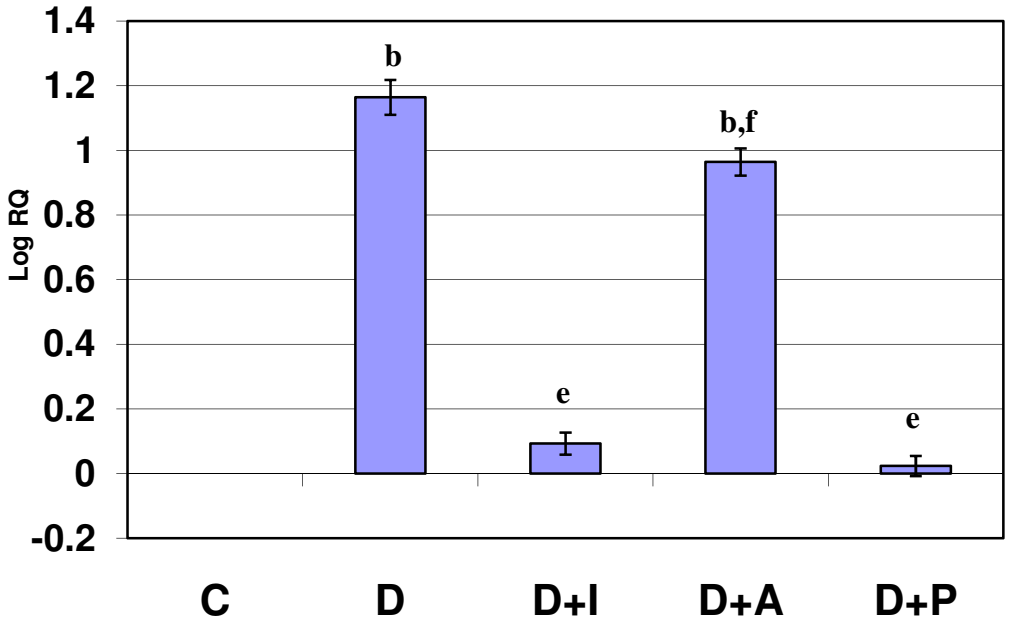


Table-47
Real Time amplification of GLUT-3 mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	1.16 ± 0.04^{b}
D+I	0.09 ± 0.03^{e}
D+A	$0.94 \pm 0.04^{b,f}$
D+P	0.02 ± 0.001^{e}

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [b] $p<0.01$when compared with control;[e] $p<0.01$,[f] $p<0.05$, when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-48
Real Time amplification of CREB mRNA in the brain stem of control and experimental rats

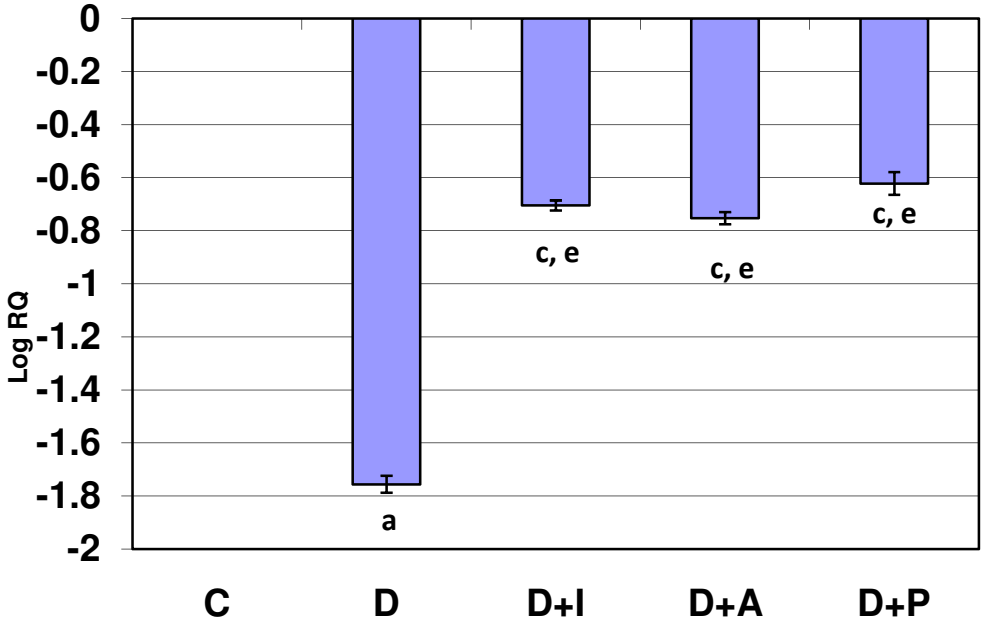


Table-48
Real Time amplification of CREB mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	-1.75 ± 0.02^{a}
D+I	$-0.70 \pm 0.02^{c,e}$
D+A	$-0.75 \pm 0.02^{c,e}$
D+P	$-0.62 \pm 0.04^{c,e}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$ when compared with control;[e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-49
Real Time amplification of phospholiase-C mRNA in the brain stem of control and experimental rats

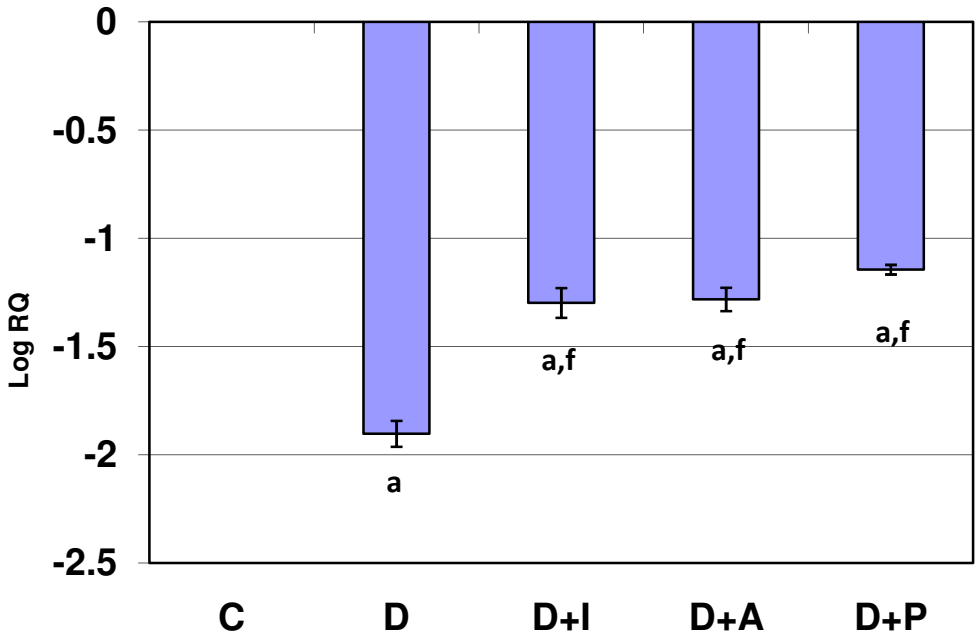


Table-49
Real Time amplification of phospholiase-C mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	-1.94 ± 0.02^{a}
D+I	$-1.29 \pm 0.03^{a,f}$
D+A	$-1.28 \pm 0.03^{a,f}$
D+P	$-1.14 \pm 0.01^{a,f}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-50
Real Time amplification of TNF- α mRNA in the brain stem of control and experimental rats

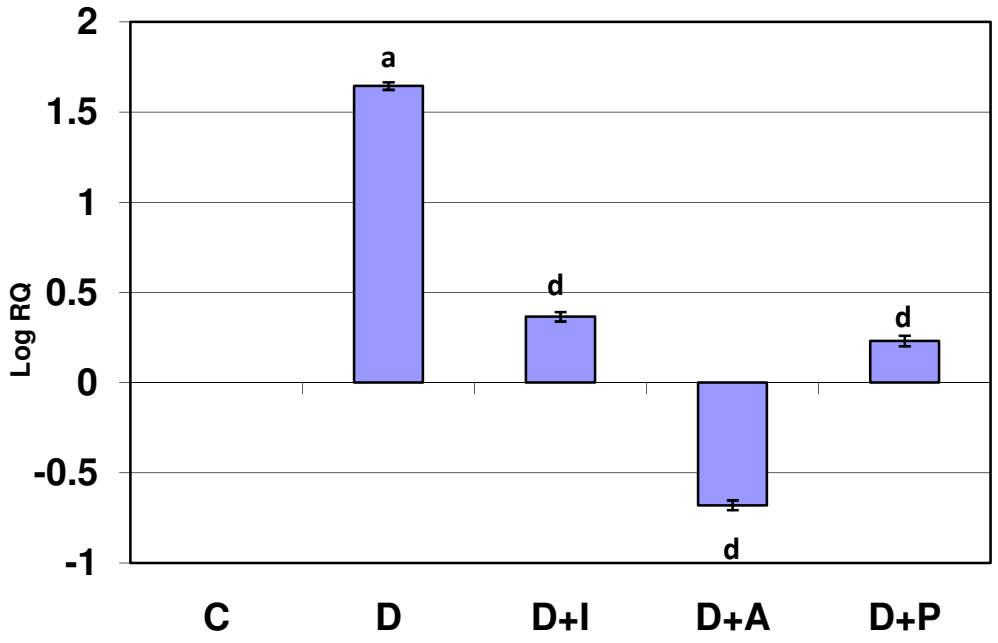


Table-50
Real Time amplification of TNF- α mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	1.64 ± 0.02^{a}
D+I	0.36 ± 0.02^{d}
D+A	-0.67 ± 0.02^{d}
D+P	0.23 ± 0.02^{d}

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-51
Real Time amplification of Akt -1 mRNA in the brain stem of control and experimental rats

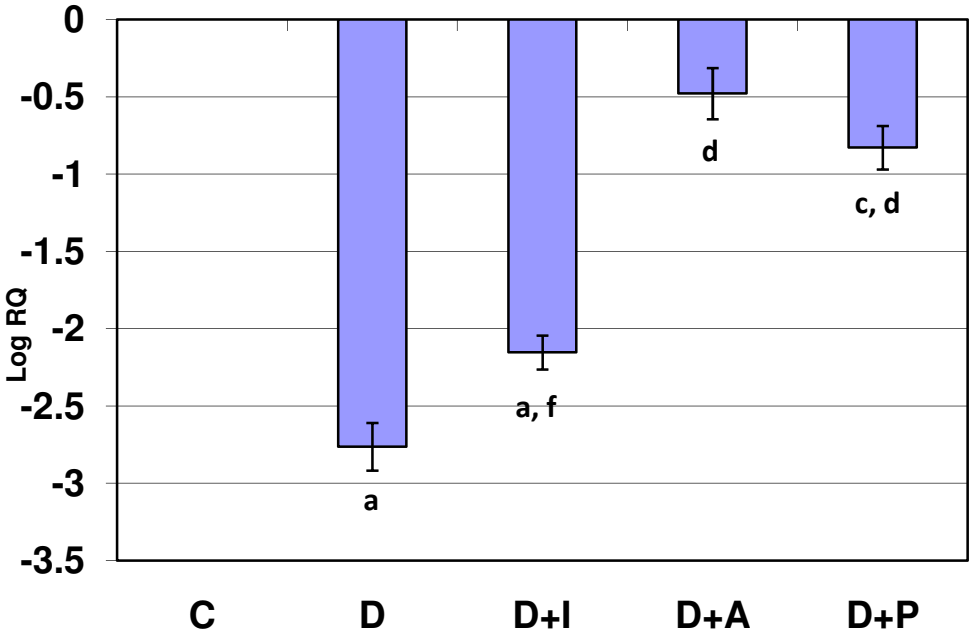


Table-51
Real Time amplification of Akt- 1 mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	-2.76 ± 0.08^{a}
D+I	$-2.15 \pm 0.06^{a,f}$
D+A	-0.47 ± 0.06^{d}
D+P	$-0.82 \pm 0.04^{c,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$ when compared with control;[d] $p<0.001$, [f] $p<0.05$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-52
Real Time amplification of caspase-8 mRNA inthe brain stem of control and experimental rats

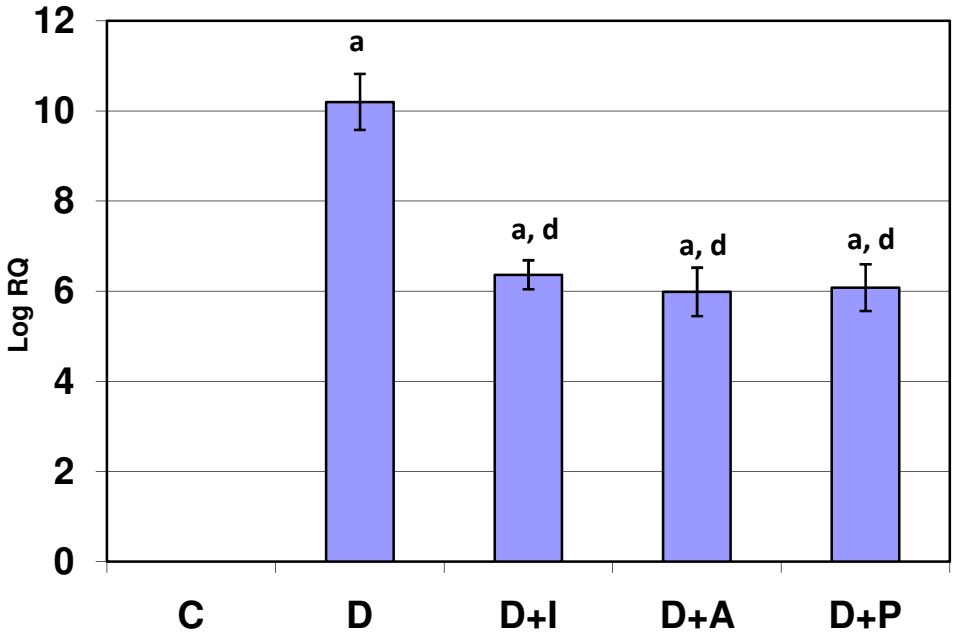


Table-52
Real Time amplification of caspase-8 mRNA in the brain stem of control and experimental rats

Experimental groups	Log RQ
C	0
D	10.19 ± 0.30^{a}
D+I	$6.36 \pm 0.21^{a,d}$
D+A	$5.98 \pm 0.26^{a,d}$
D+P	$6.07 \pm 0.28^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-53
cAMP content in the brain stem of control and experimental rats

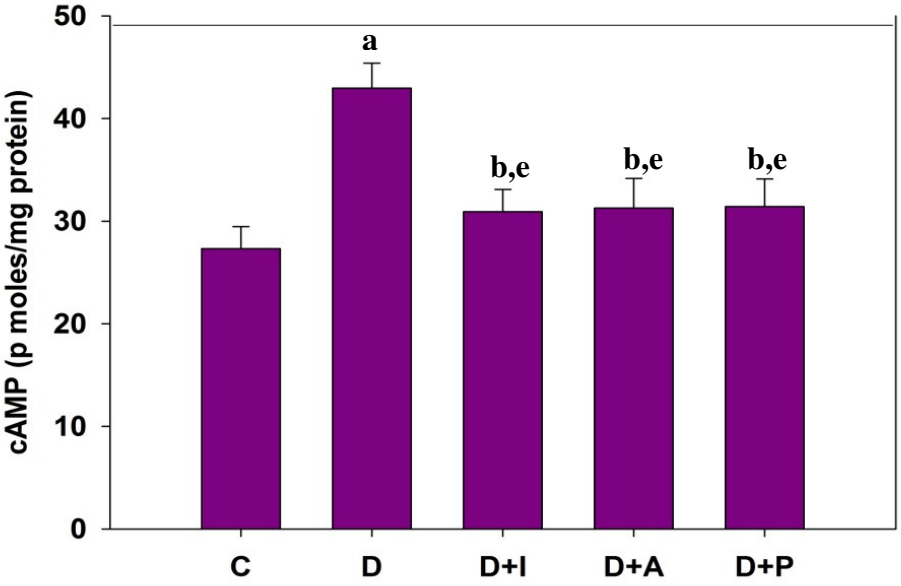


Table-53
cAMP content in the brain stem of control and experimental rats

Experimental groups	cAMP (pmoles/mg protein)
C	26.35± 2.02
D	40.17± 1.97[a]
D+I	33.71 ± 1.80[b,e]
D+A	34.46 ± 2.40[b,e]
D+P	34.28 ± 2.02[b,e]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [b] $p<0.01$when compared with control; [e] $p<0.01$when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-54
cGMP content in the brain stem of control and experimental rats

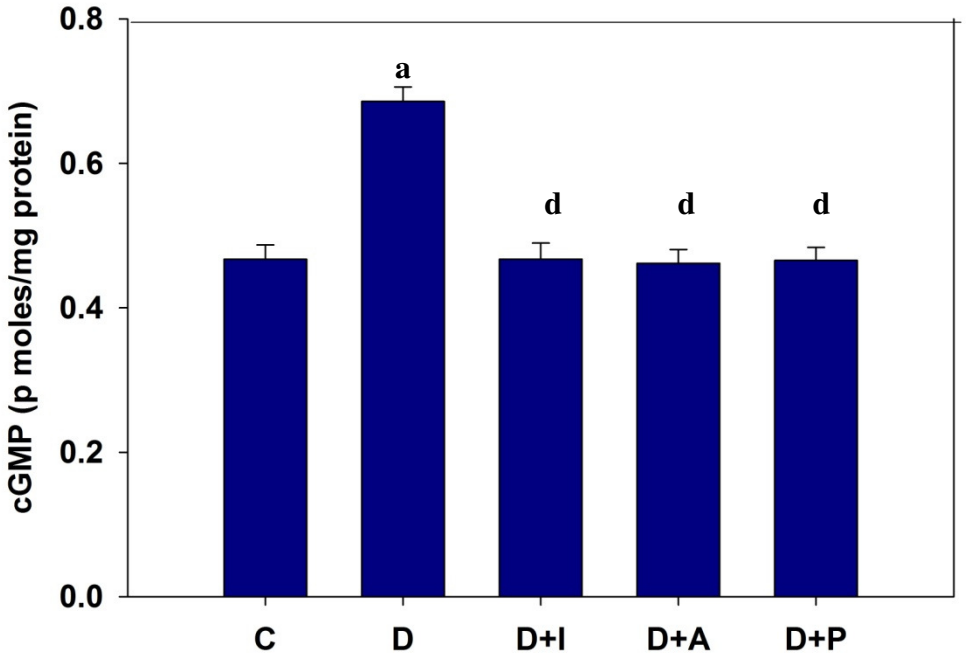


Table-54
cGMP content in the brain stem of control and experimental rats

Experimental groups	cGMP (pmoles/mg protein)
C	0.46± 0.02
D	0.68 ± 0.02[a]
D+I	0.46± 0.02[d]
D+A	0.46± 0.01[d]
D+P	0.46± 0.01[d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] p<0.001when compared with control;[d] p<0.001when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-55

IP_3 content in the brain stem of control and experimental rats

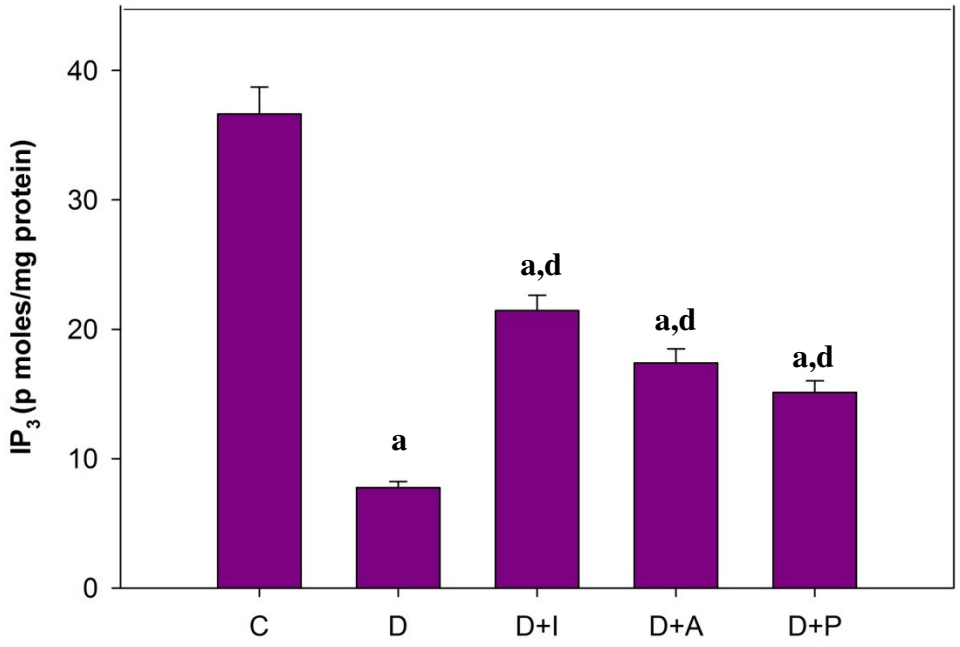


Table-55

IP_3 content in the brain stem of control and experimental rats

Experimental groups	IP_3 (pmoles/mg protein)
C	36.64± 2.09
D	7.76± 0.48[a]
D+I	21.44± 1.19[a,d]
D+A	17.40± 1.08[a,d]
D+P	15.12± 0.90 [a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-56
Confocal imaging of dopamine D_1 receptor expression in the brain stem of control and experimental rats

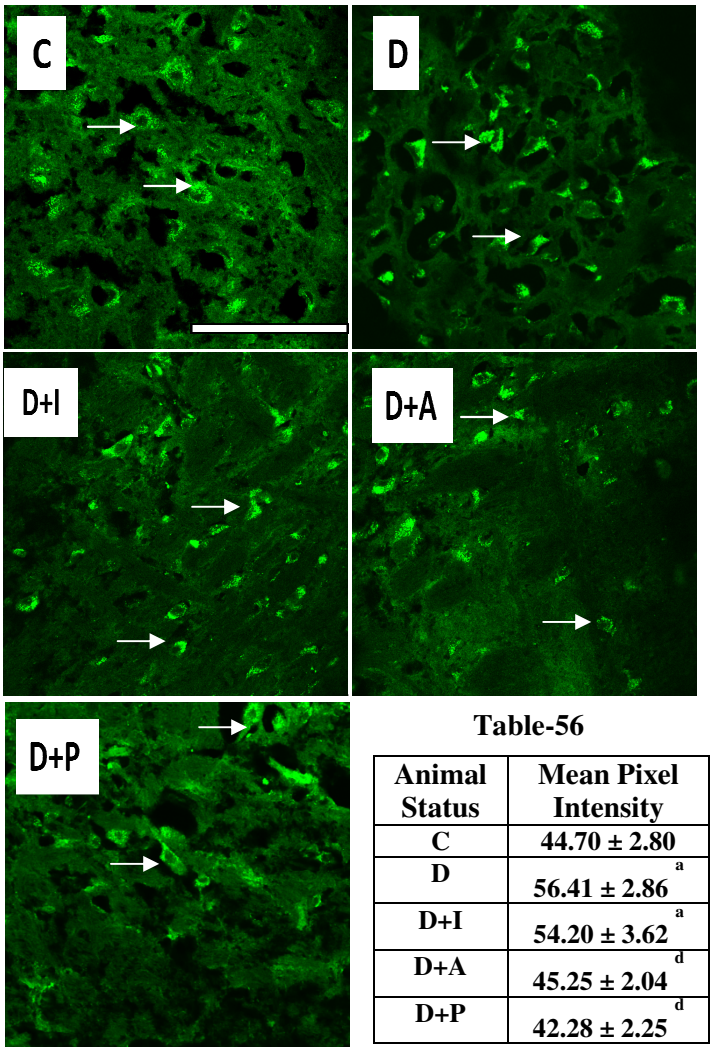


Table-56

Animal Status	Mean Pixel Intensity
C	44.70 ± 2.80
D	56.41 ± 2.86 [a]
D+I	54.20 ± 3.62 [a]
D+A	45.25 ± 2.04 [d]
D+P	42.28 ± 2.25 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control; [d]$p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats. Scale bars represent 200 µm
→ in white shows dopamine D_1 receptor

Figure-57
Confocal imaging of Dopamine D_2 receptor expression in the brain stem of Control and Experimental Rats

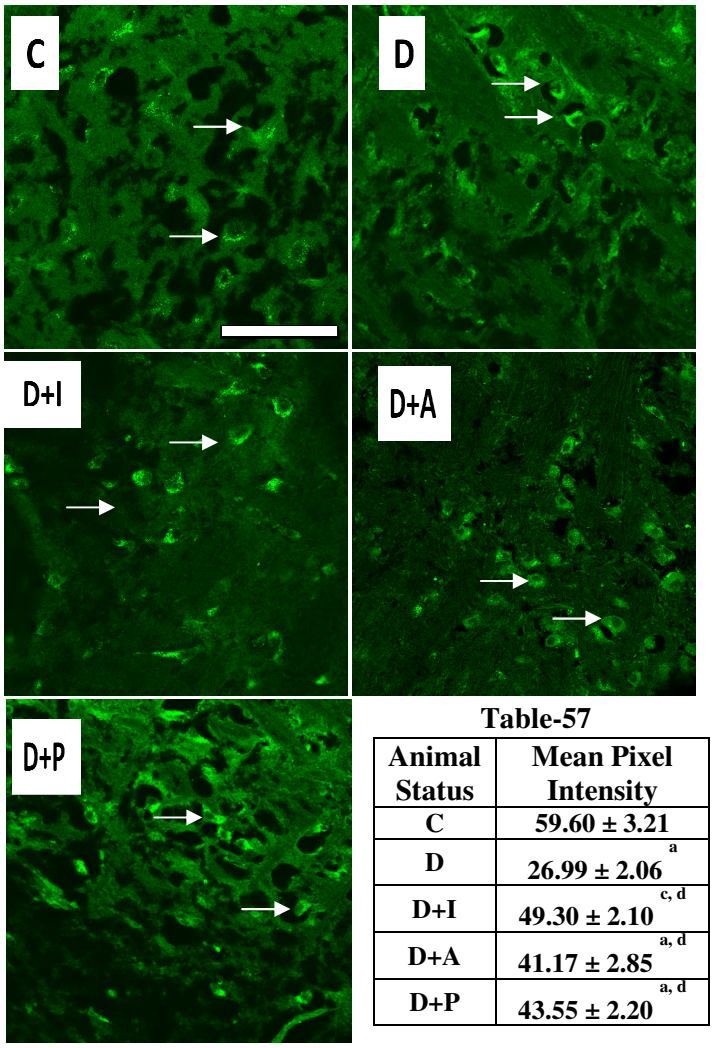


Table-57

Animal Status	Mean Pixel Intensity
C	59.60 ± 3.21
D	26.99 ± 2.06 [a]
D+I	49.30 ± 2.10 [c, d]
D+A	41.17 ± 2.85 [a, d]
D+P	43.55 ± 2.20 [a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats. Scale bars represent 200 μm
→ in white shows dopamine D_2 receptor

Figure-58

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH23390 in the pancreas of control and experimental rats

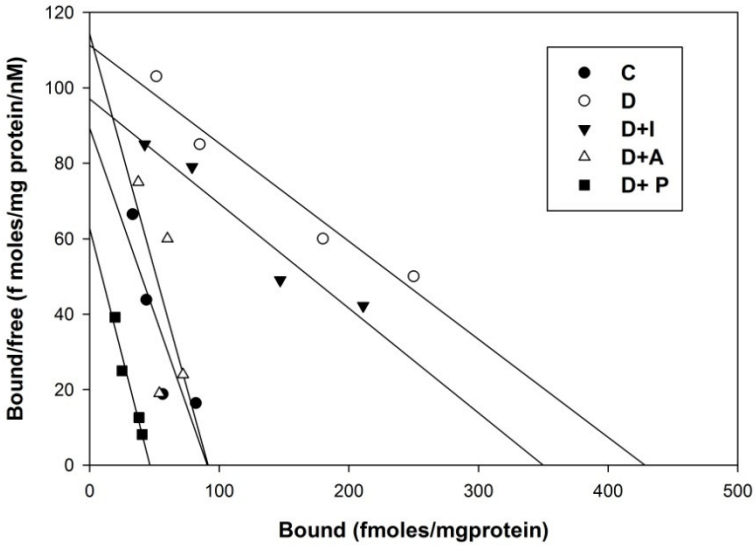


Table-58

Scatchard analysis of dopamine D_1 receptor using [^{3}H]SCH 23390 binding against SCH23390 in the pancreas of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	90.60± 7.50	1.02 ±0.04
D	427.10 ± 14.68 [a]	3.83 ± 0.05 [a]
D + I	348.90 ± 12.50 [a,e]	3.58 ± 0.04[a]
D + A	91.70 ± 6.42[d]	0.81 ± 0.03[a,d]
D + P	46.90 ± 3.72[a,d]	0.75 ± 0.02[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. B_{max} – Maximal binding; K_d – Dissociation constant

[a] $p<0.001$when compared with control;[d] $p<0.001$, [e] $p<0.01$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-59

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride inthe pancreas of control and experimental rats

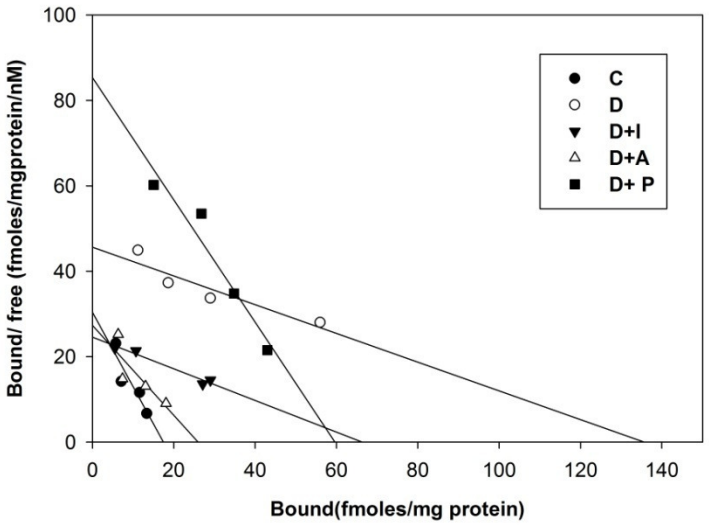


Table-59

Scatchard analysis of dopamine D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride inthe pancreas of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	17.49± 1.03	0.57 ±0.03
D	135.35 ± 3.60 [a]	2.97 ± 0.01 [a]
D + I	65.94 ± 3.57 [a,d]	2.61 ± 0.04[a]
D + A	25.62 ± 2.10[d]	0.95 ± 0.02[a,d]
D + P	60.05 ± 3.80[a,d]	0.70 ± 0.02[b]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] $p<0.001$, [b] $p<0.01$when compared with control;[d] $p<0.001$when compared with diabetic group.
C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-60
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the pancreas of control and experimental rats

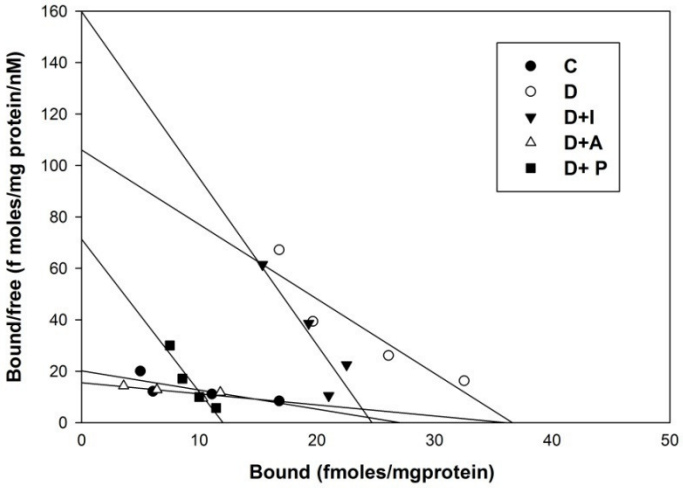


Table-60
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the pancreas of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	26.66 ± 2.06	1.36 ±0.04
D	36.66 ± 3.00[b]	0.34 ± 0.02 [a]
D + I	24.58 ± 2.07[e]	0.16 ± 0.01[a]
D + A	35.04 ± 3.02 [b]	1.17 ± 0.04[d]
D + P	12.08 ± 2.51[b,d]	0.16 ± 0.01[a]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
B_{max} – Maximal binding; K_d – Dissociation constant
[a] p<0.001, [b] p<0.01when compared with control;[d] p<0.001,[e] p<0.01when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-61
Real Time amplification of dopamine D_1 receptor mRNA in the pancreas of control and experimental rat

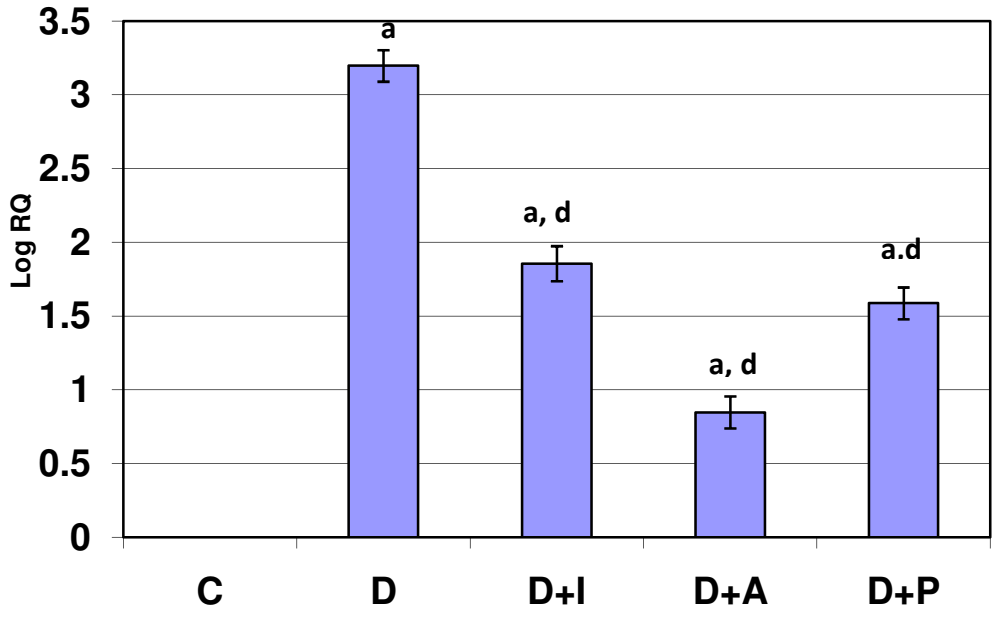


Table-61
Real Time amplification of dopamine D_1 receptor mRNA in the pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	3.19 ± 0.10^{a}
D+I	$1.85\pm0.10^{a,d}$
D+A	$0.84\pm0.06^{a,d}$
D+P	$1.58\pm0.10^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$when compared with diabetic group.
C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-62
Real Time amplification of dopamine D_2 receptor mRNA in the pancreas of control and experimental rats

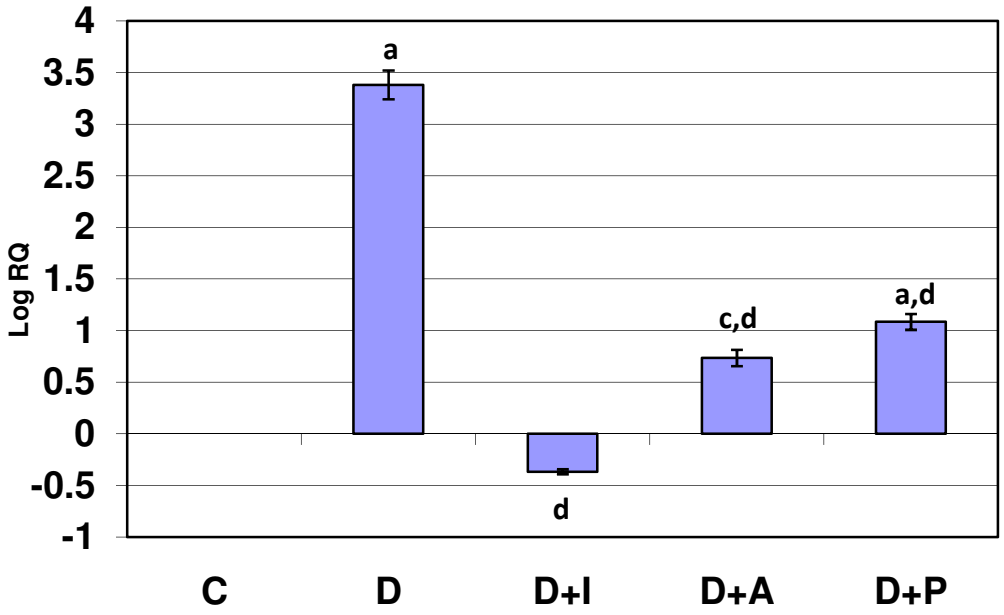


Table-62
Real Time amplification of dopamine D_2 receptor mRNA in the pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	3.37 ± 0.13^{a}
D+I	-0.36 ± 0.02^{d}
D+A	$0.73\pm 0.06^{c,d}$
D+P	$1.08\pm 0.07^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$; [c] $p<0.01$ when compared with control;[d] $p<0.001$when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-63
Real Time amplification of GLUT-2 mRNA in the pancreas of control and experimental rats

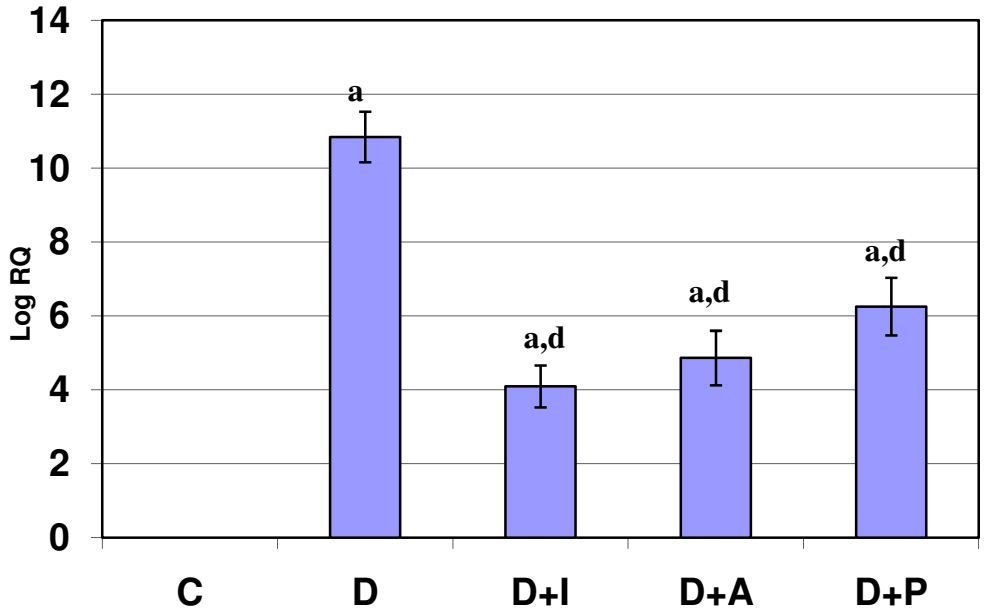


Table-63
Real Time amplification of GLUT-2 mRNA in the pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	10.84 ± 0.48^{a}
D+I	$4.09 \pm 0.36^{a,d}$
D+A	$4.86 \pm 0.43^{a,d}$
D+P	$6.25 \pm 0.53^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-64
Real Time amplification of IGF-1 mRNA in the pancreas of control and experimental rats

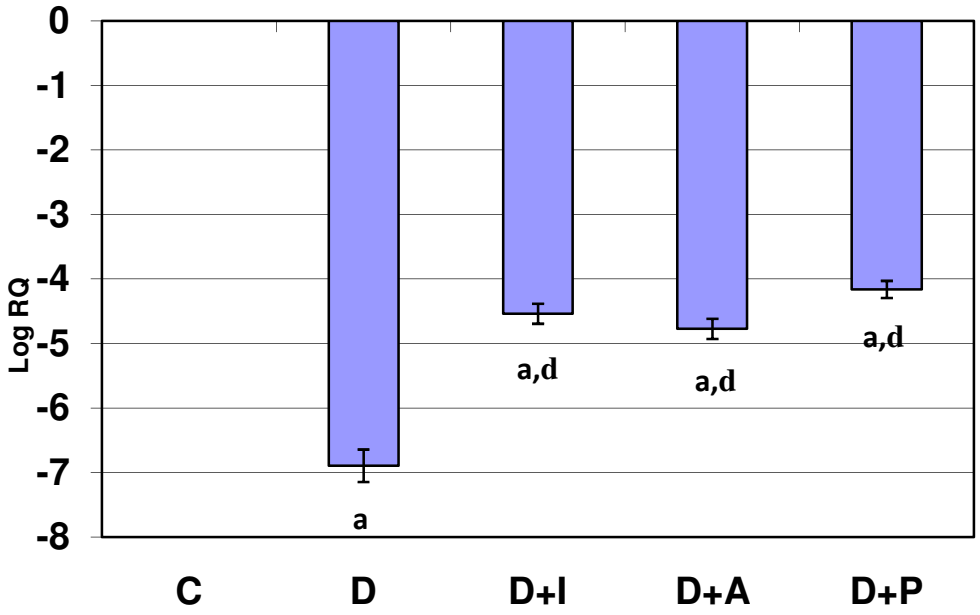


Table-64
Real Time amplification of IGF-1mRNA inthe pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	-6.89 ± 0.25^{a}
D+I	$-4.53 \pm 0.15^{a,d}$
D+A	$-4.77 \pm 0.15^{a,d}$
D+P	$-4.16 \pm 0.13^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-65
Real Time amplification of CREB mRNA in the pancreas of control and experimental rats

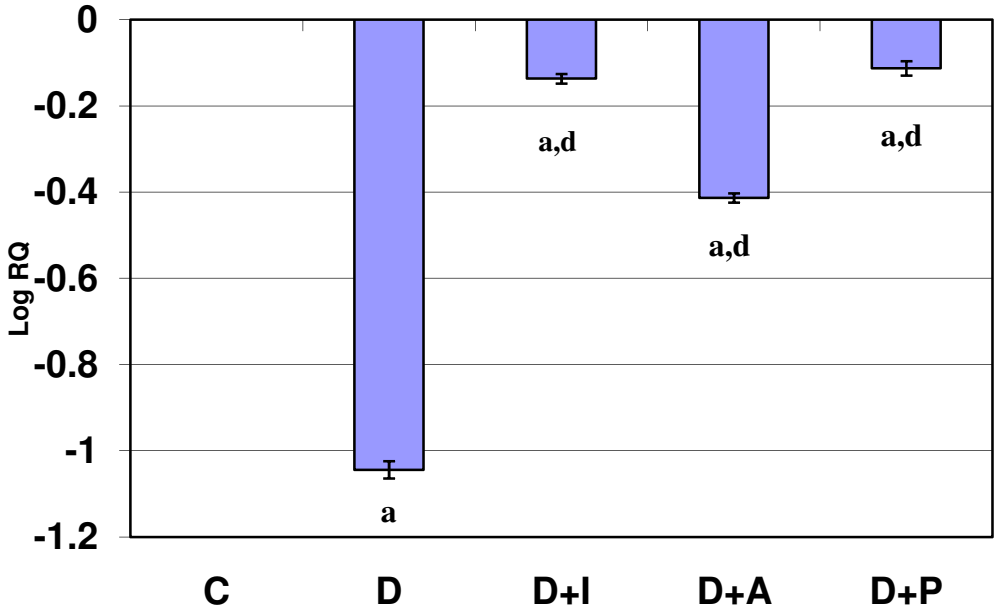


Table-65
Real Time amplification of CREB mRNA in the pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	-1.04 ± 0.005^{a}
D+I	$-0.13 \pm 0.003^{a,d}$
D+A	$-0.41 \pm 0.006^{a,d}$
D+P	$-0.11 \pm 0.004^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-66
Real Time amplification of NeuroD1 mRNA in the pancreas of control and experimental rats

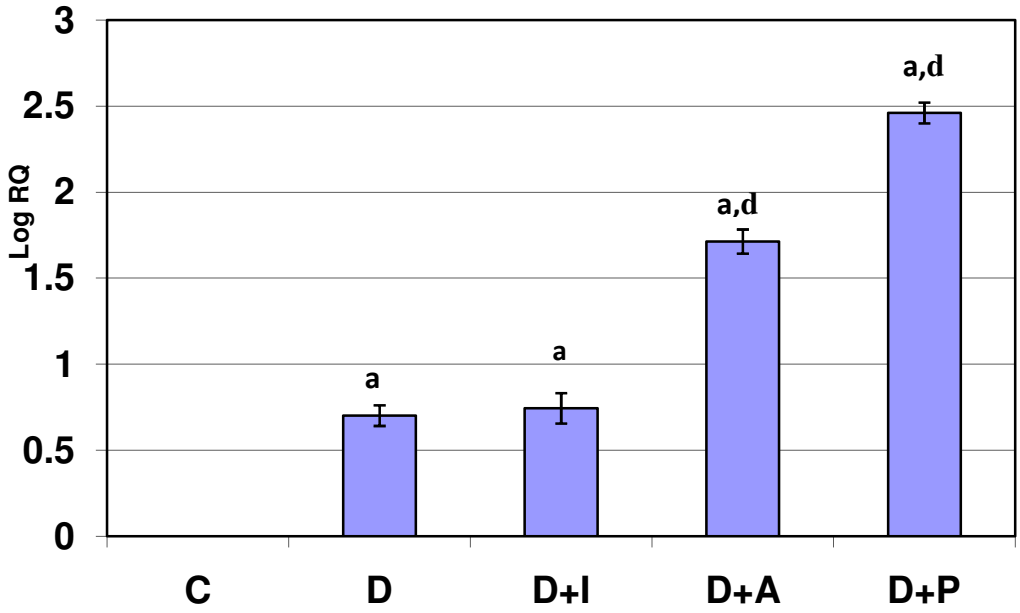


Table-66
Real Time amplification of NeuroD1 mRNA in the pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	0.70 ± 0.06^{a}
D+I	0.74 ± 0.04^{a}
D+A	$1.71 \pm 0.07^{a,d}$
D+P	$2.46 \pm 0.06^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-67
Real Time amplification of PDX-1 mRNA in the pancreas of control and experimental rats

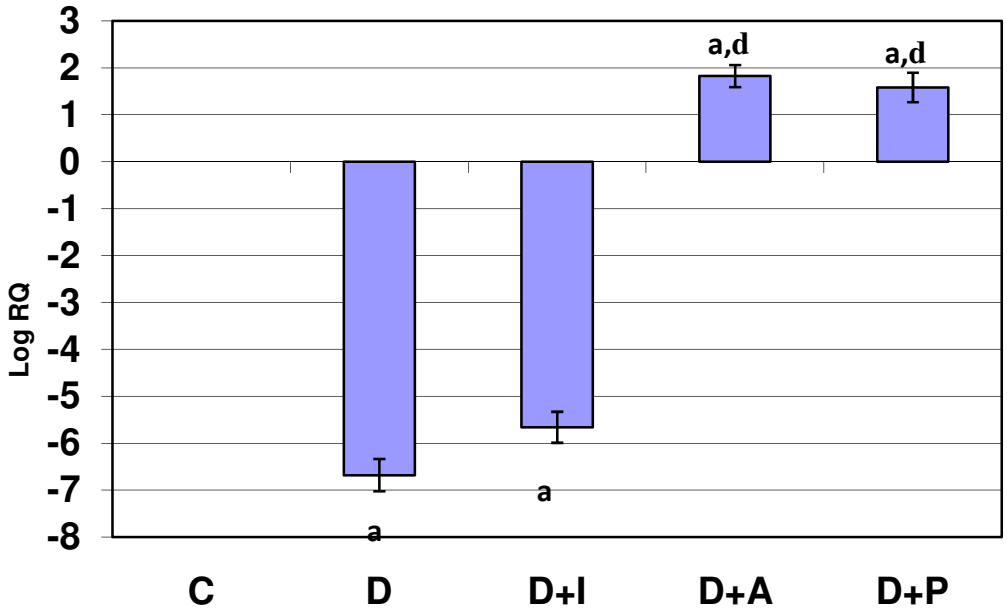


Table-67
Real Time amplification of PDX-1 mRNA in the pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	-6.68 ± 0.24^{a}
D+I	-5.66 ± 0.23^{a}
D+A	$1.82 \pm 0.13^{a,d}$
D+P	$1.58 \pm 0.14^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-68
cAMP content in the pancreas of control and experimental rats

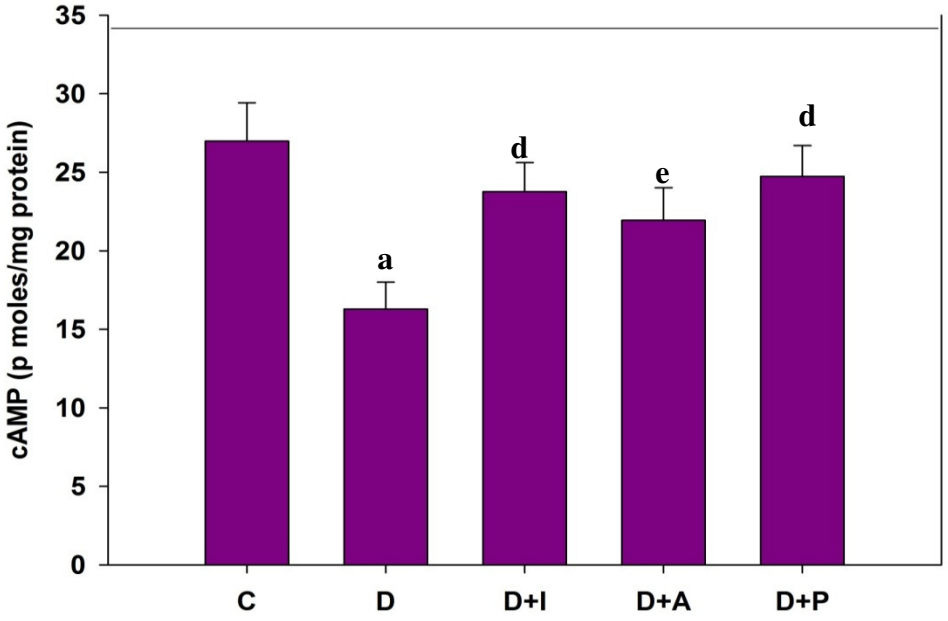


Table-68
cAMP content in the pancreas of control and experimental rats

Experimental groups	cAMP (pmoles/mg protein)
C	26.98± 2.45
D	16.29± 1.71 [a]
D+I	23.76 ± 1.85 [d]
D+A	21.94 ± 2.08 [e]
D+P	24.74 ± 1.96 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$,[e] $p<0.01$when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-69
cGMP content in the pancreas of control and experimental rats

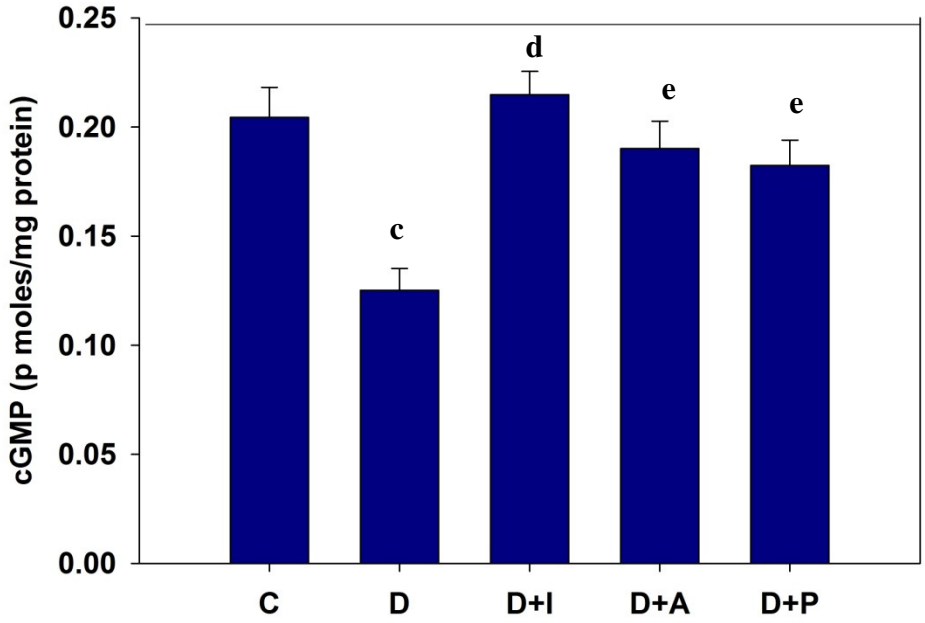


Table-69
cGMP content in the pancreas of control and experimental rats

Experimental groups	cGMP (pmoles/mg protein)
C	0.20± 0.01
D	0.12± 0.01[c]
D+I	0.21± 0.01[d]
D+A	0.19± 0.01[e]
D+P	0.18± 0.01[e]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [c] $p<0.05$ when compared with control; [d] $p<0.001$; [e] $p<0.01$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-70

IP_3 content in the pancreas of control and experimental rats

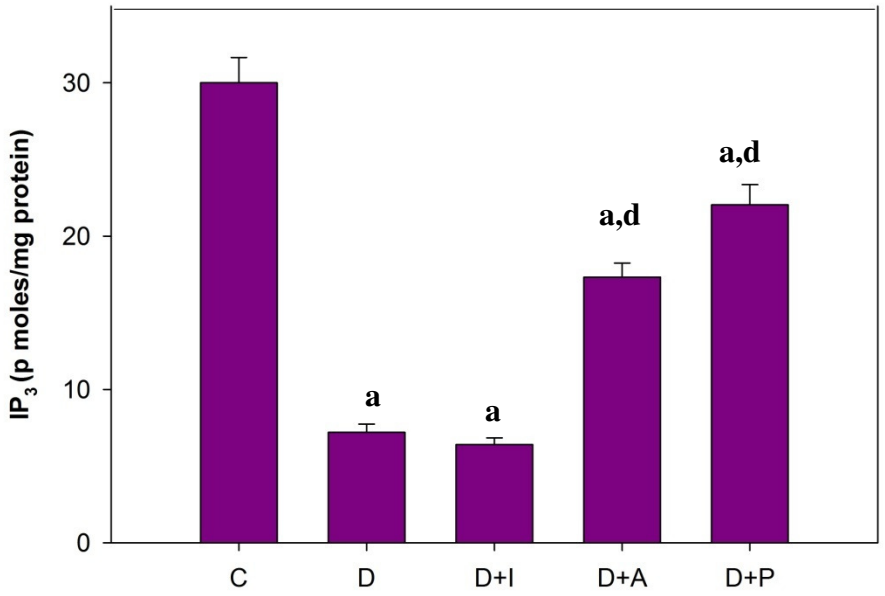


Table-70

IP_3 content in the pancreas of control and experimental rats

Experimental groups	IP_3 (pmoles/mg protein)
C	30.00± 1.65
D	7.20± 0.54[a]
D+I	6.40± 0.44[a]
D+A	17.32 ± 0.91[a,d]
D+P	22.04 ± 1.32[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-71
Confocal imaging of dopamine D_1 receptor expression in the pancreas of control and experimental rats

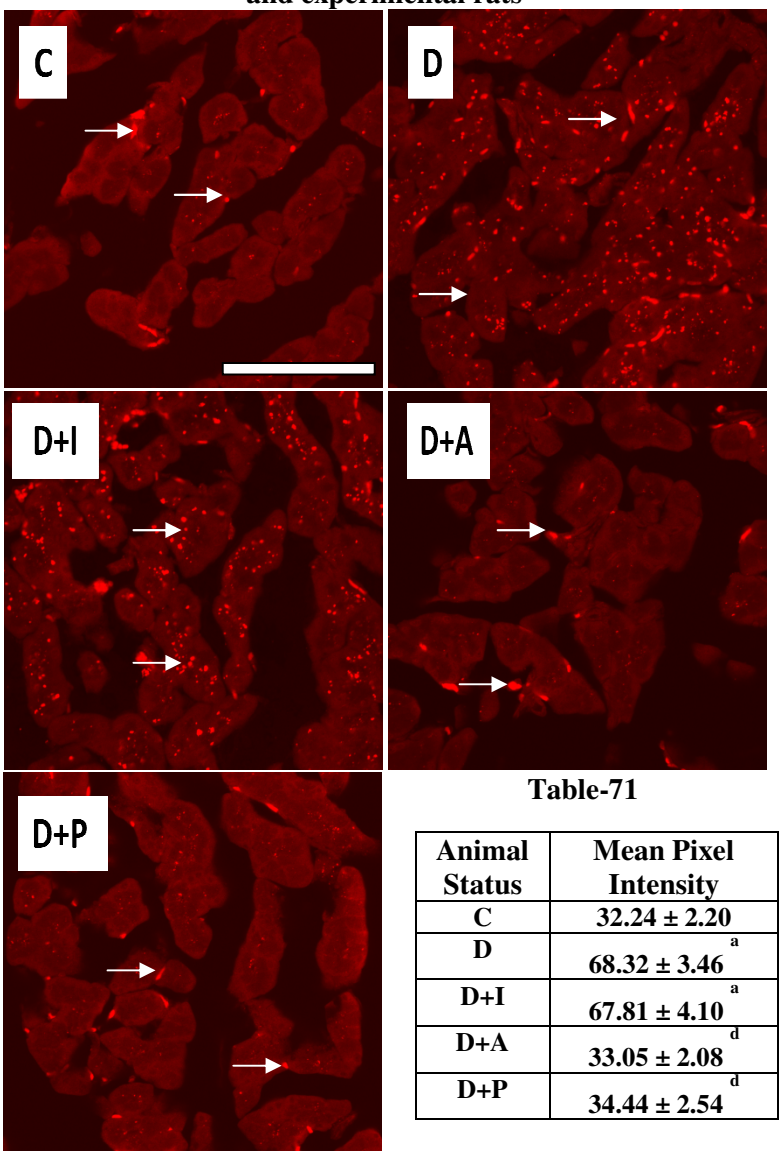


Table-71

Animal Status	Mean Pixel Intensity
C	32.24 ± 2.20
D	68.32 ± 3.46 [a]
D+I	67.81 ± 4.10 [a]
D+A	33.05 ± 2.08 [d]
D+P	34.44 ± 2.54 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control; [d] $p<0.001$ when compared with diabetic.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.Scale bar- 150 µm

Figure-72
Confocal imaging of dopamine D_2 receptor expression in the pancreas of control and experimental rats

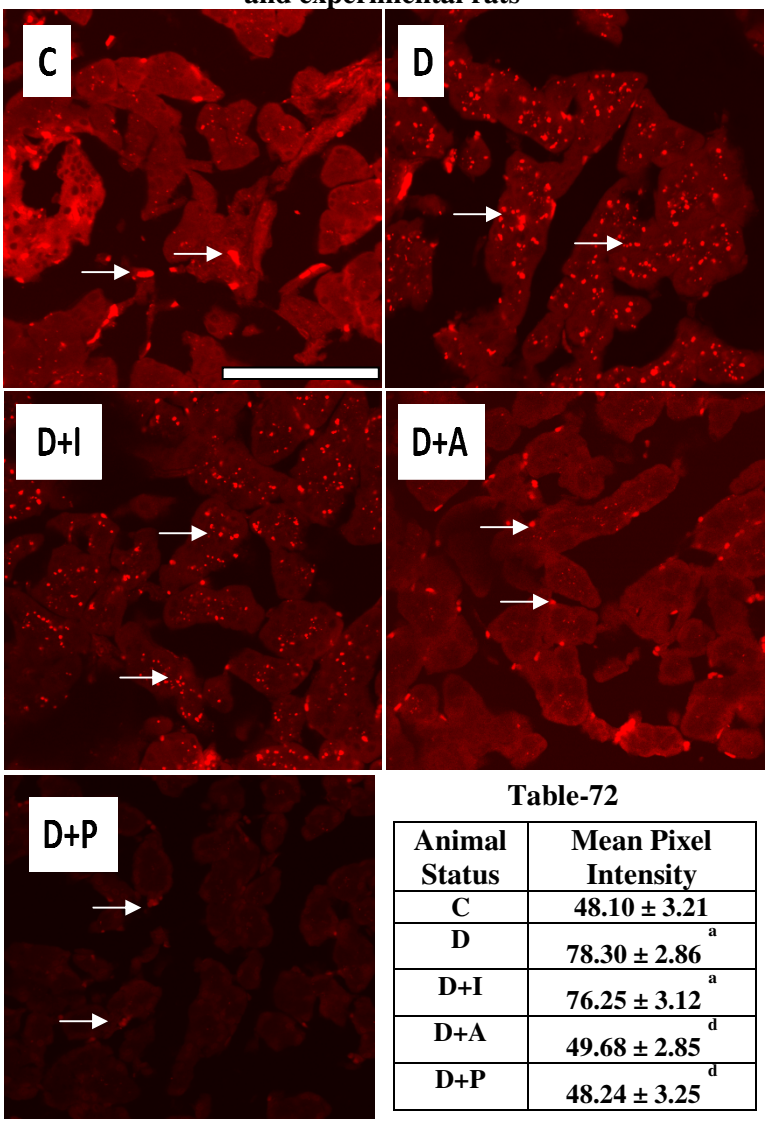


Table-72

Animal Status	Mean Pixel Intensity
C	48.10 ± 3.21
D	78.30 ± 2.86 [a]
D+I	76.25 ± 3.12 [a]
D+A	49.68 ± 2.85 [d]
D+P	48.24 ± 3.25 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.[a] $p<0.001$when compared with control; [d] $p<0.001$when compared with diabetic. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic, D+A- *Aegle marmelose* treated diabetic, D+P- pyridoxine treated diabetic.Scale bar-150 µm ⟶ in white shows dopamine D_2 receptor

Figure-73
BrdU imaging in the pancreas of control and experimental rats

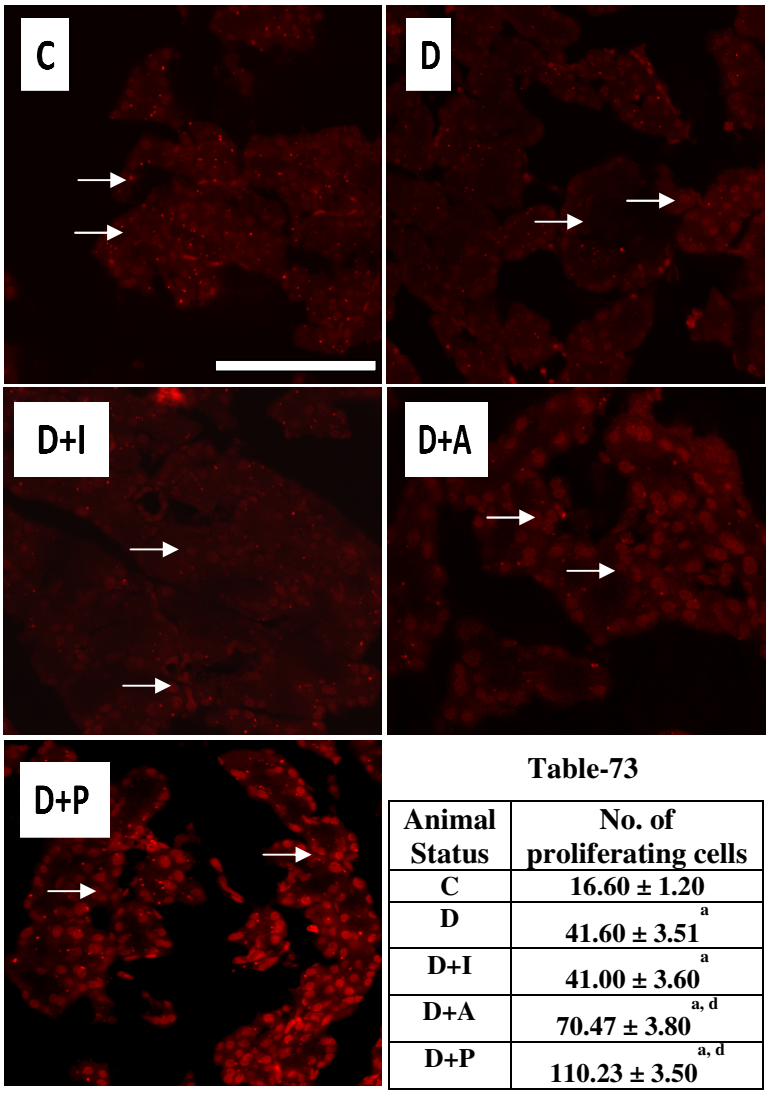


Table-73

Animal Status	No. of proliferating cells
C	16.60 ± 1.20
D	41.60 ± 3.51[a]
D+I	41.00 ± 3.60[a]
D+A	70.47 ± 3.80[a, d]
D+P	110.23 ± 3.50[a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.[a] $p<0.001$when compared with control; [d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.Scale bars represent 150 µm
→ in white shows BrdU positive cells

Figure-74
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the liver of control and experimental rats

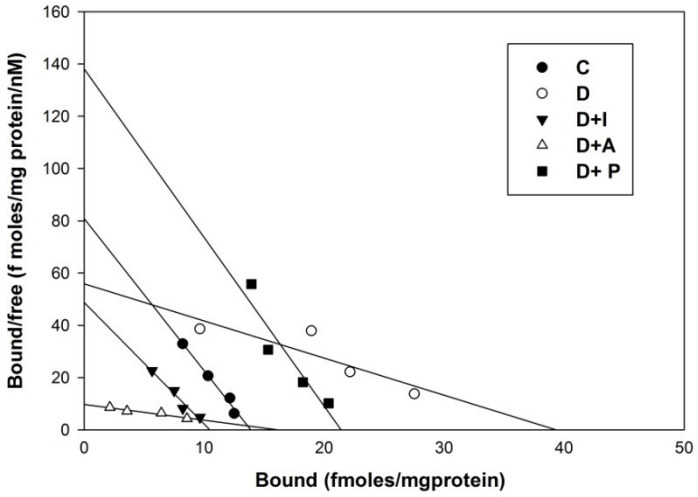


Table-74
Scatchard analysis of total dopamine receptor using [^{3}H] dopamine binding against dopamine in the liver of control and experimental rats

Experimental groups	Bmax (fmoles/mg protein)	Kd (nM)
C	13.85± 1.50	0.17 ±0.01
D	39.06 ± 3.00 [a]	0.70 ± 0.01 [a]
D + I	10.52 ± 1.73 [d]	0.21 ± 0.02 [d]
D + A	16.04 ± 1.46[d]	1.60 ± 0.10[a,d]
D + P	21.40 ± 1.44[c,d]	0.15 ± 0.01 [d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$,[C] $p<0.05$ when compared withcontrol;[d] $p<0.001$ when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-75
Real Time amplification of insulin receptor mRNA in the liver of control and experimental rats

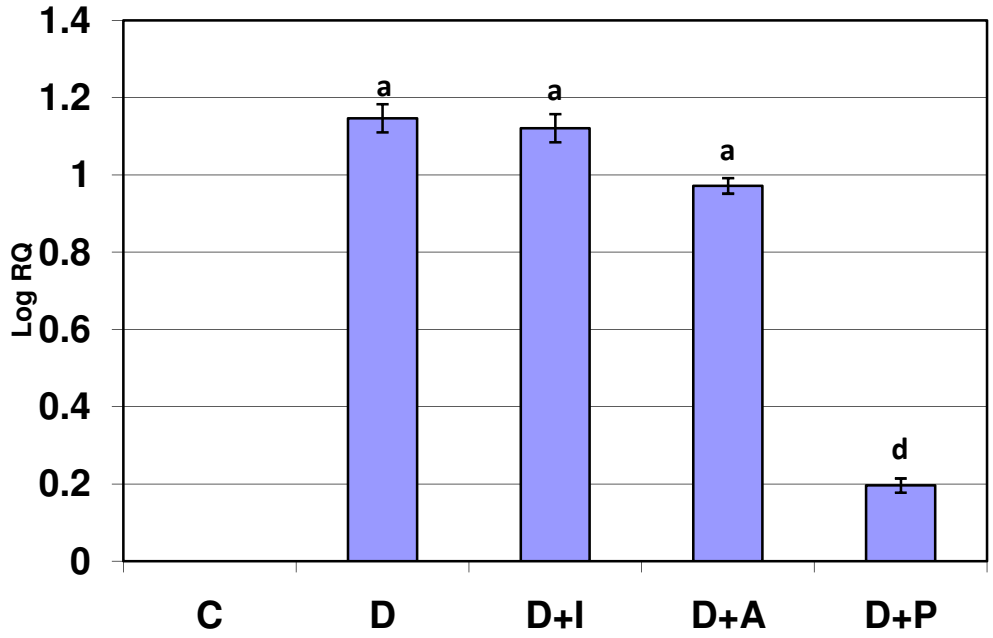


Table-75
Real Time amplification of insulin receptor mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	1.14± 0.03[a]
D+I	1.12± 0.03[a]
D+A	0.97± 0.02[a]
D+P	0.19± 0.01[d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats

Figure-76
Real Time amplification of GLUT-2 mRNA in the liver of control and experimental rats

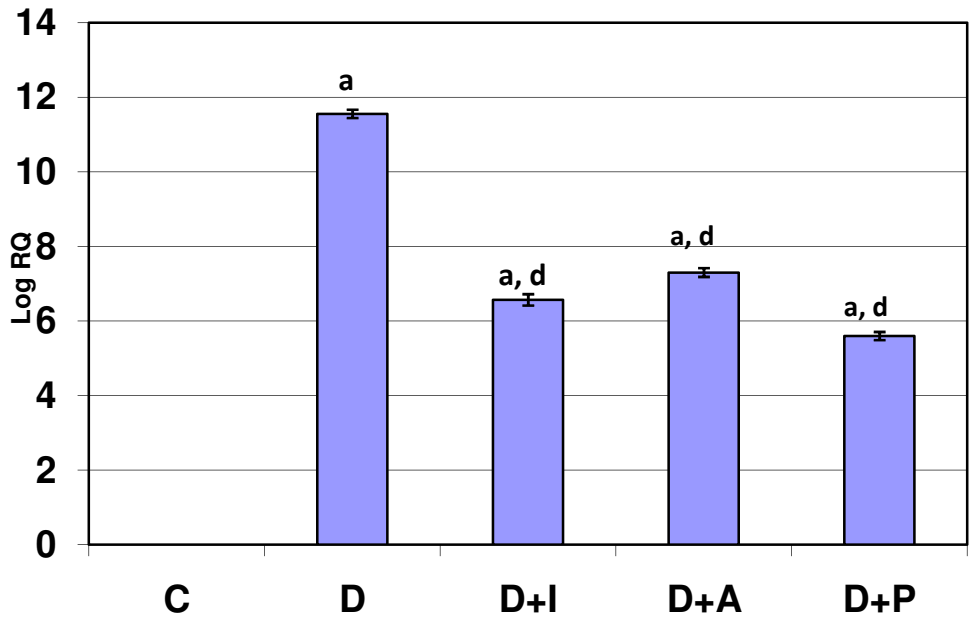


Table-76
Real Time amplification of GLUT-2 mRNA in the pancreas of control and experimental rats

Experimental groups	Log RQ
C	0
D	11.55± 0.11[a]
D+I	6.56± 0.12[a, d]
D+A	7.30± 0.11[a, d]
D+P	5.59± 0.10[a, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-77
Real Time amplification of IGF-1 mRNA in the liver of control and experimental rats

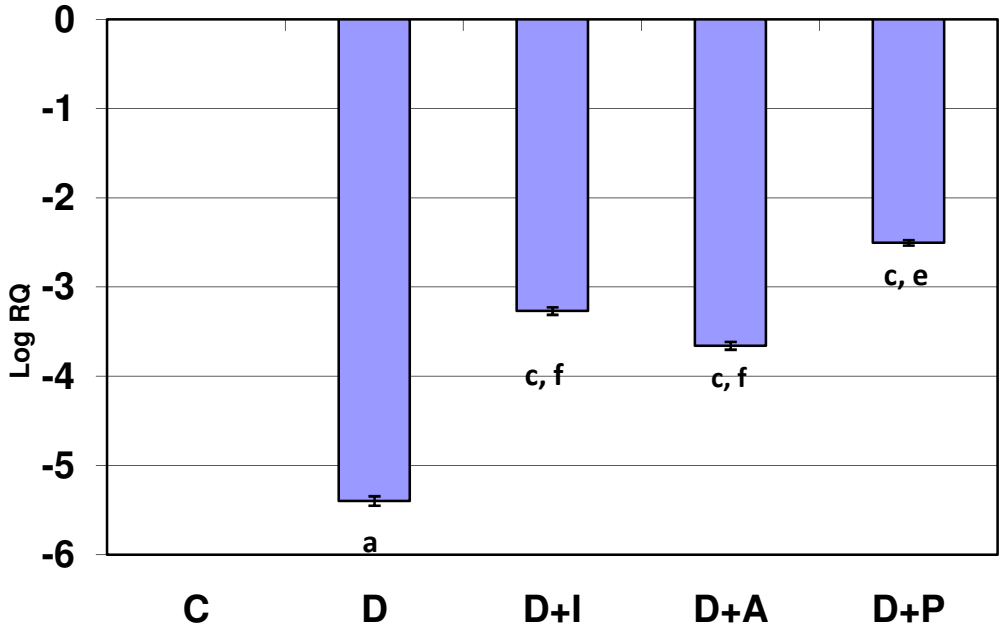


Table-77
Real Time amplification of IGF-1 mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	-5.39 ± 0.05^{a}
D+I	$-3.26 \pm 0.04^{c,f}$
D+A	$-3.65 \pm 0.04^{c,f}$
D+P	$-2.50 \pm 0.02^{c,e}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$ when compared with control;[e] $p<0.01$, [f] $p<0.05$when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-78
Real Time amplification of CREB mRNA in the liver of control and experimental rats

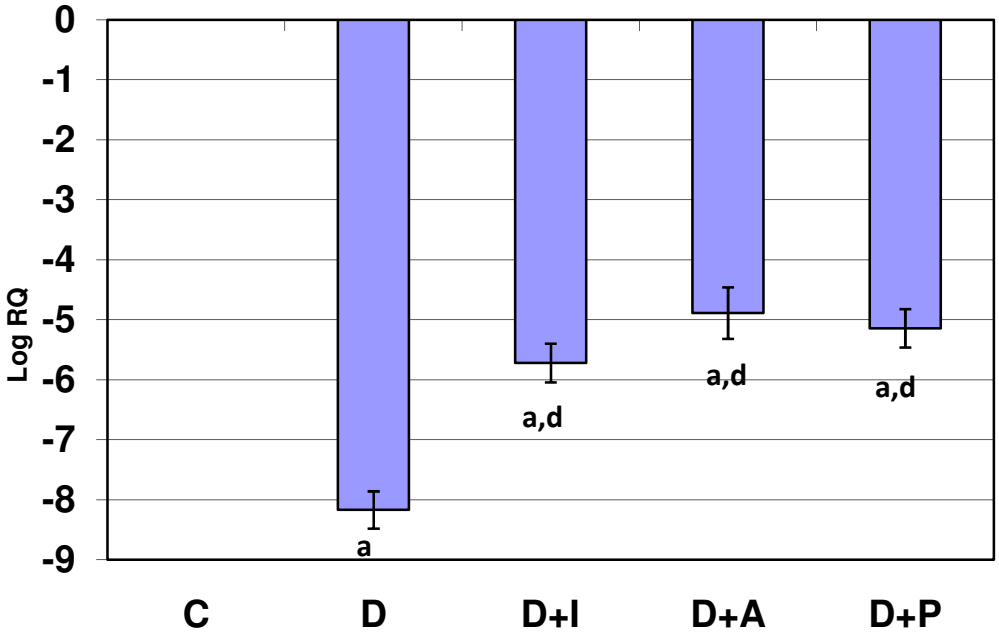


Table-78
Real Time amplification of CREB mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	-8.16 ± 0.15^{a}
D+I	$-5.71 \pm 0.14^{a,d}$
D+A	$-4.88 \pm 0.16^{a,d}$
D+P	$-5.14 \pm 0.13^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- Control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-79
Real Time amplification of phospholipase-C mRNA in the liver of control and experimental rats

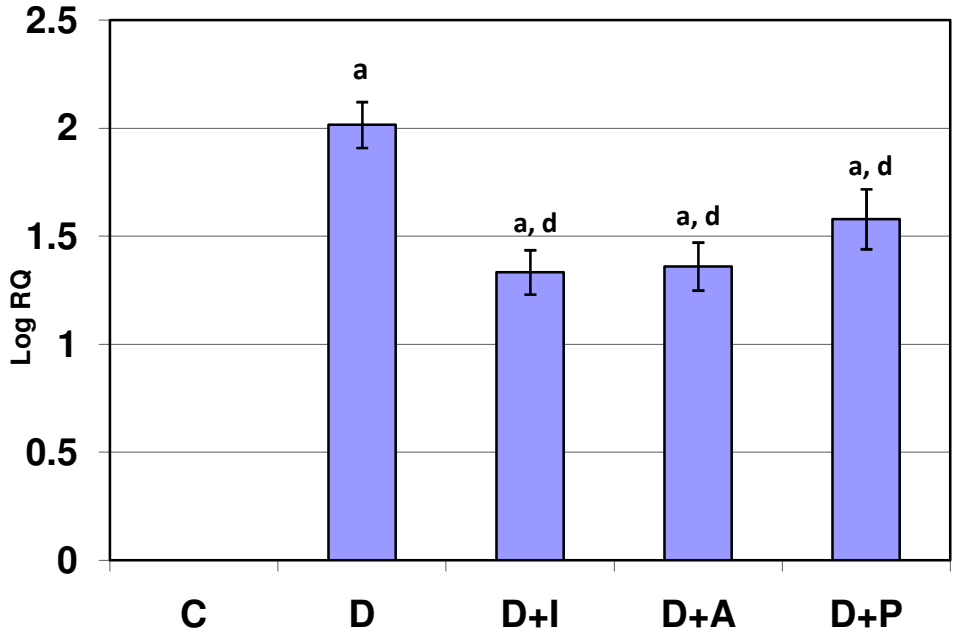


Table-79
Real Time amplification of phospholipase-C mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	2.01 ± 0.02^{a}
D+I	$1.33 \pm 0.02^{a,d}$
D+A	$1.36 \pm 0.02^{a,d}$
D+P	$1.57 \pm 0.03^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-80
Real Time amplification of TNF-α mRNA in the liver of control and experimental rats

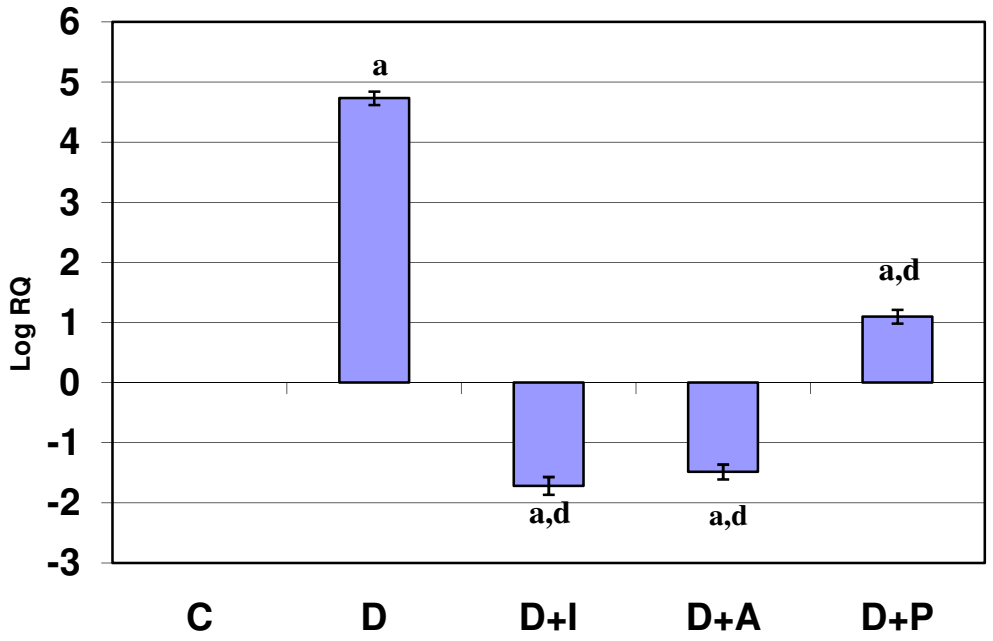


Table-80
Real Time amplification of TNF-α mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	4.73 ± 0.11^{a}
D+I	$-1.71 \pm 0.14^{a,d}$
D+A	$-1.48 \pm 0.12^{a,d}$
D+P	$1.09 \pm 0.10^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-81
Real Time amplification of NFκB mRNA in the liver of control and experimental rats

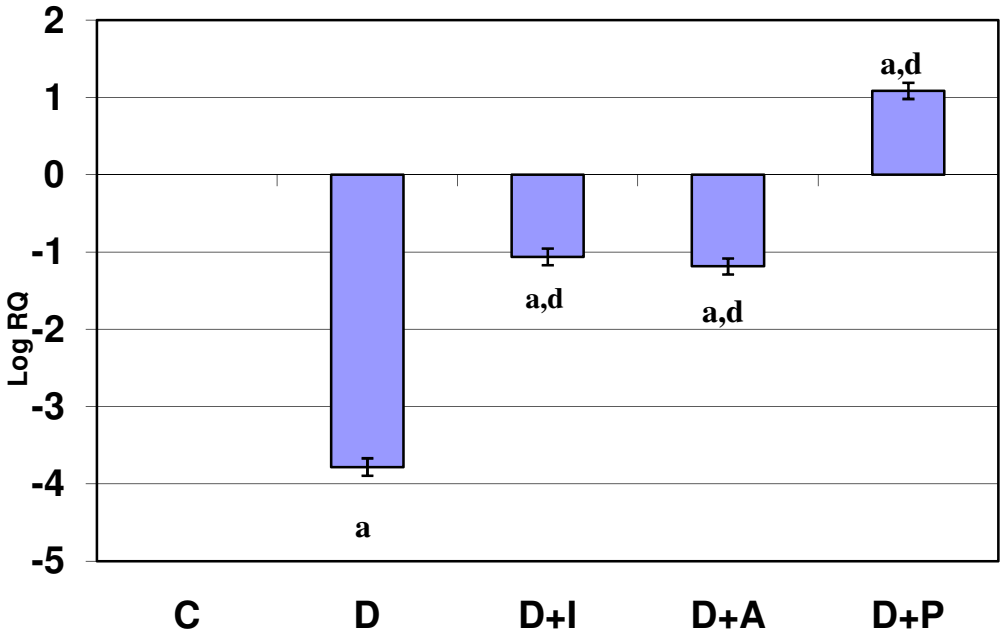


Table-81
Real Time amplification of NFκB mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	-3.78 ± 0.13^{a}
D+I	$-1.06 \pm 0.10^{a,d}$
D+A	$-1.18 \pm 0.10^{a,d}$
D+P	$1.08 \pm 0.10^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-82
Real Time amplification of Akt-1 mRNA in the liver of control and experimental rats

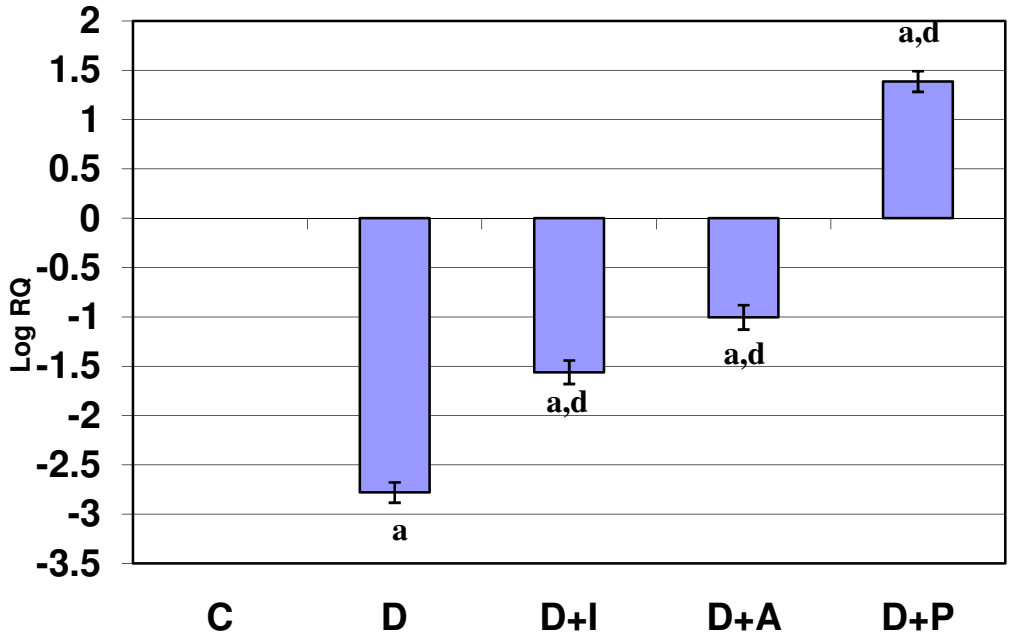


Table-82
Real Time amplification of Akt-1mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	-2.78 ± 0.10^{a}
D+I	$-1.56 \pm 0.11^{a,d}$
D+A	$-1.00 \pm 0.12^{a,d}$
D+P	$1.38 \pm 0.10^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-83
Real Time amplification of Caspase-8 mRNA in the liver of control and experimental rats

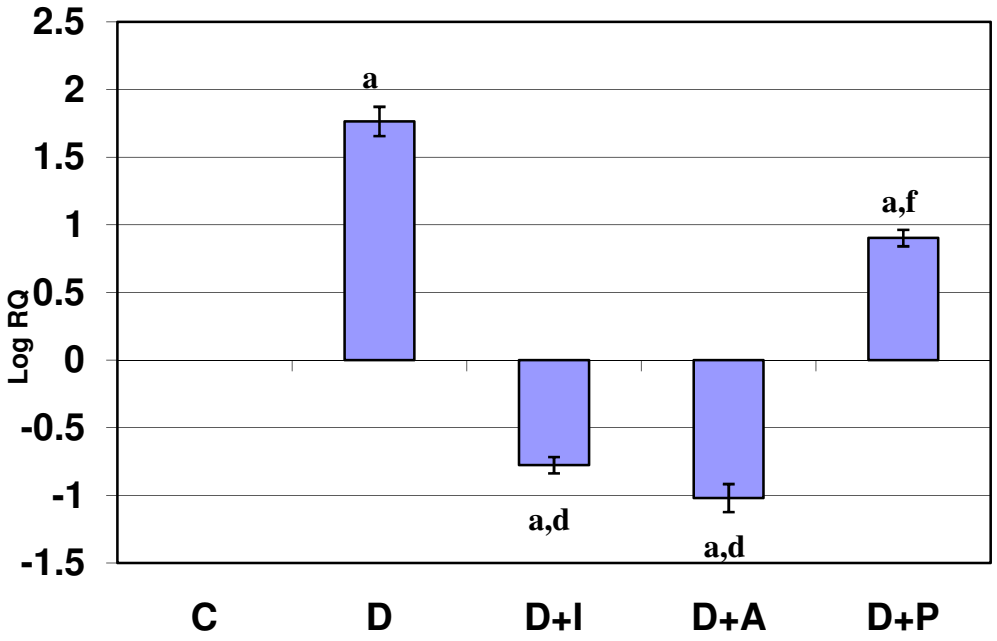


Table-83
Real Time amplification of Caspase-8 mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	1.76 ± 0.10^{a}
D+I	$-0.77 \pm 0.06^{a,d}$
D+A	$-1.01 \pm 0.10^{a,d}$
D+P	$0.90 \pm 0.06^{a,f}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$; [f] $p<0.05$when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-84
Real Time amplification of SOD mRNA in the liver of control and experimental rats

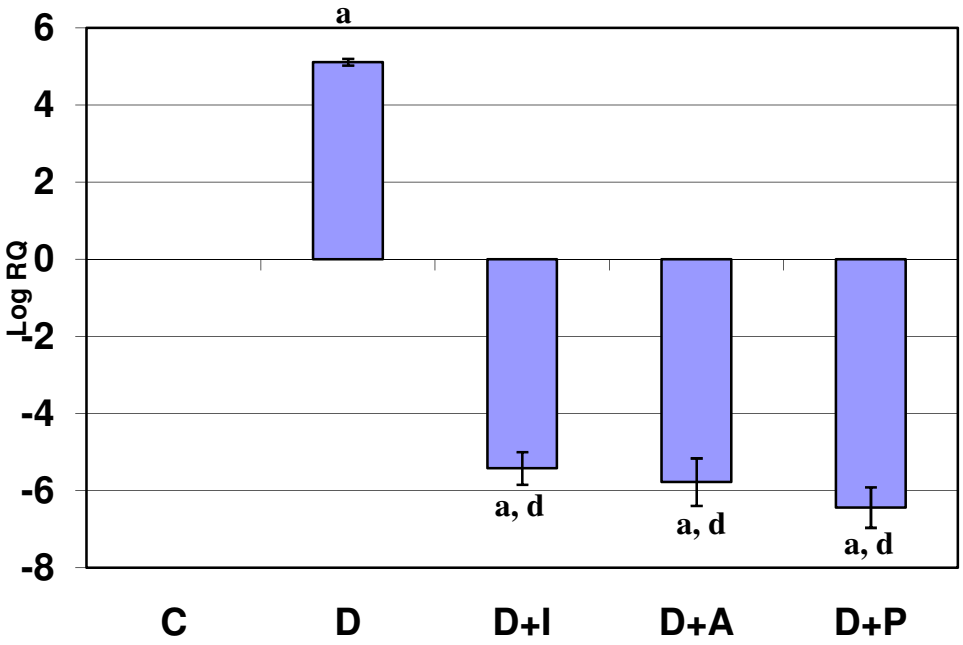


Table-84
Real Time amplification of SOD mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	5.10 ± 0.06^{a}
D+I	$-5.42 \pm 0.20^{a,d}$
D+A	$-5.77 \pm 0.24^{a,d}$
D+P	$-6.43 \pm 0.20^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-85
Real Time amplification of GPx mRNA in the liver of control and experimental rats

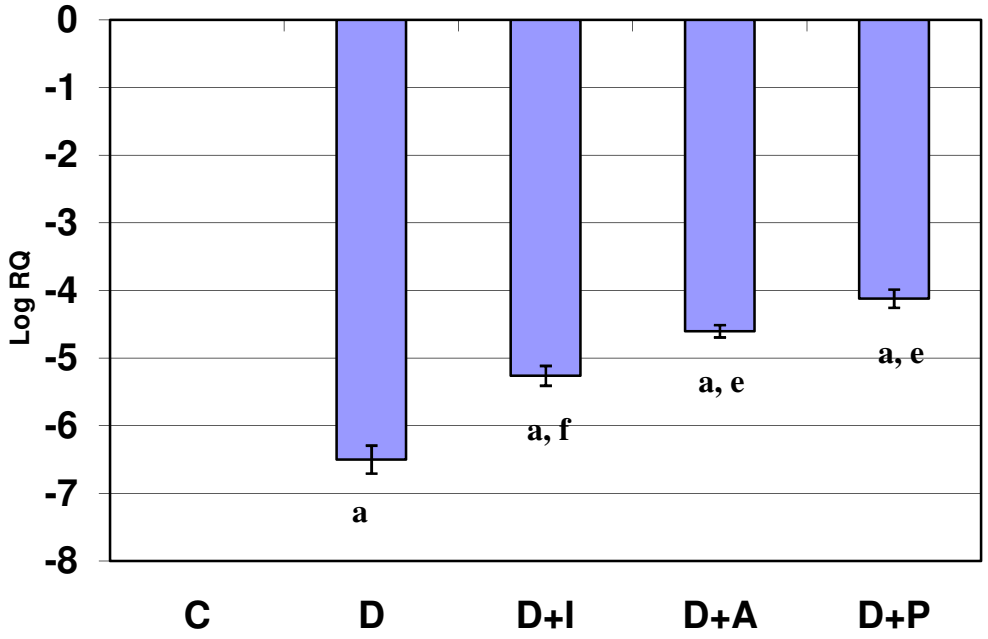


Table-85
Real Time amplification of GPx mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	-6.49 ± 0.06^{a}
D+I	$-5.26 \pm 0.05^{a,f}$
D+A	$-4.60 \pm 0.04^{a,e}$
D+P	$-4.12 \pm 0.04^{a,e}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[e] $p<0.01$,[f] $p<0.05$when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-86
Real Time amplification of BAX mRNA in the liver of control and experimental rats

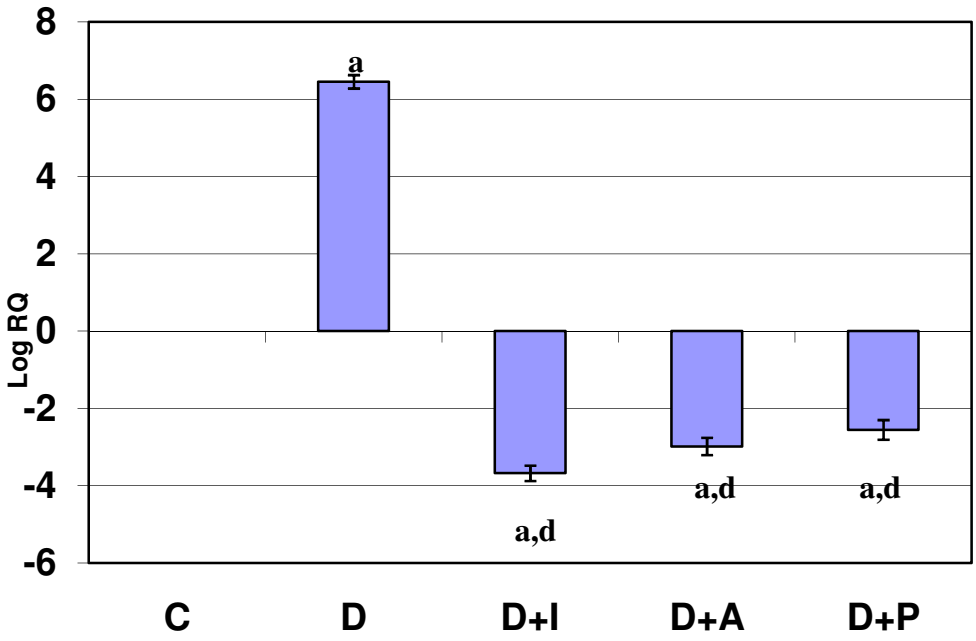


Table-86
Real Time amplification of BAX mRNA in the liver of control and experimental rats

Experimental groups	Log RQ
C	0
D	6.45 ± 0.06^{a}
D+I	$-3.67 \pm 0.05^{a,d}$
D+A	$-2.98 \pm 0.04^{a,d}$
D+P	$-2.55 \pm 0.05^{a,d}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$ when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-87
cAMP content in the liver of control and experimental rats

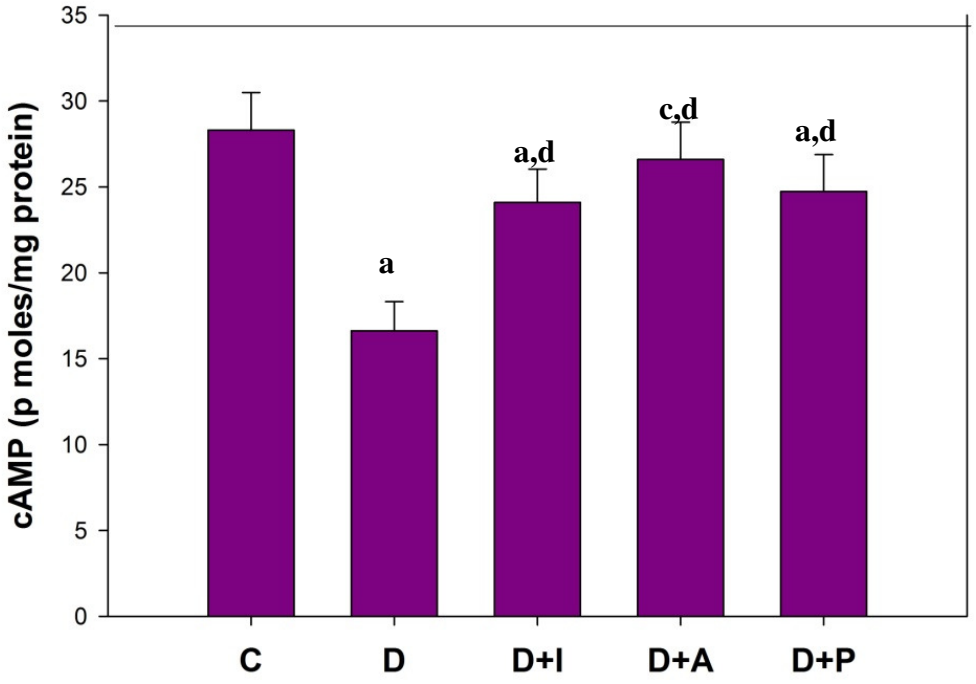


Table-87
cAMP content in the liver of control and experimental rats

Experimental groups	cAMP (pmoles/mg protein)
C	28.13 ± 2.19
D	16.62 ± 1.70[a]
D+I	24.10 ± 1.94[a,d]
D+A	26.60 ± 2.17[c,d]
D+P	24.74 ± 2.14[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$, [c] $p<0.05$ when compared with control;[d] $p<0.001$when compared with diabetic group.C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-88
cGMP content in the liver of control and experimental rats

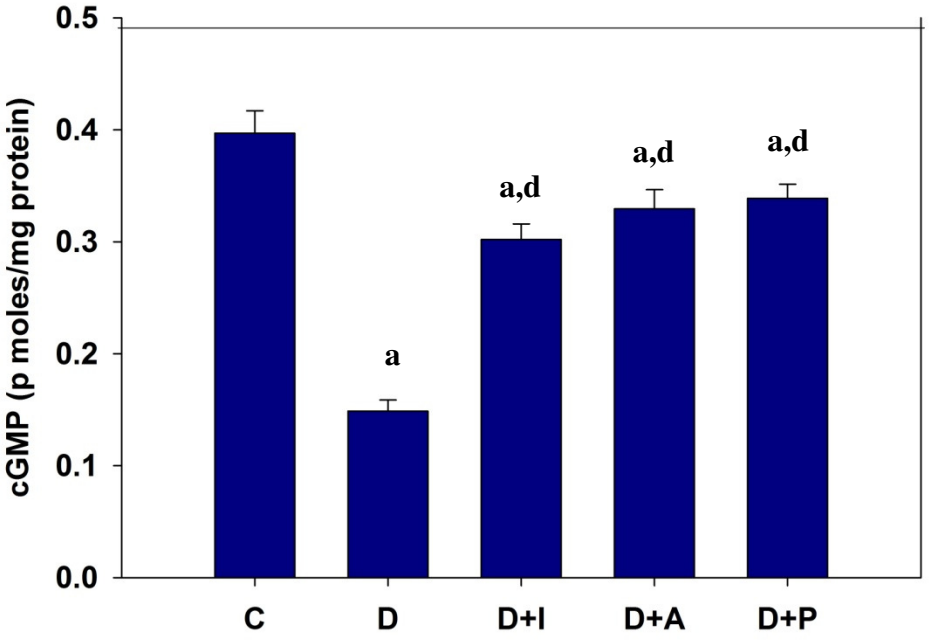


Table-88
cGMP content in the liver of control and experimental rats

Experimental groups	cGMP (pmoles/mg protein)
C	0.39± 0.03
D	0.14± 0.01[a]
D+I	0.30± 0.02[a,d]
D+A	0.32± 0.26[a,d]
D+P	0.33± 0.02[a,d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats. [a] $p<0.001$ when compared with control;[d] $p<0.001$when compared with diabetic group. C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Figure-89

IP_3 content in the liver of control and experimental rats

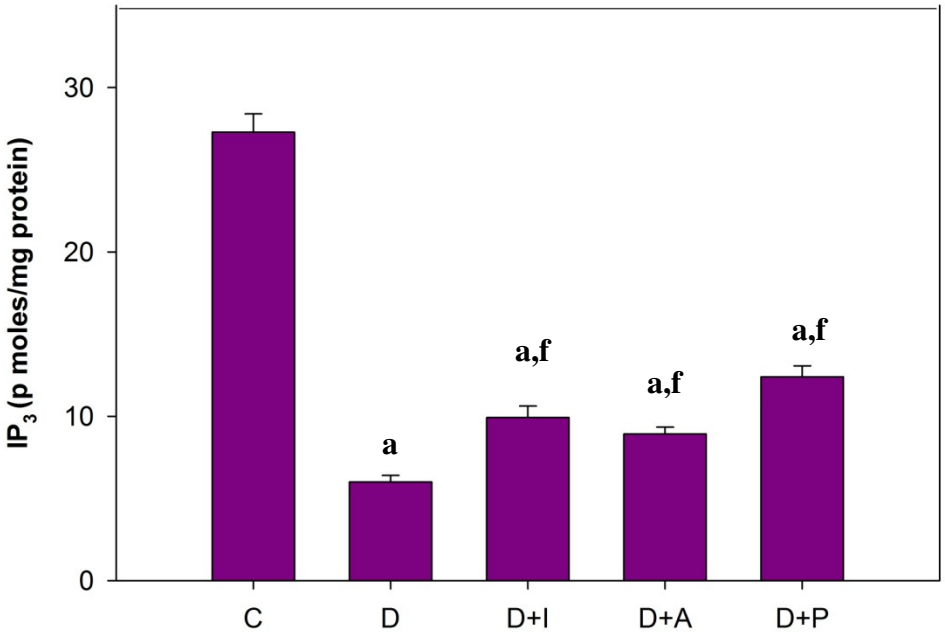


Table-89

IP_3 content in the liver of control and cxperimental rats

Experimental groups	IP_3 (pmoles/mg protein)
C	27.28± 1.12
D	6.00 ± 0.40^{a}
D+I	$9.92\pm 0.70^{a,f}$
D+A	$8.92\pm 0.42^{a,f}$
D+P	$12.40\pm 0.67^{a,f}$

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 4-6 rats.
[a] $p<0.001$when compared with control;[f] $p<0.05$when compared with diabetic group.
C- control rats, D- diabetic rats, D+I- Insulin treated diabetic rats, D+A- *Aegle marmelose* treated diabetic rats, D+P- pyridoxine treated diabetic rats.

Discussion

Diabetes mellitus, a major endocrine disorder, has become a severe health problem in the world. It is a chronic disease characterized by relative or absolute deficiency of insulin, resulting in glucose intolerance. The incidences type 2 diabetes mellitus rising at epidemic proportions. It is characterized by disturbances in carbohydrate, lipid and protein metabolism and insulin resistance concomitantly with deficits of pancreatic β cell mass and insulin secretion relative to metabolic demands. Type 2 diabetes is often associated with obesity and hypertension. Prolonged exposure to chronic hyperglycaemia in diabetes can lead to various complications, affecting the neurological, cardiovascular, renal and visual systems (Brownlee, 2001). There is increasing awareness that diabetes has an impact on the central nervous system, with reports of impaired learning, memory, mental flexibility and problem solving being more common in both type 1 and type 2 diabetic subjects than in the general population (Ryan *et al.,* 1985; Reaven *et al.,* 1990; Ryan &Williams, 1993; McCarthy *et al.,* 2002; Desrocher & Rovet, 2004; Cukierman *et al.,* 2005; Manschot *et al.,* 2006; Biessels *et al.,* 2008). The various neurotransmitter systems, including, serotonergic, cholinergic, glutamatergic and GABAergic, undergo a significant change in diabetes (Gireesh *et al*., 2008; Antony *et al*., 2010; Anu *et al*., 2010; Kumar *et al*., 2010).

BLOOD GLUCOSE, INSULIN LEVEL AND BODY WEIGHT

The streptozotocin induced diabetic rat serves as an excellent model to study the molecular, cellular and morphological changes in brain induced by stress during diabetes. Non insulin dependent diabetes is characterized by progressive destruction of β cell resulting in insulin deficiency and hyperglycemia (Ahren, 2000). Hyperglycaemia occurs as a result of increased glycogenolysis, decreased glycogenesis, increased gluconeogenesis, impaired glucose transport across membranes and almost complete suppression of the conversion of glucose into fatty

acids. Hyperglycaemic state during diabetes is due to the increased gluconeogenic pathway, which is physiologically less sensitive to the inhibition by insulin (Burcelin *et al.,* 1995). There was a significant increase in blood glucose level and decrease in the circulating insulin level of diabetic rats when compared to control group. The decreased circulating level of insulin in diabetic condition resulted in the increase of blood glucose level. Insulin, *Aegle marmelose* leaf extracts and pyridoxine treatment significantly increased circulating insulin with a significant decrease in blood glucose level. Previous reports confirmed the glucose lowering activity of *Aegle marmelose* leaf extract and pyridoxine in diabetic rats (Ponnachan *et al.,* 1993; Nair *et al.,* 1998; Abraham *et al.,* 2010). Previous studies at molecular level showed diabetes-induced changes of the cholinergic activity and the regulatory role of insulin on binding parameters and gene expression of total and muscarinic M1 receptors (Gireesh *et al.,* 2009). It is also reported that there is decrease in total muscarinic and muscarinic M1 receptors during diabetes which is up regulated by insulin and *Aegle marmelose* leaf extract treatment (Gireesh *et al.,* 2008). The increase in insulin levels in pyridoxine and *Aegle marmelose* leaf extract treated diabetic rats attribute to the stimulation of the surviving β-cells by the plant extract, which in turn exerts an anti-hyperglycaemic action. Available Reports show that anti-diabetic plants are known to increase circulating insulin levels (Lamela *et al.,* 1985). Thus, it can be suggested that the treatment induce the release of insulin thereby potentiating its effect. A possible mechanism of action is that the *Aegle marmelose* stimulate the residual pancreatic β-cell function or produced the anti-hyperglycaemic effect through enhanced peripheral utilization of glucose.

Diabetic rats also showed a significant decrease in body weight. Hyperglycemia and decreased body weight during diabetes are similar with previous reports (Junod *et al.,* 1969; Kumar *et al.,* 2010). Moreover, reports suggest that alterations in plasma insulin or glucose levels influence food intake and food preferences (Rodin, 1985). Dopamine receptor alterations in diabetic condition is

suggested to have affected the feeding behaviour. It is supported by the fact that, genetically engineered dopamine-deficient mice fail to initiate feeding and consequently die of starvation, unless L-DOPA, the precursor of dopamine, is provided daily (Zhou & Palmiter, 1995). Treatment with insulin, *Aegle marmelose* and pyridoxine significantly reduced body weight loss in diabetic rats, indicated the role of *Aegle marmelose* and pyridoxine in reducing diabetic complications.

CNS DOPAMINERGIC SYSTEM

Dopamine is intimately involved in the regulation of energy balance. Dopamine plays an important role in the complex physiology driving meal initiation and termination. Moreover, dopaminergic neurotransmission profoundly affects glucose and lipid metabolism (Meier & Cincotta, 1996). Dopamine release in response to food intake induces satiety and reward (Meguid *et al.,* 2000). Genetically engineered dopamine deficient mice fail to initiate feeding and consequently die of starvation, unless L-DOPA, the precursor of dopamine is provided daily (Zhou & Palmiter, 1995). Modulation of dopamine D_2 receptor activity profoundly affects energy homeostasis in humans and animals. Drugs that block dopamine D_2 receptor enhance appetite and induce weight gain in animals and humans (Baptista *et al.,* 1987, 2002; Ader *et al.,* 2005; Newcomer *et al.,* 2005). In addition dopamine D_2 receptor agonists improve glucose and lipid metabolism in patients with hyper prolactinemia and acromegaly (Chiba *et al.,* 1982; Hainer *et al.,* 1985; Rau *et al.,* 1993; Yavuz *et al.,* 2003; Dos Santos Silva *et al.,* 2011). Short term administration of dopamine D_2 receptor agonist-bromocriptine ameliorates various metabolic anomalies in obese humans without affecting body weight and longer term treatment improves glycemic control and serum lipid profiles in patients with type 2 diabetes (Cincotta *et al.,* 1999; Kok *et al.,* 2006). Recently, timed-release bromocriptine (Cycloset), a sympatholytic dopamine D_2 receptor agonist has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of type 2 diabetes. Dopamine levels are low

during the insulin-resistant state and increase to normal following return of the insulin-sensitive state (Luo *et al.,* 1998; 1999). Further, selective destruction of dopaminergic neurons in the suprachiasmatic nuclei causes severe insulin resistance and animal models of non seasonal obesity (i.e., ob/ob mouse Zucker fatty rat high energy–fed male Sprague-Dawley rats) have reduced dopamine levels in ventromedial hypothalamus and lateral hypothalamic nuclei (Oltmans, 1983; Levin & Dunn-Meynell, 1997; Luo *et al.,* 1997; Meguid *et al.,* 2000).

DOPAMINERGIC RECEPTOR EXPRESSIONS

STRIATUM

Disruptions in striatal dopaminergic signaling are thought to underlie a variety of psychomotor disorders including drug abuse, schizophrenia, Tourette's syndrome and Parkinson's disease (Hornykiewcz, 1973; Meltzer & Stahl, 1976; Sandor, 1993; Nestler &Aghajanian, 1997). Dopamine is involved in the regulation of energy balance. Alterations in dopaminergic neurotransmission are involved in the pathogenesis of type 2 diabetes (Lacković *et al.,*1990). Evidence suggests lower basal dopamine levels in the ventral striatum of streptozotocin-induced diabetic rats (Murzi *et al.,* 1996). The present study showed decreased expression of total dopamine receptors, dopamine D_1 receptors and increased expression of dopamine D_2 receptors in the striatum of diabetic condition. Hyperglycemia in rats is reported to decrease dopaminergic activity in the striata suggesting the up-regulation of dopamine receptors possibly due to the decreased dopamine metabolism (Ho *et al.,* 1994). Dopamine D_2 receptor density has been reported to be increased in the striatum of diabetic rats (Lozovsky *et al.,* 1981; Trulson & Hummel, 1983; Serri *et al., 1985).* The metabolic abnormalities during diabetes in the striatum cause alterations in dopaminergic neurons by decreasing their firing rate (Sailer, & Chiodo, 1980; Sailer, 1984).

Dopamine D_2 receptor up regulation observed in the present study is a compensatory mechanism to meet decreased dopamine D_1 receptor expression. Moreover, our previous studies showed decreased dopamine content in STZ induced diabetic rats (Shankar *et al.,* 2007). In the present study also, diabetes associated dopamine depletion have altered the expression dopamine receptors in striatum. During dopamine depletion there is increased activity of indirect pathway neurons (striatopallidal neurons, which express mainly dopamine D_2 receptor) and decreased activity of direct pathway neurons (striatonigral neurons, which express predominantly dopamine D_1 receptor). Dopamine D_2-type receptors have higher affinity for dopamine than dopamine D_1 receptors and D_1-mediated effects emerge earlier during dopamine depletion and small changes in dopamine levels could affect preferentially the activation of low affinity versus high affinity receptors, while dopamine D_2 receptor mediated effects become more prominent as the disease progresses (Richfield *et al.,* 1989; Jenner, 1995).

Insulin treatment significantly reversed dopamine D_1, D_2 receptor mRNA expressions and increased dopamine D_2 receptor binding sites in the striatum. Reports suggest that peripheral insulin injection to non diabetic rats increased the dopamine level in nucleus accumbens (Potter *et al.,* 1999). In the present study insulin treatment have augmented dopamine level and that played a significant role in the modulation of dopamine receptor expressions. *Aegle marmelose* treatment significantly increased the binding sites of total dopamine receptors, dopamine D_1 receptors and reversed the dopamine D_2 receptor binding parameters and mRNA expression of both receptor subtypes compared to diabetic condition. In pyridoxine treated rats, total dopamine and dopamine D_1 receptor binding parameters - B_{max} and K_d were significantly reversed towards control compared to diabetic condition. B_{max} and K_d values of dopamine D_2 receptor were significantly decreased in pyridoxine treated rats compared to diabetic group. Confocal imaging of dopamine D_1, D_2 receptor

expression studies are also in accordance with real time and receptor assay data. Confocal imaging studies in striatum showed decreased dopamine D_1 receptor expression and increased dopamine D_2 receptor expression in diabetic condition and showed reversal in treatment groups. Although the processes and mechanisms underlying the neuro protective effects of pyridoxine on dopamine receptor expressions remain to be elucidated, several reports suggest, the antioxidant properties of pyridoxine in this process. Moreover, pyridoxine is a cofactor for enzymes involved in a variety of metabolic pathways. Pyridoxine is involved in glucose homeostasis through glycogenolysis, glycolysis and glucose transport through its role in insulin and glucagon hormone action.

CEREBRAL CORTEX

The dopaminergic innervation of the cerebral cortex has been well characterized and dopamine receptor-mediated effects have also been demonstrated in the cortex (Bannon *et al.,* 1982; Penit-Soria *et al.,* 1987). Prefrontal cortex is a cortical area involved in selecting and retaining information to produce complex behaviours (Arianna *et al*., 2007). Somatosensory cortex has important role in food intake and obesity. Imaging studies reported activation of the somatosensory cortex in normal weight subjects with exposure to visual images of low caloric foods and with satiety (Tataranni *et al.,* 1999; Killgore *et al.,* 2003). Wang *et al.,* (2002) had reported higher than normal baseline metabolism in the somatosensory cortex of obese subjects.

Dopaminergic modulation of pre frontal cortex neural activity is regulated by multiple dopamine receptor subtypes. In animals, the meso prefrontal dopaminergic system is particularly vulnerable to stress. Dopamine D_1 receptor activation has both excitatory and inhibitory effects on synaptic activity in the prefrontal cortex, augmenting excitatory NMDA mediated synaptic responses on pyramidal cells, but also enhancing GABAergic transmission (Zheng *et al.,* 1999; Seamans *et al.,* 2001a, b; Tseng & O'Donnell, 2004). In contrast, D_2 receptors, which reside on pyramidal cells, local

circuit interneurons and presynaptic terminals, reduce the excitability of pre frontal cortex projection neurons while at the same time attenuating GABAergic activity (Vincent *et al.,* 1993; Sesack *et al.,* 1995; Seamans *et al.,* 2001a; Trantham-Davidson *et al.,* 2004; Tseng & O'Donnell, 2004). Actions of dopamine at D_1 family dopamine receptors are essential to working memory function in both the human and nonhuman primate prefrontal cortex (Sawaguchi & Goldman-Rakic, 1991, 1994; Williams & Goldman-Rakic, 1995; Murphy *et al.,* 1996; Mu¨ller *et al.,* 1998). Dopaminergic inputs to the prefrontal cortex region are important for its function, specific lesions of this mesocortical projection have been shown to impair performance on cognitive tasks as severely as ablation of the prefrontal cortex itself (Brozoski *et al.,* 1979; Simon *et al.,* 1980).

Food consumption increases brain dopamine levels in animals and humans (Hernandez & Hoebel, 1988, 1990; Small *et al.,* 2003). The reinforcing value of food is related to activity of the dopaminergic system. Individual differences in food reinforcement related to individual differences in dopaminergic activity. Cerebral cortical dopamine metabolism is reported to decrease because increased glucose during diabetes affects the dopaminergic activites such as working, memory and stress response (Kwok & Juorio, 1986; Tarn & Roth, 1997). Efflux of dopamine in the prefrontal cortex is reported to stimulate hunger and food intake and a perturbation in dopamine release affect memory-based search behaviour and feeding (Ahn & Phillips, 2002; Phillips *et al.,* 2004). Our results showed that in diabetic group, dopamine D_1 receptor binding parameters - B_{max} and K_d were significantly increased with an up regulation in dopamine D_1 receptor gene expression, whereas dopamine D_2 receptor binding parameters - B_{max} and K_d were significantly decreased and gene exression was down regulated. Confocal imaging studies of dopamine D_1 and D_2 receptor expressions in cerebral cortex demonstrated the increased expression dopamine D_1 receptors and decreased expression of dopamine D_2 receptors in diabetic condition and treatment groups showed significant reversal. Confocal imaging studies confirm

the receptor binding results and gene expression data. Dopamine activity is related to the density of dopamine receptors. Dopamine D_1 receptors are located post synaptically on the cortical neurons and the decreased dopamine level in the PFC induced by electrolytic lesion up regulates the dopamine D_1 receptor density in the PFC (Tassin *et al*., 1978, 1982). Similarly in the present study decreased dopamine level in the cerebral cortex augmented the expression of dopamine D_1 receptors. The D_1 family of dopamine receptors are more abundant in the prefrontal cortex than D_2 family receptors (Farde *et al.,* 1987; Goldman-Rakic *et al.,* 1990; Lidow *et al.,* 1991). Dopamine's role in inhibitory control is well recognized and its disruption contributes to behavioral disorders of discontrol such as obesity. Blocking of D_2 receptors increases food intake and raises the risk for obesity (Allison *et al.,* 1999). Modifying brain dopamine levels influences energy intake, with dopamine agonists reducing energy intake and dopamine antagonists increasing energy intake and body weight (Leddy et al., 2004; Wellman, 2005). In the present study decrease in dopamine D_2 receptors in the cortex showed to affect metabolism and feeding behaviour in diabetic rats. Imbalance in dopamine receptor expressions and dopamine depletion is suggested to be the cause for cortical dysfunction during diabetes. The functional connectivity between the striatum and the somatosensory cortex was recently corroborated in the human brain by functional imaging studies, which documented co-activation of the somatosensory cortex with that of the dorsal striatum (Postuma & Dagher, 2006). Striatal dopaminergic dysfunctions observed in the present study, have played a critical role in the modulation of cortical expression of dopaminergic receptors and dopaminergic system. It is supported by the fact that, somatosensory cortex influences brain dopamine activity including striatal dopamine release (Rossini *et al.,* 1995; Huttunen *et al.,* 2003; Chen *et al.,* 2007). There is also evidence that dopamine modulates the somatosensory cortex in the human brain (Kuo *et al.,* 2007).

In the present study total dopamine receptors were increased accompanied with an increase in its affinity in cerebral cortex of diabetic rats. This increased

number of dopamine receptors could account for the behavioural supersensitivity to dopamine agonist as a result of damage in the dopamine functions (Cresse *et al*., 1977). The increased total dopamine receptors account for the increased expression of dopamine D_1 receptors in the cerebral cortex because dopamine D_1 receptors are the predominant receptor subtype in the cecrebral cortex. Our results showed that treatment with insulin, *Aegle marmelose* and pyridoxine reversed the altered dopamine receptor expressions when compared with diabetic rats. Diabetes and its complications are related with oxidative stress. Our previous studies suggest that *Aegle marmelose* and pyridoxine treatment normalises oxidative stress in diabetic rats (Abraham *et al.,* 2010 a,b). Although, the mechanism of action of *Aegle marmelose* is not established, its antioxidant property accounts for the restoration of dopaminergic receptor expressions. Reports suggest that peripheral insulin injection to non diabetic rats increased the dopamine level in nucleus accumbens (Potter *et al.,* 1999). In the present study insulin treatment have augmented dopamine level and that played a significant role in the modulation of dopamine receptor expressions. We have reported the anti-hyperglycemic and neuroprotective activity of pyridoxine in normalizing diabetic related stress and anxiety in diabetic rats (Nair *et al.,* 1998; Abraham *et al.,* 2010). Recent reports suggest antioxidant properties of pyridoxine (Matxain *et al.,* 2006). Moreover, Pyridoxine acts as coenzyme in biosynthesis of neurotransmitters-γ-aminobutyric acid, dopamine, serotonin and it is important for development and function of the central nervous system (Dakshinamurti *et al.,* 1988; Ernahrungswiss, 1996). In the current study, pyridoxine's effects are mediated *via* resetting of dopaminergic and sympathetic tone within the CNS through its co enzymatic activity in the biosynthesis of dopamine.

BRAINSTEM

Chronic changes in glycemia induce alterations in brain glucose metabolism in rodents (McCall *et al.,* 1982; Nagy *et al.,* 1994). The brain stem is a complex rostral

continuation of the spinal cord and contains several collections of cell bodies. Brain stem receives sensory information from the face and contains the motor nuclei innervating the muscles of the face, eyes and the cranial parasympathetic system. In addition, much of the specialized sensory information originating from the cochlea and vestibular labyrinth, eyes, taste buds, cardiovascular, respiratory and digestive systems directly reaches the brain stem, where it is further processed. The sensory input and motor output of the brain stem is carried by cranial nerves.

Brain stem is an important part of the brain in monitoring the glucose status and the regulation of feeding (Guilford *et al.,* 2000). In the central nervous system, glucose regulates the activity of glucose-sensitive neurons present in the brain stem and the hypothalamus. Glucose sensing neurons have a critical role in regulating glucose and energy homeostasis through secretion of endocrine pancreas hormones, regulation of liver glucose production, feeding behaviour and energy expenditure (Marty *et al.,* 2007). These are glucose-excited neurons, which increase their firing rate with elevation in extracellular glucose concentrations and glucose-inhibited neurons, which are activated by a decrease in extracellular glucose concentration or by cellular glucoprivation (Routh, 2002; Yang *et al.,* 2004). Both types of neurons are widely distributed in the brain stem regions, in particular in the nucleus of the solitary tract (NTS), the area postrema and the dorsal motor nucleus of the vagus and it is involved in the control of energy homeostasis and food intake (Adachi *et al.,* 1984; Mizuno & Oomura, 1984; Yettefti *et al.,* 1997; Dallaporta *et al.,* 1999).

The dorsal motor nucleus of the vagus nerve is located in the brain stem. It is connected to the endocrine pancreas exclusively *via* vagal fibres and has a role in neurally mediated insulin release. The nucleus of the solitary tract in the caudal brain stem of the rat is the primary neuro anatomical site receiving visceral afferent information from postoral regions of the alimentary tract critical for the negative feedback control of food intake. The majority of published data demonstrate that the transmission of meal-related visceral afferent feedback signals to the brainstem is

mediated by the afferent vagus nerve, which terminates in the NTS. The finding that a small group of dopaminergic neurons in the caudal diencephalon projected to the spinal cord and brain stem indicated that dopamine was also involved in modulating the functions of these lower parts of the central nervous system.

Our results showed differential expression pattern dopamine receptor subtypes in brain stem of diabetic rats. D_1 receptor binding sites are significantly increased with a significant increase in affinity whereas D_2 receptor binding sites are decreased with a decrease in affinity. Our previous studies demonstrated adrenergic, serotonergic and dopamine D_2 receptor function alterations in the brainstem of diabetic rats (Abraham & Paulose, 1999; Padayatti & Paulose, 1999; Paulose *et al.,* 1999; Eswar *et al.,* 2007). Total dopamine receptor binding parameters - B_{max} and K_d were significantly decreased in diabetic condition. Adverse effects of hyperglycemia on brain function are not limited to higher centers but also involve the brain stem (Jones *et al.*, 1990). Real time PCR analysis demonstrated the up regulation of dopamine D_1 receptor gene expression and down regulation of dopamine D_2 receptor gene expression in the brain stem of diabetic rats. Confocal imaging studies using receptor specific antibodies, also showed a similar expression pattern for dopamine D_1 and D_2 receptors in the brain stem. These results indicate imbalance in dopaminergic receptor expressions and suggest impaired dopaminergic activity and dopamine related functions in the brain stem of hyperglycaemic rats. Our results showed that treatment with *Aegle marmelose* and pyridoxine reversed the altered dopamine receptor expressions when compared with diabetic rats. Resetting of doapminergic tone and activity in the striatum and cerebral cotex by *Aegle marmelose* and pyridoxine treatment has a significant role in the modulation of dopaminergic receptor expressions in the brain stem.

PANCREAS

Exocrine pancreas is an important source of non neuronal dopamine in the body (Mezey *et al.,* 1996). The pancreas contains markers usually associated with catecholaminergic and neuroendocrine cells. Pancreatic exocrine cells that produce the digestive enzymes synthesize and release dopamine into the duodenum. Dopamine appears to have a beneficial effect in acute pancreatitis (Sikiric *et al.,* 1988; Nishiwaki *et al.,* 1988; Shen *et al.,* 1990). The effects of dopamine on exocrine pancreatic secretion have been studied in several animal species (Hashimoto *et al.,* 1977; Furuta *et al.,* 1978). While a strong stimulatory effect on secretion was found in dogs the effect was less pronounced in other species and is unclear in humans (Hashimoto *et al.,* 1971; Valenzuela *et al.,* 1979; Iijima *et al.,* 1983; Takeuchi *et al.,* 1990). Anatomical studies suggest that the vagal efferent fibers originating from the nucleus ambiguus and dorsal motor nucleus of the brain stem directly innervate the pancreas through the parahypothalamic ventricular nucleus and have a role in insulin release (Bereiter *et al.,* 1981). Adrenergic and serotonergic innervations reach the pancreas through the brain stem (Smith & Davis, 1983, Lowey *et aI.,* 1994). Substantia nigra, an important autonomic area involved in controlling islet growth and development. It plays a role in modulating the outflow of both sympathetic and parasympathetic signals which ultimately reach the islets (Smith & Davis, 1983).

Insulin secretion from the pancreatic islets is controlled by the central nervous system through sympathetic and parasympathetic nerves (Ahren *et al.,* 2000). Pancreatic islets receive innervations from both divisions of the autonomic nervous system, and pancreatic endocrine secretion is partly controlled by the autonomic nervous system (Liu *et al.,* 2001). Dopamine in the peripheral nervous system modulates insulin secretion from the pancreatic islets (Nogueira *et al.,* 1994). Reports suggest that pancreatic β-cells express dopamine receptors and modulate the insulin secretion through its receptors (Davis *et al.,* 1985; Rubi *et al.,* 2005).

The results of the current study demonstrated increased dopamine D_1 and D_2 receptor binding parameters in the pancreas of diabetic rats compared to control. Total dopamine receptor binding parameters were also significantly increased in diabetic condition. In the present study pancreatic dopamine D_1 and D_2 receptor gene expression was up regulated in diabetic condition. Confocal imaging studies in the pancreas, showed increased expression of dopamine D_1 and D_2 receptor subtypes. *Aegle marmelose* and pyridoxine treatment significantly restored the altered expression dopamine receptor subtypes. Low concentrations of dopamine in pancreatic islets have decreased insulin secretion in diabetic condition (Shankar *et al.,* 2006). In the vicinity of pancreatic β cells, dopamine might be released from neurons innervating pancreatic islets (Bereiter *et al.,* 1981). In the present study, diabetes associated dopamine depletion resulted in the increased expression of dopamine receptor subtypes in the pancreas. Increased expression of dopamine receptor subtypes, modulated insulin secretion in diabetic rats. D_2 receptors are predominant isoform of dopamine receptors in pancreas. Our previous studies suggests, dopamine significantly stimulated insulin secretion at low concentration (10^{-8} M) in the presence of high glucose (20 mM) and higher concentrations of dopamine ($10^{(-7)}$-$10^{(-4)}$) inhibited glucose-induced insulin secretion in the presence of both 4 mM and 20 mM glucose. Dopamine exerts a differential effect on glucose-induced insulin secretion through dopamine D_2 receptor and it is essential for the regulation of glucose-induced insulin secretion by pancreatic islets *in vitro* (Shankar *et al.,* 2006). In contrary to the present data, reports suggest that dopamine inhibits glucose-stimulated insulin secretion, most likely *via* D_2-receptors that are expressed in pancreatic β-cells (Rubi *et al.,* 2005).

Dopamine D_2 receptors are modulators of insulin secretion and mice lacking dopamine D_2 receptor display an impaired glucose metabolism (Pijl *et al.,* 2003; Garcia *et al.,* 2010). Reports suggest that modulation of dopamine D_2 receptor activity profoundly affects energy homeostasis in humans and animals. Dopamine D_2 receptor

agonist, Bromocriptine is approved pharmaco therapeutic agent for the treatment of type 2 diabetes and improves glucose intolerance, insulin secretion and insulin stimulated glucose disposal in humans and animals (Cincotta *et al.,* 1993; Luo *et al.,* 1998; Pijl *et al.,* 2000). Altered dopaminergic activity observed in the central nervous system influenced the dopaminergic activity and insulin secretion in diabetic condition. Although, the mechanism of action of *Aegle marmelose* and pyridoxine is not established, its antioxidant property account for the restoration of dopaminergic activity through dopamine receptors in diabetic condition.

LIVER

Disrupted dopamine signaling has been implicated in diabetes. Glucose and energy metabolism is tightly regulated by the central nervous system. The CNS regulates hepatic glucose production through sympathetic pathways and integrates information on fuel availability through leptin, ghrelin, insulin, glucagon-like pepide-1 and other hormonal signals. Diabetic and obese patients have impaired responses to these signal pathways and display elevations in hepatic glucose production, insulin resistance and impaired pancreatic β-cell function (Defronzo, 2009). The metabolic control pathways of the CNS are modulated, in part, by dopaminergic signaling. Agents that block dopamine activity, such as antipsychotic medications are associated with impaired metabolism, weight gain, insulin resistance and dyslipidemia (Tschoner *et al.,* 2007).

In the present study, total dopamine receptor binding parameters- B_{max} and K_d were significantly increased and results suggest increased expression of dopamine receptors in diabetic condition. Diabetes is associated with severe dopamine depletion and this resulted in the up regulation of dopamine receptors. Insulin, *Aegle marmelose* and pyridoxine treatment significantly reduced the expression of dopaminergic receptors. Results suggest that *Aegle marmelose* and pyridoxine has a crucial role in restoring the dopaminergic tone. The central nervous system dopamineric pathway has

a significant role in the dopaminergic receptor expressions and glucose metabolism. Reports suggest that systemic and intracerebral bromocriptine administration in insulin-resistant animals lead to a decrease in elevated ventromedial hypothalamus noradrenergic and serotonergic levels, with a resultant decline in hepatic glucose production/gluconeogenesis, reduced adipose tissue lipolysis and improved insulin sensitivity (Cincotta & Meier, 1995; Luo *et al.,* 1998; Luo *et al.,* 1999; Scislowski *et al.,* 1999). Similar mechanism happened in the present study. The mechanism of action of *Aegle marmelose* and pyridoxine, through dopamine synthesis modulated dopaminergic pathways and maintained glucose and energy metabolism.

Insulin receptor expressions in experimental animals

Glucose homeostasis is of critical concern to the brain, since glucose is its primary fuel. Insulin is a glucoregulatory hormone in the periphery that functions in the CNS to regulate glucose levels and brain insulin play crucial roles in metabolism and food uptake (Gerozissis, 2003; Porte *et al.,* 2005). Insulin is an important hormone involved in the regulation of glucose metabolism in peripheral tissues and its role of in the central nervous system has been studied far less than its functions in the periphery. Brain insulin is one of the numerous neuromodulators of energy homeostasis (Woods *et al.*, 2000).

Insulin shows metabolic, neurotrophic, neuromodulatory and neuroendocrine actions in the brain (Snyder & Kim, 1980; Gasparini *et al.*, 2002). Reports suggest central insulin has a major role in the regulation of brain glucose metabolism (Hoyer *et al.*, 1993; Henneberg & Hoyer, 1995). Insulin signaling therefore has the potential to regulate the organization and stability of the neuronal cytoskeleton. Only in the last decade or so has insulin been considered vital in the brain, where it is now recognized as being involved in many cerebral functions, including plasticity, cognition, and neuroprotection (Gasparini *et al.,* 2002). Insulin receptors are widely expressed in the rodent and human brain (Unger *et al.,* 1991). When stimulated by insulin, they trigger

several signaling pathways, including the phospholipase Cγ, mitogen-activated protein kinase and PI_3K pathways.

The interaction of insulin with target cells is *via* its receptor located in the plasma membrane (Czech, 1985; Kahn, 1985; Goldfine, 1987). Brain insulin receptors are similar to those expressed peripherally (Wozniak *et al.,* 1993). In the present study insulin receptors were down regulated in striatum and up regulated in liver of diabetic rats. Reduced expression of insulin receptor suggests hypoinsulinemic and hyperglycemic condition of the body. Similar reports clearly demonstrated the down-regulation insulin receptor mRNA expression in frontoparietal brain cortex and hippocampus after STZ administration (Grünblatt *et al.,* 2004, 2007). Reports suggest reduced expression of insulin receptor in insulin resistant obese animal models (Zhou *et al.,* 2009). Dysregulation of insulin secretion or reduction in the levels of both insulin and its receptor in the brain was reported in aging, obesity, diabetes, and serious mental disorders in humans in postmortem studies and in animal models *in vitro* and *in vivo* (Zaia & Piantanelli, 1996; Fro¨ lich *et al.*, 1997; Biessels *et al.*, 2000, 2002; Gerozissis *et al.*, 2001). Ubiquitous deletion of the insulin receptors gene in mice led to marked hyperglycemia and the mice died within the first week of life (Joshi *et al.,* 1996). The increased liver insulin receptor gene expression in the present study, agree with previous results of increased insulin receptor mRNA levels quantified by slot blot, Northern blot analysis and increased insulin receptor gene transcription in the liver of streptozotocin-induced diabetic rats (Sechi *et al.,* 1992; Tozzo & Desbuquois, 1992). In the present study Insulin, *Aegle marmelose* and pyridoxine treatment restored altered gene expression of insulin receptor in the striatum. Pyridoxine treatment significantly restored the altered expression in the liver. Our previous results suggest anti hyperglycemic activity of *Aegle marmelose* and pyridoxine treatment in diabetic rats (Nair *et al.,* 1998; Abraham *et al.,* 2010). Antihyperglycemic activity of *Aegle marmelose* and pyridoxine restored insulin receptor gene expression in the present study.

GLUT 3 and GLUT 2 expressions

The brain responds to large changes in plasma glucose and initiates compensatory responses to maintain glucose homeostasis. The families of facilitative GLUT proteins are responsible for the entry of glucose into cells throughout the periphery and the brain (Maher *et al.,* 1994; Vannucci *et al.,* 1997). The expression, regulation and activity of glucose transporters play an essential role in neuronal homeostasis, because glucose represents the primary energy source for the brain (Lund & Anderen, 1979; Pardridge, 1983). Although many isoforms of glucose transporters have been identified in the brain, GLUT-3, the neuron-specific glucose transporter, is solely responsible for the delivery of glucose into neurons in the central nervous system. Our study investigated the effect of brain glucose utilization in diabetes associated neuronal damage. The results of the current study showed increased expression of GLUT-3 in the striatum, cerebral cortex and brainstem of diabetic rats. GLUT-3 mRNA expression was increased as a response to increase glucose utilization in diabetic condition. Previous studies examining the regulation of GLUT-3 expression in the brain during diabetic conditions have provided equivocal results and demonstrated increased expression of GLUT-3 mRNA and protein expression in hippocampus of diabetic rats subjected to stress (Reagan *et al.,* 1999). Insulin, *Aegle marmelose* and pyridoxine significantly improved the glucose transport system in brain regions of diabetic rats by the modulation of GLUT-3 gene expressions in striatum, cerebral cortex and brain stem. The maintenance of glucose level by insulin, *Aegle marmelose* and pyridoxine treatment account for the reduced expression of GLUT-3. Our findings suggest a modulation of GLUT 3 expression in the brain regions with *Aegle marmelose* and pyridoxine supplementation which consecutively normalise the glucose transport in CNS.

GLUT-2 is a facilitative glucose transporter located in the plasma membrane of the liver, pancreatic, intestinal, kidney cells play key roles in the handling of dietary sugars. Regulation of GLUT-2 gene expression is complex and has been the matter of

several studies focused on tissue specificity. Briefly, refeeding after a fast or low- *vs.* high-carbohydrate diets modulates GLUT-2 expression in the intestine, kidney, liver, and pancreas (Miyamoto *et al.,* 1993; Thorens *et al.,* 1996). GLUT-2 is a glucose-sensitive gene in liver cells (Rencurel *et al.,* 1996).Our results suggest increased GLUT-2 gene expression in liver and pancreas of diabetic rats. Low insulin and high glucose levels in streptozotocin-induced diabetic rodents increase GLUT-2 expression in the intestine and liver suggesting that glycemia and insulinemia control GLUT-2 expression (Miyamoto *et al.,* 1991; Yamamoto *et al.,* 1991). Hyperglycemic condition in the diabetic rats increased GLUT-2 gene expression. Insulin, *Aegle marmelose* and pyridoxine treatment maintained normoglycemia and modulated the expression of GLUT-2. Our findings suggest a modulation of GLUT-3 in the brain regions and GLUT-2 expression liver and pancreas, with *Aegle marmelose* and pyridoxine supplementation which consecutively normalise the glucose transport in CNS.

CREB expressions in brain and pancreas

CREB is a critical factor in many important functions in the nervous system, including neurogenesis and neuronal survival, development and differentiation, as well as neuroprotection, axonal outgrowth and regeneration, synaptic plasticity (Mioduszewska *et al.,* 2003; Persengiev & Green, 2003; Dragunow, 2004; Barco & Kandel, 2006)**.** Genes whose transcription is regulated by CREB include: c-fos, BDNF, tyrosine hydroxylase and neuropeptides such as somatostatin, enkephalin, VGF and corticotropin-releasing hormone (Lauren, 2005). It is a crucial neurotrophic factor and possess pro-survival and/or differentiation effects on several neuronal populations and synaptic plasticity (Thoenen, 2000). CREB is a transcription factor which is a downstream target of cyclic AMP signaling. Our results demonstrated the down regulation of CREB in corpus striatum, cerebral cortex, brain stem, pancreas and liver of STZ-induced diabetes rats. Eventhough, the level of cAMP was increased in brain regions of diabetic rats, but the CREB expression was down

regulated. In diabetes, there is an abnormal metabolic environment including high glucose, high free fatty acids and either high insulin or insulin deficiency. Accumulation of ROS has been reported in human and rodent diabetic vessels (Inoguchi *et al.,* 1994; Karasu *et al.,* 1997; Inoguchi *et al.,* 2000; Karasu, 2000). There is a suggestion in the neuronal literature that oxidative stress in a negative modulator of CREB DNA binding and also that ROS interferes with acute signaling to CREB. High glucose can increase intracellular ROS by numerous mechanisms including activation of PKC, AGE accumulation and release from mitochondria (Nishikawa *et al.,* 2000). In neuronal tissue, CREB regulation by nerve growth factor and insulin-like growth factor-1 is essential for neuronal plasticity, full axonal development, memory consolidation and neuroprotection (Shimomura *et al.,* 1998; Pugazhenthi *et al.,* 1999; Spaulding, 1993). The CREB play a pivotal role in neuroplasticity. It binds to certain DNA sequences called cAMP response elements and thereby, increases or decreases the transcription of the downstream genes (Spaulding, 1993). CREB plays a pivotal role in dopamine receptor-mediated nuclear signaling and neuroplasticity (Finkbeiner, 2000). Multiple lines of evidence define a role for CREB in proliferation and differentiation of certain cells and tissues (Heasley et al., 1991; Spaulding, 1993; Iyengar, 1996). Our results showed that *Aegle marmelose* and pyridoxine treatment significantly increased the gene expression of CREB. This study demonstrated that *Aegle marmelose* and pyridoxine possess regulatory effect in the CREB expression, which is crucial in maintaining the normal neuronal functions and neuroplasticity.

Phospholipase- C expression in brain

In the phospho inositol signaling pathway, agonist-induced interaction of cell surface receptors with G proteins activates the enzyme phospho inositol-specific phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate into IP_3 and DAG (Berridge & Irvine, 1989). PLC has been categorized

into three major families: PLC β, PLC δ, and PLC γ (Cockcroft &Thomas, 1992). All Phospholipase C isozymes recognize PIP_2 as a substrate and carry out Ca^{2+} dependent hydrolysis of inositol lipids; however, these isozymes are differentially regulated and expressed (Cockcroft &Thomas, 1992). In the CNS, neurotransmitter receptor coupling to phospholipase C has been extensively documented in [^{3}H] inositol-labeled tissue slices and synaptosomes obtained from animal brains (Fisher & Agranoff, 1987; Stephens & Logan, 1989; Chandler & Crews, 1990). In the present study, we observed, down regulation of phospholipase C gene expression in corpus striatum, cerebral cortex, brain stem and liver of diabetic rats. Down regulation of the Phospholipase C in brain regions and peripheral organs during diabetes contribute to the impaired signal transduction of G-protein coupled neurotransmitter receptors. Altered phospholipase C expression fails to modulate the activity of downstream proteins important for cellular signaling. Defective expression of phospholipase C results in low levels of IP_3 causing the impaired release of Ca^{2+} and bring down the level of intracellular calcium and thus failed to execute the normal neuronal function in brain regions and results in defective insulin secretion in the pancreas. Insulin, *Aegle marmelose* and pyridoxine treatment significantly reversed the expression pattern compared with diabetic condition. These results suggest neuromodulatory role of *Aegle marmelose* and pyridoxine in diabetic condition.

APOPTOTIC AND NEURONAL SURVIVAL FACTORS

Hyperglycemia is associated with a number of physiological changes, the most profound effects are seen in the brain. Hyperglycemia results in oxidative stress leading to an increased production of reactive oxygen species and lipid peroxidation (Ha & Lee, 2000). Oxidative stress seems to play a central role in neuronal damage (Bonnefont-Rousselot, 2002). Diabetic patients have too much of the free radical-induced damage but do not have enough antioxidant defenses (Martin-Gallan *et al.,* 2003). Type 2 diabetes mellitus shows many neurological complications. The

incidence of neurologic disorders appears to be higher in persons with type 2 diabetes mellitus, suggesting that shared mechanisms, such as insulin dysregulation, may underlie these conditions (Craft & Watson, 2004).

Substantial evidence has revealed that acute hyperglycaemia have disruptive effects on the central nervous system (Gispen & Biessels 2000). The direct glucose toxicity in the neurons is caused by increased intracellular glucose oxidation which leads to an increase in production of reactive species in diabetic rats (Nishikawa *et al.,* 2000). Evidence for classic apoptosis is seen in the striatum, brain stem and liver of diabetic rats.

TNF-α expression in brain regions and liver

Emerging evidence suggests impairment of neuronal function or loss of neurons in diabetes. Oxidative stress seems to play a central role in neuronal damage (Bonnefont-Rousselot, 2002). Brain injury appears to result from a number of processes that are initiated when blood glucose concentration is altered. Many cytokines have been suggested to participate in neurodegeneration and neurotoxicity. TNF-α is a major mediator of apoptosis and inflammation (Chen & Goeddel, 2002; Wajant *et al.,* 2003). Increased expression of TNF-α have been observed before neuronal death (Little & O'Callagha, 2001). TNF- α, the most widely studied cytokine, plays many roles as a signaling and as an effector molecule in both physiology and pathophysiology of the central nervous system (Munoz-Fernandez & Fresno, 1998). TNF- α is released during various inflammatory diseases of the CNS, being synthesized by microglia, astrocytes and some populations of neurons (Lieberman *et al.,* 1989; Chung *et al.,* 2005). TNF-α expression was significantly up regulated in corpus striatum, brain stem and liver, whereas it is significantly down regulated in cerebral cortex of diabetic rats. Increased TNF-α expression in brain regions suggests neuronal damage and it is supported by previous reports. Under

chronic hyperglycemia, endogenous TNF-α production is accelerated in microvascular and neural tissues, which may cause increased microvascular permeability, hypercoagulability and nerve damage, thus initiating and promoting the development of characteristic lesions of diabetic microangiopathy, polyneuropathy and encephalopathy (Satoh *et al.,* 2003; Brands *et al.,* 2004). Uncontrolled diabetes is reported to significantly enhance the TNF-α level (Sharma *et al.,* 2007). A significant reduction of TNF-α levels in cortical region observed in our study, indicate the spatial variation in the vulnerability of brain parts to diabetes related brain complications. Earlier study in the cortical region by Dinga *et al.,* (2005) reported an increased expression of cell adhesion molecule and proinflammatory cytokines in mediating diabetes-augmented brain damage caused by transient cerebral ischemia, where pro-inflammatory cytokines were increased without any change in TNF- α level. Insulin, *Aegle marmelose* and pyridoxine treatment significantly reduced the expression of TNF- α and rendered neuroprtotective effect. Previous studies suggest antioxidant and neuroprotective role of pyridoxine and *Aegle marmelose* in normalizing diabetic related oxidative stress (Abraham *et al.,* 2010a). Reports suggest, pyridoxine supplementation, down regulate serine protease inhibitor clade A member 3 (SPI-3) and TNF-α mRNA expression in HT-29 human colon cancer cells (Yanaka *et al.,* 2011). Antioxidant properties of *Aegle marmelose* and pyridoxine played a crucial role in this modulation.

Caspase- 8 expression in brain regions and liver

A critical role in initiation and progression of apoptosis has been attributed to members of the caspase family (Alnemri *et al.,* 1996). Caspases are a family of cysteine proteases that regulate apoptosis (Baumgartner *et al.,* 2009; Inoue *et al.,* 2009; Yi & Yuan, 2009). Caspases are synthesized as precursors (procaspases) that are activated by enzymatic cleavage (Degterev *et al.,* 2003; Inoue *et al.,* 2009). Cleavage of procaspases liberates large (~20 kDa) and small (~10 kDa) subunits that

heterodimerize to form catalytically active proteases (Degterev *et al.,* 2003; Guo *et al.,* 2004; Baumgartner *et al.,* 2009). Caspase activation is considered a commitment to cell death (Cohen, 1997). In caspase dependent cell death, caspase-8 activation is the most upstream event, and caspase-3 activation is the critical downstream event. Caspase-8 is prototypical initiator caspase of the extrinsic cell death pathway.

Caspase-8 has been localized throughout the brains of Alzheimer's patients and has been shown to mediate neuronal apoptosis (Matsui *et al.,* 2006; Miyoshi *et al.,* 2009; Vaisid *et al.,* 2009). Caspase-8 is further implicated in Parkinson's disease. Elevated caspase-8 levels were observed in human postmortem brain and in the MPTP model of Parkinson's disease (Hartmann *et al.,* 2001; Viswanath *et al.,* 2001). Our results showed enhanced gene expression of caspase 8 in striatum, brain stem and liver and it's gene expression is down regulated in cerebral cortex of diabetic rats. Cortical region showed inhibition in the expression of caspase-8 expression and suggests spatial variation in the vulnerability of brain parts to diabetes associated apoptosis. Increased TNF-α expression, observed in the diabetic condition has contributed to the increased caspase-8 expression. Activation of the caspases represents a pivotal step in the cell death signaling cascade. In the present study insulin, *Aegle marmelose,* pyridoxine treatment showed significant down regulation of caspase-8 expression compared to diabetic group. It suggests the anti apoptotic modulation of *Aegle marmelose* and pyridoxine in reducing diabetes related complications. Inhibition of caspase activation protects against neuronal loss in several animal models of brain diseases involving hypoxic ischaemia, brain trauma and Parkinsons's disease (Schulz *et al.,* 1998; Cutillas *et al.,* 1999; Depino *et al.,* 2003). Given the involvement of oxidative stress in diabetic complications, supplementation with antioxidants could be of interest, by allowing a delay in the appearance or in the development of vascular complications (Hayoz *et al.,* 1998).

NFκB gene expression in brain regions and liver

NFκB is ubiquitously expressed in peripheral and brain cells and regulates the expression of a wide variety of genes involved in cell survival, growth, stress responses, immune and inflammatory processes (Baldwin, 1996; Shimada *et al.,* 2001; Weih & Caamañ, 2003). NFκB controls the expression of genes that regulate a broad range of biological processes in the CNS such as synaptic plasticity, neurogenesis and differentiation (Hayden & Ghosh, 2008). NFκB is widely expressed in the central nervous system. NFκB is a ubiquitous transcription factor comprising at least five DNA binding protein subunits. Classic NFκB consists of two subunits, p50 and p65, which can bind to DNA as a homo- or heterodimer (Baeuerle & Henkel, 1994). At least two different cellular forms of NFκB exist: an inactive, non-DNA binding cytoplasmic form that is bound by inhibitor proteins IκBa and IκBf3 and an activated form that appears when IκB is released. Activated NFκB translocates to the nucleus where it interacts with DNA-binding sites to regulate gene expression (Baldwin *et al.,* 1996). After activation, NFκB induces the expression of genes encoding cytokines, inflammatory enzymes, cell adhesion molecules, cell surface receptors and acute-phase proteins.

In the present study NFκB gene expression is up regulated in corpus striatum and down regulated in cerebral cortex and liver of diabetic rats. NFκB is also a modulator of apoptosis and ROS production. Increased expression of NFκB occurs *via* phosphorylation of IκB, due to impaired insulin signaling, with disinhibition of NFκB (Xu *et al.,* 2001; Pierson *et al.,* 2002). NFκB plays a central role in the initiation of the inflammatory cascade with the activation of TNF-α, interleukins and C-reactive protein (Yerneni *et al.,* 1999; Luppi *et al.,* 2008; Sima *et al.,* 2009). Increased expression of NFκB and other apoptotic factors in the striatum, suggests activation apoptotic signaling in diabetic rats. Reports suggest that, chronic or acute inflammation induced by diabetic metabolism exacerbate oxidative stress and increase

NFκB activation (Ckless *et al.,* 2007). Several studies proposed that activation of NFκB could mediate neuronal cell death (Qin *et al.,* 1998; Nakai *et al.,* 2000). Furthermore, it should be noted that in the present study increased gene expression of TNF-α suggested to have increased NFκB gene expression in diabetic condition and the possibility of neuronal death.

Basal levels of NFκB activity are normally present within neuronal nuclei in the cerebral cortex, although white matter is almost completely devoid of activated NFκB. Our results indicate a reduction in cortical NFκB expression in diabetic condition. Eventhough the possibility of sensitization of brain cells to the cytotoxicity due to declined NFκB function is high, the reduced expression of TNF-α observed in our study rules out that possibility. A mechanism of NFκB function reported by Lesoualc'h *et al.,* (1998) points to increased NFκB levels in neurons resistant to oxidative cell death and an inhibition of NFκB activity reversing this resistance thereby potentiating cell death. Thus, in the present context the severe oxidative stress and declined antioxidant system in the cortical region resulted in the decreased NFκB expression. This along with the reduced TNF-α expression is suggested to be a defensive mechanism of central nervous system to counteract the fight against apoptosis or oxidative brain damage to prevent or prolongate the onset of diabetic encephalopathy. Reports suggest that impaired insulin-signaling activity acts unfavorably on the expression and translocation of NFκB and CREB with effects on proinflammatory factors and apoptosis (Li *et al.,* 2001; Sima *et al.,* 2004; Francis *et al.,* 2008). Reduced expression of NFκB in liver point to the disturbed insulin and IGF-1 signaling in diabetic rats. *Aegle marmelose* and pyridoxine treatment significantly decreased NFκB expression compared to diabetic group. Recent reports demonstrated the protective roles of pyridoxine intake against several diseases. Reports suggest that pyridoxine inhibited TNF-α-induced NFκB activation *via* suppression of IκBa degradation in HT-29 human colon cancer cells (Yanaka *et al.,*

2011). Thus in our study the modulatory role of pyridoxine is suggested to alter the expression pattern of NFκB in diabetic rats.

Akt-1 gene expression in brain regions

Akt is implicated in cellular processes such as cell survival, proliferation and growth, glucose metabolism, apoptosis, angiogenesis, transcription and migration (Scheid &Woodgett, 2003). Akt consists of three homologous members known as Akt-1, Akt-2 and Akt-3. Akt is a growth factor regulated protein kinase which contains three functionally different sites: a pleckstrin homology domain, a central catalytic domain and a C-terminal hydrophobic motif (Robertson, 2005). Binding of phosphoinositide 3-OH kinase (PI_3K) products to the pleckstrin homology domain results in Akt translocation to the plasma membrane where it is activated *via* phosphorylation by upstream kinases such as the phosphoinoside-dependent kinase 1(PDK1). Akt is an important mediator of the physiological effects of several growth and survival factors and promotes cell survival through the inhibition of apoptosis (Downward, 1998; Datta *et al.,* 1999). Within the nucleus, Akt controls expression of genes involved in cell survival *via* the transcription factors- Forkhead, NFκB and CREB (Brunet *et al.,* 2001). In the present study, the transcriptional profile of Akt-1 showed down regulation in corpus striatum, cerebral cortex and brain stem of diabetic rats. Down regulation of Akt-1 and activated expression of apoptotic factors in corpus striatum and brain stem, suggests diabetes associated neuronal loss. *Aegle marmelose* and pyridoxine treatment has modulatory role in the expression of Akt-1. Akt is a central player in insulin and growth factor signaling and a regulator of several cellular functions including cell growth and apoptosis (Hanada *et al.,* 2004). PI3K/Akt pathway promots the expression of anti-apoptotic signals and also inhibit proapoptotic gene expression (Barber *et al.,* 2001; Nakamura *et al.,* 2001). The neuroprotective function of PKB is mediated by direct phosphorylation of known regulators of apoptosis, such as the pro-apoptotic mitochondrial protein- Bad (Datta *et al.,* 1997; del

Peso *et al.,* 1997) and the transcription factor FOXO as well as the pro-survival transcription factors CREB and NFκB (Du & Montminy, 1998; Biggs *et al.,* 1999; Brunet *et al.,* 1999; Kane *et al.,* 1999; Kops *et al.,* 1999; Brunet *et al.,* 2001).

BDNF gene expression in corpus striatum

BDNF is important in differentiation, survival and plasticity of the CNS. BDNF belongs to the family of neutotrophins. The cellular actions of BDNF are mediated through TrkB tyrosine kinase receptor and by p75 neurotrophin receptor (Chao, 2003). Binding of dimeric BDNF causes dimerization of TrkB receptor and autophosphorylation of intracellular tyrosine residues. Activation of TrkB receptor leads to signaling cascades involving activation of Ras/ERK pathway, PI_3K and Phospholipase Cγ (Roux & Barker, 2002). The Ras pathway regulates neuronal survival and differentiation through downstream signaling that includes c-RAF/B-Raf/ERK1/ERK2.

BDNF signaling through PI_3K plays an important role in survival of neurons and the downstream signaling includes serine/threonine kinases 3 phosphoinositide dependent kinase-1 (PDK1) and Akt (Jones *et al.,* 1991; Vanhaesebroeck & Alessi, 2000). Akt activated by PDK1 in turn activates substrates involved in neuronal survival such as Bcl-2, Caspase-9, IκB kinase glycogen synthase kinase-3 and Forkhead family members (Roux & Barker, 2002). Phospholipase C-γ activation leads to increased levels of IP_3 and DAG (Vetter *et al.,* 1991). IP_3 increases cytoplasmic Ca^{2+} concentrations and DAG activates PKCδ (Corbit *et al.,* 1999). p75 neurotrophin receptor signaling is involved in cell survival, neurogenesis, cell cycle effects and apoptosis during developmental cell death and after nervous system injury (Roux &Barker, 2002). Pro-apoptotic p75 neurotrophin receptor triggered cell death has been observed during stress, inflammation and injury conditions (Chao *et al.,* 2006). p75 neurotrophin receptor signaling cascades include JNK, NFκB, NADE and RhoA (Roux & Barker, 2002).

BDNF is a survival gene contains cAMP response element. It is a crucial neurotrophic factor and possess pro-survival and/or differentiation effects on several neuronal populations and synaptic plasticity (Thoenen, 2000). CNS neurons are supported by several neurotrophic and transcription factors. BDNF is a potent trophic factor supports striatal cells and promotes survival and/or differentiation of GABAergic neurons *in vitro* (Mizuno *et al.,* 1994; Ventimiglia *et al.,* 1995). In the present study, BDNF expression is significantly down regulated in the striatum of diabetic condition. Activation of apoptotic factors -TNF-α, caspase-8 and NFκB resulted in the decreased expression of BDNF in the corpus stiatum of diabetic rats. Insulin, *Aegle marmelose* and pyridoxine treatment to diabetic rats significantly increased the BDNF expression. It is suggested that there is diabetic associated neuronal complication and neuronal loss in the striatum. Treatment groups showed significant reversal in BDNF expression and suggests neuroprotective role of *Aegle marmelose* and pyridoxine.

SECOND MESSENGERS IN BRAIN REGIONS, PANCREAS AND LIVER

Second messenger systems are complexes of regulatory and catalytic proteins, which are activated by first messengers to form second messengers. The signaling from the neurotransmitters is carried to the cell nucleus by second messengers like cAMP, cGMP and IP_3. Their expression and changes play a major role in the signaling cascade. Neurons use many different second messengers as intracellular signals. Second messengers relay signals received at receptors on the cell surface to target molecules in the cytosol and/or nucleus. Three major classes of second messengers are (1) cyclic nucleotides (e.g., cAMP and cGMP), (2) inositol trisphosphate (IP_3) and diacylglycerol (DAG), (3) calcium ions (Ca^{2+}). The signal transduction in metabotrophic neurotransmitters occur through activation of second messengers, whereas ionotrophic neurotransmitters act through ligand gated ion channels. The

changes in neurotransmitter level and its receptor should agree with a concomitant change in second messenger for effective signal transduction.

cAMP and cGMP content in brain, pancreas and liver

cAMP is produced when G-proteins activate adenylyl cyclase in the plasma membrane. cAMP produced by adenylyl cyclase, activates PKA by binding to the regulatory subunits in ways that result in the release and nuclear translocation of active catalytic subunits (Meinkoth *et al.,* 1993). cAMP stimulates the proliferation of many cell types, but in some cases cAMP inhibits cellular proliferation (Pastan *et al.,* 1975; Bokoch, 1993; Dugan *et al.,* 1999; Hagemann & Rapp, 1999; Zwartkruis & Bos, 1999; Wang *et al.,* 2000). An essential element of the signalling cascade leading to synaptic plasticity is the intracellular second messenger molecule cGMP. cAMP and cGMP regulate the activity of neurons throughout the central nervous system, controlling metabolic processes, electrical signaling and synaptic physiology (Greengard & Costa, 1970; Bloom, 1975; Nathanson, 1977).The cyclic nucleotides cAMP and cGMP are involved in a number of intracellular processes such as signal transduction, gene transcription, activation of kinases and regulation of channel function (Burns *et al*., 1996).

In the present study cAMP content was significantly increased in striatum, cerebral cortex and brain stem whereas it was significantly decreased in the liver and pancreas of diabetic rats. cGMP contents were significantly decreased in the striatum, cerebral cortex, pancreas and liver and it was significantly increased in brain stem of diabetic rats. Reports suggest that lesions of the nigrostriatal dopaminergic projections induce an increase in cAMP levels associated with increased basal adenylate cyclase activity in dopamine-denervated rat striata suggesting an imbalance of D_1 *vs*. D_2 receptor functions (Hossain & Weiner, 1993; Tenn & Niles, 1997). Furthermore, the loss of dopamine leads to decreased levels of cGMP suggesting that modulation of the second messenger system in dopamine-denervated rat striata (Sancesario *et al.,* 2004).

In the present study diabetic associated dopaminergic depletion and imbalance in the expression pattern of dopamine D_1, D_2 receptors attributed to the increased the content of cAMP and decreased content of cGMP in the striatum and cerebral cortex. Dopamine and cAMP regulate a diverse array of neuronal functions ranging from ion conductance and synaptic plasticity to gene expression (Lohmann *et al.,* 1997; Oh *et al.,* 1997; Kaupp & Seifer, 2002; Cooper, 2003; Ha˚kansson *et al.,* 2004; Fernandez *et al.,* 2006). cAMP and cGMP regulate the activity of neurons throughout the central nervous system, controlling metabolic processes, electrical signaling and synaptic physiology (Greengard & Costa, 1970; Bloom,1975; Nathanson,1977).

cAMP stimulates the expression of numerous genes *via* the PKA-mediated phosphorylation of CREB and its paralogs ATF-1 and CREM at a single site corresponding to Ser 133 in CREB (Gonzalez & Montminy, 1989). CREB family members also undergo Ser 133 phosphorylation in neurons in response to Ca^{2+} entry through voltage-sensitive L-type calcium channels, in part *via* the calcium/calmodulin dependent kinase IV (West *et al.,* 2001). In the present study insulin, *Aegle marmelose* and pyridoxine treatment significantly increased cAMP content. cAMP and growth factor signaling *via* CREB promotes pancreatic β cell survival (Jhala *et al.,* 2003). In the present study, *Aegle marmelose* and pyridoxine treatment increased cAMP content and suggest its potential in pancreatic β cell proliferation. Hepatic glucose overproduction is a common feature of diabetes mellitus (DeFronzo et al., 1982; Fery, 1994). The intracellular cAMP content regulates not only the activity of key enzymes of glucose metabolism in hepatocytes, but also enzyme gene expression (Pilkis & Granner, 1992).

IP_3 content in brain, pancreas and liver

IP_3 is a ubiquitous second messenger that functions by binding to IP_3 receptors on the endoplasmic reticulum membrane to cause liberation of sequestered Ca^{2+} (Berridge, 1998, 2002). The resultant cytosolic Ca^{2+} transients serve numerous

signaling functions in neurons, including modulation of membrane excitability synaptic plasticity and gene expression (Fujii *et al.,* 2000; Miyata *et al.,* 2000; Nishiyama *et al.,* 2000; Mellstrom & Naranjo, 2001; Yamamoto *et al.,* 2002; Stutzmann *et al.,* 2003). In the present study IP_3 contents were significantly decreased in corpus striatum, cerebral cortex, brain stem, pancreas and liver of diabetic rats. Decreased expression pattern of dopamine D_2 receptors observed in the present study, have played an important role in the down regulation of phospholipase-C with a decrease in IP_3 content. IP_3 is a ubiquitous second messenger that functions by binding to receptors (IP3Rs) on the endoplasmic reticulum (ER) membrane to cause liberation of sequestered Ca^{2+} (Berridge, 1998, 2002). In the phosphoinositide signaling pathway, agonist-induced interaction of cell surface receptors with G proteins activates the enzyme phosphoinositol-specific phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate into IP_3 and DAG (Berridge & Irvine, 1989). Decreased gene expression of phospholipase- C enzyme observed in different brain regions and liver is responsible for the decreased content of IP_3 in diabetic rats. IP_3 mobilizes Ca^{2+} from intracellular sources after binding with IP_3 receptors, DAG activates the phosphorylating enzyme protein kinase C (Dekker *et al.,* 1995; Berridge *et al.,* 1998). These events mediate cellular activation and subsequent biological responses such as neurotransmitter release, cell growth, differentiation, neuronal development and gene expression (Nishizuka, 1988; Berridge & Irvine, 1989). The neuronal intracellular calcium has an important role in the regulation of synaptic plasticity (Barbara, 2002). Moreover, disruptions in this pathway are implicated in neurodegenerative disorders (Abe, 1997; Mattson *et al.,* 2000; LaFerla, 2002). IP_3 has an important role in insulin secretion. Insulin secretion is largely a Ca^{2+}-dependent process and restricted increases in intracellular Ca^{2+} have been related to impairment of glucose-stimulated insulin release (Boschero *et al.,* 1990). Decreased IP_3 observed in the pancreas of diabetic rats results in decreased insulin levels. *Aegle marmelose* and pyridoxine treatment significantly increased IP_3 content in corpus

striatum, brain stem, pancreas and liver. *Aegle marmelose* and pyridoxine has a crucial role in the modulation of second messenger cascade in diabetic rats.

Fluoro Jade- C staining in corpus striatum and brain stem

Fluoro Jade labels neurons that degenerate in response to a variety of insults, including excitotoxic events (Schmued *et al.,* 2005). Fluoro-Jade C exhibits the greatest affinity for degenerating neurons and therefore results in staining with the highest resolution and contrast of the Fluoro-Jade dyes. In the present study fluorojade staining revealed degenerating neurons in the striatum. Fluoro Jade-C accumulation is seen in striatum of diabetic rats. Diabetic and insulin treated group showed significantly increased number of Fluoro Jade positive neurons in the striatum. Reports suggest hypoglycemic brain injury due to insulin therapy in diabetic patients (Davis *et al.,* 1998). In the present study, also the increased number of degenerating neurons suggested is due to the insulin treatment associated brain injury. Flouro jade positive neurons were significantly less in striatum of pyridoxine and *Aegle marmelose* treated rats. Reports suggest that pyridoxine ameliorates degenerative diseases and protect against neurotoxicity (Dakshinamurti *et al.,* 2001; Hwang *et al.,* 2007).Our previous results suggest the neuroprotective role of pyridoxine and *Aegle marmelose* in normalising diabetic related stress and anxiety in diabetic rats (Abraham *et al.,* 2010). The neuroprotection is through the antioxidant properties of pyridoxine as suggested by previous reports (Jain & Lim, 2001). Our findings proved that, *Aegle marmelose* and pyridoxine supplementation has a potential role in modulation of diabetes mediated neuronal loss and help in neuronal survival in striatum. Therefore, *Aegle marmelose* and pyridoxine serve as neuroprotective agents for the treatment of diabetic related neurodegeneration.

GPx and SOD gene expression in liver

Diabetes causes a variety of functional and structural disorders in the central and peripheral nervous systems (Biessels *et al.,* 1994). High glucose produces ROS as a result of glucose auto-oxidation, metabolism and the development of advanced glycosylation end products. Oxidative stress helps the progression and the development of diabetes and its complications (Ha & Lee, 2000). The level of antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes (Baynes, 1991; Porte & Schwartz, 1996).

The liver plays a unique role in glucose homeostasis and it is organs that both consumes and produce substantial amounts of glucose. Diabetes-associated hyperglycemia and hypoinsulinemia lead to impairment of hepatic glucose and lipid metabolism. Interest in development of novel antidiabetic agents has been fuelled by the intense complications due to therapeutic treatment of diabetes and associated liver failure (de Marco *et al.,* 1999; Maritim *et al.,* 2003). Antioxidant enzymes- SOD and GPx, have an important role in maintaining physiological levels of free radicals by hastening the dismutation of free radicals and eliminating organic peroxides and hydro-peroxides (Pari & Latha, 2005). GPx has been regarded as a major determinant of hepatic antioxidant status and it protects the tissue from highly reactive hydroxyl radicals by catalyzing the reduction of hydrogen peroxides.

In the present study GPx gene expression significantly down regulated whereas SOD gene expression up regulated in diabetic condition. SOD has ability to directly neutralize a number of free radicals and reactive oxygen and nitrogen species, it stimulates several antioxidant enzymes that increase its efficiency as an antioxidant. In the present study SOD gene expression was significantly up regulated to compensate with the oxidative stress induced by hyperglycemia. Kakkar *et al.,* (1995) reported the increased activities of total SOD and Cu-Zn-SOD in liver after 1week of induction of diabetes. Increase in SOD activity could be due to its induction by

increased production of superoxide (O_2^-) which has been implicated in cell dysfunction (Wiseman &Halliwell, 1996). Insulin, *Aegle marmelose* and pyridoxine treatment significantly modulated the expression of SOD and GPx in liver of diabetic rats. Anti oxidant properties of *Aegle marmelose* and pyridoxine treatment is responsible for this modulation. Reports suggest that the antioxidant phytochemical such as flavonoids, alkaloids, sterols, tannins, phlobotannins and flavonoid glycosides present in the leaf extract possess free radical scavenging activity (Rajadurai *et al.,* 2005). Eugenol and Marmesinin compounds in *Aegle marmelose* have independently shown their activity against oxidative stress (Vidhya & Devaraj, 1999). Recent studies show antioxidant properties of pyridoxine (Jain & Lim, 2001; Stocker *et al.,* 2003). The present study suggests antioxidant properties of *Aegle marmelose* and pyridoxine in protecting the liver from diabetes associated oxidative stress.

BAX gene expression in liver

BAX is a pro-apoptotic protein allowing apoptosis to occur through the intrinsic, damage-induced pathway and amplifying that one occurring through the extrinsic, receptor mediated pathway. BAX is present in viable cells and activated by pro-apoptotic stimuli. BAX has multiple functions: it releases different mitochondrial factors such as cytochrome c, second mitochondria-derived activator of caspase (SMAC) it regulates mitochondrial fission, the mitochondrial permeability transition pore; it promotes Ca^{2+} leakage through ER membrane (Ghibelli & Diederich, 2010). Cytosolic BAX translocates to mitochondria upon death stimulus, promoting cytochrome c release (Gross *et al*., 1998). BAX mediated cell death relates with mitochondrial permeability transition (Jin & El-Deiry, 2005). The expression of proapototic protein BAX can be taken as an index of cell death.

Hyperglycaemia during diabetes, causes increased production of oxygen free radicals from glucose autoxidation and protein glycosylation (Wolff *et al.,* 1987; Hunt *et al.,* 1990). In the present study pro-apoptotic BAX gene expression was

significantly up regulated in the liver of diabetic rats and indicates the mitochondria mediated apoptosis in diabetic rats induced by hyperglycemic stress. Reports suggest that, metabolic or oxidative stress activates or induces expression of the proapoptotic BCL-2 family member BAX (Ho *et al.,* 1997; Khaled *et al.,* 1999; Belaud-Rotureau *et al.,* 2000; Nakamura & Sakamoto, 2001). Insulin, *Aegle marmelose* and pyridoxine treatment significantly reversed the gene expression of BAX. Reports, suggest antioxidant properties of *Aegle marmelose* and pyridoxine (Jain & Lim, 2001; Stocker *et al.,* 2003; Rajadurai & Prince, 2005). In the present study, the antioxidant properties of *Aegle marmelose* and pyridoxine reversed gene expression of BAX in diabetic rats.

NeuroD1 and PDX-1 gene expression in the pancreas

Pancreatic β cells are sensitive to stress. During the development of type 2 diabetes, pancreatic β cells become progressively unable to produce and secrete sufficient insulin to prevent hyperglycemia (Genuth *et al.,* 2003; Muoio & Newgard, 2008; Rutter & Parton, 2008). Identifying strategies to maintain euglycemia is essential to limit diabetes and its destructive consequences (Borowiak & Melton, 2009; Halban *et al.,* 2010). New drugs for preserving and restoring pancreatic β-cell function are critically needed for the treatment of diabetes. Insulin gene transcription is regulated by the cooperation of a group of glucose-sensitive transcription factors expressed in a tissue-restricted manner (Ohneda *et al.,* 2000, Aramata *et al.,* 2005). Among the most important of these transcription factors are NeuroD1 and PDX-1 which activate the insulin gene promoter synergistically and are essential for glucose-stimulated insulin gene transcription. Mutations in PDX-1 and NeuroD1 have been linked to maturity-onset diabetes of the young (MODY) and are classified as MODY4 and MODY6 genes, respectively (Habener *et al.,* 1998; Vaxillaire & Froguel, 2006). PDX-1 is a major player of pancreas formation and it is present in the β cells. PDX-1 is considered to be the master regulator of pancreatic development and β-cell differentiation. PDX-1 has a dual role as an inducer of the endocrine lineage from

ductal epithelial cells and in the maturation of β-cells (Hill & Duvillie, 2000). NeuroD1 is required for the development of neurons and neuroendocrine cells in other organs including lung, intestine and pancreas (Kageyama *et al.,* 1997; Chae *et al.,* 2004). In the adult, NeuroD1 functions primarily in pancreatic β cells.

In the present study NeuroD1 gene expression was significantly up regulated in *Aegle marmelose* and pyridoxine treatment groups compared to diabetic condition. The decreased insulin level and hyperglycemic condition existed in the body, augmented the baseline expression of NeuroD1 in diabetic and insulin treated rats. PDX-1 expression was significantly down regulated in diabetic and insulin treated rats and *Aegle marmelose* and pyridoxine treatment showed significant increase in PDX-1 expression. The results suggest the regeneration of pancreas through PDX-1 and NeuroD1 *Aegle marmelose* and pyridoxine treatment.

BrdU imaging in pancreas

BrdU is a thymidine analog and during BrdU administration experiments, it is incorporated into newly synthesized DNA strands of actively proliferating cells. BrdU positive cells were significantly increased in *Aegle marmelose* and pyridoxine treated groups compared to diabetic condition. It suggests the regeneration of the pancreas in diabetic rats through *Aegle marmelose* and pyridoxine treatment. Enhanced expression of pancreatic transcription factors-PDX-1 and NeuroD1 observed in the present study, played a crucial role in the regeneration process. Reports suggest that, cAMP and growth factor signaling *via* CREB promotes pancreatic β cell survival (Jhala *et al.,* 2003). In the present study, increased expression of cAMP, CREB and IGF-1 expressions, suggest its potential in pancreatic β cell proliferation. Several strategies have been described for generating β cells from stem cells, pancreatic duct cells and other differentiated cell types (Borowiak & Melton, 2009). The majority of these strategies involved the heterologous expression of groups of transcription factors and hormonal factors that induce β-cell differentiation (Kobinger *et al.,* 2005; D'Amour *et*

al., 2006; Kroon *et al.,* 2008; Zhou *et al.,* 2008; Zhang *et al.,* 2009). A small molecule was identified that induced pancreatic progenitors from embryonic stem cells (Chen *et al.,* 2009). Compounds that enhanced β-cell proliferation also has been reported (Wang *et al.,* 2009). The plant alkaloid, conophylline, induces β-cell differentiation from rat pancreatic acinar cells and fetal pancreatic tissue over 3–6 weeks (Kawakami *et al.,* 2010).

Earlier studies, from our laboratory have proved the functional regulation of the central neurotransmitter receptor subtypes during diabetes, pancreatic regeneration, cell proliferation and insulin secretion (Sudha & Paulose 1998; Abraham & Paulose 1999; Biju, 2003; Mohanan *et al.,* 2005; Kaimal *et al.,* 2007; Gireesh *et al.,* 2008). The leaves of *Aegele marmelose* were reported as a source of alkaloidal-amide Aegeline, was found to have anti-hyperglycemic activity as evidenced by lowering the blood glucose (Narender *et al.,* 2007; Abraham *et al.,* 2010). The antidiabetic effect of coumarins in *Aegele marmelose* potentiates insulin secretion from existing β cells in the islet of Langerhans (Kamalakannan & Prince, 2003). A possible mechanism of action is that antidiabetic compounds in *Aegle marmelose* reduces hyperglycemia mediated oxidative stress and the stimulate the proliferation of residual pancreatic β-cells and enhances insulin secretion and thereby glucose regulation in the body. We have reported the anti-hyperglycemic and neuroprotective activity of pyridoxine in normalizing diabetic related stress and anxiety in diabetic rats (Nair *et al.,* 1998; Abraham *et al.,* 2010). Recent reports suggest antioxidant properties of pyridoxine (Matxain *et al.,* 2006). Moreover, Pyridoxine acts as coenzyme in biosynthesis of neurotransmitters- γ-aminobutyric acid, dopamine, serotonin and it is important for development and function of the central nervous system (Dakshinamurti *et al.,* 1988; Ernahrungswiss, 1996). In the present study, pyridoxine's effects are mediated through its co enzymatic activity in the biosynthesis of dopamine. Anti hyperglycemic and antioxidant activity of pyridoxine is suggested to have reduced oxidative stress and enhanced the proliferation of existing pancreatic β cells. The regenerating

property of *Aegle marmelose* and pyridoxine has therapeutic role in diabetes management.

Summary

1. Streptozotocin induced diabetic rats were used as model to study the expression patterns of dopamine receptor subtypes, insulin receptors, IGF-1, GLUT-3, GLUT-2, CREB, second messenger enzyme phospholipase- C, apoptotic factors TNF- α, caspase-8, BAX, neuronal survival factors - Akt-1, NFκB, BDNF and status of second messengers- cAMP, cGMP, IP_3, antioxidant enzymes- GPx and SOD. Experiments were designed to study the neuroprotective role and mechanism of actions of *Aegle marmelose* and pyridoxine in diabetes management.

2. Antihyperglycemic activity of *Aegle marmelose* and pyridoxine were evaluated by the measurement of blood glucose and circulating insulin level in experimental rats. Diabetic rats showed increased blood glucose and decreased insulin level. Insulin, *Aegle marmelose* and pyridoxine treatments to diabetic rats significantly reversed the blood glucose and circulating insulin level.

3. Dopamine D_1, D_2 receptor and total dopamine receptor binding studies were done in corpus striatum, cerebral cortex, brain stem, pancreas and liver of control and experimental rats.

4. Dopamine D_1, D_2 receptor subtypes showed differential expression pattern. Dopamine D_1 receptors were increased in cerebral cortex, brain stem and pancreas whereas corpus striatum showed decreased dopamine receptors in diabetic rats. The gene expression studies of dopamine D_1 receptors were up regulated in cerebral cortex, brain stem and pancreas and down regulated in

corpus striatum. *Aegle marmelose* and pyridoxine treatment reversed the altered expression of dopamine D_1 receptor number and gene expression.

5. Dopamine D_2 receptors were increased in corpus striatum, pancreas and decreased in cerebral cortex, brain stem of diabetic rats. The gene expression studies of dopamine D_2 receptors were up regulated in corpus striatum, pancreas and down regulated in cerebral cortex, brain stem of diabetic rats. *Aegle marmelose* and pyridoxine treatment reversed the altered expression of dopamine D_2 receptor number and gene expression.

6. Total dopamine receptors were increased in cerebral cortex, pancreas and liver while corpus striatum, brain stem showed decreased expression of total dopamine receptors in diabetic rats. *Aegle marmelose* and pyridoxine treatment significantly reversed the altered expression of total dopamine receptors.

7. Insulin receptor status was analysed using Real Time PCR in corpus striatum and liver. Insulin receptor mRNA expression was down regulated in corpus striatum and up regulated in liver. Insulin, *Aegle marmelose* and pyridoxine treatment reversed the altered gene expression of insulin receptor.

8. GLUT-3 mRNA expressions were studied in corpus striatum, cerebral cortex and brain stem. GLUT-3 mRNA expressions were up regulated in diabetic rats compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment reversed the disrupted GLUT-3 gene expression in brain regions.

9. GLUT-2 mRNA expressions in pancreas and liver showed up regulation compared to control. Significant reversal of GLUT-2 mRNA expression was

observed with insulin, *Aegle marmelose* and pyridoxine treatment to diabetic rats.

10. IGF-1 gene expressions were down regulated in corpus striatum, pancreas and liver of diabetic rats. *Aegle marmelose* and pyridoxine treatment to diabetic rats ameliorated the altered expression pattern.

11. Gene expressions of CREB were down regulated in corpus striatum, cerebral cortex, brain stem, pancreas and liver of diabetic rats. Insulin, *Aegle marmelose* and pyridoxine treatment to diabetic rats significantly reversed the decreased expression pattern.

12. Second messenger enzyme - phospholipase C showed a decreased expression in diabetic brain regions - corpus striatum, cerebral cortex, brain stem and increased gene expression in liver. Treatment groups showed significant reversal when compared with diabetic group.

13. TNF- α mRNA expressions were up regulated in corpus striatum, brain stem, pancreas, liver and down regulated in cerebral cortex of diabetic rats. All treatments groups showed significant reversal in pancreas, liver and brain regions except cerebral cortex.

14. Caspase-8 gene expressions were up regulated in corpus striatum, brain stem, pancreas, liver and down regulated in cerebral cortex of diabetic rats. Insulin, *Aegle marmelose* and pyridoxine treatment to diabetic rats reversed caspase-8 expression in all regions except cerebral cortex.

15. NFκB gene expression was up regulated in corpus striatum and down regulated in cerebral cortex and liver of diabetic rats. Treatment groups

showed significant reversal in NFκB gene expression in corpus striatum and liver whereas in cerebral cortex significant reversal was not observed.

16. Gene expression of Akt-1 was down regulated in corpus striatum, cerebral cortex and brain stem of diabetic rats. Insulin, *Aegle marmelose* and pyridoxine treatment to diabetic rats showed significant reversal in brain regions except cerebral cortex.

17. BDNF gene expression was down regulated in striatum of diabetic rats. All treatment groups showed significant reversal in BDNF expression.

18. cAMP contents were significantly increased in corpus striatum, cerebral cortex, brain stem and decreased in pancreas and liver of diabetic rats. Insulin, *Aegle marmelose* and pyridoxine treatment reversed cAMP contents in diabetic rats.

19. cGMP contents were significantly decreased in corpus striatum, cerebral cortex, liver and increased in pancreas and brain stem of diabetic rats. Insulin, *Aegle marmelose* and pyridoxine treatment reversed cGMP contents in diabetic rats.

20. IP_3 contents were significantly decreased in brain regions- corpus striatum, cerebral cortex, brain stem, pancreas and liver of diabetic rats. *Aegle marmelose* and pyridoxine treatment increased IP_3 contents in corpus striatum, brain stem, pancreas and liver. In cerebral cortex IP_3 content was reversed by *Aegle marmelose* treatment but pyridoxine treatment did not show reversal in IP_3 content.

21. Fluoro Jade positive neurons were significantly increased in striatum of diabetic group. *Aegle marmelose* and pyridoxine treatment to diabetic rats showed significant reduction in Fluoro Jade positive neurons.

22. Anti oxidant enzymes - GPx and SOD showed differential expression pattern in liver of diabetic rats. GPx gene expression was down regulated whereas SOD gene expression was up regulated in diabetic group. All treatment groups showed significant reversal in GPx and SOD gene expression.

23. BAX gene expression in liver was up regulated in diabetic condition compared to control. Insulin, *Aegle marmelose* and pyridoxine treatment to diabetic rats showed significant reversal in BAX gene expression.

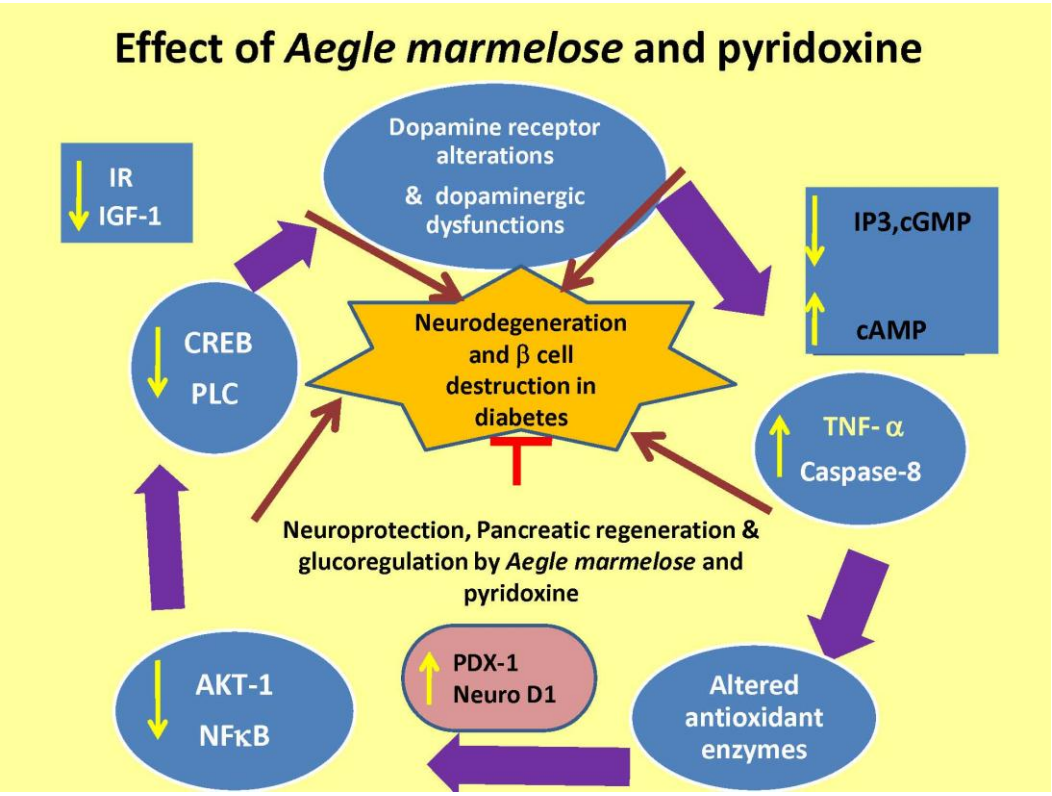
24. BrdU positive cells were significantly increased in pancreas along with increased gene expression of PDX-1 and NeuroD1 in *Aegle marmelose* and pyridoxine treated rats compared to diabetic group.

In the present study, we summarize, that dopaminergic receptor subtypes showed differential expression pattern in brain regions, pancreas and liver and it has significant role in glucose metabolism. Regulators of glucose metabolism and insulin signaling- Insulin receptor, GLUT 3, GLUT 2, IGF-1 were altered in diabetic condition. CREB, Phospholipase- C, second messengers, antioxidant enzymes, apoptotic factor alterations were seen in diabetic rats. *Aegle marmelose* and pyridoxine treatment enhanced the expression pancreatic regeneration markers- PDX-1, NeuroD1 and pancreatic β cell proliferation in diabetic rats. *Aegle marmelose* and pyridoxine treatment decreased the expression of apoptotic factors and increased the expression of neuronal survival factors in diabetic rats. Thus the results suggest the the

therapeutic role of *Aegle marmelose* and pyridoxine in ameliorating CNS dysfunctions and glucose regulation.

Conclusion

Diabetes mellitus, a chronic metabolic disorder results in neurological dysfunctions and structural changes in the CNS. Antioxidant therapy is a challenging but necessary dimension in the management of diabetes and neurodegenerative changes associated with it. Our results showed regional variation and imbalance in the expression pattern of dopaminergic receptor subtypes in diabetes and its role in imbalanced insulin signaling and glucose regulation. Disrupted dopaminergic signaling and increased hyperglycemic stress in diabetes contributed to the neuronal loss. Neuronal loss in diabetic rats mediated through the expression of pattern of GLUT-3, CREB, IGF-1, Akt-1, NFκB, second messengers- cAMP, cGMP, IP_3 and activation of apoptotic factors factors- TNF-α, caspase-8. Disrupted dopaminergic receptor expressions and its signaling in pancreas contributed defective insulin secretion in diabetes. Activation of apoptotic factors- TNF- α, caspase-8 and defective functioning of neuronal survival factors, disrupted second messenger signaling modulated neuronal viability in diabetes. Hyperglycemic stress activated the expression of TNF-α, caspase-8, BAX and differential expression of anti oxidant enzymes- SOD and GPx in liver lead to apoptosis. Treatment of diabetic rats with insulin, *Aegle marmelose* and pyridoxine significantly reversed the altered dopaminergic neurotransmission, GLUT3, GLUT2, IGF-1 and second messenger signaling. Antihyperglycemic and antioxidant activity of *Aegle marmelose* and pyridoxine enhanced pancreatic β cell proliferation, increased insulin synthesis and secretion in diabetic rats. Thus our results conclude the neuroprotective and regenerating ability of *Aegle marmelose* and pyridoxine which in turn has a novel therapeutic role in the management of diabetes.


Effect of *Aegle marmelose* and pyridoxine
Dopamine receptor alterations & dopaminergic dysfunctions
IR
IGF-1
IP3,cGMP
cAMP
CREB
PLC
Neurodegeneration and β cell destruction in diabetes
TNF- α
Caspase-8
Neuroprotection, Pancreatic regeneration & glucoregulation by *Aegle marmelose* and pyridoxine
AKT-1
NFκB
PDX-1
Neuro D1
Altered antioxidant enzymes

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Publications

1. **Anitha M**, Abraham Pretty Mary, C S Paulose. **(2012)**. Disturbed brain glucose metabolism and dopamine receptor expression in diabetic rats: Restoration by pyridoxine – *European journal of pharmacology.* (in press)
2. **Anitha M**, Nandhu M S, Anju T R, Jes Paul and C S Paulose. **(2011).** Targeting Glutamate mediated excitotoxicity in Huntington's disease: Neural progenitors and parial glutamate antagonist, Memantine a new therapeutic strategy. *Medical Hypotheses,* 76(1):138-40.
3. Anju T R, **Anitha M**, Chinthu R, Paulose C S **(2012)**. Cerebellar $GABA_A$ receptor alterations in hypoxic neonatal rats: Role of glucose, oxygen and epinephrine supplementation. *Neurochemistry international* (in press).
4. Anju TR, Binoy J, **Anitha M**, Paulose CS **(2012).** Striatal GABA Receptor Alterations in Hypoxic Neonatal Rats: Role of Glucose, Oxygen and Epinephrine Treatment . *Neurochem Res* 37(3): 629-38
5. Sherin A, Anu J, Peeyush KT, Smijin S, **Anitha M**, Roshni BT, C S Paulose (2011). Cholinergic and GABAergic Receptor Functional Deficit in the Hippocampus of Insulin Induced Hypoglycemic and Streptozotocin Induced Diabetic Rats. *J. neuroscience*., 202:69-76.
6. Nandhu MS, Paul J, Kuruvilla KP, **Malat A,** Romeo C, Paulose CS. (2011). Enhanced glutamate, IP3 and cAMP activity in the cerebral cortex of unilateral 6-hydroxydopamine induced Parkinson's rats:Effect of 5-HT, GABA and bone marrow supplementation. *J Biomed Sci.* 15;18:5.
7. Abraham PM, Kuruvilla KP, Mathew J, **Malat A**, Joy S, Paulose CS (2010). Alterations in hippocampal serotonergic and INSR function in streptozotocin induced diabetic rats exposed to stress: neuroprotctive role of pyridoxine and Aegle marmelose. J Biomed Sci. 17:78 1-15.

8. Rengarajan S, Parthasarathy C, **Anitha M**, Balasubramaniam K. (2007). Diethylhexyl phthalate impairs insulin binding and glucose oxidation in Chang liver cells. Toxicol In vitro 21: 99-102.
9. **Anitha Malat,** Chinthu Romeo, Roshni Baby Thomas, Shilpa Joy and C S Paulose **(2012)**. Striatal neuronal degeneration in streptozotocin induced diabetic rats: Neuroprotective role of pyridoxine- *Metabolic Brain Disease* (under review).

Abstracts/ Scientific Presentations

1. **Anitha Malat**, Pretty Mary Abraham and C.S.Paulose. Serotonergic receptor down regulation in brain stem of Streptozotocin induced Diabetic Rats: Antagonism by pyridoxine and insulin. Society of Biological Chemists (India) 78th Annual meeting, October 30 - November 1, 2009 National Centre for Cell Science.
2. **Malat Anitha,** Abraham Pretty Mary, Joy Shilpa and C. S. Paulose. Down regulation of serotonin receptors and its transporter expression in the pancreas of streptozotocin induced diabetic rats: Effect of pyridoxine and *Aegle marmelose.* Indian Ageing congress held at Banaras Hindu University (BHU), Varanasi, India 12-14 November, 2010.
3. Chinthu Romeo, **Anitha. M,** Jayanarayanan. S, Korah. P. Kuruvilla, Smijin Soman, C.S. Paulose. Enhanced malate dehydrogenase, glutamate dehydrogenase, arginase and cholesterol in herbal formulation treated rats: A molecular study. UGC sponsored state level seminar on modern methods in herbal drug development, Kerala. (July28-29, 2010)
4. Shilpa Joy, **Anitha. M**, C. S. Paulose. $GABA_{B,}$ cAMP AND CREB functional regulation in partially hepatectomised rats. GABA-Chitosan nanoparticles induced hepatocyte proliferation. Kerala science congress (January, 2011).
5. Naijil George, **Anitha Malat,** Korah P Kuruvilla, C.S. Paulose. Novel role of Vitamin D3 in the prevention of Diabetogenesis in Rats. National conference

on Emerging Trends in Biotechnologists (India), Acharya Nagarjuna University, Guntur. (September, 2011)

6. **Anitha Malat,** Jayanarayanan S, Nandhu. M. S and C S Paulose. *Aegle marmelose* modulates pancreatic regeneration through PDX-1 expression in streptozotocin induced diabetic rats. 8thIBRO World Congress of Neuroscience, Florence (July14-18th, 2011).

Awards

1. Travel Grant from IBRO to attend 8thIBRO World Congress of Neuroscience, Florence (July14-18th, 2011).
2. IBRO YOUNG INVESTIGATOR FELLOWSHIP in connection with 8th IBRO World Congress of Neuroscience, Florence (2011)