Muscarinic M1, M3 and $GABA_B$ receptor subtypes functiona regulation in streptozotocin-induced diabetic rats: Effects of Baclofen, Vitamin E and Gymnemic acid

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Certificate

This is to certify that the thesis entitled **"Muscarinic M1, M3 and $GABA_B$ receptor subtypes functional regulation in streptozotocin-induced diabetic rats: Effects of Baclofen, Vitamin E and Gymnemic acid"** is a bonafide record of the research work carried out by Mr.Ajayan M.S, under the guidance and supervision of Late Dr. C.S. Paulose, FIMSA, FGSI, FAMS, Professor Emeritus, UGC-BSR fellow (15/02/1953-29/03/2016) in the Department of Biotechnology, Cochin University of Science and Technology.

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I hereby declare that the thesis entitled "**Muscarinic M1, M3 and $GABA_B$ receptor subtypes functional regulation in streptozotocin-induced diabetic rats: Effects of Baclofen, Vitamin E and Gymnemic acid"** 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 Late Dr. C.S. Paulose, FIMSA, FGSI, FAMS, Professor Emeritus, UGC-BSR fellow (15/02/1953-29/03/2016), Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other title or recognition from any University / Institution.

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Ajayan M. S

Dedicated to my beloved Parents...

ABBREVIATIONS

AC	Adenylyl Cyclases
ACh	Acetylcholine
AChE	Acetylcholine esterase
AD	Alzheimers disease
AGEs	Advanced glycation end products
Bax	BCL-2-associated X protein
B_{max}	Maximal binding
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cAMP	Cylic adenosine monophosphate
Caspases	Cysteine-dependent aspartate-specific protease
ChAT	Choline acetyltransferase
CNS	Central Nervous System
CPM	Counts per minute
CREB	cAMP response element-binding protein
Ct	Crossing threshold
DA	Dopamine
DAMP	Deoxy acetyl methyl piperidine
DEPC	Di ethyl pyro carbonate
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EPI	Epinephrine
EPSCs	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
FITC	Florescent isothiocyanate
G protein	Guanosine nucleotide-binding proteins
GABA	Gamma amino butyric acid

$GABA_B$	Gamma amino butyric acid B receptor
GAD	Glutamic acid decarboxylase
GLUT 2	Glucose transporter type 2
GLUT 3	Glucose transporter type 3
GLUT 4	Glucose transporter type 4
GPCR	G protein-coupled receptor
GPx	Glutathione peroxidase
HBSS	Hang's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF	Insulin-like growth factor
INS	Insulin
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	NH_2-terminal Jun kinases
K_d	Dissociation constant
Leu	Leucine
LTD	Long term depression
mAChRs	muscarinic acetylcholine receptors
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
nAChRs	Nicotinic acetylcholine receptors
NF-κB	Nuclear factor-kappa B
Nrf2	Nuclear factor erythroid-2-related factor-2
P	Level of significance
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Triton X- 100
PCR	Polymerase Chain Reaction
PFC	Prefrontal cortex
PKA	Protein kinase A
PKC	Protein kinase C

PLC	Phospholipase C
QNB	Quinuclidinylbenzilate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell park memorial institute
SEM	Standard error of mean
Ser	Serine
SOD	Superoxide dismutase
STZ	Streptozotocin
Thr	Threonine
TNF-α	Tumour necrosis factor-α
Tyr	Tyrosine
VDR	Vitamin D receptor
VICC	Voltage - insensitive calcium channels

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Introduction

Diabetes is a group of metabolic diseases characterized by high levels of blood sugar (hyperglycemia). It results from defects in insulin production and/or insulin action, and impaired function in the metabolism of carbohydrates, lipids and proteins which leads to long term health complications (Oktayoglu *et al*., 2009). Diabetic retinopathy, which affects the blood vessels in the retina, causes visual symptoms including reduced vision and blindness (Rafieian-Kopaei & Nasri, 2014; Bahmani *et al*., 2014). Diabetic nephropathy usually leads to changes in the kidney tissue, loss of progressively larger amounts of protein in the urine and chronic kidney disease (Rouhi & Ganji, 2013). Diabetic neuropathy commonly causes tingling, numbness and pain in the feet. It also increases the risk of skin damage due to altered sensation. These are related to uncontrolled hyperglycemia (Tesfaye & Gill, 2011). According to a report from World Health Organization, about 220 million people have type 2 diabetes mellitus. Its incidence is increasing rapidly, and it is expected to increase to more than 365 million by 2030 (Nasri, 2013). This increase is due to the general population aging, as well as eating and physical activity habits across the world with a parallel increase in obesity (Van dijk *et al*., 2011). The non-insulin-dependent diabetes mellitus is a chronic disease characterized by hyperglycemia and insulin resistance (Merzouk *et al*., 2003). So diabetes is one of the most debilitating conditions in patients affecting a substantial proportion of the world's population. Diabetes can predispose an individual to metabolic and cardiovascular disturbances, and obesity. These pathologies are accompanied by vascular complications (Forbes & Cooper, 2013).

Type 1 diabetes mellitus is a multifactorial autoimmune disease characterized by destruction of pancreatic β-cells, culminating in absolute insulin deficiency (Frese & Sandholzer, 2013). It is now well known that the clinical onset of the disease represents the end stage of an immunological process that occurs over a course of months to years (Vendrame *et al*., 2004). Type 1 diabetes

is most probably caused by an auto-immune destruction of insulin-producing β-cells mediated by cytotoxic T cells recognizing specific cell associated antigens (Bolli, 2001). Type 1 diabetes affects males and females equally and decreases life expectancy by an estimated 13 years. An estimated 5-15% of adults diagnosed with type 2 diabetes actually have type 1 diabetes or latent autoimmune diabetes of adults (LADA) (Livingstone *et al*., 2015). An estimated 387 million people have diabetes worldwide, of which type 1 diabetes accounts for between 5% and 10%. Diabetic complications continue to be a major cause of morbidity and mortality in persons with type 1 diabetes (Maahs *et al*., 2010; Borchers *et al*., 2010).

The identified genes related with diabetes code for β-cell mass, β-cell function, pro insulin and insulin molecular structure, insulin receptors, hepatic synthesis of glucose, glucagon synthesis, and cellular responsiveness to insulin stimulation (Nolan *et al*., 2011). The treatment of diabetes mellitus is based on different potential compounds that works on diverse mechanism of actions including stimulation of insulin secretion, decrease of hepatic gluconeogenesis, improve in insulin receptor sensitivity and hindrance of digestion and absorption of carbohydrate, respectively. Insulin, secreted by the β-cells of the pancreas, lowers the concentration of glucose in blood by inhibiting hepatic glucose production and stimulating the uptake and metabolism of glucose by muscle and adipose tissue (Davis & Granner, 1996). In the rat model for diabetes, regulation of glucose homeostasis is linked with maintenance of pancreatic insulin mRNA expression. When pre-diabetic or mildly glucose intolerant rats were challenged with a diabetogenic agent, maintenance of normal glucose levels is linked with an increase in insulin mRNA expression. When this adaptive response failed, hyperglycemia worsened (Giddings *et al*., 1985). After administration of streptozotocin (STZ) and alloxan, a marked reduction in insulin mRNA level was observed (Mulder *et al*., 1995). As the sugar level increased, the quality of life decreases in diabetic retinopathy patients. It may be due to greater glucose flux and possibly poor diabetes control (Gendy *et al*., 2012).

In the STZ-induced type 1 diabetic rats, the course of development of peripheral and central neurophysiological changes differs. The agents activating pathways downstream of glucose metabolism or generating second messengers-which are coupled to potentiation of insulin secretion, may work effectively as β-cell based therapies for diabetes. Appropriate profiles of insulin release will allow tight glycemic control to minimize the complications that arise from long-term hyperglycemia (Shanta Persaud & Peter Jones, 2008). In diabetes mellitus, post absorptive hepatic glucose production is increased, which positively correlates with fasting plasma glucose concentration. Between gluconeogenesis and glycogenolysis, gluconeogenesis appears to be drastically increased in diabetes mellitus (Consoli, 1992).

Cholinergic signaling plays a significant role in a wide diversity of central nervous system (CNS) and peripheral functions including learning, memory and attention mechanisms, motor control, nociception, regulation of sleep-wake cycles, cardiovascular function, renal and gastrointestinal functions, and many others. A wide variety of central nervous system disorders including Alzheimer's disease, Parkinson's disease, schizophrenia, epilepsy, sleep disorders, neuropathic pain and others involve malfunction of cholinergic transmission. Evidence suggests that cholinergic neurotransmission in the forebrain regions and cholinergic involvement in learning and memory are mediated primarily by muscarinic receptors (Levey, 1993). The altered choline acetyltransferase and cholinesterase activities, and choline concentration have been suggested to be the reasons for the super sensitivity to muscarinic agonists in the myocardium of the STZ-induced type 1 diabetic rats (Carrier & Aronstam, 1987). In addition, cardiac M2-muscarinic receptor (M2-MR) gene expression was increased in the STZ-induced type 1 diabetic rats (Liu *et al*., 2005). Alterations in the atrial muscarinic system in the STZ-induced type 1 diabetic rats have been suggested to be a consequence of impaired functional muscarinic receptor-G protein coupling (Lee *et al*., 2008).

Five subtypes of muscarinic acetylcholine receptors (mAChR) have been identified: M1, M3 and M5 receptors are preferentially coupled to G-protein and

stimulate phospholipase C, and M2 and M4 receptors are coupled to G-protein associated with the inhibition of adenylate cyclase (Ravikumar & Sastry, 1985). Muscarinic acetylcholine receptors are extensively distributed in the striatum, brain stem, cortex and midbrain, and their levels are relatively lower in the cerebellum and hippocampus. M1 receptors are concentrated mainly in the forebrain regions while M2 receptors dominate in the thalamus (Schlumpf *et al*., 1991). The selective distribution of M1 receptors in the forebrain and the deleterious effects of M1 antagonists on memory and learning indicate a primary role of this subtype of muscarinic receptors in cognition, attention mechanisms, and sensory processing (Langmead *et al*., 2008).

$GABA_B$ receptors are metabotropic G-protein-linked receptors, the chief neuro-inhibitory neurotransmitter in mammalian systems. $GABA_B$ receptors are obligatory heterodimers with two homologous subunits (G_{B1} and G_{B2}) required for functioning, are widely expressed and distributed in the CNS (Kaupmann *et al*., 1998), and can activate diverse intracellular pathways (Guyon & Leresche, 1995; Laviv *et al*., 2011). $GABA_B$ receptors are also expressed on cells of the immune system with a possible link to the inflammatory response (Tian *et al*., 2004; Rane *et al*., 2005). $GABA_B$ receptors are the site of action of the muscle relaxant, baclofen, and are insensitive to drugs that modulate $GABA_A$ receptors (Bormann, 2000). When GABA binds to the $GABA_B$ receptor, there is an increase in potassium conductance and a decrease in voltage-dependent calcium currents, resulting in hyperpolarization of the neuron and inhibition of neurotransmission (Ong & Kerr, 2000). *In vivo* studies suggest an important role for $GABA_BR$ in regulating β-cell function. For instance, in apparent contradiction, $GABA_{B1}$ R-deficient mice (global knockout of B1 subunit) displayed improved glucose tolerance, increased pancreatic insulin content, and elevated glucose-stimulated insulin secretion, associated with enlarged islets and insulin resistance (Crivello *et al*., 2013; Bonaventura *et al*., 2008).

The cell membrane lipid peroxidation is involved in the etiology of neurodegenerative disorder related with diabetes; lipid peroxides may cause oxidative damage to the myelin sheath surrounding the nerve. Therefore, oxidative

stress may predispose diabetic patients to the development of neuropathy by a mechanism involving increased lipid peroxidation (Dickinson *et al*., 2002). The prolonged hyperglycemia causes increased protein glycation, which has been known to be a source of free radicals (Ceriello, 1999). The reactive oxygen species (ROS) and the products of advanced glycosylation are important in the onset and development of complications in chronic diabetes. Reduced lipid peroxidation and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications (Armstrong *et al*., 1996). During oxidative stress, the balance between degeneration and regeneration shifts toward more degeneration (Vijayakumar *et al*., 2012).

During the antioxidant therapy, oxidative stress may be reduced and the balance shifts towards regeneration, antioxidant can inhibit the free radical-induced endoneural damage and these can also improve the antioxidant tone in the diabetic individual in whom the antioxidant capacity is defective because of the active polyol pathway (Ilango *et al*., 2012). GPx, GSH, and vitamin E are all low in the kidney and lens of diabetic rats when compared to non-diabetic controls and that these effects may be improved with exercise and supplementation with vitamins C and E. It has been observed that rats with STZ-induced type 1 diabetes has lower SOD1 and catalase activity than control rats (Sindhu *et al*., 2004), but the activity of the Gpx was found to be no different between groups. Treatment with insulin or antioxidants appeared to normalize these trends. It has been observed that the livers of diabetic rats on a vitamin E supplemented diet had increased SOD 1 activity over controls on a standard diet (Kinalski *et al*., 2000). Moreover, antioxidant treatment was shown by immunohistochemical analysis to preserve the number of insulin-positive β-cells. The proper maintenance of antioxidant defenses might be helpful for slowing progression of diabetes itself by sustaining functional pancreatic β-cells.

The enzymatic antioxidant activities such as superoxide dismutase, catalase and glutathione peroxidase decrease in liver, kidney and heart tissues of patients with diabetes mellitus, the levels of the reactive oxygen species such as

superoxide anion radicals increase (Giugliano *et al*., 1995). The significant increase in endogenous prooxidant activity and the decrease in antioxidants have been shown to contribute to the oxidative stress in diabetes. A marked decrease in glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) activities have been reported in diabetic animals (Abdollahi *et al*., 2013; Rafieian-Kopaei & Nasri, 2013). Treatment with probucol, which has antioxidant activity resulted in a significant improvement in myocardial activities of catalase, SOD and GSHPx (antioxidant enzymes) providing evidence that diabetic cardiomyopathy was associated with an antioxidant deficit (Nasri & Rafieian-Kopaei, 2013; Ardalan *et al*., 2014). Gpx-1 is a key-enzyme implied in defense against oxidative stress and its super expression accelerates the ROS decrease, diminishing phosphatase inhibition and consecutively of insulin receptor phosphorylation, leading to insulin resistance (Mcclung *et al*., 2004).

Akt, also known as protein Kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes including cell growth, survival, proliferation and metabolism (Gonzalez & McGraw, 2009). Akt is a major mediator of the insulin effect on glucose metabolism (Whiteman *et al*., 2002). Akt is stimulated by a variety of growth factors including insulin itself (Whiteman *et al*., 2002). The importance of Akt in β-cells comes from studies in mice deficient in different Akt isoforms. Mice lacking Akt-1 have normal glucose homeostasis but impaired foetal and postnatal growth (Cho *et al*., 2001).

The homeo domain transcription factor pancreatic duodenal homeo box (Pdx-1) is a major mediator of insulin transcription and a key regulator of the β-cell phenotype. Heterozygous mutations in Pdx-1 are associated with the development of diabetes in humans (Kathryn *et al*., 2010). The complete deficiency of Pdx-1 is associated with pancreatic agenesis and partial deficiency leads to severe β-cell dysfunction, and increases β-cell death and diabetes both in rodent and human. Chronic hyperglycemia and dyslipidemia are major features of diabetes, cause β-cell dysfunction *via* reduced Pdx-1 expression (Fujimoto & Kenneth, 2009). The reduced signaling through the insulin/insulin-like growth factor (IGF) pathway appears to be an important common mechanism leading to

the decreased Pdx-1 expression that is consistently present in states of β-cell failure (Chang Chen *et al*., 2008).

Bax belongs to a family of proteins which share homology with Bcl-2 in several highly conserved regions. The Bcl-2 proteins are involved in the regulation of apoptosis, and act to promote or suppress cell death. Bax levels were increased in human diabetic retinas that had high levels of glucose (Abou-Seif & Youssef, 2004). The expression of Bax was increased in the cortex of the kidney of diabetic animal models (Li *et al*., 2004). An over expression of Bax leads to programmed cell death.

NF-κB is a transcription factor required by dorsal root ganglia neurons (DRG) for survival and plasticity, and regulates transcription of antioxidant proteins (e.g. MnSOD). The decrease in NF-κB activity in DRG contributes to pathological phenomena observed in cultured DRG neurons from diabetic rats (Saleh *et al*., 2013). NF-κB is also a critical mediator of inflammatory events in other cell types and tissues, and is implicated in damage caused by arthritis and other diseases when activated as part of an inflammatory axis, as it occurs in non-neuronal cells of peripheral nerves. For example, NF-κB DNA binding activity and expression of the p65 subunit are significantly up regulated in the sciatic nerve of STZ-induced type 1 diabetic mice at 6-9 months (Bierhaus *et al*., 2004; Toth *et al*., 2008).

Tumor necrosis factor alpha (TNF-α) is a potential therapeutic target for onset of diabetes. Paradoxically, long-term treatment with TNF-α as well as short-term treatment with anti-TNF-α prevent the later development of diabetes in non-obese mice (NOD) (Jacob *et al*., 1990; Yang *et al*., 1994). TNF-α is an inflammatory cytokine, strongly correlated with insulin resistance and chronic inflammation (Hotamisligil *et al*., 1994). TNF-α may serve as an inflammatory biomarker and as an important risk indicator for the future development of diabetes and may prove a novel target for therapeutic intervention (Shoelson *et al*., 2006). Increased TNF-α production has been observed in adipose tissue derived from obese rodents or human subjects and implicated as a causative factor in obesity-associated insulin resistance and the pathogenesis of diabetes (Aguirre,

2000). TNF-α might play a dual role in type 1 diabetes. For instance, TNF and a TNFR2 agonist can selectively kill human auto reactive CD8 T cells (Ban *et al*., 2008). A potential mechanism through which diabetes may increase apoptosis is the excessive production of the TNF-α pleiotropic cytokine that plays an important role in inflammation and immunity (Taylor, 2001).

CREB belongs to the basic-leucine-zipper family of transcription factors and binds the 8 bp palindromic sequence TGACGTCA known as the c-AMP-response element (CRE) (Jung, 2008). Tissue-specific regulation of CREB-targeted genes depends on recruitment of a coactivator known as CREB-binding protein (CBP) (Barrera & Ren, 2006). CREB must be phosphorylated by kinases such as PKA, PKC and MAPK for dimerisation, binding of CBP, and increased affinity for the CRE (Kreisberg *et al*., 1996; Brivanlou *et al*., 2002). The role of CREB in the development of diabetic nephropathy includes transcriptional upregulation of FN, USF2 and decorin (DCN) genes (Wahab, 2000; Shi, 2008).

One of the most vital cellular nutrient transports in eukaryotic cells is the transport of glucose across plasma membrane which is catalyzed by a family of glucose transporter proteins (GLUT). GLUT 3 plays an important role in the alterations of placental function observed in diabetic pregnancies (Boileau *et al*., 1995). GLUT 3 has found to be present in early gestation, proposing its important role in glucose uptake (Brown *et al*., 2011). The four- to five fold increase in GLUT 3 mRNA and protein expression in placentas of diabetic rats suggests that GLUT 3 could be responsible for the enhanced glucose uptake and glycogen accumulation. The 30% increase in the placental weight of diabetic rats is unlikely to account for the fourfold increase in GLUT 3 expression and the fivefold increase in glucose uptake and glycogen accumulation (Thomas *et al*., 1990). So GLUT 3 glucose transporter regulates the placental glucose transport and utilization.

Phospholipase C (PLC) activity is known to influence cardiac function. The PLC β3 in the cardiac cell plasma membrane (sarcolemma, SL) was isolated from diabetic rat hearts. The total SL PLC was decreased in diabetes and was

associated with a decrease in SL PLC β3 activity. Immunofluorescence in frozen diabetic left ventricular tissue sections revealed to be due to a decrease in PLC β3 protein abundance. So a defect in PLC β3-mediated signaling processes may contribute to the cardiac dysfunction seen during diabetes (Paramjit *et al*., 2004).

Antioxidant substances, such as vitamin E, play an important role in the reduction of the neurological damage caused by diabetes mellitus. Vitamin E, a fat-soluble vitamin that prevent damage to lipids by oxygen free radicals (Parks & Traber, 2000). The highly-reactive species attack lipids within membranes or lipoproteins; they start out the chain reaction of lipid peroxidation. Vitamin E interferes with this chain reaction, eg. it acts as a chain breaking inhibitor of lipid peroxidation. Vitamin E is an antioxidant that has been confirmed to improve insulin action. It is also important in blood pressure and glucose homeostasis, related with the involvement of free radicals in both essential hypertension and diabetes mellitus. Vitamin E, inhibits lipid peroxidation and interferes with the process of cytokine-mediated cytotoxicity by acting as a free oxygen scavenger (Paredes *et al*., 2002). Furthermore, vitamin E has been shown to be effective in preventing severe complications in long-standing type 1 diabetic patients (Rosen & Toeller, 1999). Vitamin E ameliorates oxidative stress in type 1 diabetes patients and improves antioxidant defense system (Gupta *et al*., 2011). Diabetes has been reported to induce alterations in the antioxidative defense capacity in brain, which could result in increased risk of peroxidative damage. This increase in lipid peroxidation during diabetes is due to peroxidation of polyunsaturated fatty acids, leading to the degradation of phospholipids, which is considered as an index of cellular deterioration (Mallick *et al*., 2011).

Vitamin E supplementation has been associated with a significant decline in protein oxidation, lipid peroxidation and enhancement in the antioxidant defense system. Vitamin E may promote beneficial effects on diabetic complications through the attenuation of oxidative stress (Rafieian-Kopaei & Baradaran, 2013). The possible participation of dietary antioxidants, such as vitamins, in ameliorating the diabetic state and retarding the development of diabetes complications (Sheikh-Ali *et al*., 2011; Cuerda *et al*., 2011). The balance

between the rate of free radical generation and elimination is important. Excess cellular radical generation can be harmful; however, if there is a significant increase in radical generation, or a decrease in radical elimination from the cell, oxidative cellular stress ensues (Valko *et al.*, 2007). There is convincing experimental and clinical evidence that the generation of reactive oxygen species (ROS) increases in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress (Rosen *et al.*,2001). Vitamin E is effective in preventing severe complications in long-standing type 1 diabetic patients (Rosen & Toeller, 1999).

The process of neuropathic pain and other physiologic usual pains are different. GABAergic system, a pain relief mechanism, produces inhibitory neurotransmitter in CNS. Baclofen has excitatory synaptic effect on GABA receptors so used as an anti-nociceptive drug in the management of trigeminal neuralgia. Baclofen and GABA drugs affect pancreas *in vivo* to increase plasma insulin and reduce blood glucose in diabetic rats (Moulin, 1996). Baclofen may be effective in delayed onset of type 1 diabetes (TID) of non-obese diabetic mice by stimulating GABA activity (Beales *et al.*, 1995). The treatment of non-obese diabetic (NOD) mice with a $GABA_BR$ agonist delayed the onset of type 1 diabetes and showed an improvement in β-cell survival and proliferation (Tian *et al.*, 2013; Ligon *et al.*, 2007). GABA administration limited β-cell apoptosis in a model of human islet transplant. A significant reduction in the percentage of apoptotic cells concomitant with an increased frequency of $insulin^+$ β-cells in human islets was evident as reported in mice treated with GABA, baclofen, or muscimol (Tian *et al.*, 2013). GABA, baclofen, and muscimol reduced the percentage of apoptotic islet cells dose-dependently in mice. In mice treated with GABA or $GABA_A/_BR$-specific agonists, the percentage of newly replicated β-cells reached approximately 3%, suggesting that oral GABA treatment promotes β-cell replication (Tian *et al.*, 2013). Baclofen is an effective drug which is helpful for quieting the created pain in dormant phase as a model of diabetic pain; therefore,

in the treatment of the diabetic painful neuropathy baclofen can be an alternative drug.

The need for new therapies for glycemic control is apparent due to the fact that the commercially available anti-diabetic drugs are associated with side effects affecting blood lipid levels and/or systemic blood pressure, limited efficacy, and tolerability and significant mechanism-based side effects which may lead to increase in morbidity and mortality (Aguilar, 2011; Prabhakar *et al*., 2014; Alexiou & Demopoulos, 2010). Administration of *Gymnema sylvestre* extract to diabetic rats increased superoxide dismutase activity and decreased lipid peroxide by either directly scavenging the reactive oxygen species, due to the presence of various antioxidant compounds, or by increasing the synthesis of antioxidant molecules (albumin and uric acid) (Vasi & Austin, 2009). Leaves of *Gymnema* suppress the taste of sugar upon chewing; hence these are also used to lower the intense desire of consuming sugary products (Saneja *et al*., 2010; Rani *et al*., 2012). Gymnemic acid, depresses appetite and causes weight reduction, restores pancreas function, and has anti-tooth decaying effect. It possesses different therapeutic activities like suppression of taste buds activity for sweetness, inhibition of intestinal glucose absorption and lowers the plasma glucose levels (Patel *et al*., 2012; Solanki *et al*., 2013).

The gymnemic acids of *Gymnema sylvestre* have been reported to have direct action on pancreatic β-cells and may bring about the regeneration or repair of β-cells leading to increase in serum insulin levels (Shanmugasundaram *et al*., 1990; Persaud *et al*., 1999; Kanetkar *et al*., 2007). *Gymnema montanum* leaves, a plant related to *Gymnema sylvestre* has been reported to revert alloxan-induced liver damage to normal state by its hypoglycemic action and stimulatory insulin release by the β-cells (Ananthan *et al*., 2003). The secondary metabolite production from plant tissue culture has been known as a significant resource for new drug improvement and clinical research in the fields of pharmacology and medicine. Plant extracts of *Gymnema sylvestre* has reported use as a remedy for diabetes mellitus. It has also been used broadly in the form of fractions and isolated compounds as potential bioactive molecules including gymnemic acid and

gymnemagenin. Gymnemic acid is reported to augment glucose uptake and utilization (Daisy *et al*., 2009). It also improves the function of pancreatic β-cells and may also reduce glucose absorption in the gastrointestinal tract.

Diabetes brings about ischemic damages in various brain regions because of increased oxidative stress caused by hyperglycemia. Increased reactive oxygen species (ROS; e.g. superoxide anion, hydroxyl radicals, peroxynitrite, hydrogen peroxide) can alter neuronal function because of neuronal death through protein oxidation, DNA damage, elevated non-enzymatic glycosylation, peroxidation of membrane lipids (Hawkins & Davies, 2001). Diabetes arises as a result of deteriorating β-cell function, poor compliance to treatment, weight gain, reduced exercise, dietary changes, or illness.

The antidiabetic effect of baclofen, Vitamin E and gymnemic acid were examined.

(1) Evaluation of DNA and protein profiles using radioactive incorporation studies for DNA and protein synthesis.

(2) Gene expression studies of Akt-1, Pdx-1 and NF-κB for pancreatic β-cell proliferation and differentiation.

(3) Evaluation of the expression of antioxidant enzymes, SOD and GPx, and apoptotic mediators like Bax, Caspase 8 and TNF-α for antioxidant and antiapoptotic actions.

(4) The expression of $GABA_B$ and insulin receptor in brain regions and pancreas for cell signaling pathways.

In the case of diabetes, this network involves insufficient islet β-cell and adipose-tissue responses to chronic fuel surplus, which results in a so-called nutrient spillover and finally, overall metabolic stress. The final damage includes multiple organs (Nolan *et al.*, 2011). The receptor number and binding affinity of total muscarinic, muscarinic M1, muscarinic M3, GABAergic receptor subtypes were studied in cerebral cortex, cerebellum, brain stem and pancreas of experimental rats. The mRNA expression of muscarinic and GABAergic receptor subtypes were determined using Real Time PCR. Immunohistochemistry studies using confocal microscope were carried out to substantiate receptor density and

gene expression results. Cell signaling changes in the brain regions and pancreas to diabetic conditions were evaluated by examining the gene expression profiles of $GABA_B$ receptor, CREB, phospholipase C, insulin receptor and GLUT. The neuro biochemical and gene expression studies reveal that the action of these hypoglycemic drugs will lead to the control of blood glucose level, proliferation of pancreatic β-cells, improved action of antioxidant activities and restored the function of specific markers in molecular signaling. This study has established the interaction of antidiabetic substances such as Baclofen, Vitamin E and Gymnemic acid with muscarinic and GABAergic receptors and evaluated their therapeutic role in the management of diabetes.

OBJECTIVES OF THE PRESENT STUDY

In the present work we studied the potential of anti-diabetic compounds; Baclofen, Vitamin E and Gymnemic acid treatment to enhance β-cell proliferation and reduce neuronal damage in STZ-induced type 1 diabetic rats. For achieving the aim, muscarinic M1, M3 and $GABA_B$ receptors functional regulation, gene expression of growth factors, neuronal survival and apoptotic factors during treatment with Baclofen, Vitamin E and Gymnemic acid in diabetic rats were studied. The objectives are

1. To study the body weight and blood glucose level of Baclofen, Vitamin E and Gymnemic acid in STZ-induced diabetic animal model.
2. To study the gene expression of β-cell regeneration markers- Pdx-1, apoptotic markers- Bax, Caspase 8, TNF-α and cell signaling molecules, NF-κB and Akt-1 in the pancreas of experimental rats using Real Time PCR.
3. To measure the total muscarinic, muscarinic M1 and muscarinic M3 receptor subtypes binding parameters in cerebral cortex, cerebellum, brain stem and pancreas of control, diabetic, insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rats.
4. To measure the GABAergic binding parameters in cerebral cortex, cerebellum, brain stem and pancreas of control, diabetic, insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rats.
5. To analyze the expression of acetylcholine esterase, choline acetyltransferase in the cerebral cortex, cerebellum, brain stem and pancreas of control, diabetic, insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rats using Real Time PCR.
6. To analyze the expression of muscarinic M1, Muscarinic M3 expression in the cerebral cortex, cerebellum, brain stem and pancreas of control,

diabetic, insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rats using Real Time PCR.

7. To investigate cell signaling alterations by gene expression studies of Insulin receptor, $GABA_B$ receptor, GLUT 3, CREB and Phospholipase C in the cerebral cortex, cerebellum, brain stem and pancreas of control, diabetic, insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rats using Real Time PCR.
8. To examine the oxidative stress in cerebral cortex, cerebellum, brain stem and pancreas of experimental rats by assessing antioxidant and peroxidation activities of control, diabetic, insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rats.
9. To study the localization and expression status of muscarinic M1, muscarinic M3, in the brain slices of cerebral cortex, cerebellum, brain stem and pancreas of experimental diabetic rats using specific antibodies in Leica Scanning Confocal Microscope.
10. To study the nuclear density of cerebral cortex, cerebellum, brain stem and pancreas of experimental diabetic rats by TO-PRO®-3-iodide stain using Leica Scanning Confocal Microscope.

Literature Review

Diabetes mellitus (DM) has become a major global health issue over the past few decades. The prevalence of diabetes has been increased worldwide and has reached alarming levels in many countries around the world. It is estimated that 415 million people (6.4% of the adult population worldwide) were affected by diabetes in 2015, and the number is projected to increase to 642 million by 2040. Accordingly, it is predicted to become the 7th leading cause of death in the world by the year 2040 (Zimmet *et al*., 2014). Diabetes is a group of metabolic disorders in which the blood sugar is higher than normal level either because the production of insulin is not enough (type 1 diabetes mellitus) or the cells do not properly respond to the insulin (type 2 diabetes mellitus) (Shoback & Gardner, 2011). Diabetes not only affects the carbohydrate metabolism, but also is associated with various central and peripheral complications. (Sheetz & King, 2002). Diabetic patients suffer from hyperglycemia due to a failure of glucose regulation and are subject to a variety of complications such as retinopathy, kidney disease, cardiovascular disease, diabetic foot ulcers, diabetic enteropathy, and neuropathy (Shi & Hu, 2014; Daddio *et al*., 2015).

Type 1 diabetes (T1D) is an autoimmune disease characterized by insulitis and islet β-cell loss. At the onset of T1D, more than 70% of β-cells are destroyed, whereas the residual β-cells most likely represent the only reservoir for the regeneration of islet β-cell mass (Patel & Israni, 2013). Type 1 diabetes usually starts in people younger than 30 and is therefore also termed juvenile-onset diabetes, even though it can occur at any age (Patterson *et al*., 2014). Type 1 diabetes is a chronic autoimmune disorder that precipitates in genetically susceptible individuals by environmental factors (Atkinson & Eisenbarth, 2001). Type 1 diabetes mellitus is one of the most common autoimmune disorders in children, with a 3% annual increase in the global incidence rate since the 1980s (Rawshani *et al*., 2014). The metabolic abnormalities of type 1 diabetes, such as hyperglycemia and hypoglycemia may have a damaging effect on the central

nervous system and be associated with significant long-term neurological complications (Ching Chou *et al*., 2015). The risk of diabetic nephropathy in type 1 diabetes is fourfold higher in children whose mothers have type 1 diabetes than in those without a parent with diabetes, indicating a role for epigenetics in the development of kidney disease (Thorn *et al*., 2007).

The identification of substances that mediate or mimic the action of insulin could lead to the development of novel compounds which may be of clinical use in the treatment of persons having disorders of glucose metabolism, such as impaired glucose tolerance, elevated blood glucose associated with diabetes and insulin resistance (Larner *et al*., 1997). It is characterized by high blood glucose, a progressive decline in β-cell function and chronic insulin resistance (Defronzo, 1988). The dietary recommendations for diabetes have widened, with the appreciation that fiber rich food such as leafy vegetables would be able to enhance the glycemic control of patients with diabetes and thereby prevent the progression of the disease into diabetic complications (Attanayake *et al*., 2015).

Relationship of pancreatic β-cells and diabetes

Diabetes mellitus is a group of metabolic disorders characterized by high blood sugar levels over a prolonged period resulting from either destruction or impairment of insulin secreting pancreatic β-cells and insulin action in target tissues (Kawser *et al*., 2016). In type 1 diabetic patients, the majority of β cells are lost to an autoimmune attack. In advanced type 2 diabetic patients, the number of β cells is often lower than the non-diabetic individuals, and the function of the remaining β cells is attenuated (Costes *et al*., 2013). Insulin injections and drug treatments act only to reverse hyperglycemia, but do not increase the number of β cells. Diabetes is associated with insulin resistance initially and later, as the function of the β-cell decreases, insulin deficiency (Cerasi, 2000). Type 2 diabetes is characterized both by abnormalities of insulin secretion progressively leading to secretion failure as well as insulin resistance of all major target tissues (Haring, 1999). Although insulin resistance is important in the early stages of type 2

diabetes, the failure in adequate β-cell compensation leads to the progression to the diabetic state. Compensation for insulin resistance is through increased secretion per β-cell or by an increase in β-cell mass through neogenesis or replication of the existing β-cells (Withers *et al*., 1998). The β-cell mass is normally tightly maintained through a balance of β-cell birth (β-cell replication and islet neogenesis) and β-cell death through apoptosis. Chronic exposure to hyperglycemia, alone is capable of initiating apoptotic β-cell death in cell cultures and animal models and has also been observed in humans through autopsy (Marroqui *et al*., 2015). Most of the increase in β-cell mass with insulin resistance is probably due to increased β-cell number, but β-cell hypertrophy may also contribute (Weir & Bonner-Weir, 2004).

The pancreas and glucose control

Insulin released from the β-cells is considered as a putative mediator of glucose-inhibited glucagon secretion (Elliott *et al*., 2015). Central to the development and progression of both type 1 and type 2 diabetes are the pathophysiological changes that happen in the pancreas and in particular, the insulin-secreting β-cells. In type 1 diabetes, the β-cells undergo cell death primarily as a result of necrosis caused by an autoimmune response (Robertson *et al*., 2003; Robertson & Harmon, 2006). The resulting depletion of functional β-cells leads to the type 1 diabetes phenotype. Even though chemically induced, many animal studies have indicated a role for oxidative stress in development of STZ-induced type 1 diabetes, as antioxidants have been found to slow or prevent pancreatic complications after administration of this agent (Takatori *et al*., 2004). Unlike type 1 diabetes, type 2 diabetes begins as a disease of insulin sensitivity, not insulin secretion. Therefore, type 2 diabetics often retain functional β-cells for some time after the onset of the disease (Robertson & Harmon, 2006).

Over time and with development of diabetes, capabilities for appropriate insulin secretion are often diminished. This appears to be because the disease state induces significant oxidative stress in the pancreas of rat models for diabetes (Ihara *et al*., 1999). The blood glucose concentration is monitored by glucose-

sensing cells in the portal vein area and in different regions of the brain, resulting in parasympathetic stimulation of insulin release in hyperglycemia and glucagon release in hypoglycemia, as well as sympathetic inhibition of insulin and stimulation of glucagon secretion in hypoglycemia (Thorens, 2014). The oxidative stress is accompanied by a substantial decrease in the number of insulin-secreting β-cells in the pancreas (Jin *et al*., 2008). The mechanisms by which oxidative stress gradually decreases insulin secretion over time appears to be through increased β-cell death and dysfunction in the transcriptional regulation of insulin *via*, Pdx-1 (pancreatic and duodenal homeobox 1) (Tanaka *et al*., 2002; Harmon *et al*., 2005). Interestingly, low glucose also promotes oxidative stress and consequential apoptosis, a pathway suppressed by vitamin E (Cai *et al*., 2007).

Role of insulin in diabetes mellitus

Diabetes continues to place a major burden on the health care system of the United States and worldwide. Diabetes involves two major defects: decreased insulin production from the pancreas and increased insulin resistance. Many patients with diabetes have decreased insulin production which requires exogenous insulin therapy in order to manage their disease. (Wesley & Jennifer, 2016). Insulin initiates its physiological effects by binding to a high affinity specific receptor located on the plasma membrane. The receptor is saturable, and both the binding capacity and the biological activity of insulin are highest at a plasma insulin concentration of 20 to 30 μU/ml. After binding to the receptor, insulin transmits its signal to the interior of the cell through a second messenger that influences enzymatic processes. Thus, the hormone probably carries out its actions without entering the cell (Kibiti, 2006). The insulin production is directly proportional to the amount of sugar (carbohydrate) utilized. The more sugar one consumes, the more insulin the body will have to produce, but, the tiny pancreatic β-cells were never designed to produce this level of insulin (Poitout & Robertson, 2002).

The chronic hyperglycemia arising from diabetes mellitus accompanies long-term damage, dysfunction, and failure of various organs, especially the eyes,

kidneys, nerves, heart and blood vessels. Pathogenesis of diabetes mellitus underlies autoimmune destruction of the pancreatic β-cells leading to insulin deficiency and bio-signaling derangements that are consequent to insulin resistance or insensitivity. It is still obscure which abnormality is the primary cause of the hyperglycemia (Anon, 2004). Insulin regulates blood glucose levels by its effects on the liver and skeletal muscles. Normal blood glucose levels are maintained by a balance between hepatic glucose production and glucose utilization by the peripheral tissues. Type 1 diabetes is characterized by the destruction of most of the functional β-cell mass. This destruction usually results in an absolute insulin deficiency, thus leading to the dependency of individuals suffering from type 1 diabetes on exogenous administration of insulin (Welters & Lammert, 2014). Some individuals with type 1 diabetes still have the capability to typically secrete low amounts of endogenous insulin. Although the amount of endogenously secreted insulin is not sufficient for the body's needs of insulin, it is important to maintain this endogenous insulin secretion, as it is associated with less long-term complication (Atkinson *et al*., 2014).

^{125}I-insulin binding to the receptor was impaired in both obese and obese type 2 diabetic patients, and partially purified insulin receptor from isolated abdominal skeletal muscle strips from non-obese diabetic patients, have also shown decreased ^{125}I-insulin binding and insulin receptor kinase activity as compared to control subjects (Maegawa *et al*., 1991). Significantly, kinetic defects in the activation of the insulin receptor that were observed in adipocytes from obese and obese diabetic patients, could be corrected by a 2-weeks intensive insulin treatment (Ciaraldi *et al*., 1991). In adipocytes, a reduction in ^{125}I-insulin binding was observed in adipose tissue from diabetic patients irrespective of their degree of obesity and in insulin resistant subjects as well as a reduction of insulin-stimulated IR kinase activity in adipose tissue from diabetic patients (Sinha *et al*., 1987). So insulin regulates hepatic gluconeogenesis and promotes glucose catabolism by the skeletal muscles. Hyper insulinemic hypoglycemia is characterized by a dysregulation of insulin secretion from pancreatic β-cells leading to inappropriate insulin secretion in spite of low blood glucose

concentrations (Guemes & Hussain, 2015). As glucose is the primary fuel for the brain, inappropriate insulin secretion at low blood glucose concentrations endangers the fuel supply, which can lead to severe brain injury (Rozenkova *et al*., 2015).

Regulation of Insulin Secretion

Insulin is secreted in a pulsatile fashion, which is considered to have important physiological functions with regards to the amount of insulin secreted and the effectiveness of insulin signaling in its target tissues (Satin *et al*., 2015). Insulin secretion from pancreatic β-cells can be influenced and modulated by other factors such as hormones and neurotransmitters that enhance or inhibit insulin secretion including autocrine signaling and paracrine interactions within the islet (Rorsman & Braun, 2013). The primary function of β-cells is to synthesize and secrete insulin, which accounts for over 10% of a β- cell's total protein content. To understand how therapeutic agents can act on β-cells to stimulate insulin secretion, it is first necessary to understand how β-cells normally respond to elevations in blood glucose levels with regulated insulin release. The major stimulus for insulin secretion is glucose that enters β-cells *via* glucose transporters (Folias & Hebrok, 2014). Thus, glucose is transported into β-cells by the high-capacity glucose transporter (GLUT 2, GLUT 1 and GLUT 3 in humans), and the first stage of metabolism is *via* a pancreas-specific glucokinase that generates glucose-6-phosphate. The importance of this enzyme in insulin secretion has been demonstrated by recognizing that glucokinase gene mutations are responsible for some cases of maturity-onset diabetes of the young (Velho *et al*., 1997). In β-cells, the main stimuli for insulin release are elevated blood glucose levels following a meal (Komatsu *et al.*, 2013).

In β-cells, the initial step of glucose metabolism, which is the phosphorylation of glucose, is catalyzed by glucokinase, an isoenzyme of the hexokinase. It has a lower affinity to glucose than other hexokinases and exhibits half-maximal activity at glucose concentrations in the millimolar range thus being a rate-limiting step in insulin secretion (Folias & Hebrok, 2014; Lenzen, 2014;

Rorsman & Braun, 2013). Further glycolytic and oxidative metabolism of glucose results in the generation of adenosine triphosphate (ATP), which closes ATP-sensitive potassium (K^+) channels in the plasma membrane. The ensuing reduction in K^+ efflux depolarizes the β-cell plasma membrane, leading to an opening of voltage-dependent calcium (Ca^{2+}) channels. This allows Ca^{2+} in the extracellular fluid to enter β-cells down its concentration gradient and stimulate exocytotic release of stored insulin through interactions with Ca^{2+}- sensitive proteins such as Ca^{2+}/calmodulin-dependent protein kinases and syntaptotagmins (Persaud & Howell, 2003). Endocrine cells secrete their respective hormones in response to external signals, such as nutrient intake or stress, *via*, humoral, neural or hormonal signaling pathways. The underlying molecular process that translates the stimulus into the actual hormone release is called stimulus-secretion coupling which is known as the stimulus-dependent exocytosis of a particular substance, such as glucose-stimulated β-cell insulin release (Ashcroft *et al*., 1994). Inadequate insulin secretion disrupts glucose homeostasis at all ages, including infancy. Much attention has been paid to the mechanisms causing excessive insulin secretion in congenital hyper-insulinism and insufficient secretion in monogenic neonatal diabetes (Stanley, 2016).

Neurotransmitters role in diabetes

Acetylcholine, a classical neurotransmitter that also functions as a non-neuronal paracrine signal, activates muscarinic receptors that play a key role in maintaining many metabolic functions, including glucose homeostasis. There is strong evidence that cholinergic mechanisms are important for function and survival of the endocrine pancreas, the *islet of Langerhans* (Gilon & Henquin, 2001). Activation of muscarinic receptors leads to improved insulin secretion from pancreatic islets (Duttaroy *et al*., 2004; Boschero *et al*., 1995). In understanding the function of acetylcholine in the brain, a special emphasis has been placed on the importance of acetylcholine for memory and learning with a focus on a specific role of the cholinergic forebrain system in attention (Kozak *et al*., 2006). A deficit in the function of the cholinergic system is thus likely to result in

cognitive impairment. As neurocognitive impairment is frequently associated with schizophrenia, and has been shown to be worsened by exposure to muscarinic antagonists, an involvement of the cholinergic system in the pathophysiology of this illness seems possible (Minzenberg *et al*., 2004; Ellis *et al*., 2006). Activation of the muscarinic receptors results in a slower but potentially more sustained response than activation of nicotinic ion channels. The understanding of the cholinergic system is complicated by the fact that both nicotinic and muscarinic cholinergic neurotransmission contribute to its function (Lucas-Meunier *et al*., 2003). Insulin mediates its action primarily by binding to insulin receptors. The insulin receptor is a receptor tyrosine kinase and assembles as tetramer but can also form a functional hybrid receptor with the highly related insulin-like growth factor receptor 1 (Bedinger & Adams, 2015).

Acetylcholine signaling in diabetes

The STZ-induced type 1 diabetes mellitus and insulin-induced hypoglycemia both leads to a significant increase of the binding of Gq-coupled M3-mAChR in the cerebral cortex and cerebellum but the extent of changes induced by hypoglycemia was extensively higher compared to diabetes mellitus, which indicates the harmful effect of recurrent hypoglycemia on cholinergic system in the brain (Antony *et al*., 2010b; Peeyush *et al*., 2011; Sherin *et al*., 2011). The changes in the number and activity of the metabotropic and ionotropic acetylcholine receptors have been implicated in the pathophysiology of many diseases of the CNS, including cognitive impairment. It was shown that in the cerebral cortex, hypothalamus and brain stem of STZ-rats the number of Gq-coupled M1-mAChRs and the expression of genes encoding M1-mAChR were decreased with an augment in affinity of the receptor to agonists, and the binding parameters of the M1-mAChR were reversed to near control by the treatment with insulin (Gireesh *et al*., 2008; Peeyush *et al*., 2011). The natural ligand for mAChRs is acetylcholine, which is released from cholinergic nerve endings and non-neuronal cells. Acetylcholine-mediated activation of the receptors is responsible for G -protein activation and the downstream signaling pathway that

follows activation of signals (Kurowski *et al*., 2015). In the cerebral cortex of the diabetic and control rats with insulin-induced long-term hypoglycemia the maximal binding of M1-mAChRs and their expression were decreased to a greater extent compared with diabetic animals with hyperglycemia (Sherin *et al*., 2011).

At the same time, in the cerebellum and corpus striatum of both diabetic rats and hypoglycemic diabetic and control rats the binding parameters and gene expression of M1-mAChRs was, on the contrary, increased (Antony *et al*., 2010b). This indicates that the alterations in the initial steps of M1-mAChR signaling in the diabetic brain are area-specific. This allows a conclusion that the imbalance in glucose homeostasis affects acetylcholine metabolism and cholinergic muscarinic neurotransmission in the brain, and changes the expression and function of cholinergic receptors. The study of 7-week- and 90-week-old STZ-rats showed that in the brain stem of both groups of animals the number of M1-mAChRs was significantly decreased whereas the number of M3-mAChRs greatly increased compared to their respective controls, and the insulin treatment reversed the binding parameters of M1- and M3-mAChRs to near control level (Balakrishnan *et al*., 2009).

In the cerebral cortex of 7-week-old STZ rats the number of M1-mAChRs decreased by 28 %, while the number of M3-mAChRs increased by 30 %. In the cerebral cortex of 90-week-old diabetic rats the number of M1- and M3-mAChRs increased by 43 and 23 %, respectively, and the level of acetylcholine was significantly increased compared to control (Savitha *et al*., 2010). These alterations of M1 and M3-mAChR expression correlate with cholinergic hypo function in short-term and prolonged STZ-induced type 1 diabetes mellitus. It should be noted that M1- and M3-mAChRs are abundantly expressed in the brain regions involved in cognition, including the cerebral cortex, hippocampus and striatum (Porter *et al*., 2002). As a rule, most animal models of obesity and hyper insulinemia are associated with increased vagal cholinergic activity that is strongly associated with the M3-mAChR expressed in the brain and the peripheral tissues (Gautam *et al*., 2008). The role of M3 receptor, M3 KO mice that lacked the

receptor selectively in pancreatic β-cells were generated and these demonstrated impaired glucose tolerance and reduced insulin secretion (Nakajima *et al*., 2013).

A better understanding of the function and involvement of mAChRs in pathological conditions can enable the development of therapeutics to target diseases and disorders of the CNS and periphery. Phe181 also interacts with QNB and this is the only amino acid in the orthosteric binding pocket of M2 that differs from all the other mAChR sub-types. The rest of mAChRs have a leucine residue in the homologous position e.g. Leu225 in M3 (Haga *et al*., 2012; Kruse *et al*., 2013). Along with insulin, vitamin D3 and curcumin in particular, which differ in the chemical nature and the mechanism of action are also capable of restoring the functions of cholinergic system in the diabetic brain. Vitamin D3, as well as insulin, markedly recovers the altered gene expression of M1- and M3-mAChRs in the cerebral cortex and cerebellum of STZ-induced type 1 diabetic rats and binding parameters of these receptors to near control (Kumar *et al*., 2011). Vitamin D3-induced improvement of the cholinergic system and glucose homeostasis in the diabetic brain is due to the influence of vitamin D3 on activity of pancreatic M3-mAChR followed by enhanced synthesis and secretion of insulin and reduction of the neuronal disorders in diabetes mellitus (Peeyush *et al*., 2011). It was found, in addition, that vitamin D3 restores the disrupted expression of IR in the cerebral cortex of diabetic rats. Curcumin possesses powerful anti-diabetic properties and has the ability to modulate mAChRs thereby ameliorating the impaired cognitive functions in diabetes mellitus (Peeyush *et al*., 2011).

Ionotropic nicotine acetylcholine receptors are also involved in the pathogenesis of neurodegenerative processes in diabetes mellitus. Note that the stimulation of nicotinic acetylcholine receptors and mAChRs provokes opposing physiological and behavioral responses, which is due to the existence of multiple nicotinic and muscarinic receptor subtypes and their diverse anatomical distributions in the Central Nervous Sytem. For example, nicotine administration inhibits food intake, increases metabolic rate, and leads to reduced adiposity (Li *et al*., 2003), while the activation of M3-mAChRs induces hyperphagia and obesity (Gautam *et al*., 2008). The transgenic mice over-expressing the M3 receptor in β-

cells displayed enhanced glucose tolerance and increased insulin release (Nakajima *et al*., 2013). α7-Nicotinic receptors highly expressed in the course of brain development are implicated in memory, attention and information processing (Picciotto *et al*., 2000).

In the cerebral cortex of STZ-induced type 1 diabetic rats the expression of α7-nicotinic receptors was markedly increased. The receptors significantly influenced the activity within the cortex circuitry, and diabetes mellitus-associated deregulation of this activity could contribute to disorders involving the cerebral cortex (Peeyush *et al*., 2011). Alongside with the increase in α7-nicotinic receptors expression, in the cerebral cortex of diabetic rats were revealed the increased acetylcholine esterase (AchE) and the decreased choline acetyl transferase (ChAT) mRNA levels, which indicate fast acetylcholine degradation and a subsequent down stimulation of acetylcholine receptors causing undesirable effects on cognitive functions. These changes in the expression of AchE and ChAT in diabetes mellitus led to a reduction of cholinergic neurotransmission efficiency due to a decrease in acetylcholine levels in the synaptic cleft, thus contributing to progressive cognitive impairment and other neurological dysfunctions in diabetes mellitus.

Insulin therapy and curcumin substantially regularize the increased expression of AchE and ChAT, and significantly revert up regulation of α7-nicotinic receptor in the cortex of STZ-induced type 1 diabetic rats improving the cognitive functions, such as learning and memory. The absence of M3-mAChR protects the animals against experimentally or genetically induced obesity and obesity-associated metabolic deficit and greatly ameliorates the impairments in glucose homeostasis and insulin sensitivity. The M3-mAChR-deficient mice are largely protected against obesity-associated glucose intolerance, insulin resistance, hyper insulinemia, and hyperglycemia triggered by a high-fat diet, chemical disruption of hypothalamic neurons by gold-thioglucose, and genetic disruption of the leptin gene. Therapeutic importance offering a potential approach in the treatment of diabetes with the identification of compounds that can selectively activate M3 receptors (Kruse *et al*., 2014). These data favor the fact that the M3-

mAChR and other subtypes of mAChRs can represent a potential pharmacologic target for the treatment of diabetes mellitus, obesity and associated neurological disorders.

Muscarinic acetylcholine receptors

Anticholinergics are used in the treatment of a variety of conditions. Some of the important conditions are: chronic obstructive pulmonary disease (COPD), asthma, motion sickness, dizziness, toxicity by organophosphorus insecticides or compounds like muscarine, conditions inducing high blood pressure and symptoms due to Parkinsonism. Atropine, falling under anticholinergic class of drug, consists of l- as well as d- forms of hyoscyamine and it's action is solely due to the *levo* form. Atropine counteracts the actions of acetylcholine and other esters of choline, thus is also termed as antimuscarinic agent (Venkatesh *et al*., 2016).The mAChRs received their name by their ability to bind muscarine, a product of the mushroom *Amanita muscarina*. mAChRs associate to G proteins and consist of five different receptors (M1–M5). A single neuron can express more than one mAChR subtype; for example, hippocampal pyramidal neurons have all five types of mAChRs (Levey *et al*., 1995). M1 receptors were first inactivated by genetic manipulation (Hamilton *et al*., 1997). The Experiments on the M1 knockout mice revealed that seizure activity by muscarinic stimulation was mostly connected to M1 receptors. In the corpus striatum, M1 receptors might play a role in the early stages of Parkinson's disease by increasing the dopamine release (Wess, 2004). The long term potentiation in hippocampus is also reduced in M1 knockouts that exhibit a mild cognitive deficit in behavioral tests, suggesting the role of M1 receptors in learning and memory-related processes (Anagnostaras *et al*., 2003). M2 receptor function has been associated with muscarinic stimulation-induced tremor and akinesia. M2 receptors participate in the presynaptic inhibition of transmitter release including acetyl choline release. These autoreceptors of cholinergic transmission mediate feedback inhibition in the nervous system and in the neuromuscular junction.

M3 receptors are widely expressed in the brain; the expression level is lower compared with other mAChR subtypes though. The adult M3 receptor-deficient mice exhibit a weight loss of approximately 25% (Yamada *et al*., 2001a). M3 receptors seem to play an important role in the regulation of appetite and daily food intake. So M3 mAChRs are able to form functional dimers that make the mAChR mediated function more complex *in vivo* (Wess, 2003). ACh is rapidly hydrolyzed after release, desensitization of mAChRs occurs under physiological conditions. As with a large number of G-protein coupled receptors, agonist-induced desensitization of mAChRs usually involves receptor phosphorylation (Haga & Haga, 1990). M3 receptors also mediate the contractile response of smooth muscle by cholinergic stimulation in various areas including urinary bladder, ileum, and trachea, and are involved in parasympathetic control of pupillary sphincter muscle contractility in the eye (Wess, 2003). M4 receptors participate in the auto inhibition of cholinergic axons and also occur as presynaptic hetero receptors in the regulation of dopamine release. M1-M4 receptor subtypes have emerged as crucial drug targets for medical treatment of neurological such as schizophrenia and Alzheimer's disease (Thal *et al*., 2016). M4 receptors influence locomotion through an interaction with D1 dopamine receptors.

M5 receptors were the last muscarinic receptors, but its physiological role is less understood. In addition, their low expression level in the brain and the lack of specific ligand make them difficult to study. M5 receptors mediate Ach-induced dilation of cerebral blood vessels (Yamada *et al*., 2001b) that might be important in the development of Alzheimer's disease. M5 receptor is the sole mAChR subtype expressed by the substantia nigra dopaminergic neurons. The loss of the nigrostriatal dopaminergic input is the cellular defect underlying Parkinson's disease and ligands targeting mAChRs are used in the therapy of this disease. M5 receptors are involved in the oxotremorine-induced facilitation of dopamine release in the striatum (Yamada *et al*., 2001b). The indirect effects through M4 receptors on GABAergic cells also contribute to the oxotremorine-induced facilitation. Different experimental approaches have shown that

muscarinic receptors are present in many regions of the CNS (Wei *et al*., 1994; Wess, 2004; Vilaro *et al*., 1993). Ach regulates inflammation *via* M3 receptors on structural cells (Kistemaker *et al*., 2015). The M1, M4 and M5 receptors are predominantly expressed in the CNS, while the M2 and M3 receptor subtypes are widely distributed in both the CNS and peripheral tissues (Caulfield & Birdsall, 1998; Wess *et al*., 2007). In the forebrain, including the striatum, the M1 and M4 receptors are the most abundantly expressed muscarinic receptors, whereas the expression of M2 and M3 receptors is moderate and the density of M1 receptors is low (Yasuda *et al*., 1993, Wei *et al*., 1994).

Classification of Muscarinic receptors

Muscarinic M1 receptor

Muscarinic agonist depolarization of rat isolated superior cervical ganglion, recorded extracellularly, is mediated by M1 receptors (Brown *et al*., 1980). This is probably the result of inhibition of opening of the voltage-gated M-type K1 channels in these neurons (Marrion *et al*., 1989; Bernheim *et al*., 1992), although M1 receptors can modulate other conductances which could contribute to the depolarizing response (Marsh *et al*., 1995). The muscarinic M1 receptor, in the pathophysiology of schizophrenia is a promising new class of candidate compounds and allosteric ligands, for addressing the difficulties involved in targeting system. The body of evidence presented here highlights the dysfunction of the cholinergic system in schizophrenia and that targeting this system by taking advantage of allosteric ligands is having clinically meaningful effect on different disorders (Shaun *et al*., 2016). The pharmacology of this system has not been investigated using the more recently discovered antagonists. However, the ablation of M1-current inhibition in sympathetic ganglion neurons of the M1-knockout mouse argues convincingly for the linkage (Hamilton *et al*., 1997).

Canine saphenous vein: contraction is mediated by M1 receptors, because the apparent pKB values of a range of partially selective antagonists is entirely consistent with an M1 receptor profile (O'Rourke & Vanhoutte, 1987; Sagrada *et al*., 1994; Watson *et al*., 1995). It should be noted that there is a low receptor

reserve associated with the contraction. Most agonists, notably those of low intrinsic efficacy, act as antagonists. The M1- and M4-subtype selective agonists are infused into the insular cortex, showing that the activation of mAChRs can produce anxiolytic effects while their inhibition increases anxiety. In this sense, cholinergic effects on experimental anxiety appear to be *via*- and locally-dependent, emphasizing a complex and poorly understood mechanism (Li *et al*., 2014).

The M1 receptor subtype is expressed throughout the forebrain, including the neocortex, dorsal striatum, nucleus accumbens (NAcc), and hippocampus (Weiner *et al*., 1990; Levey *et al*., 1995; Hersch *et al*., 1994). M1 receptors have been implicated in many functions of the CNS. For example, pharmacological and genetic studies support a role of M1 receptors in cognitive functions like learning and memory, especially in the acquisition phase (Robinson *et al*., 2011). M1 and TrkB receptors cooperate and add their respective individual effects to increase axonal elimination rate even more, the effect of the M2 receptor is largely independent of both M1 and TrkB receptors. Thus both, cooperative and non-cooperative signaling mechanisms contribute to developmental synapse elimination (Laura *et al*., 2016). In the striatum, M1 receptors are co expressed with D2 dopamine receptors by GABAergic projection neurons, suggesting that activation of M1 receptors may oppose D2 receptor-mediated neuronal inhibition (Bernard *et al*., 1992; Di Chiara *et al*., 1994).

Muscarinic M2 receptor

Guinea-pig heart: Activation of muscarinic receptors in these preparations produces a decrease in force of contraction and (in non-paced tissues) a reduction in the rate of beating. These things are probably the consequence of inhibition of voltage-gated Ca^{2+} channels and activation of inwardly rectifying K^{+} channels, respectively. Broad studies with many antagonists have defined this response as being mediated by the M2 receptor (Caulfield, 1993). M2 receptors can mediate both negative and positive inotropic responses in the left atrium of the reserpinized

rat, that the latter effect being insensitive to pertussis toxin (Kenakin & Boselli, 1990).

It has also been recommended that a M1 muscarinic receptor stimulates phospholipase C, and increases Ca^{2+} currents in pertussis toxin-treated guinea-pig and rat ventricular myocytes (Sharma *et al*., 1997). Supporting evidence for this contention was that subtype-specific antibodies detected M1 receptor protein in myocytes, and reverse transcriptase polymerase chain reaction detected significant m1 mRNA (Gallo *et al*., 1993; Sharma *et al*., 1997). The development of mAChR allosteric modulators to improve subtype selectivity little has been reported on the development of radio labeled modulators to study the actual allosteric binding pocket. With the characterization of the M2/M4 mAChR allosteric modulator radioligand [^{3}H] LY2119620, great strides were taken to further the understanding of mAChR allosteric binding sites (Croy *et al*., 2014). The effect of pirenzepine in antagonizing the muscarinic stimulation of phospholipase C extrapolated to an apparent pKB value of approximately 9.5, which is not consistent with any known muscarinic receptor (Gallo *et al*., 1993).

M3 receptor

Guinea-pig ileum: The muscarinic receptors mediating contraction of guinea-pig ileum (and indeed of many other smooth muscle preparations) are defined pharmacologically as M3 (Eglen *et al*., 1996). There is a large population of M2 receptors in many smooth muscles, and it seems likely that they are involved in antagonizing the relaxant effects of agents that elevate cAMP (Thomas *et al*., 1993; Eglen *et al*., 1994). M3 receptors in guinea-pig ileum also stimulate the opening of cation-selective channels that depolarize the muscle cells (Bolton & Zholos, 1997). Several studies have indicated that the receptor mediating relaxation of vascular smooth muscle (*via* release of relaxing factors from endothelial cells) is M3 (Eglen & Whiting, 1990; Caulfield, 1993; Van Zwieten & Doods, 1995) but there is also evidence for differences in pKB values for selective antagonists in blocking the relaxant responses in some blood vessels. The bencycloquidium bromide (BCQB; a novel M3 receptor antagonist) might be

used in a potential therapy for inflammation in cigarette smoke-induced pulmonary diseases (Zhang *et al*., 2015).

There also have been implications that smooth muscle M3 receptors from diverse tissues may be heterogeneous. Thus, compounds such as zamifenacin, darifenacin, and p-F-HHSiD have been reported to distinguish between muscarinic agonist responses in tissues such as trachea, ileum, and urinary bladder (Eglen *et al*., 1996). Muscarinic AChRs, mainly M1 subtypes, are distributed in rat mesenteric arteries and the activation of M1 and M3, but not M2, which may be located on calcitonin gene-related peptide(CGRP)-ergic nerves releases CGRP, thereby causing an endothelium-independent vasodilatation (Panot *et al*., 2016). Acetylcholine contributes to allergen-induced smooth muscle mass and airway remodeling *via* M3-AChR, and that the level of M3-AChR expression in a murine model of chronic asthma can be used to predict bronchial airway remodeling. It is necessary to characterize the expression, functional state, and ratio of the three subtypes of muscarinic receptors in the lung tissue of healthy individuals and asthmatic patients, which would be beneficial for a more informed and effective therapy of respiratory disorders (Zhao *et al*., 2016).

M4 receptor

Rabbit anococcygeus muscle: In preparations in which the tone has been raised by histamine, muscarinic agonists relax the pre-contraction. This apparently is an exclusively presynaptic effect, involving the release of an inhibitory non-adrenergic and non-cholinergic neurotransmitter, probably nitric oxide (Gross *et al*., 1997). Muscarinic antagonists inhibit the relaxation, the pKB values indicating that M4 receptors mediate this response (Gross *et al*., 1997). There is a low receptor reserve for this response because agonist potencies are low and several agonists of low intrinsic efficacy act as antagonists. The neuroblastoma-glioma hybrid cell line NG108–15 expresses M4 mRNA (Peralta *et al*., 1987) and M4 receptors can be detected readily in radioligand binding assays (Lazareno *et al*., 1990). Muscarinic acetylcholine receptor (mAChR) subtypes (M1, M2 and M4), adenosine receptors (AR; A1 and A2A) and tropomyosin-related tyrosine kinase B

(TrkB) receptors are involved in the control of synapse elimination in the mouse NMJ (Nadal *et al*., 2016). Inhibition of adenylyl cyclase activity by muscarinic agonists in rat corpus striatum probably is mediated by M4 receptors (Caulfield, 1993; Olianas *et al*., 1996). However, a 3- to 10- fold discrepant value for the pKB of methoc- tramine has been reported (Onali & Olianas, 1995).

M5 receptor

The presence of the M5 protein and its mRNA in the brain and periphery is known (Weiner *et al*., 1990; Flynn *et al*., 1997), but it has not yet been possible to delineate a whole-tissue response whose location and pharmacology match that predicted for the expressed gene product. There have been a number of studies of the function of the cloned M5 receptor so that this gene product does correspond to a functional receptor. However, it has been shown only recently that the pKB values for several selective antagonists in blocking function in cells transfected with the M5 gene agree with the binding affinities measured in membranes from the same cell line (Watson *et al*., 1998). There is evidence that the A2058 human melanoma cell line expresses only M5 receptors (Kohn *et al*., 1996). This may provide a useful model for an endogenous M5 receptor in a human cell line, but it also should be noted that the coupling mechanisms in this cell line are somewhat unusual. Another potentially useful system is the eosinophilic leukemia cell line (EoL-1) where M5 (and M3) receptors can be induced on differentiation with interferon (Mita *et al*., 1996).

Pharmacology and mechanism of Muscarinic receptors

All mAChR subtypes are susceptible to allosteric modulation; the binding of the allosteric modulator to the allosteric binding site results in an alteration in the conformation of the classical binding site leading to a change in the affinity of the receptor for classical muscarinic agonists and antagonists. There are also allosteric agents with positive cooperation on the binding of mAChR antagonists, such as strychnine (Lazareno & Birdsall, 1995). Other compounds such as brucine, vincamine and alcuronium are able to allosterically modify the binding of

mAChR agonists (Jakubik *et al*., 1997). Atropine, scopolamine, pirenzepine, and pilocarpine bind on the same binding site as the agonist but produce different conformational changes in the receptor structure that ultimately leads to the inhibition of the receptor function (Van Koppen & Kaiser, 2003). Most currently used insecticides are neurotoxic chemicals that target a limited number of sites and insect cholinergic neurotransmission is the major target. A potential target for insecticide development is the mAChR, which is a metabotropic G-protein-coupled receptor. Insects have A- and B-type mAChRs and the five mammalian mAChRs are close to the A-type (Xia *et al*., 2016).

In the Central Nervous System, the presynaptic mAChR (M2) inhibits cholinergic function as a muscarinic autoreceptor. Antagonism of this receptor by scopolamine results in release of choline into the extracellular space (Sarter & Parikh, 2005). It is well known that mAChRs play an important role in spike frequency adaptation in central neurons (Nicoll *et al*., 1990). The galantamine, a third generation cholinesterase inhibitor used in the therapy of Alzheimer's disease, could dose dependently reduce the after hyperpolarization after a burst of action potentials and the spike frequency accommodation of hippocampal CA1 neurons (Oh *et al*., 2005). Larger trains of back propagating action potentials, exhibiting adaptation, were shown to be subject to modulation by mAChRs suggesting that dendritic integration can be modified by mAChRs (Tsubokawa & Ross, 1997).

The cholinesterase blocker physostigmine and cholinomimetics evokes theta wave activity in the hippocampus through a muscarinic mechanism in blood vessels (Olpe *et al*., 1987; Konopacki *et al*., 1988). The rhythm is believed to be critical for the temporal coding or decoding of active neuronal ensembles and the modification of synaptic weights. The administration of a non-convulsive dose of pilocarpine may also alter the expression of hippocampal NMDARs in the rat, suggesting that the activation of mAChRs can modify anxiety through hippocampal plastic changes and cell excitability (Hoeller *et al*., 2016). Muscarinic antagonists, such as scopolamine or atropine, impair cognitive abilities in humans (Drachman, 1977). It has been shown recently that transient activation

of M1-mAChRs induces Ca^{2+} release from intracellular stores *via* IP3 and subsequent activation of a SK-type Ca^{2+} activated K^{+} conductance showing that ACh can directly inhibit neocortical pyramidal neurons through Ca^{2+} mobilization (Gulledge & Stuart, 2005). In the sensory system, presence of mAChRs has been shown on vestibular hair cells to evoke transmitter release from these cells (Derbenev *et al*., 2005).

The effectors of the cholinergic transmission by muscarinic and nicotinic systems cooperate in the Central Nervous Sytem. So the mAChRs are metabotropic receptors and mediate slow responses with significant delay, nicotinic facilitation, following fast activation of nAChRs, can be sustained for up to 2 hours and muscarinic inhibition seems to be more transient in certain cells (Girod & Role, 2001). Nicotinic agonists depolarize striatal interneurons and induce firing through non-α 7 nAChRs, which, together with presynaptic inhibition through muscarinic receptors, form a dual cholinergic control on spiny inter neurons of the striatum (Koos & Tepper, 2002). The main threads of current theories of nicotinic functions in the CNS include the following: (1) nAChRs may modulate, rather than mediate, fast synaptic transmission (McGehe & Role, 1995), (2) desensitization of the nAChRs, i.e., loss of function, is a key factor in the effect of nicotine during smoking and also shape the nAChR-mediated activity in normal cholinergic transmission extending the computational power of the neuron, and (3) nAChRs directly release transmitters from presynaptic boutons skipping postsynaptic secondary modulations (Vizi & Lendvai, 1999). The association of autoantibodies with immune markers suggests that they activate B and T cells expressing β-adrenergic and Muscarinic acetylcholine receptors. Dysregulation of acetylcholine and adrenergic signaling could also explain various clinical symptoms of chronic fatigue syndrome (Madlen *et al*., 2016).

The nicotinic enhancement of memory and learning function is constructed on the level of cellular synaptic plasticity (Levin & Rezvani, 2000). The M1 and M4 receptors have been associated with learning, memory, and cognition and have emerged as attractive targets for the treatment of various central nervous system disorders, including Alzheimer's disease, schizophrenia,

and drug addiction (Kruse *et al*., 2014). Nicotine selectively improves the cognitive performance (especially those involving attentional processes) in deprived smokers and in cases with impaired cognition (Freedman *et al*., 1995), such as Alzheimer's disease (Rezvani & Levin, 2001; Newhouse *et al*., 2004). So the cholinergic system plays an important role in regulating cognitive functions in the brain regions facilitating on an enough assembly of metabotropic and ionotropic receptors.

Gamma amino butyric acid signaling in diabetes

γ-Aminobutyric acid (GABA) is one of the most abundant inhibitory neurotransmitters in the central nervous system. Recently, it has been reported that GABAergic signaling pathways are found in various non-neuronal tissues including the immune system and play a functional role (Yoon Seok Roh *et al*., 2016). GABAergic inhibitory function in the cerebral cortex is of great significance in the regulation of excitability and responsiveness of cortical neurons. GABA inhibition is mediated both by $GABA_A$ receptors, which open membrane chloride channels and stabilize the membrane potential below firing threshold, and $GABA_B$ receptors, which act *via*, G proteins to reduce transmitter release from presynaptic terminals. The inhibitory GABA-releasing interneurons mediate the function of excitatory glutamatergic neurons in the brain regions, which contributes significantly to the control of glutamate content in brain regions and prevents glutamate toxicity induced in the brain of hypo- and hyperglycemic diabetic rats. The genetically modified α-cells can regenerate and convert into β-like cells *in vivo* holds great promise for diabetes research. However, to eventually translate these findings to human, it is crucial to discover compounds with similar activities. The identification of GABA as an inducer of α-to-β-like cell conversion *in vivo* is very promising. GABA-induced cell-mediated β-like cell neogenesis could therefore represent an unprecedented hope toward improved `therapies for diabetes (Ben-Othman, 2016).

Disruption of GABAergic inhibition induces seizures leading to neuronal damage and, therefore, pathophysiology of many seizure disorders is the result of

alteration of GABA receptor function (Antony *et al*., 2010a). It was shown that the synaptic level of GABA and its release in the diabetic brain are slightly changed or remain unchanged. The extracellular basal level of GABA at dentate gyrus of STZ-induced type 1 diabetic rats 12 weeks after the induction of diabetes mellitus showed no changes (Reisi *et al*., 2009). The content of vesicular GABA transporter was significantly decreased in hippocampal synaptosomal membranes in two week diabetes mellitus, although only minor changes in the release of GABA and in the loading capacity of GABA transporters were found (Baptista *et al*., 2011).

In the cerebellum of STZ-induced type 1 diabetic rats with hyperglycemia the gene expression of $GABA_{A\alpha1}$ subunit and glutamate decarboxylase was decreased and these molecular alterations were exacerbated by recurrent hypoglycemia (Sherin *et al*., 2010). The gene expression of CREB, a stimulus-inducible transcription activator implicated in the activation of protein synthesis essential for long-term memory and seizure formation, was significantly down regulated in diabetes mellitus and recurrent hypoglycemia. Since CREB up regulates endogenous $GABA_{A\alpha1}$ transcription, the decreased expression of CREB in the cerebellum of hypoglycemic and hyperglycemic rats led to the attenuation of GABAergic system and, as a result, to exocitotoxic damage of neuronal cells (Sherin *et al*., 2010). GABA mostly acts *via* the $GABA_A$ receptor located on α-cells to eventually induces a down regulation of Arx expression/ectopic expression of Pax4 and thereby promote their conversion into β-like cells (Al-Hasani *et al*., 2013; Courtney *et al*., 2013). In the cerebellum, where GABA receptors are involved in control of coordination and motor learning and, like in the cerebral cortex, play a critical role in neuronal excitability and modulation of synaptic neurotransmission (Luján, 2007). This indicates that the changes of GABA signaling, characteristic of the diabetic brain, are due to the alterations in the level and functional activity of GABA receptors and downstream signal components of GABA-regulated intracellular cascades.

Actually, the GABA binding and the gene expression of the subunits of $GABA_A$ and $GABA_B$ receptors were decreased in the cerebral cortex of diabetic

rats compared to control animals. In the diabetic hypoglycemic rats having two episodes of insulin-induced hypoglycemia in the course of 10 days GABA binding and expression of GABA receptor subunits were reduced to a greater extent in comparison with diabetic hyper/euglycemic animals. This is the evidence that hypoglycemia augments the adverse effects of hyperglycemia on GABAergic system, and the impairments of functions of GABAergic neurons in the diabetic cerebral cortex are intensified in hypoglycemia. The expression of glutamate decarboxylase, the rate-limiting enzyme of GABA synthesis, which is used as a marker of GABAergic activity, was also significantly down regulated in diabetes mellitus and hypoglycemia exacerbated the altered expression (Antony *et al*., 2010a). Preventive or curative therapies for type 1 diabetes must be multifactorial, GABA therapy could be coupled to a mild immunomodulatory approach, aiming to both restore β-cell mass and to adapt the immune system to these neo-formed β-cells (Lord & Greenbaum, 2015). It follows that both hypo- and hyperglycemia in diabetes mellitus decline GABAergic neuroprotective function in the cerebral cortex and cerebellum, which accounts for increased resistance of these brain areas to subsequent neuronal damage.

Gamma Amino Butyric Acid (GABA) Receptors

^{1}H magnetic resonance spectroscopy (MRS) provides a powerful tool to measure gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the human brain (Ian *et al*., 2016). It is located in about 30% of cerebral neurons, and affects almost all neuronal activities (Gladkevich *et al*., 2006). In inflammatory disorders, one of the mechanisms by which gut commensals might putatively affect the disease course is altered through production of GABA, which has been shown to have immune- regulating features (Auteri *et al*., 2015). In the neuron, GABA is synthesized in the cytosol from its precursor glutamate by glutamate decarboxylase (GAD), and then transported into the synaptic vesicles against a proton electrochemical gradient (Thomas-Reetz & Decamilli, 1994). Exocytosis of synaptic vesicles is triggered when the voltage-gated calcium channels (VGCCs) open, resulting in a transient rise in cytosolic

calcium. GABA is released into the synaptic space, and exerts its effect through binding to corresponding receptors (Thomas-Reetz & Decamilli, 1994). The electrophysiological experiments in the 1960s had clearly demonstrated hyperpolarizing actions of GABA in neuronal preparations (Curtis & Watkins, 1960; Krnjevic & Schwartz, 1967). It took many years until experimental confirmation could be provided supporting the existence of specific GABA receptors, which might mediate this hyperpolarizing action of GABA.

The administration of GABA to STZ- induced type 1 diabetic treated rats preserved pancreatic tissue with improved insulin secretion, improved glucose level and minimized oxidative stress in brain tissues. It could be concluded that GABA might protect the brain from oxidative stress and preserve pancreas tissues by adjusting glucose and insulin levels in diabetic rats and might decrease the risk of neurodegenerative disease in diabetes (Eltahawy *et al*., 2016). The specific binding sites representing GABA receptors were demonstrated using [^{3}H] GABA (Peck *et al*., 1973). A discovery that a specific binding site of the benzodiazepine tranquilizing drugs was connected with the GABA receptor which facilitates the research related to the characterization of GABA receptors enormously (Haefely *et al*., 1975). This research was further advanced by the synthesis of 4, 5, 6, 7-tetrahydroisoxazolo [5, 4-c] pyridine-3-ol (THIP) and a large number of other GABA receptor agonists and antagonists (Krogsgaard-Larsen *et al*., 2002; Frolund *et al*., 2004). These studies ultimately led to the cloning of a large family of GABA-receptor subunits, the combination of which into complexes of five subunits forms functional receptors, which on agonist (GABA) binding flux Cl^- through the membrane (Jensen *et al*., 2005).

Thus, it was established that certain GABA responses could be mimicked by the lipophilic GABA analog Baclofen, an effect that could not be blocked by the classical GABA receptor antagonist bicuculline (Bowery *et al*., 1980). This led to the nomenclature $GABA_A$ receptors for the Cl^- channel-forming receptors and $GABA_B$ for this new class of receptor that did not gate an ion channel. The $GABA_B$ receptor originally identified as a functional entity being activated by GABA and Baclofen in a bicuculline insensitive manner (Bowery *et al*., 1980). It

was made known to be coupled with G proteins and adenylate cyclase, the response of which leads to either an activation of K^+ Channels with a subsequent increase in K^+ conductance and a hyperpolarization effect or a decrease in conductance of presynaptic Ca^{2+} channels resulting in a decreased transmitter release (Deisz, 1997). The role of GABA in the regeneration of pancreatic β-cells where its interaction with GABA receptors in islet β-cells produces membrane depolarization and Ca^{2+} influx, leading to the activation of PI3-K/Akt–dependent growth and survival pathways, thus preserving β-cells. Moreover, GABA causes membrane depolarization and enhances insulin secretion (Purwana *et al.*, 2014). The cloning of this receptor has confirmed that it belongs to the 7TM super family of receptors and it has been shown to form a heteromeric complex of $GABA_B$ subunits to be functionally active (Bettler & Brauner-Osborne, 2004). The recent knockout studies have revealed that mice devoid of the $GABA_B$ subunit exhibit epileptic seizures and such animals show lack of $GABA_B$-mediated responses (Prosser *et al.*, 2001).

Classification of Gamma Amino Butyric Acid (GABA) Receptors

$GABA_A$ receptors are ionotropic receptors that gate chloride channels, while $GABA_B$ receptors are G-protein coupled receptors. These receptors can be differentiated on the basis of agonist and antagonist selectivity. $GABA_A$ receptors are antagonized by bicuculline and insensitive to baclofen, whereas $GABA_B$ receptors are activated by baclofen and insensitive to bicuculline. GABA receptors in rat cerebellum that is insensitive to both bicuculline and baclofen are known as $GABA_C$ receptors (Drew *et al.*, 1984; Chebib & Johnston, 2000). Subsequent molecular biological and neuropharmacological studies showed that $GABA_C$ receptors were homomeric ionotropic GABA receptors made up of r-subunits that had been first cloned from the retina, and having a distinct pharmacological agonist and antagonist profile (Bormann & Feigenspan, 1995; Johnston, 1996). More recently, the International Union of Basic and Clinical Pharmacology recommended that $GABA_C$ receptors were a subtype of $GABA_A$ receptors and

should be classified as $GABA_A$ receptors (Olsen & Sieghart, 2008, Alexander *et al*., 2011).

$GABA_A$ receptor

$GABA_A$ receptors with different subunit combinations possess different pharmacological properties. The fast inhibitory actions of GABA are mainly mediated by $GABA_A$Rs in the brain. The existence of multiple ligand-binding sites and a lack of structural information have hampered the efficient screening of drugs capable of acting on $GABA_A$Rs (Kei *et al*., 2016). In the CNS, a functional $GABA_A$R is mostly found in a configuration containing two α-subunits, two β-subunits, and one γ-subunit (Mody & Pearce, 2004). In the adult brain $GABA_A$R is the most prevalent receptor, and upon binding GABA, it exerts an inhibitory effect manifested by hyperpolarization of the cell membrane (Owens & Kriegstein, 2002). However, during brain development, GABA induces depolarizing effects, acting as the principal excitatory transmitter (Ludwig *et al*., 2003; Represa & Ben-Ari, 2005). Many of these subunits are found in islets or β-cell lines, and it appears that the β, γ configuration also represents a functional $GABA_A$R in the islet cells. $GABA_A$ receptor subunit expression is differentially regulated during brain development, with each subunit exhibiting a unique regional and temporal developmental expression profile.

The small molecules capable of interacting with the orthosteric GABA recognition site, a wide variety of different allosteric modulators for $GABA_A$Rs have been reported. Among these are benzodiazepine derivatives, which are potent for their sedative-hypnotic and anxiolytic effects. Barbiturates, etomidate, propofol, and neurosteroids, which are all widely used as anesthetics, can also function as allosteric modulators, despite the fact that they have different interaction sites from that of benzo-diazepines (Sieghart, 2015). The Propofol belongs to a class of drugs that are used for anesthesia. It is the most widely used intravenous general anesthetic (Yip *et al*., 2013). It works positively to modulate the $GABA_A$ receptors and enhance the effect of GABA on these receptors (Yip *et al*., 2013; Krasowski *et al*., 1998). Etomidate is another type of drugs that is used

to induce anesthesia and sedation (Guo *et al*., 2014). Etomidate can also work as a modulator of $GABA_A$ receptors and it enhances the effects of $GABA_A$ receptors (Feng *et al*., 2014). Some of the 21 $GABA_A$ subunits dominate expression during embryonic development (e.g. α2, α3, and α5), whereas others dominate post natally or in the adult brain (Laurie *et al*., 1992; Fritschy *et al*., 1994). For example, the expression of the α1 subunit is low at birth, but increases during the first postnatal week, whereas the α2 subunit decreases progressively (Fritschy *et al*., 1994). Additionally, the α5 subunit is found throughout pre- and postnatal development (Killisch *et al*., 1991). The β2/3 subunits are ubiquitously expressed during development, indicating their association with α subunits in distinct receptor subtypes (Fritschy *et al*., 1994). The γ1 and γ3 subunits expression levels drop markedly during development, whereas γ2 expression is widespread and remains mostly constant throughout development (Fritschy *et al*., 1994). Although the significance of the differential expression of $GABA_A$ receptor subunits is not completely understood, it seems that subunit switching in certain brain regions is essential for normal development (Culiat *et al*., 1994; Gunther *et al*., 1995).

$GABA_B$ receptor

$GABA_B$ receptors are composed of two subunits: $GABA_{B1}$ that has two isoforms $GABA_{B1a}$ and $GABA_{B1b}$ and the other subunit is $GABA_{B2}$ (Filip *et al*. 2014). The *in situ* hybridization and immunohistochemical studies have defined a pattern of early and strong $GABA_{B1}$ receptor expression in discrete brain regions during embryonic development (López-Ben- dito *et al*., 2002b, 2003, 2004b; Kim *et al*., 2003; Martin *et al*., 2004; Panzanelli *et al*., 2004). $GABA_{B1}$ receptor mRNA is intensely expressed at E11 and at E12, it is detected in the hippocampal formation, cerebral cortex, intermediate and posterior neuroepithelium, and the pontine neuroepithelium (Kim *et al*., 2003; Martin *et al*., 2004). Furthermore, the most widely studied isoforms of the $GABA_{B1}$ subunit, $GABA_{B1a}$ and $GABA_{B1b}$, seem to be developmentally regulated, with $GABA_{B1b}$ being the most abundant isoform in the adult, while $GABA_{B1a}$ dominates during postnatal development (Fritschy *et al*., 1999). $GABA_B$ receptors that are present at the presynaptic

membrane work to inhibit voltage-activated Ca^{2+} channels and decrease GABA, glutamate and other neurotransmitters release, while $GABA_B$ receptors located at the post synaptic membrane stimulate inwardly rectified potassium channels and hyperpolarize the neuron (Gassmann & Bettler, 2012). Strong stimulus is needed to activate $GABA_B$ receptors in the brain slice (Jurado-Parras *et al*., 2016).

$GABA_{B2}$ receptor mRNA and protein are not detected at the same time period, as the expression of the $GABA_{B1}$ subunit, whose isoforms greatly exceed that of the $GABA_{B2}$ subunit during embryonic development but equalizes in most regions in the adult brain (Kim *et al*., 2003; López-Bendito *et al*., 2002b, 2004b; Martin *et al*., 2004; Panzanelli *et al*., 2004). It is well known that GABA plays an important role in modulating brain repair (Hiu *et al*., 2016). Thus it is likely that the $GABA_{B1}$ subunit is more important than the $GABA_{B2}$ subunit in the early development of the CNS. Indeed, it appears that $GABA_B$ receptor subunits are not coordinately regulated during development. Despite the fact that functional $GABA_B$ receptor requires hetero-dimerization of $GABA_{B1}$ and $GABA_{B2}$ subunits, the expression of each of them is under independent control during embryonic development (Martin *et al*., 2004).

Pharmacology and mechanism of GABAergic receptors

It has been reported that in the presence of glucose at high concentrations (over 10 mmol/L), $GABA_BR$ displays an inhibitory effect on insulin secretion, whereas in the presence of lower glucose levels it has no effect (Gu *et al*., 1993; Brice *et al*., 2002). The $GABA_BR$ agonist baclofen inhibited glucose-stimulated insulin secretion in wild-type but not in $GABA_BR$-knockout *islets* (Bonaventura *et al*., 2008). At this point, it appears that this receptor can have inhibitory or stimulatory effects on β-cell functions under various circumstances, and this merits further investigation. GABA is produced by both myenteric plexus and mucosal endocrine-like cells and thus functions as a neural and endocrine mediator in the gastrointestinal (GI) tract. Reduced GABA levels suppress regulatory T cells and enhance Th17-type immunity and thereby facilitate inflammation (Auteri *et al*., 2015). It is interesting to note that in addition to

GABA-receptor signaling, GABA catabolism also contributes to insulin secretion. GABA is catabolized in the GABA-shunt pathway after its transamination with 2-oxoglutarate by GABA transaminase into succinic semialdehyde and glutamate (Pizarro-Delgado *et al*., 2009).

It is reported that both α-ketoisocaproic acid and glucose promote GABA metabolism and stimulate insulin secretion in a GABA-shunt-dependent fashion. This mechanism appears to be important, especially in rendering β-cells more competent in the face of a restriction in the metabolic flow of the citric acid cycle set by low2-oxoglutarate dehydrogenase activity in response to high glucose stimulation (Pizarro-Delgado *et al*., 2010). GABA induces membrane depolarization and Ca^{2+}-dependent activation of cell-growth and survival pathways involving PI3K/Akt (Soltani *et al*., 2011; Purwana *et al*., 2014). The intestinal microbiome alterations (including GABA-producing bacteria) specific to Behcet's disease (BD), an autoinflammatory multisystemic disorder, suggesting that GABA concentration changes might potentially participate in pathogenesis of BD with (neuro-Behcet's disease, NBD) or without CNS involvement (Consolandi *et al*., 2015). GABA therapy preserves β-cell mass and prevents diabetes in type 1 diabetes mouse model (Represa & Ben-Ari, 2005).

Oxidative Stress and Complications of Diabetes

Oxidative stress is thought to be increased in a system where the rate of free radical production is increased and/or the antioxidant mechanisms are impaired (Irshad & Chaudhari, 2002). Increased oxidative stress is a widely accepted factor in the development and progression of diabetes and its complications (Ramakrishna & Jailkhani, 2007). A well established correlation exists between development of macro and microvascular disease in diabetes mellitus (Heistad Donald, 2005). Several lines of evidence indicate that in diabetes advanced glycation end products (AGEs) form in the eye and contribute to diabetic complications. This is most prominent in the retina where antioxidant enzymes, transcription factors and mitochondrial proteins have been found to undergo glycation which may impair their functions. Thus protein glycation may

contribute to oxidative stress in the diabetic eyes (Milne & Brownstein, 2013). The production of various reactive oxygen and nitrogen species (ROS and RNS, respectively) has been shown to occur in diabetic tissues including the retina (Behl *et al.*, 2016). Reactive oxygen species has also been implicated in diabetes-associated angiogenesis in the retina and is considered important contributors to diabetic retinopathy (Giordano *et al*., 2015). Vascular endothelial cells are an important target of hyperglycemic damage, but the mechanisms underlying this damage are not fully understood. An early marker of such damage is the development of an endothelial dysfunction (Coesention & Luscher, 1998). The chronic complications of diabetes mellitus affect many organ systems and are responsible for the majority of morbidity and mortality associated with the disease (Powers, 2008). There is considerable evidence that hyperglycemia causes many of the major complications of diabetes including nephropathy, retinopathy, neuropathy, and macro- and microvascular damages (Defronzo, 1997). Chronic hyperglycemia is often blamed for generation of free radical-mediated oxidative stress (Tse *et al*., 2015). Moreover, study also explored that oxidative stress has a significant role on production of advanced glycation end products (AGEs) (Pazdro & Burgess, 2012). It is highly suggested that NAD(P)H-mediated oxidative stress helps in disease progression of diabeic neuropathy (Jha *et al*., 2014).Oxidative stress resulting from increased production of ROS (or their inadequate removal) plays a key role in the pathogenesis of late diabetic complications (Brownlee, 2001; West, 2000).

In uncontrolled diabetes, the level of superoxide dismutase, the enzyme responsible for inactivating the superoxide radical, along with the levels of the antioxidants vitamin E and α-lipoic acid are decreased (Maxwell *et al*., 1997; Hartnett *et al*., 2000). There is also some evidence that a deficiency in erythrocyte catalase, an enzyme responsible for the removal of H_2O_2, is associated with increased frequency of diabetes (Goth & Eaton, 2000). Although our understanding of how hyperglycemia-induced oxidative stress ultimately leads to tissue damage has advanced considerably in recent years (nishikawa *et al*., 2000), effective therapeutic strategies to prevent or delay the development of this damage

remain limited (Jacot & Sredy, 1999). Oxidative stress is not only associated with complications of diabetes, but has been linked to insulin resistance *in vivo* (defined as a subnormal response to a given amount of insulin) (Ceriello, 2000). *In vivo*, studies in animal models of diabetes indicate that antioxidants, especially lipoic acid, improve insulin sensitivity. Oxidative stress may play a role in the pathogenesis of both type 1 and type 2 diabetes mellitus and its impact on lens transparency. Oxidative stress results mainly by an increased production of free radical and a sharp reduction of antioxidant defense (Lopez-Tinoco *et al*., 2013). Several clinical trials have also demonstrated improved insulin sensitivity in insulin-resistant and/or diabetic patients treated with the antioxidants vitamin C, lipoic acid, vitamin E and glutathione (Jacob *et al*., 1999). The enhanced oxidative stress and changes in antioxidant capacity, found in both clinical and experimental diabetes, are thought to be the main cause of chronic diabetic complications. High levels of free radicals cause damage to vital cellular components such as proteins, membrane lipids, and nucleic acids, and finally lead to cell death (Yorek *et al*., 2014).

Oxidative Stress and β-Cell Dysfunction in diabetes

The prevalence of diabetes is markedly increased; the incidence of diabetic complications and the cost of treatment remain major issues throughout the world. Recent studies showed that β-cell deficit is a core feature of diabetes. Even in the obese subjects with diabetes, β-cell function and mass are reduced (Saisho, 2015). Preservation or recovery of β-cell mass (BCM) is, therefore, an important therapeutic strategy for diabetes. In adult humans, BCM increases by approximately 20%–50% in obese non-diabetic individuals in the Caucasian population (Saisho *et al*., 2013). β-cell is particularly susceptible to the damages inflicted by oxidative stress. Through the concerted efforts of GLUT 2 (the high Km glucose transporter), glucokinase (the glucose sensor), and glucose metabolism, β-cells are responsible for sensing and secreting the appropriate amount of insulin in response to a glucose stimulus (Guillam *et al*., 2000). Although this process involves a complex series of events, mitochondrial

metabolism is crucial in linking stimulus to secretion (Maechler *et al.*, 1997). Therefore, the ability of ROS and RNS to damage mitochondria and significantly attenuate insulin secretion is not surprising (Laybutt *et al.*, 2002). The following sections discuss the impact of physiological inducers of oxidative stress including hyperglycemia, FFA, and their combination on β-cell function.

Genetic factor is associated with diabetes susceptibility through reduced islet number. Genome-wide association studies have currently detected genetic loci associated with diabetes, most of which are assumed to relate to the β-cell, also indicating the importance of β-cells in the pathogenesis of diabetes (Jonsson *et al*., 2013). Increasing pressure on β-cells to synthesise and secrete more insulin is an inexorable consequence of the progression of diabetes as glycemic control progressively wanes. The chronic oxidative stress that develops with pre-diabetes and early diabetes is associated with increasing serum insulin, which is typically accompanied by decreasing intracellular stores of insulin in β-cells (Hasnain *et al*., 2014). Many studies have reported that β-cell dysfunction is the result of 1) chronic exposure to hyperglycemia, 2) chronic exposure to FFA, and 3) a combination of chronic hyperglycemia and FFA. Furthermore, these effects appear to be dependent upon the oxidative stress induction of the NF-κB and additional stress-sensitive targets (Weir *et al.*, 2001). There is some evidence that activation of NF-κB is mostly a proapoptotic event in β-cells (Cardozo *et al*., 2001). There is considerable evidence that chronic hyperglycemia in patients with type 2 diabetes contributes to impaired β-cell function (Robertson *et al.*, 2000). However, evidence for a direct toxic effect of glucose *in vitro* has been conflicting. This conflicting evidence is due, in large part, to the definition of toxicity along with differences, sometimes subtle, in experimental design. Moreover, recent data suggest that the combined effects of elevations in glucose and FFA, acting by the generation of ROS, may be particularly toxic (Poitout & Robertson, 2002).

A deleterious cycle of β-cell dysfunction involving oxidative and endoplasmic reticulum stress, impaired secretory pathway function, β-cell apoptosis and local *islets* inflammation can progressively develop in diabetes

(Donath *et al*., 2013; Eizirik *et al*., 2013; Montane *et al*., 2014). Oxidative stress results from increased ROS and/or RNS (Joseph *et al*., 2003). Examples of ROS include charged species such as superoxide and the hydroxyl radical and uncharged species such as hydrogen peroxide and singlet oxygen. The possible sources of oxidative stress in diabetes might include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants, such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defense enzymes such as superoxide dismutase and catalase (Haskins *et al*., 2003). ROS generated by high glucose is causally linked to elevated glucose and other metabolic abnormalities important to the development of diabetic complications.

Hyperglycemia, hyperinsulinemia, and insulin resistance enhance free radical generation and thus contribute to oxidative stress in non-insulin dependent diabetes mellitus (Paolisso *et al*., 1999). Streptozotocin (STZ), a widely used chemical to develop a diabetic animal model, can result in excessive ROS production, lipid peroxidation, and DNA damage by formation of several kinds of free radicals including superoxide anion, hydroxyl radical, and hydrogen peroxide (Goyal *et al*., 2016; Salgueiro *et al*., 2016). Several prior studies demonstrate that exposure to STZ results in pancreatic β-cell dysfunction and apoptosis (Lei *et al*., 2012). It has been suggested that the STZ treatment might successfully mimic the oxidative stress scenario of diabetes in pancreatic cells (Wu & Yan, 2015). Oxidative stress linked with hyperglycemia may lead to a reduced number of glucose transporters and impairment of insulin signaling. Oxidative stress can even have adverse effects on cell insulin secretion (Kaneto *et al*., 1999). Therefore, oxidative stress resulting from hyperglycemia and insulin resistance can worsen non-insulin dependent diabetes mellitus by promoting further insulin resistance and decreased insulin secretion.

Lipid peroxidation and antioxidant activities in diabetes

Diabetes may occur by either the inefficient or no insulin production, resistance to insulin and by microbial infection. It has also been substantiated that the immune system is responsible for direct or indirect role in all the three reasons of diabetes. The main problem associated with diabetes mellitus are the elevation of blood glucose levels due to impaired metabolism, and the generation of harmful free radicals as a result of the use of lipids for energy production. In cases of diabetes, the auto-oxidation of glucose increases and during the conversion of oxidized glucose into glucose acid, free radicals are generated (Alam *et al*., 2014). Diabetic patients have also shown an increased ageing i.e. the processes or the characteristics and symptoms present during ageing (Wolff, 1993). Ageing is generally linked with the lipid peroxidation by ROS and so the diabetic has a role in lipid peroxidation. Immune reactions involve ROS, which also causes lipid peroxidation. Although many enzymes/substances are available in the body which have the ability to control these radicals such as vitamin C, superoxide dismutase and glutathione peroxidase (Akkus *et al*., 1996).

Glutathione peroxidase (GPx), which is a soluble selenoprotein, reduces H_2O_2 and organic hydroperoxides to H_2O and the corresponding alcohols using reduced glutathione as an essential co-substrate (Kobayashi *et al*., 2002). To date, five GPx isoenzymes have been identified. GPx-1 (a cytosolic form of GPx) is the most abundant and ubiquitous intracellular isoform (Arthur, 2000). GPx-1 mRNA is expressed in vascular endothelial cells where laminar shear stress upregulates the expression and enzymatic activity (Takeshita *et al*., 2000). The heterozygous deficiency of GPx-1 leads to endothelial dysfunction and to significant structural vascular and cardiac abnormalities (Forgione *et al*., 2002). These findings suggest that GPx-1 is a key enzyme in protecting vessels against oxidative stress and atherogenesis. The increase in oxygen free radicals in diabetes could be due to an increase in blood glucose levels, which upon auto-oxidation generate free radicals. STZ has been shown to produce oxygen free radicals (Ivorra *et al*., 1989). A combination of hypertension and diabetes mellitus is known to be accompanied by higher oxidative stress than that observed in the individual disorder alone. The

antioxidant vitamins; vitamin C, vitamin E levels and lipid peroxidation status in hypertensive patients and diabetic hypertensive patients receiving vitamin supplements, insulin and lipid lowering drugs (Ekeanyanwu *et al*., 2016). Lipid peroxide-mediated tissue damages have been observed in the development of type I and type II diabetes mellitus (Feillet-Coudray *et al*., 1999). It has been shown that supra physiological glucose concentrations result in high levels of intracellular peroxide concentrations in isolated *islets* damaging β-cell function (Tanaka *et al*., 2002).

In diabetic patients, the autoxidation of glucose results in the formation of hydrogen peroxide which inactivates SOD (Fajans, 1995). Therefore, the accumulation of hydrogen peroxide may be one of the explanations for decreased activity of SOD in these patients. The primary catalytic cellular defense that protects cells and tissues against potentially destructive reactions of superoxide radicals and their derivatives is the Cu/Zn-SOD. It has been observed that SOD can be rapidly induced in some conditions when cells or organisms are exposed to oxidative stress (Michiels *et al*., 1994). The highest SOD activity in red blood cells at the onset of diabetes and subsequent decrease in its activity have been reported (Domingues *et al*., 1998). Evidence is accumulating to support the idea that there is a close relationship between the processes of oxidation, glycation, lipids content and antioxidants may specifically inhibit glycation of proteins (Davie *et al*., 1992).

Diabetic neuropathy is one of most common complication of diabetes. The suggested factor for the development of diabetic neuropathy is increased lipid peroxidation which is a result of enhanced free radical generation and decreased antioxidant defense (Shilpashree *et al*., 2016). Redox activity of endogenous antioxidant agents can be helpful in designing the useful therapy of antioxidants in diabetic neuropathy. Another endogenous antioxidant is melatonin, which is a neurohormone synthesized by the pineal gland and is involved in regulation of circadian rhythms and also possesses a powerful antioxidant capacity *in vitro*. *In vivo*, the concentrations of melatonin are relatively low and its antioxidant action can be attributed to its modulation of secretion of other antioxidants (Negi *et al*.,

2011; Reiter *et al*., 2005). The significant correlation between total antioxidant capacity and clinical characteristics of diabetic patients including their blood levels of glucose and glycated hemoglobin suggests that the measurement of total antioxidant capacity in diabetic patients can be a marker of glycemic control (Rahbani- Nobar *et al*., 1999).

Treatment strategies for diabetes

Changes in human behaviour and lifestyle such as nutritional habits over the last century have resulted in a dramatic increase in the incidence of diabetes worldwide (Zimmet *et al*., 2001). The life style management is apparently the corner stone of management of diabetes mellitus. It is recognized as being a vital part of diabetes and cardiovascular disease prevention. Meta-analyses demonstrate that lifestyle interventions, including diet and physical activity, led to a 63% reduction in diabetes incidence in those at high risk. Lifestyle modification programs have demonstrated encouraging improvement in risk factors for diabetes; however, the effect on diabetes incidence has not been reported (Rebecca *et al*., 2009). The dietary management of diabetes mellitus is a balance of lifestyle management. It has a positive outcome on long term health and quality of life. Dietary management aims at most favorable metabolic control by establishing a balance between food intake, physical activity, and medication to keep away from complications. In type 2 diabetes, the dietary objective is for improved glycemic and lipid levels and weight loss as appropriate (Piero *et al*., 2006).

Different oral hypoglycemics have been in use to aid in maintenance of blood glucose level at the requisite threshold in diabetics through distinct mechanisms (Inzucchi, 2002). A genetic-environmental interaction is thought to be the cause of type 1 diabetes, but it is still unclear. While diagnosed in both children and adults, diagnosis of the disease is most commonly made at the age of 12 years with winter and fall seasonal distribution. Type 1 diabetes mellitus affects 0.17 percent of children in the United States and 10 percent of all the people with diabetes. The staple in the pharmacologic management of type 1 diabetes is the self-administration of insulin (Jones *et al*., 2010).

Antioxidant therapy could prevent a disturbance in the mechanism of protection against the harmful cellular and biomolecular effects that led to elevations in the cell function. Since diabetes is associated with increased oxidative stress as a consequence of persistent hyperglycemia, supplementation with vitamin E, and thus regulation of glycemia, could have a protective effect against lipid peroxidation in diabetes. Vitamin E has been found to prevent microvascular complications of diabetes. Indeed in animal models it decreases hyperglycemic- induced protein kinase C activation and D- acetyl glycerol levels, which have been associated with abnormalities in the retinal, renal and vascular tissues in diabetes (Bursell *et al*., 1999).

The application of traditional medicines for diabetes and associated complications, such as diabetic neuropathy (DN), has received increasing attention. In a recent study was to investigate the potential ameliorative effect of *Gymnema sylvestre* (Gs) in a rat model of diabetic neuropathy was investigated (Amal Jamil Fatani *et al*., 2015). Most of the plant extracts exhibited hypoglycemic, hypolipidemic, and antioxidant effects in animals as well as in humans, which may be helpful in approaches to treating diabetes and associated complications. The diabetic rats received an alcoholic extract of *Gymnema sylvestre* (100 mg/kg/day) for 1 month and the second week, the mean blood sugar level was lower among animals receiving the *Gymnema* extract (74 mg/dL) than among the control group (106 mg/dL). Patients received 200 mg *Gymnema* powder twice daily in addition to their usual doses of insulin, mean glycosylated hemoglobin (HbA 1c) decreased significantly from baseline (12.8 to 9.5%) at 6 months in a controlled trial of patients with type 1 diabetes (Shanmugasundaram *et al*., 1990). 22 patients were given *Gymnema sylvestre* extract along with their oral hypoglycemic drugs. All patients demonstrated improved blood sugar control (Mozersky, 1999). Because many medicinal plants constitute a rich source of bioactive chemicals that are largely free from adverse effects and have excellent pharmacological actions, they could lead to the development of new classes of possibly safer antidiabetic agents.

Antioxidant activity of Vitamin E

Animal studies have demonstrated that vitamin E plays a part in reducing lipid peroxidation (Kutlu *et al*., 2005) and thereby protecting and maintaining the lens of the diabetic eye (Naziroglu *et al*., 1999). Vitamin E status has been demonstrated to be of importance in human diabetics as well. Plasma vitamin E to lipid ratio was lower in diabetics than controls, and this effect was even more pronounced in diabetics with neuropathy (Ziegler *et al*., 2004). In this study, plasma vitamin E to lipid ratio was also inversely related to an assessed score of neuropathy. Central nervous system is also significantly affected by diabetes, which is further demonstrated by a link between diabetes and an increased risk for dementia (Biessels *et al*., 2002). Membrane fluidity, which is vital for proper nerve conduction, decreases due to oxidative stress and subsequent peroxidation of long-chain polyunsaturated fatty acids in diabetic rat brain and can be improved with antioxidant treatment (Siddiqui *et al*., 2005). Vitamin E acts against peroxidation of polyenoic acids of biological membranes. Tocopherols have the ability to donate hydrogen. They stop radical chain reactions by transferring hydrogen from the phenolic group of the free radical peroxide. The resulting phenoxy-radical may react with vitamin C. Vitamin C is involved in the antioxidant protection of cells as it reduces tocopherol radical (Lovercam *et al*., 2013 ; Horky *et al*., 2013).Vitamin E fed to diabetic rats lowered oxidative stress, increased antioxidant enzyme expression, and increased membrane fluidity in brain (Hong *et al*., 2004). This is consistent with results showing diabetes to reduce membrane fluidity in other tissues of diabetic animals (Rhee *et al*., 2005). Proper regulation of membrane fluidity may lead to improvements in other outcomes of brain function.

Vitamin E is a well-documented fat-soluble antioxidant and has been shown to inhibit free radical-induced damage to sensitive cell membranes (Panda & Cherian, 2014; Rengaraj & Hong, 2015). The reduction of the antioxidant capacity generates more H_2O_2 and other reactive intermediates such as hydroxyl radicals. Therefore, not only lipids but also proteins, carbohydrates and nucleic acids are affected by alteration of the oxidant and antioxidant systems. Increased

free radical production and reduced activity of antioxidant defense systems in diabetes and hence tissue damage is facilitated (Zimmet *et al*., 2005). Dietary supplementation with antioxidant nutritional factors such as micronutrients and vitamins could be used as a novel strategy in both prevention and control of type 2 diabetes mellitus (Dragana Nikolic *et al*., 2014). The treatment of diabetic rats with vitamins C, E and beta-carotene results in a significant reduction in the level of thiobarbituric acid reactive substances (TBARS) and glutathione peroxidase (GSH-Px) activity, an increase in Superoxide Dismutase (SOD) activity, while Catalase (CAT) activity does not change. Vitamins C and E, lower the level of TBARS and GSH-Px, while the activity of CAT and SOD were increased (Kedziora-Kornatowska *et al*., 2003). Vitamin E and C (VEC) helped alleviation of the renal degeneration by protecting the glomerular structures from oxidative injury. Concomitant administration of VEC would be more effective in preventing the complications of diabetes (Ganesh *et al*., 2012). The excessive doses of vitamin E may result in side effects. The preterm septic neonates are particular by susceptible to a wide spectrum of different morbidities such as respiratory distress syndrome, broncho-pulmonary dysplasia, periventricular leukomalacia, severe intraventricular hemorrhage, cerebral palsy, vision and hearing impairments. The development of these morbidities has been related to oxidative stress (Bajcetic *et al*., 2014).

Vitamin E was found to be excellent for strengthening the antioxidative defense system, reducing the generation of ROS and damaging oxidative substances, and maintaining membrane fluidity in the brain of diabetes-induced rats. Vitamin E was found to reduce the accumulation of ROS, such as superoxide radical, decreased the generation of oxidative damage substances, such as the carbonyl value, increased the membrane fluidity lowered by oxidative damage, and significantly improved lipid composition (Jung-Hee Hong *et al*., 2004). In addition to the antinflammatory and antioxidative properties, vitamin E shows other properties, such as the modulation of the expression of genes encoding proteins involved in signaling (Cardenas & Ghosh, 2013). In addition, vitamin E is also involved in the uptake, transport and degradation of tocopherols, as well as

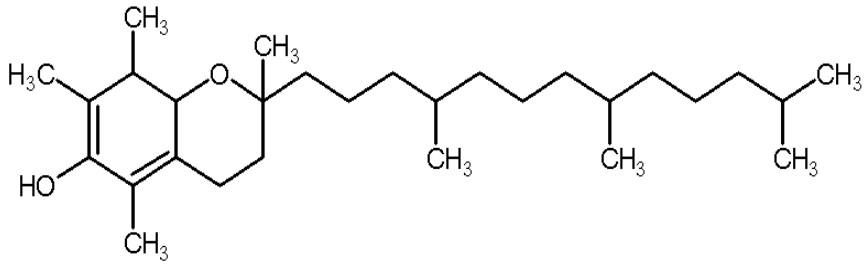
the uptake of lipoproteins and the storage and export of lipids such as cholesterol (Cardenas & Ghosh, 2013). An increase in lipid damage and a reduction in the activity of antioxidant enzymes SOD, CAT, glutathione peroxidase, glutathione reductase and glucose 6-phosphatedehydrogenase in the liver and pancreas of alloxan-induced diabetic rats were reported (Oloyede *et al*., 2015). In STZ-induced type 1 diabetic rats, hydrogen sulfide, which has antioxidant activity, presented antidepressant- like and anxiolytic-like effects, reduced lipid damage and increased SOD and glutathione enzyme activities in the hippocampus (Tang *et al*., 2015).

Antidiabetic activity of Vitamin E

Chronic hyperglycemia resulting from diabetes has profound effects on nearly every system of the body. The toxic effects of hyperglycemia may result from accumulation of non-enzymatically glycosylated products (Singh *et al*., 2014). The treatment with antioxidant vitamins, especially vitamin E, is of special interest, and given the potential protective activity of antioxidants, vitamin E consumption may prevent the development of type 1diabetes mellitus (Bonnefont-Rousselot *et al*., 2000; Knekt *et al*., 1999; Matheus *et al*., 2008). Vitamin E was able to reduce food and water intake in diabetic rats. This could be due to the correction of glycemia (Bonnefont-Rousselot *et al*., 2000), which was associated with an improved metabolic state in those animals. In recent studies the effect of Vitamin E on blood glucose and its possible interactions with oral antidiabetic agents (i.e. glimepiride, gliclazide and metformin) in rabbits have been reported. The study was performed on albino rabbits in which hyperglycemia was induced by giving glucose. Vitamin E significantly lowered the blood glucose level but on co-administration with oral antidiabetic agents, it antagonized the effect of the latter drugs (Chandra Veer Singh & Rakesh Verma, 2016). It is, therefore, advisable not to give inadvertent amount of Vitamin E to diabetic patients just because antioxidants are helpful to diabetes and does not produce adverse effects. Adipose tissue weight and hepatic glycogen content decreased in the experimental STZ-induced type 1 diabetes in rats (Ruperez *et al*., 2008). The body weight was

restored in the presence of vitamin E in the STZ-induced type 1 diabetic rats, demonstrating its anti-diabetogenic effect.

Structure of Vitamin E



Vitamin E, a membrane-bound, lipid-soluble antioxidant, has been shown to protect biological membranes against injury induced by the reactive oxygen species (Vannucchi *et al.*, 1999) and to block glycation of proteins by inhibiting MDA formation (Jain *et al*., 1996). Vitamin E includes eight molecules composed by a chromanol ring and a phytol side chain having same functions: four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ) (Gornas, 2015). Vitamin E treatment ameliorated the reduced ATPase activity, high lipid peroxidation and high glycosylated protein levels in diabetes. Vitamin E treatment led to a significant inhibition in blood glucose, protein glycosylation and lipid peroxidation, which in turn prevented abnormal activity of the enzyme in the brain (Das Evcimen *et al*., 2004). Vitamin E is also known to protect ATPase activity in microsomal fraction of the brain and hyperglycemia-induced oxidative damage (Kowluru *et al.*, 2000). Vitamin E has been further shown to protect biological membranes against injury induced by the reactive oxygen species (Jain *et al*., 1996; Vannucchi *et al*., 1999; Kinalski *et al*., 2000).

It is well known that tocopherol incorporates itself into lipid membranes. In a variety of phospholipid bilayers of differing composition, it's potential antioxidant capabilities were demonstrated using neutron diffraction, NMR spectroscopy, and UV/VIS absorbance (Marquardt *et al*., 2015). Vitamin E also

accelerates diacylglycerol kinase activity, thereby decreasing levels of diacylglycerol, which is an allosteric activator of protein kinase C (Azzi *et al*., 2000). Increased protein kinase C activity apparently impairs insulin action by phosphorylating serine or threonine residues on insulin receptor and insulin receptor-1 proteins (Griffin *et al*., 1999). This decreases insulin-stimulated, phosphatidylinositol 3-kinase-catalyzed phosphorylation of tyrosine residues in these proteins, which is required for effective insulin action. Recent evidence suggests that vitamin E may influence the activity of these enzymes by decreasing the curvature of plasma membranes (Bradford *et al*., 2003). The diabetic rats that received vitamin E had lower glucose levels than the rats treated with Streptozotocin alone. This finding indicates that vitamin E may have a role in preventing hyperglycemia (Paolisso *et al*., 1993). The vitamin E reduces plasma glucose levels and they proposed that vitamin E may have a role in the modulating insulin action (Paolisso *et al*., 1993). These results along with the findings presented here indicate that vitamin E may modulate glucose metabolism.

Diabetic animals that received vitamin E (2%) supplementation exhibited a significant reduction in glycemia and glycated hemoglobin compared groups diabetic and diabetic animals that received vitamin E, indicating a positive effect of this antioxidant on blood glycemia (Shirpoor *et al*., 2007). The study performed with diabetic rats that received vitamin E (300 mg/kg body weight), observed a significant reduction in blood glucose and glycated hemoglobin in treated diabetic animals compared with untreated diabetic animals. Another study also performed with diabetic rats supplemented with vitamin E (650 mg/kg body weight) demonstrated that glucose blood levels were reduced in supplemented diabetic animals compared with untreated diabetic animals (Belai *et al*., 1991). Diabetes-induced oxidative stress generates reactive oxygen species and an inbalance among antioxidants (Bhor *et al*., 2004). The altered activity in primary antioxidant enzymes (e.g., catalase, superoxide dismutase, and glutathione peroxidase) and an augment in lipid peroxidation and carbonyl protein content, thus ensuring the occurrence of oxidative stress in diabetic rats.

The vitamin E supplementation reduced blood sugar levels in diabetics. The administration of 900 mg/day of vitamin E for four months to diabetes mellitus patients significantly improved glucose tolerance. Vitamin E also inhibited glycosylation of proteins when administered at doses of 600 or 1200 IU/day; the higher dose was slightly more effective than the lower dose (Pinckney *et al*., 1999). Additional newer research has also suggested other positive effects on diabetes mellitus from vitamin E supplementation. For instance, one study found that 1,000 IU daily supplementation of vitamin E led to improved circulation to tissues in patients with diabetes mellitus after three months. A significant body of evidence indicates that vitamin E may help prevent heart disease, one of the main complications of diabetes. One study of tocotrienols (mixed forms of vitamin E) reduced total cholesterol by 30% and low density lipoprotein by 42% in patients with diabetes mellitus.

The study suggests that the antioxidant effects of vitamin E help to reduce the oxidation of low density lipoprotein particles. A recent study also found that vitamin E supplementation decreased several markers of thrombosis in patients with diabetes mellitus. A different study found that patients with diabetes mellitus given 600 mg per day of vitamin E had improvements in the health of nerves that control the functioning of the heart (Manzella *et al.*, 2001). A study found that 500 IU of vitamin E per day given to patients with diabetes reduced the levels of factors that increase the risk of developing vascular complications. An additional study found that 800 IU per day of vitamin E improved β-cell function, increased plasma insulin, and may have decreased insulin resistance in 40 patients with diabetes mellitus who took the supplement for a month (Gokkusu *et al*., 2001). It has also been shown that lipid peroxidation is involved in the cytotoxic effects of cytokines on rat islets and that the inhibition of this process protects islet beta cells from the combined cytotoxic effects of interleukin 1, tumor necrosis factor and interferon gamma (Rabinovitch *et al*., 1992). The clinical trials to evaluate the benefits of low-cost vitamin E supplementation in reducing the risk of complications in the diabetic patient population could extensively decrease the cost of caring for diabetic patients.

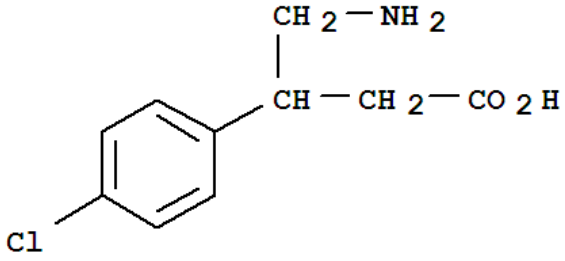
Baclofen (Gamma Amino butyric acid agonist) as a diabetic pain model

Major classes of adjuvant analgesics include antidepressants, antiepileptic drugs (AED), corticosteroids, alpha (α2) adrenergic agonists, N-methyl D-aspartate (NMDA) receptor antagonists, gamma amino butyric acid (GABA) agonists, local anesthetics, topical analgesics, benzodiazepines, neuroleptics, muscle relaxants, bisphosphonates, cannabinoids, psycho-stimulants, anti-cholinergics, calcitonin, radiopharmaceuticals and octreotide. There are numerous pharmacotherapeutic options for the management of chronic pain. Proper evaluation, along with complete assessment of pain, is crucial to provide best possible analgesic approach (Neha Gupta & Amy Allen, 2016). The pain stimulation reaches to the spinal cord, descending pain control systems and also some of the local mechanisms act on it; it then reaches to the thalamus and cortex. Disease, inflammation and hurt to the nerve system create important changes in pain pathways such as stimulation, gene adjustment and will express new molecules such as neurotransmitter enzymes and receptors (Sawynok *et al*., 2004).

The activation of $GABA_B$ receptor by Baclofen attenuates diabetic neuropathic pain, which may partly be accomplished via down regulating the expression of NMDA receptors (Liu *et al*., 2014). An increased release of glutamate and activation of the NMDA [N-methyl-D-aspartate) receptor would maintain the hyperalgesia state (Malan *et al*., 2003). Neuropathy is one of the most common complications of diabetes, with patients often suffering from severe and unremitting pain. The pain can be acute in onset, resolving within around 6 months, or more chronic with symptoms lasting for many years (Thomas & Scadding, 1987). A number of agents that are effective in the clinic have been shown to partially alleviate mechanical hyperalgesia in this model (Courteix *et al*., 1994). However, some clinically effective treatments like the tricyclic anti-depressants are ineffective in the STZ-induced type 1 diabetic rats, and agents such as morphine that do not provide symptomatic relief in the patient effectively increase mechanical pain threshold in the diabetic rats (Courteix *et al*., 1994; Calcutt & Chaplan, 1997).

Structure of Baclofen

Chemical name: 4-Amino-3-(4-chlorophenyl) butanoic acid



The treatment with non-sedative doses of the prototypic $GABA_B$ receptor agonist, Baclofen, suppressed several alcohol-related behaviors – including alcohol drinking, operant alcohol self-administration, and alcohol-induced conditioned place preference in rats, mice, and monkeys (Agabio & Colombo, 2014; Phillips & Reed, 2014). Baclofen is also used as an anti-inflammatory drug which essentially manages pain by central mechanism compared to diabetic neuropathy which acts peripherally (Terrence *et al*., 1985). Alternative animal models of neuropathic pain conditions may involve selection of an animal that naturally possesses a painful disease condition providing neuropathic pain and its symptoms in diabetes. Animals may be modified to possess a pain condition due to a disease in a variety of ways for example by administration of Streptozotocin to induce a diabetic neuropathy (Courteix *et al*., 1993).

The effect of Baclofen as a non-opiate, analgesic drug on the increased pains in the quiescent phase as the model of diabetic pain is investigated. The method is experimental, evaluating the pain level through conducting the formalin test in 3 groups of rats. The first group was divided to control (injection normal saline) and diabetic (injection alloxan 100 mg/kg) which were tested, after one to four weeks from the beginning of diabetes, the second one was divided to a new control and diabetic group, and before performing formalin test, the Baclofen (10 mg/kg) was injected to them (Elaheh Nooshinfar *et al*., 2010). Due to the results of this study it seems that diabetes, with the changes in the central and peripheral

pathways of the pain. These changes are accompanied with declining the internal anti pain systems such as GABAergic, which can be treated with Baclofen. Baclofen may be recommended as an effective drug to comfort painful diabetic neuropathy.

Herbal extract Gymnemic acid (*gymnema sylvestre*) for treatment of diabetes

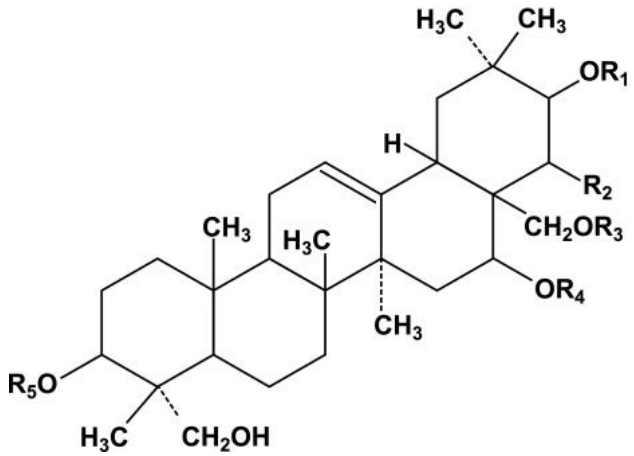
Gymnema sylvestre is an antidiabetic plant, a large tropical liana, native to central, southern and western India and is also found growing in Africa and Australia. It possesses antimicrobial, antihypercholesterolemic, hepatoprotective and sweet suppressing activities. *Gymnema* leaf contains more than 20 saponin glycosides. The major saponin fraction comprises of gymnemic acid which is a complex mixture of at least nine closely related acidic glycosides. The herb's bioactive ingredient, gymnemic acid, extracted from leaves and roots, helps to lower and balance blood sugar levels (Nidhi *et al*., 2016). *Gymnema sylvestre* is an important herb belonging to the family *Asclepiadaceae*. It is a slow growing, perennial, woody climber, distributed throughout the India, in dry forests up to 600m height. Gymnemic acid is a effective bioactive compound extracted from *Gymnema sylvestre* and has been used traditionally in ayurvedic medicine to treat diabetes mellitus. It is believed to reduce one's ability to detect sweetness on the palate, and has also shown in test tube studies to increase pancreatic beta cell function (Persaud *et al*., 1999). One study in people with type 1 diabetes mellitus monitored glucose control measures in people taking 200 mg of *Gymnema* twice a day. They were found to have significant reduction in both fasting blood glucose and HbA1c, and their required insulin doses were decreased (Shanmugasundaram *et al*., 1990). *Gymnema sylvestre* leaves are reported to stimulate insulin secretion and increase glucose uptake *in vitro* and *in vivo* (Okabayashi *et al*., 1990).

Mechanism of Action of Gymnemic Acids

The main constituent of *Gymnema* is believed to be gymnemic acid, a mixture of at least 17 different saponins. Gymnemic acid formulations have been found useful against obesity, according to recent reports (Yoshikawa *et al*., 1993).

This is attributed to the ability of gymnemic acids to delay the glucose absorption in the blood. The atomic arrangement of gymnemic acid molecules is similar to that of glucose molecules. Aqueous extract of *Gymnema sylvestre* leaves have the ability to stimulate insulin secretion in mice cells and isolated human islets *in vitro* (Alromaiyan *et al*., 2013).These molecules fill the G-protein coupled taste receptor locations on the taste buds thereby preventing its activation by sugar molecules present in the food, thereby curbing the sugar craving. Similarly, Gymnemic acid molecules fill the receptor location in the absorptive external layers of the intestine thereby preventing the sugar molecules absorption by the intestine, which results in low blood sugar level (Sahu *et al*., 1996).

Structure of Gymnemic acid



The major phytoconstituents of *Gymnema sylvestre* are gymnemic acids, gudmarin and saponins. Gymnemic acid ($C_{43}H_{68}O_{14}$) is a pentacyclic triterpenoid and is the main active phytoconstituents of *Gymnema sylvestre*, exhibiting potent anti-diabetic activity (Vaidya, 2011). The heteromeric T1R2 + T1R3 GPCR complexes responsible for the initial binding of sweet tastants in humans have been shown to be directly inhibited by both the *Gymnema sylvestre* peptide gurmarin and gymnemic acids (Sanematsu *et al*., 2014). Dihydroxy gymnemic

triacetate (DGT) is a novel phytocompound isolated from the leaves of *Gymnema sylvestre*. This compound has been shown to have anti-hyperglycemic activity in Streptozotocin-induced type 1 diabetic rats (Jayaraman *et al*., 2015). The active constituent is Gymnemic acid a triterpenoid saponin has significant effects on the intestinal absorption of glucose in to the blood and stimulation of β-cells of pancreas in the production of Insulin (Achyuth *et al*., 2016).

Antihyperglycemic activity of gymnemic acid

The major bioactive constituents of *Gymnema sylvestre* are a group of oleanane type-triterpenoids known as gymnemic acid. Gymnemic acid has been reported as antihyperglycemic, normoglycemic and antihyperlipidemic in *in vitro* studies (Sugihara *et al*., 2000; Daisy *et al*., 2009). The antibacterial, antioxidant activity and in *vitro* propagation studies of *Gymnema sylvestre* in the pharmaceutical trade due to its use as a remedy for diabetes and also as a tonic of the nerves and as a laxative (Vani *et al*., 2016). Gymnemic acid elicit antihyperglycemic by increasing serum insulin levels due to regeneration of pancreatic cells, stimulating insulin release and inhibition of glucose absorption (Shanmugasundaram *et al*., 1990; Persaud *et al*., 1999; Masayuki *et al*., 1997). *Gymnema sylvestre* leaves have been found to cause hypoglycemia in laboratory animals and have established a useful herbal medicine to help treat adult onset of diabetes mellitus (NIDDM). Aqueous extract of *Gymnema sylvestre* altered the elevated glucose, lipid, insulin levels and also improved the histopathology of liver in dexamethasone-induced insulin resistance rats suggesting that *Gymnema sylvestre* has anti-diabetic and hypolipidimic activity (Kumar *et al*., 2015).

Gymnema leaf extract was administered to a diabetic patient; there was stimulation of the pancreas by virtue of which there was an increase in insulin release (Persaud *et al*., 1999). Gymnemic acid is obtained from the natural resource and has got antioxidant property. Administration of gymnemic acid in a dose of 250 mg/kg and 500 mg/kg body weight orally showed promising neuroprotective effect by reducing cerebral infarct size as well as improved all antioxidant levels showing activity against oxidative stress (Kiran *et al*., 2016).

Gymnemic acid is known to have a good effect for curing of diabetes by blocking sugar binding sites and hence not allowing the sugar molecules to accumulate in the body. Normal and STZ-induced type 1 diabetic rats were treated with either a 50% ethanolic extract of Gymnema leaves (GS3, 20 mg/day/rat), a purified residue of GS3 (GS4, 20 mg/day/rat), or no intervention for up to 95 days (Shanmugasundaram *et al*., 1988). Investigating the capacity of a GS extract (GS4) to stimulate insulin release from rodent insulin-secreting cell lines and isolated rat *islets* it was found that the presence of high levels of gymnemic acids led to unregulated insulin release by exerting deleterious effects on the β-cell plasma membrane rather than regulated, reversible insulin secretion (Persaud *et al*., 1999).

Cell signaling and functional markers

Diabetes mellitus, a syndrome characterized by hyperglycemia, affects cellular integrity and signal transduction. The diabetic stress resulted in an altered gene expression of different cellular markers as an adaptive response which resulted in the activation and regulation of apoptotic pathways in the brain regions. The cortical changes triggered a marked reduction in the β-cell proliferation indicated by the DNA synthesis data, even though protein synthesis was significantly high compared to control. Thus, diabetes resulted in a series of changes in the molecular signaling as the effort to ameliorate the hyperglycemic condition, which in most cases resulted in activation of cell death pathways. .

In diabetes, treatment with antioxidant enzymes has been demonstrated to preserve β-cell function, increase insulin sensitivity, protect the vascular endothelium, and ameliorate polyneuropathy. Diabetes mellitus, a syndrome characterized by defective insulin signaling manifests with a wide range of complications in the overall molecular signaling. The cell survival pathway mediated by Akt signaling through NF-κB and apoptotic pathways activated by TNF-α and Caspase 8 in the brain regions and pancreas plays significant role in controlling the proliferative capability of pancreatic β-cells. The gene expression

pattern of these markers in the brain regions and pancreas is important to understand the overall regulation of cellular survival under diabetic condition.

Akt/PKB

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a major effector of the PI3K signaling pathway, activated by numerous growth factors and hormones such as insulin. PKB is central to multiple signaling pathways and transduces extracellular signals to dictate cellular responses towards proliferation, migration, anti-apoptosis, and maintenance of metabolic homeostasis (Gongda *et al*., 2015). In mammals, Akt is ubiquitously expressed and is associated with the regulation of cellular proliferation, metabolism, cell growth and cell death. Akt has been widely studied for its central role in physiology and disease, in particular cancer where it has become an attractive pharmacological target (Harshani *et al*., 2016). This protein kinase regulates several biological processes including cellular growth, proliferation, survival and metabolism in multiple organs, and has also been involved in tumorigenesis. In mammals, three Akt isoforms encoded by three separate genes have been identified. These isoforms share 85% sequence homology (Woodgett, 2005; Du & Tsichlis, 2005). The three homologous isoforms are ie, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ (Manning, 2010). Distinct roles for each isoform, with Akt1/PKBα linked to cell survival, Akt2/PKBβ with cell-substrate metabolism, and Akt3/PKBγ with brain development have been identified (Hawley *et al*., 2005). PKB is a key enzyme mediating the metabolic actions of insulin (Whiteman *et al*., 2002; Fayard *et al*., 2005). Akt regulates multiple cellular functions including proliferation survival, and growth during embryonic development and adult tissue homeostasis (Chuang *et al*., 2014).

The Akt2/mice develop diabetes due to reduction in insulin-stimulated glucose uptake in peripheral tissues and β-cell failure, similar to diabetes in humans (Cho *et al*., 2001; Garofalo *et al*., 2003). Akt regulates adult stem cell proliferation, migration and apoptosis and its deregulation has been implicated in the progression of cancer, diabetes, and aging (Segrelles *et al*., 2014). PI3K/Akt

signaling pathway and PTEN gene have been known to be closely related to hypoxia-ischemia brain injury and involved in neonatal hypoxic ischemic brain damage (HIBD) (Dan Yao *et al*., 2016). The mutation of the human Akt 2 has been described in a family with severe insulin resistance and diabetes, indicating a crucial role of Akt/PKB signaling in human insulin sensitivity (George *et al*., 2004). Akt 3 null mice do not show growth retardation but exhibit reduced cell size and number in the brain (Tschopp *et al*., 2005). Overexpression of a constitutively active form of Akt 1 in β-cells induces increase in both their size and number. Glucose tolerance is improved and the animals are protected against STZ-induced type 1 diabetes (Bernal-Mizrachi *et al*., 2001; Tuttle *et al*., 2001).

The β-cell lines have also shown the importance of Akt for cell survival. Activation of Akt/PKB signaling mediates anti-apoptotic effects induced by glucose, GLP-1, GIP, IGF-1, glucose and insulin (Brubaker & Drucker, 2004; Kim *et al*., 2005). The PI3K/Akt signaling pathway is a classic anti-apoptosis, urge-survival signal transduction pathway in the cell. It has been reported to play an important role in protecting the brain against ischemic, in angiogenesis and anti-apoptosis (Qazi *et al*., 2013). Autonomous apoptosis occurs in the majority of pathogen-specific effector CD8+ T cells through the PKB/FoxO axis, whereas a small percentage will develop into memory CD8+ T cells for rapid response to secondary stimulation (Kim & Suresh, 2013). Extensive studies have revealed that PKB stimulates aerobic glycolysis in many types of cancer cells (Ran *et al*., 2013). *In vitro* experiments revealed that these alterations were not due to abnormalities in intracellular calcium, suggesting a distal insulin secretory defect involving the exocytotic machinery. No alteration in β-cell mass was observed in these animals on a normal or high-fat diet. It remains possible that the 20% of normal Akt still present in the pancreas of these mice is sufficient to preserve the β-cell mass by compensation (Elghazi *et al*., 2007). These data thus confirmed the importance of Akt in β-cell physiology and highlighted a novel role for this protein in the final steps of insulin exocytosis.

Pdx-1

The homeodomain-containing transcription factor pancreatic duodenal homeobox 1 (Pdx-1) plays a key role in pancreatic development and β-cell function. It is a major regulator of transcription in pancreatic cells, and transactivates the insulin gene by binding to a specific DNA motif in its promoter region (Pedica *et al*., 2014). The human Pdx-1 gene is located on chromosome 13q12.1 near the Cdx2 gene. In mouse and rat, the Pdx-1 genes are localized on chromosomes 5 and 12 respectively (Gu *et al*., 2002). Pdx-1 is the primary regulator of glucose-stimulated insulin gene expression and it is thought to modulate transcription *via* phosphorylation- dependent changes in subcellular localization and interactions with co-regulators (Oliver Krasinski *et al*., 2008).

Pdx-1 is a master regulator of pancreatic β-cell development and adult β-cell function and is vital for upstream control of insulin gene transcription, endoplasmic reticulum homeostasis, β-cell survival, and mitochondrial respiration (Sachdeva *et al*., 2009). Pdx-1 plays a crucial role in the development and function of the pancreas, both in the maintenance of progenitor cells and in determination and maintenance of differentiated endocrine cells (Johannes Von *et al*., 2010). Mutations in the β-cell, Pdx-1 is associated with diabetes. All 3 mutations (C18R, d76N and R197H) resulted in reduced binding of the protein to the insulin gene promoter and decreased insulin gene transcription (Macfarlane *et al*., 1999).

In mice with a 50 percent reduction in Pdx-1, the isolated islets showed more susceptibility to apoptosis at basal glucose concentrations along with impaired ability to maintain β-cell mass with age. Its expression is shown to be down regulated during hyperglycemic condition (Robertson & Harmon, 2006). Pdx-1 also turns out to be a major player in the maintenance of an adequate pool of healthy β-cells in adults. It maintains homeostasis between β-cell neogenesis and apoptosis (Bernardo *et al*., 2008). The post-translational modifications of the Pdx-1 protein and its interaction with other regulatory proteins will be

fundamental to develop new treatments for diabetes mellitus (Alquobaili & Montenarh, 2008).

NF-κB

The NF-κB pathway, a critical regulator of apoptosis, plays a key role in many normal cellular functions. Genetic alterations and other mechanisms leading to constitutive activation of the NF-κB pathway contribute to cancer development, progression and therapy resistance by activation of downstream anti-apoptotic pathways, unfavorable microenvironment interactions, and gene dysregulation (Li Yu *et al*., 2016). Curcumin, melatonin, resveratrol and sulphoraphane have reported beneficial effects in ameliorating various functional (motor nerve conduction velocity and nerve blood flow), sensorimotor (thermal and mechanical hyperalgesia) and biochemical deficits in experimental diabetic neuropathy (Negi *et al*., 2011; Kumar *et al*., 2010). These agents also suppressed the increased activity and levels of nuclear factor kappa B (NF-κB) and associated proteins and hence protected against neuroinflammation in diabetic neuropathy (Joshi *et al*., 2013). Constitutive activation of the NF-κB pathway inhibits cell differentiation and apoptosis, promotes cell proliferation, and increases angiogenesis, cancer-related inflammation and metastatic potential. Consequently, activated NF-κB is one of the prime therapeutic targets in lymphoma cells (Rosebeck *et al*., 2014). The Nuclear factor-2 erythroid related factor-2 (Nrf2) and Nuclear factor-kappa light chain enhancer of B cells (NF-κB) individually affect many signaling cascades to maintain a redox homeostasis; additionally they interact with each other to further modulate level of key redox modulators in health and disease. The selective inactivation of NF-κB in mouse liver enhanced insulin sensitivity and suppressed hepatic gluconeogenesis also *via* inhibition of the cAMP/PKA/CREB pathway (Ke *et al*., 2015). These studies suggest that cAMP/PKA is the major positive regulator for hepatic gluconeogenesis. The activation of NF-κB linked regulatory pathways generally underlies inflammatory processes, and an increase in the nuclear translocation of NF-κB has been demonstrated in human diabetic nephropathy (Cohen *et al*., 2002; Sakai *et al*., 2005).

Increased reactive oxygen species activate NF-κB, which then acts to diminish ROS by inducing the expression of genes of pro-survival proteins including MnSOD (Mattson *et al*., 1997). Inhibition of neuronal NF-κB, conversely, intensifies insult-induced increases in mitochondrial ROS and mitochondrial depolarization in cultured CNS and PNS neurons, as well as reducing axonal growth and exacerbating neuronal death (Fernyhough *et al*., 2005; Glazner & Fernyhough, 2002; Paschen & Frandsen, 2001). The p53 tumor suppressor and transcription factor serve as one of the first lines of defense against the effects of genotoxic damage, onco gene activation, metabolic changes, and hypoxia. Upon transcriptional induction of genes encoding anti-apoptotic and antioxidant proteins, NF-κB blocks cell apoptosis along the apoptotic pathways by regulating p53 (Johnson & Perkins, 2012). The activity of poly (ADP-ribose) polymerase (PARP) was increased in the whole retinas, endothelial cells, and pericytes of diabetic rats. The retinoblastoma (Rb) protein is a major route of crosstalk between the non-canonical NF-κB pathway and p53 in chronic lymphocytic leukemia (Iannetti *et al*., 2014). NF-κB up-regulates the activity of Rb, which induces expression of the polycomb protein EZH2 in CD40L stimulated cells from chronic lymphocytic leukemia patients. PARP activation plays an important role in the diabetes-induced death of retinal capillary cells, at least in part *via* its regulation of NF-κB (Zheng *et al*., 2004). The specific agents that might regulate the crosstalk between the two central pleiotropic transcription factors, Nrf2 and NF-κB, may be one of the prospective strategies that might aid in finding newer therapeutic choices for prevention and treatment of diabetic neuropathy (Veera *et al*., 2013).

Bax

Bax can form homodimers and can heterodimerise with other Bcl-2-related proteins. The formation of heterodimers between Bax and Bcl-2 homologues with death repressor function (Bcl-2 and Bcl-Xl) leads to the inhibition of the death-promoting effects of Bax. It has been proposed that the relative expression of the different Bcl-2 families of proteins controls the

sensitivity of cells to apoptotic stimuli (Reed, 1994). Bax is the major effector in apoptotic ganglion cell death in the retina after ischaemia, excitotoxicity and axotomy, and in retinal degeneration (Isenmann *et al*., 1999; Chen *et al*., 2003). In recent studies, a significant increase in oxidative stress was demonstrated by increased lipid peroxidation with a decrease in the glutathione levels in liver of diabetic rats. Increased percentages of apoptosis with down-regulation of Bcl-2 and activation of Bax as well as the caspases-3 and 9 were demonstrated in the diabetic rats (Monera, 2016). The hypoxia stimulates apoptosis and inhibits proliferation in the presence of normal glucose, while hyperglycemia significantly attenuates the hypoxic-induced growth response (Gao *et al*., 2007). The activated transduction signals eventually trigger β-cell apoptosis through different mechanisms, including endoplasmic reticulum (ER) stress, modulation of Bcl-2 family proteins, mitochondrial permeabilization and caspase activation (Thomas, 2009; Eizirik, 2008).

A common characteristic of cell death induced after β-cell exposure to pro-inflammatory cytokines or glucolipotoxicity is Bax translocation to the mitochondria, cytochrome c release and activation of the initiator caspase-9, triggering the cleavage of executioner caspases-3 and caspase-7 (Grunnet, 2009; Gurzov, 2010). The high glucose level could lead to endothelial dysfunction by activating the expression of Bax and down regulating Bcl-2 in diabetic patients. A recent report showed that rutin supplementation restored the reduced levels of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glutathione (GSH) and decreased the level of thiobarbituric acid reactive substances (TBARS), which are formed as a byproduct of lipid peroxidation. Additionally, treatment with rutin in the diabetic retina showed anti-apoptotic activity by decreasing the intensity of Caspase 3 and increasing the level of Bcl-2 (Ola *et al*., 2015). In diabetic ketoacidosis, the high production of lactate could further aggravate endothelial dysfunction by reducing oxygen in the tissue microenvironment. The prolonged hyperglycemia induces apoptosis in the endothelial cells of diabetic ulcers, and this will further aggravate

microvasculopathy and delay tissue healing and regeneration of diabetic ulcers (Hasnan *et al*., 2010).

Caspases

β-Cell apoptosis is an important pathological mechanism of β-cell loss in type 1 and type 2 diabetes (Butler *et al.*, 2003; Rhodes, 2005). Although the instigating factors that lead to β-cell destruction in these two types of diabetes are distinct, a common cell death machinery is likely operational. Caspases are the major components of the cell suicide machinery, and the two major pathways are intrinsic or mitochondrial and extrinsic or death receptor-mediated (Danial & Korsmeyer, 2004; Woo *et al*., 1998). The STZ- induced type 1 diabetes model show that loss of Caspase 8 in β-cells protects mice from diabetes development. In a high fat diet-induced diabetes model, glucolipotoxicity from high fat diet feeding induces β-cell apoptosis, which is associated with increased Caspase 3 and Caspase 8 activity (Lupi *et al*., 2002; Marchetti *et al*., 2004).

Caspase 8 may also play a role in selectively removing vulnerable β-cells with aging; thus, in its absence, this vulnerable population of β-cells may accumulate, leading to increased β-cell death (Maedler *et al*., 2006). The Caspase 8 may play a similar role in this regard. Under physiological conditions, Caspase 8 is essential in the maintenance of β-cell mass and in regulating insulin secretion, whereas in the presence of apoptotic stimuli, such as in diabetic conditions, Caspase 8 is required for β-cell apoptosis. Apoptosis of islet beta cells occurs in immune-mediated diabetes. β-cell apoptosis was found to be the final step in the pathogenesis of diabetes (Quan *et al*., 2013). The context-specific diverse functions of Caspase 8 in controlling different facets of β-cell biology raise the importance of a clear understanding of these apoptotic molecules because these are potential therapeutic targets for prevention of β-cell apoptosis in the treatment of diabetes (Nicole Liadis *et al*., 2007).

TNF-α

TNF-α induces insulin resistance and endothelial dysfunction. A study with diabetes rats reported that TNF-α induced microvascular cell apoptosis of diabetes, and enhanced TNF-α in turn increased forkhead box O1 mRNA levels, nuclear translocation, and DNA binding in retinas. The results showed that FoxO1, which regulated cell death and prevented cell cycle progression, could induce cell apoptosis and micro vascular cell loss of diabetes (Behl *et al.*, 2009). The proinflammatory cytokines and chemokines were promoted in diabetes patients with bone fracture or in osteoblasts by the high glucose stimulation. TNF-α and high glucose synergistically reduced the viability and induced apoptosis in the osteoblast-like MG-63 cells *in vitro*. It implies the significant regulatory role of TNF-α in the delayed fracture healing in diabetes (Sun *et al*., 2016). The STZ-induced changes could be reduced or prevented by early treatment with peroxisome proliferator-activated receptor agonists in doses smaller than routinely used to treat diabetes. TNF-α down regulates certain peroxisome proliferator-activated receptors (Beier *et al*., 1997). The increased fat-derived TNF-α was a causative factor in the induction of insulin resistance and diabetes associated with obesity (David, 2000). The diabetes enhanced TNF-α levels decreased fibroblast density and proliferation, and increased fibroblast apoptosis and activation of the pro-apoptotic transcription factor, forkhead box O1 (FOXO1).

TNF-α also has a role in the development of insulin resistance; in fact, it affects insulin sensitivity by changing the phosphorylation of insulin receptor substrate-1 and interferes with the insulin signaling cascade, thereby leading to insulin resistance (Hotamisligil *et al*., 1993; Hotamisligil, 2006). TNF-α is a potent mediator of leukostasis induced by VEGF, interleukin-1α, and platelet-activating factor in the retinal vasculature and it also mediates the cell death/apoptosis of retinal neurons and vascular endothelial cells in diabetic retionpathy (Vinores *et al*., 2007). The retinal leukostasis and apoptosis mediated by TNF-α contribute to blood retinal barrier (BRB) breakdown in diabetic retinopathy. The soluble tumor necrosis factor receptors 1 and 2 (sTNFR1 and sTNFR2) contribute to experimental diabetic kidney disease, a condition which

substantially increases cardiovascular risk. The levels of sTNFRs, and their association with prevalent kidney disease, incident cardiovascular disease, and risk of mortality independently of baseline kidney function and microalbuminuria in a cohort of patients with diabetes studied (Carlsson *et al.*, 2016). The level of soluble TNF receptors increases in the serum and vitreous fluid of patients with proliferative diabetic retinopathy (Limb *et al*., 2001; Limb *et al*., 1999).

When TNF-α is blocked there is improved healing, increased fibroblast proliferation and reduced apoptosis and greater fibroblast density in the diabetic group. Fibroblast proliferation and FOXO1 activity were investigated only in a diabetes model (Michelle *et al*., 2010). It is well known that diabetes causes prolonged inflammation during wound healing (Lioupis, 2005; Singh *et al*., 2005). Over production of TNF-α is thought to contribute to a number of disease processes associated with persistent inflammation and tissue destruction (Ardizzone & Porro, 2005; Graves *et al*., 2006). TNF-α levels are elevated in non-healing ulcers and associated with impaired healing in diabetes mouse models (Goova *et al*., 2001). The soluble TNFRs have been shown to be cross-sectionally associated with lower glomerular filtration rate (GFR), and higher levels of low grade albuminuria in patients with diabetes. High levels of sTNFRs are potentially useful markers of poor prognosis in diabetes, in addition to their potential use as markers of kidney function decline, progression of chronic kidney disease and nephropathy (Carlsson *et al*., 2016) .Chronic elevation of TNF-α has been shown to impair cutaneous wound healing and to cause a decrease in collagen production, while exogenous TNF-α results in a decrease in wound strength (Buck *et al*., 1996; Salomon *et al*., 1991). Moreover, TNF-α is associated with the aetiological processes in both type 1 and type 2 diabetes, as well as diabetic complications (Moller, 2000; Uno *et al*., 2007).

Phospholipase C

A reduction in the cardiac density of α1-adrenoceptors has been demonstrated in diabetes as well as a decreased production of IP3 in response to α1-adrenoceptor stimulation (Tanaka *et al*., 1992; Tanaka *et al*., 1993). Since the α1-adrenoceptor signal is transduced to PLC β isozyme *via* Gqα, and given the role of gLC products in influencing cardiac function, to examine changes in the status of one of the major PLC β-isozymes present in the heart, PLC β3, as well as the assessment of Gqα in an experimental model of STZ-induced type 1 diabetes (James & Downes, 1997; Katan, 1998). This would provide novel information about the PLC β signaling pathway during diabetes (Rebecchi & Pentyala, 2000). The functional significance of the defective PLC β3 activity and diminished levels of IP3 is that it may constitute a mechanism for the reported reduced force of contraction, in response to α1-adrenergic stimulation, of the isolated papillary muscle, although in enhanced inotropic response to α1-adrenergic stimulation in the isolated working heart from diabetic rats (Heijnis & Zwieten, 1992).

A reduced production of PLC β3-derived DAG would affect several cellular processes (Puceat & Vassort, 1996; Kamp & Hell, 2000). While, abnormalities in other signaling pathways occur during diabetes, in particular, the β-adrenoreceptor induced increases in contractions and Ca^{2+} transients, which are markedly diminished by suggesting that an impairment of PLC β3 signaling mechanisms may also significantly contribute to a defective cardiac contractile performance during diabetes (Tamada *et al*., 1998; Ha *et al*., 1999). Furthermore, it is pointed out that depressed activities of other PLC isozymes in diabetic cardiomyopathy have also been observed (Tong *et al*., 1998; Tappia *et al*., 2000). The decreased PLC β3 activity was associated with a decrease in the abundance of PLC β3 protein, however; other mechanisms may also be involved. In this regard, an increased oxidative stress has been shown to occur during diabetic cardiomyopathy (Dhalla *et al*., 1998).

GLUT

The rate of glucose entry into the cell is determined by tissue-specific expression of one or more GLUT proteins. Among all glucose transporters, GLUT 1- 4 are most widely studied and their roles have been well documented as glucose and/or fructose transporters in different tissues and cell types. Physiology of various glucose transporters, of which there are two main types; sodium-glucose linked transporters (SGLTs) and facilitated diffusion glucose transporters (GLUT), which can be divided into many more subclasses. Transporters differ in terms of their substrate specificity, distribution and regulatory mechanisms. Glucose transporters have also received much attention as therapeutic targets for various diseases (Archana *et al*., 2016).

GLUT 1, the major membrane protein, was the first purified membrane transporter. Gene transcription of GLUT 1 is stimulated by glucose deprivation, as well as most mitogens (Baldwin & Lienhard, 1989). GLUT is found predominantly at the endothelium of barrier tissues such as blood vessels and the blood-brain barrier (BBB), however, it is expressed in many other tissues such as kidney and colon with minimal expression in the liver (Birnbaum *et al*., 1986). Medicinal plants rich in flavonoids have shown promising effects in up regulation of GLUT 1 expression levels. Berberin, the major active component of *Rhizoma Coptidis*, has been reported to enhance GLUT 1 expression and promotes its activities (Kim *et al*., 2007; Cok *et al*., 2011). Several studies have shown that an increased expression of glucose transporters in tumor cells is closely linked to the development of tumors. The crystal structure of human glucose transporter. Based on the crystal structure and other relevant biochemical data, a mechanistic model for GLUT 1, giving rise to another major breakthrough in glucose transporter research (Deng *et al*., 2014). In addition, genistein derivatives have been demonstrated promising effects in the treatment of diabetes mellitus.

GLUT 2, this glucose transporter is involved in glucose-sensing in pancreatic β-cells, liver, and hypothalamus as well as triggering the glucose-mediated insulin secretion cascade (Dupuis *et al*., 2010; Mounien *et al*., 2010). Among all glucose transporters, GLUT 2 has the lowest apparent affinity for

glucose. It has low affinity to other monosaccharaides such as galactose, mannose and fructose. However, glucosamine can be transported with a very high affinity (Uldry *et al*., 2002). Sodium-Glucose Transporter 2 (SGLT2) is a high-capacity, low-affinity glucose transporter protein responsible for the movement of glucose molecules across the basolateral membrane of epithelial cells. In kidneys, it also plays a critical role in the absorption of glucose, thereby maintaining the body's glucose homeostasis (Sasseville *et al*., 2014). Sodium-Glucose Transporter 2 (SGLT2) inhibitors are novel therapeutic treatments for improving glucose homeostasis in patients with diabetes. Through reductions in glucose reabsorption by the kidney, they lower serum glucose in patients with diabetes and they improve glucose control whether used alone or in combination with other therapies (Biff *et al*., 2016). SGLT 2 inhibitors reduce the transport maximum for glucose leading to glycosuria and reductions in the serum glucose concentrations, and over the long term, reduce glycohemoglobin levels in patients with diabetes. Recently, some patients receiving SGLT2 inhibitors have been reported to develop ketoacidosis (Henry *et al*., 2015; Taylor *et al*., 2015). A better understanding of the renal response to ketoacidotic states may provide some insight as to why this complication occurs in a subset of patients with diabetes. GLUT 2 has drawn attention as a molecule that could be involved in the pathogenesis of diabetes mellitus. Studies have proven that the GLUT 2 expression is down regulated in pancreatic β-cells, while hepatic expression of this glucose transporter is enhanced in diabetic animal models (Okamoto *et al*., 2002). Hesperidin and naringin have been demonstrated to reduce protein expression of GLUT 2 in the liver of experimental animals (Jung *et al*., 2006).

GLUT 3 also known as neuron-specific glucose transporter is expressed abundantly in mammalian neurons and trophoblasts, with less expression in the cell body (Mueckler & Thorens, 2013; Simpson *et al*., 2008). The predominant site of expression of GLUT 3 is brain. However, lower amounts are expressed in placenta, liver, heart and kidney (Mueckler & Thorens, 2013). The isolated cardiomyocytes of healthy and diabetes rodents showed that GLUT expression on the surface of cardiomyocytes is not insulin-dependent, indicating a role of basal

glucose transporter for GLUT (Waller *et al*., 2013). This tissue distribution is apparently due to the fact that GLUT 3 protein expression occurs more in tissues which exhibit high demand of glucose such as brain. The potential role of maternal hyperglycemia in the stimulation of GLUT 3 expression was evaluated by lowering maternal glycemia. Infusing phlorizin to the diabetic pregnant rats induced a two- to three fold decrease in maternal plasma glucose levels maintained for 5 days (without any concomitant change in insulin levels) (Jansson *et al*.,1994). This resulted in a two-fold decrease in GLUT 3 mRNA and protein levels and suggests that maternal hyperglycemia plays a major role in the increase of GLUT 3 mRNA and protein levels observed in diabetes. A different type of regulation is more likely to prevail in the brain since GLUT 3 protein expression has neither increased by insulinopenic diabetes in adult rats and mice nor in rat fetuses (Atkins *et al*., 1994; Nagamatsu *et al*., 1994).

GLUT 4, the presence of this glucose transporter has also been identified in the brain and kidney (Huang & Czech, 2007). GLUT 4 has also been found to play important role in the growth of malignant cells of multiple myeloma and has been proposed as important therapeutic targets (McBrayer *et al*., 2012). GLUT 4 plays a vital role in glucose-sensing although only 15% of the blood glucose is absorbed by adipose tissue and the remaining 85% by muscle in healthy individuals (Yang *et al*., 2005). Impaired translocation of intracellular GLUT 4 to the plasma membrane refers to insulin resistant. Development of insulin resistance in conjunction with impaired insulin secretion and insulin resistance in the liver plays an important role in the pathogenesis of diabetes (Zhidan *et al*., 1998). Numerous studies have suggested the role of flavonoids and phenolic compounds in enhancement of GLUT 4 expression and glucose uptake. Quercetin and procyanidins have been reported to possess anti diabetic properties by up regulation of mRNA level of GLUT 4 and its translocation to the cell membrane in adipocytes and skeletal muscle cells (Jing *et al*., 2014; Huang & Czech, 2007). The insulin resistance by suppressing GLUT 4 expression and glucose uptake in cardiomyocytes in a PKA-dependent manner, which suggests that the mechanism is present in cardiac muscle (Mangmool *et al*., 2016). Various flavonoids have

shown anti-hyperglycemic effects by increasing mRNA expression levels of GLUT 4 in murine embryonic fibroblast line (Matsuda *et al*., 2011).

CREB

The cAMP response element binding protein (CREB) is a key regulator of glucose metabolism and synaptic plasticity that is canonically regulated through recruitment of transcriptional coactivators (Sang *et al*., 2016). The CREB-regulated transcription coactivators (CRTCs) are a family of proteins that interact with phosphorylated CREB and enhance PGC-1α expression (Bruno *et al*., 2014). CREB was characterized in diabetic rat liver, since it is known to play a major role in mediating the basal transcriptional activity of the PEPCK (Phosphoenol pyruvate carboxykinase) gene as well as the cAMP-dependent stimulation of PEPCK gene transcription, the latter through the phosphorylation of serine 133 of CREB (Gerald *et al*., 1995). The PEPCK promoter contains a DNA sequence termed a cAMP response element (CRE), which acts as a binding site for the CRE-binding protein (CREB) (Roesler *et al*., 1995). CREB is phosphorylated on a specific serine residue by protein kinase A (PKA), leading to the activation of this transcription factor and concomitant stimulation of gene transcription. The BAY 11-7082 (BAY) was used as a pharmacological inhibitor of IκBα (inhibitor of kappa B alpha) phosphorylation to block NF-κB activation. BAY treatment in diabetic rats also increased the phosphorylation of CREB which indicates that the NF-κB activation inhibitor engage a CREB-regulated mechanism *in vivo* (Ashok Kumar & Shyam Sunder Sharma, 2016). Mutation of the CRE results in the loss of cAMP responsiveness, as well as a significant decrease in the basal level of PEPCK gene transcription (Liu *et al*., 1991). A critical role for CREB in the mechanisms that govern specific aspects of the β-cell response to GLP-1 receptor signaling, further validating CREB as a therapeutic target for diabetes (Soona *et al*., 2014). Thus, CREB appears to be a central player in determining the rate of PEPCK gene transcription, and might be expected to mediate the diabetes-related increase in PEPCK gene expression.

Antioxidant enzymes and lipid peroxidation enzymes

Superoxide dismutase (SOD)

Hyperglycemia produces marked oxidation impact as evidenced by a significant increase in lipid profile, lipid peroxidation products, as well as a significant decrease in total superoxide dismutase activity (Kowluru & Kanwar, 2009). Animals treated with *Boerhaavia diffusa* (100, 200, and 400 mg/kg) significantly alleviated hyperglycaemia-induced mechanical, thermal hyperalgesia and cold allodynia; restored the reduced body weight and improved the biochemical parameters such as blood sugar levels, superoxide dismutase, and total protein and attenuated the calcium concentration and lipid peroxidation in a dose dependent manner. Thus, this study concludes that *Boerhaavia diffusa* exhibits significant antidiabetic, antioxidant and neuroprotective activities against streptozotocin-induced diabetic neuropathy in rats (Sandeep *et al*., 2016). SOD 3, or extracellular superoxide dismutase (EC- SOD), is the predominant extracellular antioxidant enzyme. The individuals with the Ala40Thr EC-SOD genotype are at increased risk in developing diabetes, mainly cardiovascular complications. The Ala40Thr genotyping is a possible marker for diabetes prediction, and for monitoring disease progression (Nahed *et al*., 2016). The auto oxidation of glucose results in the formation of hydrogen peroxide (H_2O_2), which inactivates SOD (Fajans, 1995). The activity of SOD decreases in erythrocytes and also due to ageing in diabetic subjects which could be due to increase in the glycation of superoxide dismutase. The important role of superoxide dismutase and its genotype distribution in diabetic patients with and without retinopathy, suggests the need for antioxidant supplements to delay the severity of diabetic retinopathy (Ahmed *et al*., 2013).

The circulating levels of SOD 1 in adolescents with Type 1 diabetes seems to be protective against endothelial dysfunction, the low SOD 1-levels being a susceptibility marker for diabetic vascular complications (Suys *et al*., 2007). The SOD activity in peripheral blood cells is reduced in the diabetic patients with diabetic nephropathy as compared with those without diabetic complications (Ezeiruaku & Micheal, 2015). Tubular cell and podocyte apoptosis

is an early event in diabetic neuropathy, and the simultaneous release of SOD 1 and cytochrome C regulates the mitochondrial apoptosis (Li *et al*., 2009). Hypertension and the rennin-angiotensin system are key factors in diabetic neuropathy, closely related to SOD 1 levels (Tang *et al*., 1997). The fibrosis mediated by transforming growth factor-beta (TGF-β) is the corner-stone of glomerulosclerosis in diabetic neuropathy, and SOD 1 is a strong antifibrotic agent, lowering the TGF-β1-expression (Vozenin-Brotons *et al*., 2001). The insulin resistance is increased in patients with nephropathy in inversely proportion to glomerular filtration rate and seems to be the most important predictor for the development of diabetic neuropathy (Thorn *et al*., 2005; Trevisan *et al*., 1998).

Glutathione peroxidase (Gpx)

Antioxidants have been reported to prevent the development of diabetic complications. Oral magnesium treatment in diabetes has also been reported to potentiate the antioxidant defense system. The glutathione antioxidant system (total glutathione, reduced glutathione, glutathione peroxidase) was investigated in experimental diabetic rats treated orally with magnesium (Ige *et al*., 2016). Magnesium has been reported to be directly involved in intracellular antioxidant defense mechanism by increasing the activity of the enzyme, glutathione peroxidase (GPx). This enzyme has been reported to increase the rate of reaction between glutathione and free radicals, particularly toxic hydrogen peroxide (Yavuz & Mollaoglu, 2013). Glutathione peroxidase-1 (GPx1) is a major and ubiquitously expressed antioxidant enzyme present in the cytosol and mitochondria. It is involved in the detoxification of hydrogen and lipid peroxides and acts as a peroxynitrite reductase (Sies *et al*., 1997; Brigelius-Flohe *et al*., 2003). In the absence of this antioxidant enzyme, a buildup of ROS occurs that is known to damage DNA, proteins, and lipids (Sies *et al*., 1997). In mammals, glutathione peroxidase family (Gpx) is the main system of antioxidative defense (Kuzuya *et al*., 2008). Gpx-1 is the most abundant Gpx isoenzyme, being responsible for 96% of the Gpx antioxidative activity at the renal level, its expression being increased in conditions of hyperglycemia, in contrast with the

other important antioxidant enzymes (catalase, superoxide dismutase 1, superoxide dismutase 2, thioredoxine, thioredoxin reductase), which are not expressed in a different way (De Haan *et al*., 1998; Morrison *et al*., 2004).

Selenium deficiency significantly decreases the Gpx-1 activity, leading to increased oxidative stress and acceleration of renal damages in conditions of hyperglycemia (Reddi & Bollineni, 2001). In contrast to this, the selenium supplementation is accompanied by an increase in Gpx-1 expression and the diminution of renal injury in patients with diabetes (Douillet *et al*., 1996). Gpx-1 overexpression is associated with hyperglycemia, hyperinsulinemia, accumulation of adipose tissue and increase of leptin levels, in spite of the insulinomimetic effect of selenium, which is rather probable to be mediated through the intermediary of selenoproteins (Chen *et al*., 2003). Hesperidin is effective in decreasing oxidative stress by increasing the activities of antioxidant enzymes SOD and GPx and limiting lipid peroxidation process (Mostafa & Abd-Ellah, 2014). Hesperidin shows antioxidant activity and radical scavenging properties where hesperidin reduces superoxide ions in electron transfer plus concerted proton transfer reaction (Kuntic et *al*., 2014). In cinnamaldehyde treated diabetic rats, cinnamaldehyde plays antioxidant role due to decreased levels of lipid peroxidation products and increased activity of antioxidant enzymes, SOD and GPx. This activity may be due to the inhibition of glycation of these enzymes (Subash et *al*., 2014). Cinnamaldehyde has an effect on the antioxidant status of diabetic rats where cinnamaldehyde displays a strong antioxidant activity towards DPPH, hydrogen peroxide, and superoxide radicals (Haripriya *et al*., 2013). The Gpx-1 superexpression induced by vascular pressure leads to increased production of hydrogen peroxide, which, in the absence of expression, increases the activities of the other scavenger enzymes, triggering the activation of pro-atherogenic genes (Wagner *et al*., 2009). ROS production induced by hyperglycemia seems to be the key-event in pathogenic pathway activation in diabetic nephropathy. The association of Pro200Leu (known also as Pro198Leu) polymorphism in Gpx-1 gene with risk to develop diabetic neuropathy, in type 1 diabetes (T1D) (Nicolae *et al*., 2011).

Gpx is one of the antioxidant enzymes that protect, prevent or reduce the level of oxidative damage to cells in the body. Decreased activity of this antioxidant enzyme may increase the susceptibility of diabetic patients to oxidative injury. Adequacy of the enzyme help in preventing some clinical complications associated with diabetes (Ezeiruaku & Udenwoke, 2016). Under normal conditions, free radicals are formed in minute quantities and are rapidly scavenged by natural cellular defense mechanisms comprising of enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) etc. An increased production of malondialdehyde (MDA), a marker for lipid peroxidation has been found in erythrocyte membrane of diabetic patients together with depressed erythrocyte content i.e. antioxidant enzymes (AOEs) and reduced glutathione (GSH) (Nagasaka *et al*., 1989). The enzymatic antioxidant activities of superoxide dismutase, catalase and glutathione peroxidase decrease in liver, kidney and heart tissues of patients with diabetes mellitus, the levels of the ROS such as superoxide anion radicals increase (Asayama *et al*., 1989; Giugliano *et al*., 1995). These alterations suggest that free oxygen radicals and antioxidant mechanisms might play an important role in the pathogenesis of diabetes.

In the present study, we investigated the antidiabetic activities of Baclofen, Vitamin E and Gymnemic acid in STZ-induced type 1 diabetic Wistar rats. The capability of Baclofen, Vitamin E and Gymnemic acid to increase β-cell proliferation was studied by radio ligand incorporation studies. The molecular neurobiological mechanisms behind the treatment-induced β-cell compensatory response against hyperglycemia was assessed by studying the gene expression of key markers of β-cell survival, neuronal regeneration and differentiation. The GABAergic and muscarinic receptor subtype functional regulation in the cerebral cortex, cerebellum, brain stem and pancreas was studied to understand the sympathetic and parasympathetic neuronal cell signaling during diabetic and anti-diabetic conditions. The expression of key cell signaling transcriptional proteins and antioxidant enzymes were evaluated to find intracellular signaling pathways present in pancreas and brain regions. These anti-diabetic compounds modulate

the expression of various molecular targets, such as transcription factors, enzymes, cytokines, cell cycle proteins, receptors and adhesion molecules. The muscarinic and GABAergic receptor subtypes functional regulation correlated with Baclofen, Vitamin E and Gymnemic acid-mediated anti-diabetic property will facilitate an important therapeutic goal for the treatment of diabetes and its complications.

Materials and Methods

CHEMICALS USED IN THE STUDY AND THEIR SOURCES

Biochemicals

Baclofen, α-tocopherol (Vitamin E), Streptozotocin, propranolol, atropine, pirenzepine, 4-DAMP mustard (4-deoxy acetyl methyl piperidine mustard), fetal calf serum (heat inactivated), poly L lysine, collagenase type XI, bovine serum albumin fraction V, RPMI-1640 medium, HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid), Hanks Balanced Salt Solution (HBSS), ethylene diamine tetraacetic acid (EDTA), Triton X-100, Tris HCl, sucrose, D-glucose, citric acid, magnesium chloride, calcium chloride and paraformaldehyde (PFA) were purchased from Sigma Chemical Co., St. Louis, USA. Tissue freezing medium Jung was purchased from Leica Microsystems Nussloch GmbH, Heidelberger, Germany. Glucose estimation kit was obtained from Merck, New Jersey, USA. All the other analytical grade reagents were purchased locally from SRL, Mumbai, India. The bioactive compound, Gymnemic acid was purchased from Synthite Industries Ltd, Synthite Valley, Kolenchery, Kerala.

Radiochemicals

Quinuclidinyl benzilate, L-[Benzilic-4, 4'-^{3}H]-[4-^{3}H] (Sp. Activity 42 Ci/mmol), and 4-DAMP, [N-methyl-^{3}H] (Sp. Activity 83 Ci/mmol) was purchased from NEN life sciences products Inc., Boston, U.S.A. [^{3}H] baclofen (Sp. Activity 42.9 Ci/mmol), [^{3}H] Gamma aminobutyric acid (Sp. Activity 76.2 Ci/mmol), [^{3}H] thymidine (Sp. Activity 18.0 Ci/mmol) and [^{3}H] leucine (Sp. Activity 63.0 Ci/mmol) were purchased from Amersham Bioscience, USA.

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 were purchased from Applied Biosystems, Foster City, CA, USA. Muscarinic M1 receptor (Rn_00589936), muscarinic M3 receptor (Rn_00788315), choline acetyltransferase (Rn_01453446), acetyl cholinesterase (Rn_00596883), $GABA_B$ (Rn_00578911), insulin receptor (Rn_00567070), CREB (Rn_00561126), phospholipase C (Rn_01647142), SOD (Rn01477289), Bax (Rn_01480160), Pdx-1(Rn_00755591), Akt-1 (Rn_00583646), NF-κB (Rn_01399583), TNF-α (Rn_99999017), Caspase-8 (Rn_00574069), GLUT 3 (Rn_00567331), superoxide dismutase (Rn_01477289) and glutathione peroxidase (Rn_00577994) primers were used for the gene expression studies.

Confocal Dyes

Rat primary antibody for muscarinic M1 (Cat. No. 087k1395), M3 (Cat. No. 126k1205), and FITC coated secondary antibody (Cat. No. AP307R) were purchased from Sigma Aldrich and Chemicon, USA. The secondary antibody cy5® (Cat. No. ab97077) was purchased from abcam, USA. TO-PRO®-3 iodide stain (Cat. No. 642/661) was used to stain tissue sections, and was purchased from life Technologies CA, USA. Peroxide and antioxidant detection assay kits were purchased from Sigma Aldrich and Chemicon, USA.

ANIMALS

Adult male Wistar rats of 180-240g body weight purchased from Kerala Agricultural University, Mannuthy, India and Amrita Institute of Medical Sciences, Kochi, India were used for all experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care procedures were in accordance with Institutional, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA – Reg. No:

383/01/a/CPSCEA) and the National Institute of Health guidelines. This is sanctioned the project "*neurotransmitter receptor functional interrelationship in diabetic rats and other animal models*" has been approved by the IAEC. The sanctioned letter is attached herewith in this thesis.

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). Control rats were injected with citrate buffer.

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. Estimation of diabetic condition was evaluated by measuring the Fasting blood glucose level. Food pellets were removed 3 hours before blood sampling.

DETERMINATION OF ANTI-DIABETIC POTENTIAL OF BACLOFEN, VITAMIN E AND GYMNEMIC ACID

Animals used in this study were randomly divided into the following groups. Each group consisted of 6-8 animals.

a) Group 1: Control (given citrate buffer injection) (C)
b) Group 2: Diabetic (D)
c) Group 3: Diabetic rats treated with Insulin (D+I)
d) Group 4: Diabetic rats treated with Baclofen (D+B)
e) Group 5: Diabetic rats treated with Vitamin E (D+E)
f) Group 6: Diabetic rats treated with Gymnemic acid (D+G)

The insulin treated diabetic group (Group 3) received subcutaneous injections (1Unit/kg body weight) of insulin daily during the entire period of the experiment. A mixture of both Lente and Plain insulin (Abbott India) were given for the better control of glucose (Sasaki & Bunag, 1983). The last injection was given 24 hr before sacrificing the diabetic rats. D+B group received an oral dose of Baclofen (1mg/Kg body weight). D+E group received a subcutaneous injection of Vitamin E (10 mg/kg body weight). D+G group received an oral dose of gymnemic acid (200mg/kg body weight). Blood samples were collected from the tail vein at 0 hours (Before the start of the experiment), 3rd, 7th, 10th and 14th, 17th, 21th day and the glucose levels were estimated. Blood samples were collected 3hrs after the administration of morning dose. Changes in the body weight of animals were monitored on 1st day (before the start of the experiment), 7th and 14th day.

SACRIFICE AND TISSUE PREPARATION

The animals were then sacrificed on 21th day by decapitation. The cerebral cortex, cerebellum, brain stem and pancreas were dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966) and the pancreas 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.

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

The hydrogen peroxide formed in this reaction reacts with 4-amino antipyrine 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.

ISOLATION OF PANCREATIC ISLETS

Twenty one days after the beginning of STZ-induced diabetic experimental animal models, pancreatic islets were isolated from all the experimental groups by standard collagenase digestion procedure using aseptic techniques (Howell & Taylor, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers *et al*., 1985). The pancreas from the rats were aseptically dissected out into a sterile Petri dish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2 mL collagenase type XI solution (1.5 mg/mL in HBSS, pH 7.4). The collagenase digestion was carried out for 15 minutes at 37°C in a shaker with vigorous shaking (300 RPM/minute). The tissue digest was filtered through a 500 µm nylon screen and the filtrate was washed thrice by successive centrifugation and re-suspension in cold HBSS. The pancreatic islet preparation with a viability of >90%, as assessed by trypan blue exclusion was chosen for experiments.

[^{3}H] THYMIDINE AND [^{3}H] LEUCINE INCORPORATION STUDIES

150 µL of pancreatic beta cell suspension (cell density of 1.6×10^5 cells/cm^2) was added to a poly L-lysine coated glass slide. The cells were incubated for 24 hours at 37 °C in 5% CO_2 atmosphere. Before incubation, [^{3}H] leucine of specific activity 63 Ci/mmol was added to one set of culture plates for all the five experimental groups to determine the protein synthesis and [^{3}H]

thymidine of specific activity 18 Ci/mmol to the next set of plates to determine the measurement of DNA synthesis. All the experiments were done in triplicates. The cells were scrapped off from the culture plates and centrifuged at 2000 x g for 20 minutes. The supernatant was discarded and the pellet was resuspended in 50 µL, 1M NaOH and kept overnight. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

MUSCARINIC RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS

Binding studies in the Brain regions

Total muscarinic, muscarinic M1 and M3 receptor binding studies

[^{3}H] QNB and [^{3}H] DAMP binding assay in cerebral cortex, cerebellum, brain stem and pancreas were done according to the modified procedure of Yamamura & Snyder (1981). Brain tissues were homogenized in a polytron homogenizer with 20 volumes of cold 50mM Tris-HCl buffer, containing 1mM EDTA (pH 7.4). The supernatant was then centrifuged at 30,000xg for 30 minutes and the pellets were resuspended in appropriate volume of Tris-HCl-EDTA buffer.

Total muscarinic and muscarinic M1 receptor binding parameter assays were done using [^{3}H] QNB (0.1-2.5nM) and M3 receptor using [^{3}H] DAMP (0.01-5nM) in the incubation buffer, pH 7.4 in a total incubation volume of 250µL containing appropriate protein concentrations (200-250µg). The non-specific binding was determined using 100µM atropine for total muscarinic, pirenzepine for muscarinic M1 and 4-DAMP mustard for muscarinic M3 receptor. Total incubation volume of 250 µL contains 200-250µg protein concentrations. Tubes were incubated at 22°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments.

$GABA_B$ RECEPTORS BINDING STUDIES USING [^{3}H] BACLOFEN

[^{3}H] Baclofen binding to $GABA_B$ receptor in the membrane preparations were assayed (Hills *et al*., 1987). Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.4 containing 2 mM $CaCl_2$ and 0.3 - 0.4 mg protein. In saturation binding experiments, 10-100nM of [^{3}H] baclofen was incubated with and without excess of 100 µM unlabelled Baclofen. The incubations were carried out at 20ºC for 20 minutes. The binding reactions were terminated by centrifugation at 14000xg for 10 minutes. The dried pellet was resuspended and 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 a spectrophotometer at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data were 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 radio ligand 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 EXPERIMENTAL RATS

Preparation of RNA

RNA was isolated from pancreas and different brain regions–cerebral cortex, cerebellum and brain stem of experimental rats using TRI reagent from Sigma Chemical Co., St. Louis, USA.

Isolation of RNA

Tissue (25-50) mg 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 $\geq$ 1.7. The concentration of RNA was calculated by measuring absorbance at 260 nm. An absorbance of 1 unit
at 260 nm corresponds to 42 µg of RNA.

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 muscarinic M1, M3, insulin, $GABA_B$ receptors, acetylcholine esterase, choline acetyl transferase, super oxide dismutase, Bax, Caspase 8, Akt-1, Gpx, phospholipase C, GLUT 3 and CREB. 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 thermo cycling profile conditions were as follows:

50ºC -- 2 minutes --- Activation

95ºC -- 10 minutes --- Initial Denaturation

95ºC -- 15 seconds --- Denaturation

50ºC -- 30 seconds --- Annealing } 40 cycles

60ºC -- 1 minute --- 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 ($2^{-\Delta\Delta}C\ T$).

PEROXIDE DETECT™ ASSAY

Principle of the assay

Peroxide detection assay was done in order to measure the amount of peroxides in the samples. It gives an idea about the degree of free radicals present in the test samples. The principle of the test is:

$Fe2^{+}$ + Peroxides $\xrightarrow{\text{acidic pH}}$ Fe^{3+}

Fe^{3+} + Xylenol Orange (XO) $\longrightarrow$ Colored product (which can be observed at 560 nm.)

The final product is stable for several hours after the initial color development of 30 minutes. H_2O_2 standard curve was prepared by placing 0, 10, 20, 40, 60, and 80 µL of the 100 mM standard hydrogen peroxide solution. Each tube was brought up to a final volume of 100 ml with de-ionized water. 10 µL of the test sample was added. The final volume was made up to 100 µL with deionized water. 1 ml

of the working colour reagent was added to each tube, mixed, and incubated at 25°C for ~30 minutes until colour formation is complete. The absorbances of standard and test samples were measured at 560 nm in a spectrophotometer using water as a reference. Standard curve of nmoles of H_2O_2 against A_{560} was plotted and the nmoles of peroxide in the test sample were calculated.

ANTIOXIDANT ASSAY

Principle of the assay

The principle of the antioxidant assay is formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation, ABTS·+, a soluble chromogen that is green in color and can be determined spectrophotometrically at 405 nm.

$$HX\text{-}FeIII + H_2O_2 \cdot X\text{-}[FeIV{=}O] + H_2O$$

$$ABTS + \cdot X\text{-}[FeIV{=}O] \; ABTS\cdot + HX\text{-}FeIII$$

In this equation, HX-FeIII is metmyoglobin and ·X-[FeIV=O] is ferryl myoglobin

Antioxidants suppress the production of the radical cation in a concentration dependent manner and the color intensity decreases proportionally. Troloxe, a water-soluble vitamin E analog, serves as a standard or control antioxidant.

Procedure of the assay

The Trolox standards were prepared for a standard curve. ABTS Substrate working solution was prepared by adding 25 ml of 3% Hydrogen Peroxide Solution to 10 ml of ABTS Substrate Solution. In tubes for the Trolox standard curve, 10 ml of a Trolox Standard and 20 ml of Myoglobin working solution was

added. In tubes for the test Samples, 10 ml of Test Sample and 20 ml of Myoglobin working solution was added. 150 µL of ABTS substrate working solution was added to each tube and incubated for 5 minutes at room temperature. After incubation, 100 µL of Stop solution was added to each well. The endpoint absorbance at 405 nm was determined using a plate reader.

IMMUNOHISTOCHEMISTRY OF BRAIN REGIONS AND PANCREAS BY TO-PRO®-3 IODIDE STAIN USING CONFOCAL MICROSCOPE

The experimental rats were deeply anesthetized and were transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS (Chen *et al*., 2007). After perfusion, pancreas and brain regions and pancreas from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30% sucrose solution in PBS. 10 µm sections of pancreas and brain regions were cut using Cryostat (Leica, CM1510 S). The TO-PRO®-3 Iodide Stain (1:250 dilution in PBST) were added to the respective sections and incubated for 1 hr at 4 °C. After 2 hrs of incubation, the tissue slices were rinsed with PBS. The sections were observed and photographed using Confocal imaging system (Leica TCS SP5 Laser Scanning Confocal Microscope). Quantification was done using Leica application suit advanced fluorescence (LASAF) software by considering the mean pixel intensity of the image. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. The nuclear densities of the experimental groups are correlated with mean pixel intensity patterns. All the imaging parameters in the Confocal imaging system like photomultiplier tube (PMT), pinhole and zoom factor were kept the same for imaging the sections of all experimental groups.

IMMUNOHISTOCHEMISTRY OF MUSCARINIC M1, M3 RECEPTORS IN THE BRAIN REGIONS OF EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

The experimental rats were deeply anesthetized and were transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS (Chen *et al*., 2007). After perfusion, pancreas and brain regions from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30% sucrose solution in PBS. 10 μm sections of pancreas and brain regions were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked with Phosphate buffered saline with Triton X-100 (PBST) containing 5% normal goat serum for 1 hour. The primary antibodies of muscarinic M1 receptor and muscarinic M3 receptor (1:400 dilution in PBST with 5% normal goat serum) were added to the respective sections and incubated overnight at 4°C. After overnight incubation, the tissue slices were rinsed with PBS and incubated with fluorescent labelled secondary antibody prepared in PBST with 5% normal goat serum at 1:1000 dilution for 2 hours in room temperature. The sections were observed and photographed using Confocal imaging system (Leica TCS SP5 Laser Scanning Confocal Microscope). Quantification was done using Leica application suit advanced fluorescence (LASAF) software by considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of receptors specific to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system like photomultiplier tube (PMT), pinhole and zoom factor were kept the same for imaging the sections of all experimental groups.

IMMUNOCYTOCHEMISTRY OF MUSCARINIC M1, M3 RECEPTORS EXPRESSION IN THE PANCREAS OF EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Pancreatic islets were isolated from control and experimental rats by standard collagenase digestion procedures using aseptic techniques (Howell & Taylor, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers *et al.*, 1985) with the following

composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 1mM KH_2PO_4, 14.3mM $KHCO_3$ and 10mM HEPES. The pancreas was aseptically transferred to a sterile glass vial containing 2.0ml collagenase type XI solution (1.5 mg/ml in HBSS), pH 7.4. The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300rpm/minute). The tissue digest was filtered through 500 μm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS. The pancreatic islet preparation having a viability of >90% was assessed by Trypan Blue. The islets were seeded in culture wells and allowed to adhere to the plate. The islets were rinsed with PBS and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH- 7.0., for 30 minutes on ice. After fixation, the islets were washed thrice with blocking buffer containing 0.1 M phosphate buffer, pH- 7.0., 0.1% Triton X and 10% BSA. Then the islets were incubated with primary antibody for muscarinic M1, M3 receptors diluted in PBST at 1: 1000 dilution), prepared in blocking buffer with 1% serum and incubated overnight at 4°C. After the incubation, the islets were washed thrice with blocking buffer. Then the islets were incubated with secondary antibody tagged with cy5® (Cat. No. ab97077, abcam, diluted in PBST at 1: 1000 dilution)

diluted in blocking buffer with 1% serum and incubated at room temperature in dark for two hours. After incubation the islets were rinsed with blocking buffer and were observed and photographed using confocal imaging system (Leica SP 5). The specificity of the immunocytochemical procedure is validated by negative controls to ensure that the labelling method accurately identifies the antibody bound to the specific muscarinic M1, M3 receptors in the pancreatic islets.

Expressions of muscarinic M1, M3 receptors were analysed using pixel intensity method.

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

The body weight was significantly decreased ($p<0.001$) in the diabetic rats when compared to control group. After insulin treatment, Baclofen, Vitamin E and Gymnemic acid supplementation for 21 days, the body weight reversed to near the initial body weight. In D+B, there was a significantly ($p<0.001$) decreased body weight when compared to control and D+I group, but significantly increased when compared to diabetic group. D+E group showed a significantly ($p<0.001$) decreased body weight when compared to control, but significantly increased when compared to diabetic and D+I group. D+G group showed a significantly ($p<0.001$) decreased body weight when compared to control, but significantly increased when compared to diabetic and no significant change when compared to D+I group (Figure- 1, 2 & Table-1, 2).

BLOOD GLUCOSE LEVEL

Blood glucose level of all rats before streptozotocin administration was within the normal range. Streptozotocin administration led to a significantly increased ($p<0.001$) blood glucose level of diabetic group when compared to control group. Insulin, Baclofen, Vitamin E and Gymnemic acid treatments were able to significantly reverse ($p<0.001$) the increased blood glucose level to near the control level when compared to diabetic group. In D+B, there was a significantly ($p<0.001$) increased blood glucose when compared to control and D+I group, but significantly decreased when compared to diabetic group. D+E group showed a significantly ($p<0.001$) increased blood glucose when compared to control and D+I group, but significantly decreased when compared to diabetic group. D+G group showed a significantly ($p<0.001$) increased blood glucose level when

compared to control and D+I group, but significantly decreased when compared to diabetic group (Figure- 3, 4 & Table- 3, 4).

Table-1 & 2

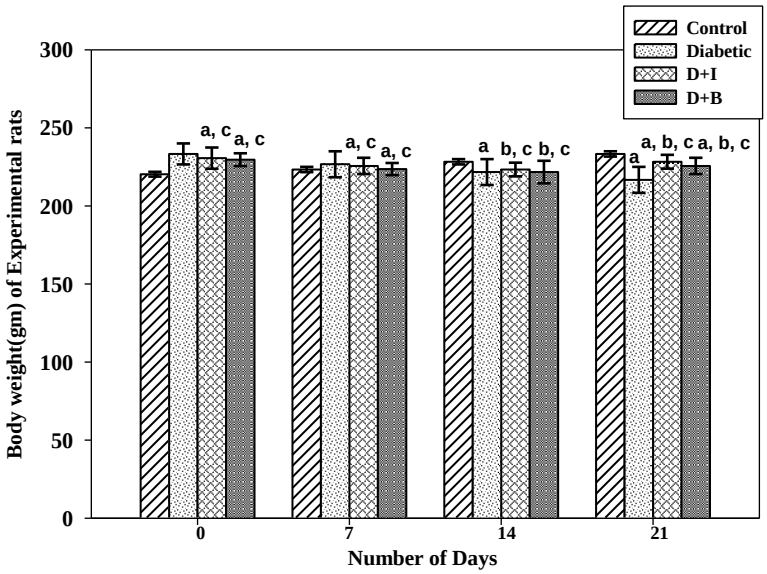
Body weight (gm) of Experimental rats

Experimental groups	0 day	7th day	14th day	21th Day
Control	**220±1.5**	**223.3±1.7**	**228.3±1.7**	**233.3±1.7**
Diabetic	**233.3±6.7**	**226.7±8.3[a]**	**221.7±8.3[a, b]**	**216.7±8.3[a, b]**
D+I	**230.6±6**	**225.6±5.2[b, c]**	**223.3±4.4[a,c]**	**228.3±4.4[b, c]**
D+B	**229.6±4.1**	**223.6±3.9[a, c]**	**221.7±7.2[a, c]**	**225.6±5.2[a, b]**
D+E	**233.3±4.4**	**226.3±4.4[b, c]**	**226.2±2.8[a, b]**	**229.6±4.4[a, b]**
D+G	**229.8±5.8**	**225.3±7.6[a, b]**	**224.9±3.7[b, c]**	**228.5±4.4[a, c]**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]$p<0.001$ when compared with control; [b]$p<0.001$ when compared with initial weight, [c]$p<0.001$ when compared with diabetic group. C- Control rats, D- Diabetic rats, D+I- Insulin treated diabetic rats, D+B- Baclofen treated diabetic rats, D+E- Vitamin E treated diabetic rats and D+ G- Gymnemic acid treated diabetic rats.

Figure-1 & 2

Body weight (gm) of experimental rats



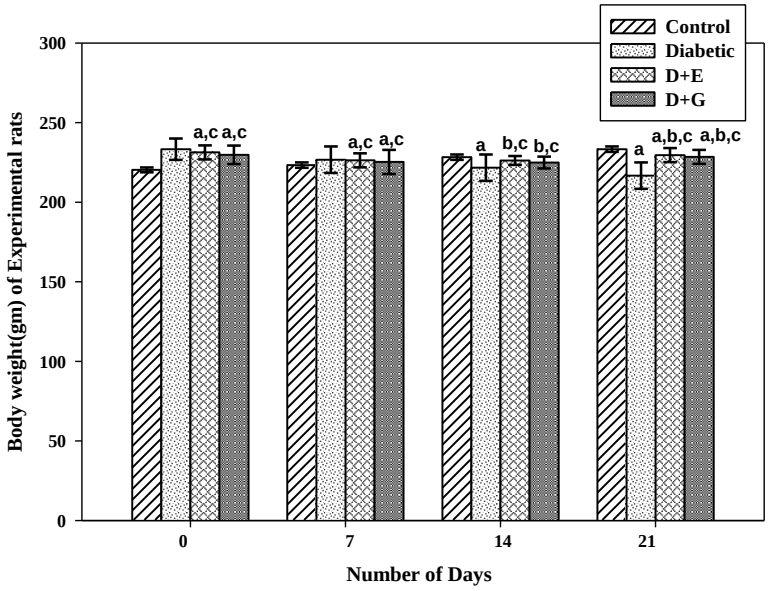
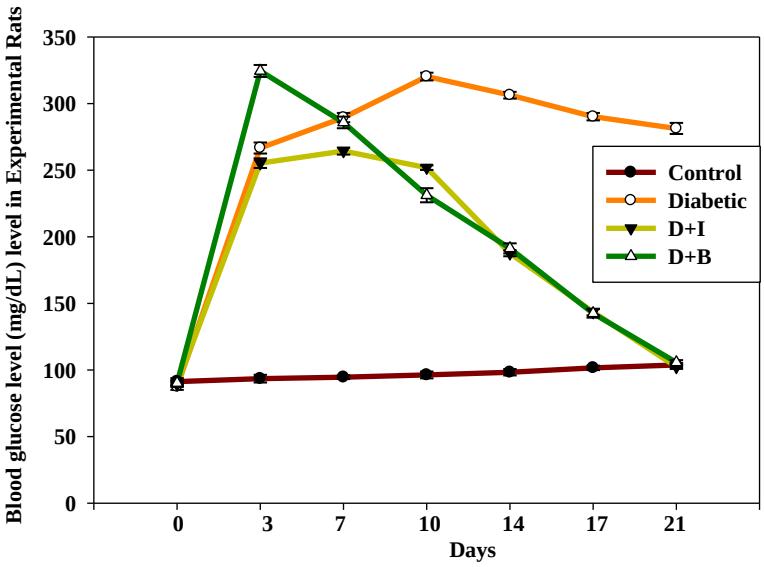


Figure- 3 & 4

Blood glucose (mg/dL) level in the Experimental rats



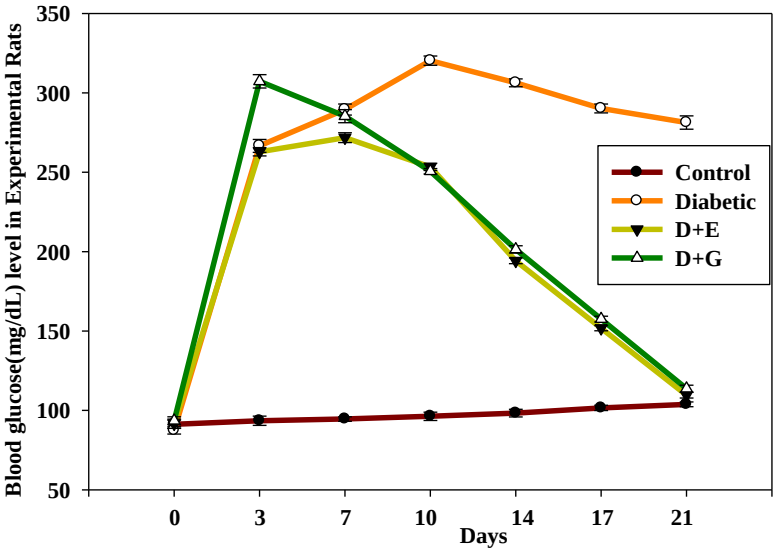


Table-3

Blood glucose (mg/dL) level in the Experimental rats

Experimental groups	0 day (before STZ injection)	3rd Day (initial)	7th day	10th day	14th day	17th day	21th day
Control	91.3±2.6	93±2.9	94.6±1.4	96.3±2.6	98.3±2.4	101.6±1.7	103±1.15
Diabetic	87.4±2.3	266.6±4.1[a]	289.5±3.5 [a]	320.3±2.9[a]	306.3±2.5[a]	290.2±2.8 [a]	281.3±4.2[a]
D+I	89.1±1.8	255±3.6[a]	264.3±2.6[a]	251±1.7[a, b]	187.6±2.2[a, b]	143.5±2.5 [a, b]	102.3±1.5[c]
D+B	90.2±2.1	324.5±4.5[a, c]	285.8±4.3[a, b]	231.2±5.3[a, c]	191.3±3.8[a, c]	142.5±3.2[a, b]	107.9±1.7[a, b]

Table-4

Experimental groups	0 day (before STZ injection)	3rd Day (initial)	7th day	10th day	14th day	17th day	21th day
Control	91.3±2.6	93±2.9	94.6±1.4	96.3±2.6	98.3±2.4	101.6±1.7	103±1.15
Diabetic	87.4±2.3	266.6±4.1[a]	289.5±3.5 [a]	320.3±2.9[a]	306.3±2.5[a]	290.2±2.8 [a]	281.3±4.2[a]
D+E	91.6±2.9	262±2.6[a, c]	271.8±3.1[a, c]	253±2.3[a, b]	194.2±1.8[a, b]	151.8±1.6 [a, b]	109.6±1.8[a, c]
D+G	93.5±2.5	307.3±4.2[a, b]	285.3±4.1[a, b]	250.6±1.7[a, c]	201.4±2.3[a, b]	157.5±1.8[a, c]	105.3±2.2[a, b]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001,[b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001,[e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Diabetic +Insulin Treated group, D+B- Diabetic + Baclofen Treated group, D+E- Diabetic + Vitamin E Treated group and D+G- Diabetic + Gymnemic acid Treated group.

Acetylcholine esterase, Choline acetyltransferase ,$GABA_B$, Insulin receptors, Akt-1, Pdx-1, Bax, Caspase 8, Glutathione peroxidase (Gpx), Superoxide dismutase (SOD), GLUT 3, Phospholipase C, CREB, NF-κB, TNF-α gene expressions and total antioxidant, total aqueous peroxide levels in the brain regions and pancreas of experimental rats.

CEREBRAL CORTEX

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the cerebral cortex of experimental rats

The total muscarinic receptor status was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist atropine. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) decreased significantly in diabetic rats compared to control group. In D+B, showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased (B_{max} ($p<0.001$) and no significant change in K_d when compared to diabetic and significantly decreased {(B_{max} ($p<0.05$) and K_d ($p<0.05$)} when compared to D+I group. D+E group showed that significantly decreased B_{max} ($p<0.05$) and no significant change in K_d when compared to control, but significantly increased (B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group {(B_{max} ($p<0.001$) and K_d ($p<0.05$)}. D+G group showed that significantly decreased (B_{max} ($p<0.001$) and no significant change in K_d when compared to control, but significantly increased {(B_{max} ($p<0.001$) and K_d ($p<0.05$)} when compared to diabetic and D+I group {(B_{max} ($p<0.05$) and K_d ($p<0.05$)} **(**Figure-5, Table-5).

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the cerebral cortex of experimental rats

Binding analysis of muscarinic M1 receptor was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The Scatchard analysis showed that the B_{max} ($p<0.001$) K_d ($p<0.001$) decreased significantly and in diabetic group when compared to control group. In D+B, showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and no significant change in K_d when compared to diabetic and significantly decreased (B_{max} ($p<0.001$) and no significant change in K_d when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) significantly decreased and no significant change in K_d when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group {(B_{max} ($p<0.001$) and K_d ($p<0.001$)}. D+G group showed that B_{max} ($p<0.05$) and K_d ($p<0.05$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.05$) and no significant change in K_d when compared to diabetic and significantly increased (B_{max} ($p<0.001$) and K_d ($p<0.05$) when compared to D+I group {Figure-6, Table-6).

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor, antagonist, 4-DAMP mustard in the cerebral cortex of experimental rats

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) increased significantly in diabetic group when compared to control group. In D+B, showed that B_{max} ($p<0.001$) increased significantly and no significant

change in K_d when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to diabetic and significantly increased B_{max} ($p<0.001$) and no significant change in K_d when compared to D+I group. D+E group showed that significantly increased B_{max} ($p<0.001$) and no significant change in K_d when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.05$) significantly decreased when compared to diabetic and significantly increased B_{max} ($p<0.001$) and no significant change in K_d when compared to D+I group. D+G group showed that B_{max} ($p<0.05$) and K_d ($p<0.001$) significantly increased when compared to control, but significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and increased when compared to D+I group {(B_{max} ($p<0.001$) and K_d ($p<0.001$)}. **(**Figure-7, Table-7).

$GABA_B$ receptor analysis

Scatchard analysis of [^{3}H] baclofen binding against baclofen to $GABA_B$ receptors in the cerebral cortex of experimental rats

Binding analysis of $GABA_B$ receptor was done using [^{3}H] baclofen. The Scatchard analysis showed that the significantly decreased B_{max} ($p<0.001$) and increased K_d ($p<0.001$) in diabetic group when compared to control. In D+B, showed that significantly decreased B_{max} ($p<0.001$) and increased K_d ($p<0.001$) when compared to control, but significantly increased B_{max} ($p<0.001$) and no significant change in K_d when compared to diabetic and significantly increased B_{max} ($p<0.001$) and K_d ($p<0.05$) when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to diabetic and significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to D+I group. D+G group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and

decreased K_d ($p<0.001$) when compared to diabetic and significantly decreased (B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to D+I group. **(**Figure-8, Table-8).

REAL TIME-PCR ANALYSIS

Real Time-PCR analysis of acetylcholine esterase in the cerebral cortex of experimental rats

Gene expression of acetylcholine esterase mRNA showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant up regulation of AchE mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and there was no significant change in D+I ($p<0.05$) group. D+E group showed a significant decrease in the mRNA levels when compared to control ($p<0.05$), significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant up regulation when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-9, Table-9).

Real Time-PCR analysis of choline acetyltransferase in the cerebral cortex of experimental rats

Gene expression of choline acetyltransferase mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant decrease of ChAT mRNA gene expression when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I group ($p<0.05$). D+G group showed a significant ($p<0.001$) down regulation when compared to control,

but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-10, Table- 10).

Real Time-PCR analysis of muscarinic M1 receptor mRNA in the cerebral cortex of experimental rats

Gene expression of muscarinic M1 receptor mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant increase of muscarinic M1 receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and ($p<0.01$) D+I group. D+G group showed a significant increase of muscarinic M1 receptor gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-11, Table-11).

Real Time-PCR analysis of muscarinic M3 receptor mRNA in the cerebral cortex of experimental rats

Gene expression of muscarinic M3 receptor mRNA showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant increase of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and up regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to ($p<0.001$) control, but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant up regulation of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), but

significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-12, Table-12).

Real Time-PCR analysis of $GABA_B$ receptor mRNA in the cerebral cortex of experimental rats

Gene expression of $GABA_B$ receptor mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant up regulation of $GABA_B$ receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed significant down regulation in the mRNA levels when compared to ($p<0.001$) control, but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant down regulation of $GABA_B$ receptor gene expression when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-13, Table- 13).

Real Time-PCR analysis of insulin receptor mRNA in the cerebral cortex of experimental rats

Gene expression of insulin receptor mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex diabetic rats compared to control. In D+B, there was a significant decrease of insulin receptor gene expression when compared to control ($p<0.05$), but significant up regulation when compared to ($p<0.001$) diabetic and significant down regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant up regulation of insulin receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic

($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group **(**Figure-14, Table- 14).

Real Time-PCR analysis of Akt-1 mRNA in the cerebral cortex of experimental rats

Gene expression of Akt-1 mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant increase of Akt-1 mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and ($p<0.001$) D+I group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to ($p<0.001$) diabetic and D+I ($p<0.001$) group. D+G group showed a significant ($p<0.001$) decrease of Akt-1 mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and there is no significant change in D+I group **(**Figure-15, Table-15).

Real Time-PCR analysis of Bax mRNA in the cerebral cortex of experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant increase of Bax mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of Bax mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group **(**Figure-16, Table-16).

Real Time-PCR analysis of caspase 8 mRNA in the cerebral cortex of experimental rats

Gene expression of caspase 8 mRNA showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant increase of caspase 8 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation ($p<0.05$) when compared to diabetic ($p<0.05$) and significant up regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant increase of caspase 8 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-17, Table-17).

Real Time-PCR analysis of Gpx (glutathione peroxidase) mRNA in the cerebral cortex of experimental rats

Gene expression of Gpx mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant increase of Gpx mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant down regulation of Gpx mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group (Figure-18, Table-18).

Real Time-PCR analysis of SOD (superoxide dismutase) mRNA in the cerebral cortex of experimental rats

Gene expression of SOD mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant decrease of SOD mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group showed a significant decrease of SOD mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-19, Table-19).

Real Time-PCR analysis of GLUT 3 mRNA in the cerebral cortex of experimental rats

Gene expression of GLUT 3 mRNA showed significant up regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant increase of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+G group showed a significant increase of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group **(**Figure-20, Table-20).

Real Time-PCR analysis of phospholipase C mRNA in the cerebral cortex of experimental rats

Gene expression of phospholipase C mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant decrease of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.001$) group. D+G group showed a significant decrease of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.05$) group **(**Figure-21, Table-21).

Real Time-PCR analysis of CREB mRNA in the cerebral cortex of experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significant decrease of CREB mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and down regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of CREB mRNA gene expression when compared with control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I group ($p<0.001$) **(**Figure-22, Table-22).

Total antioxidant activity in the cerebral cortex of experimental rats

Total antioxidant activity showed significantly decreased ($p<0.001$) in the cerebral cortex of diabetic rats when compared to control. In D+B, there was a significantly decreased antioxidant level when compared to control ($p<0.05$), but significantly increased when compared to diabetic ($p<0.001$) and significantly decreased when compared to D+I ($p<0.05$) group. D+E group showed a significantly decreased antioxidant level when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly decreased antioxidant level when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group **(**Figure-23, Table-23).

Total aqueous peroxide activity in the cerebral cortex of experimental rats

Total aqueous peroxide activity showed significantly increased ($p<0.001$) in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significantly increased aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+E group showed a significantly increased in the aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly increased aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-24, Table-24).

CONFOCAL STUDIES

TO-PRO®-3 iodide staining in the cerebral cortex of experimental rats

TO-PRO®-3 staining showed significantly decreased ($p<0.001$) nuclear density in the cerebral cortex of diabetic rats compared to control. In D+B, there was a significantly decreased nuclear density when compared to control ($p<0.05$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significantly decreased in the nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+G group showed a significantly decreased nuclear density when to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-25, Table-25).

Muscarinic M1 receptor antibody staining in the cerebral cortex of experimental rats

Muscarinic M1 receptor subunit antibody staining in the cerebral cortex showed a significantly decreased ($p<0.001$) in the mean pixel value of diabetic rats compared to control. In D+B, there was a significantly decreased muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significantly decreased in the muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly decreased muscarinic M1 receptor expression when compared to control ($p<0.05$), but significantly increased when compared to diabetic ($p<0.001$) and ($p<0.001$) D+I group **(**Figure- 26, Table- 26).

Muscarinic M3 receptor antibody staining in the cerebral cortex of experimental rats

Muscarinic M3 receptor subunit antibody staining in the cerebral cortex showed a significantly increased ($p<0.001$) in the mean pixel value of diabetic rats compared to control. In D+B, there was a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+E group showed a significantly increased in the muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group showed a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-27, Table- 27).

Figure-5

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the cerebral cortex of experimental rats

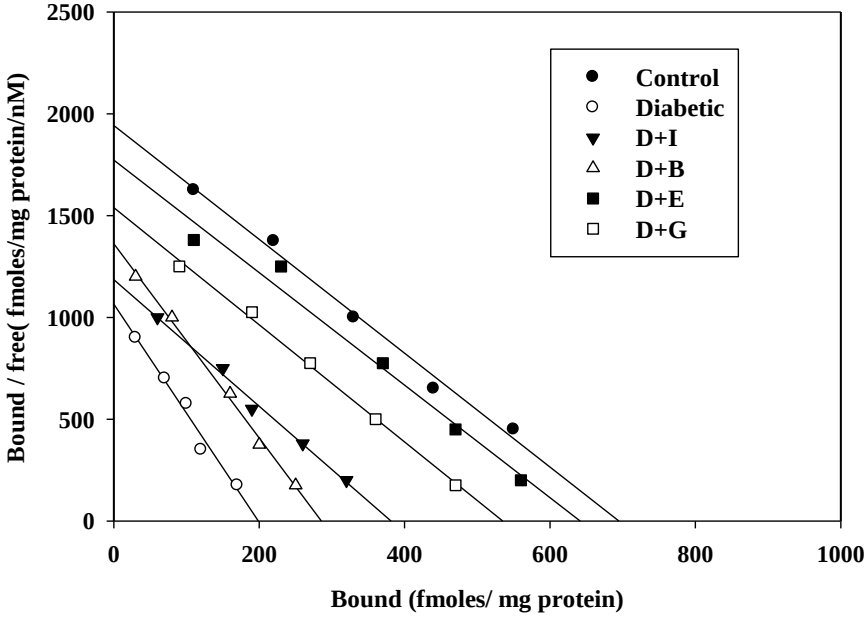


Table-5

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the cerebral cortex of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	693.81± 18.36	0.36 ± 0.07
Diabetic	200.19± 7.13[a, g]	0.19 ± 0.03[a, g]
D + I	381.47± 12.68[a, d]	0.32 ± 0.06[a, d]
D + B	285.40 ± 9.39[a, d, h]	0.21 ± 0.05[a, h]
D+E	639.63±16.54[b, d, g]	0.36± 0.07[d, h]
D+G	535.41±15.06[a, d ,h]	0.35±0.06[e, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-6
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the cerebral cortex of experimental rats

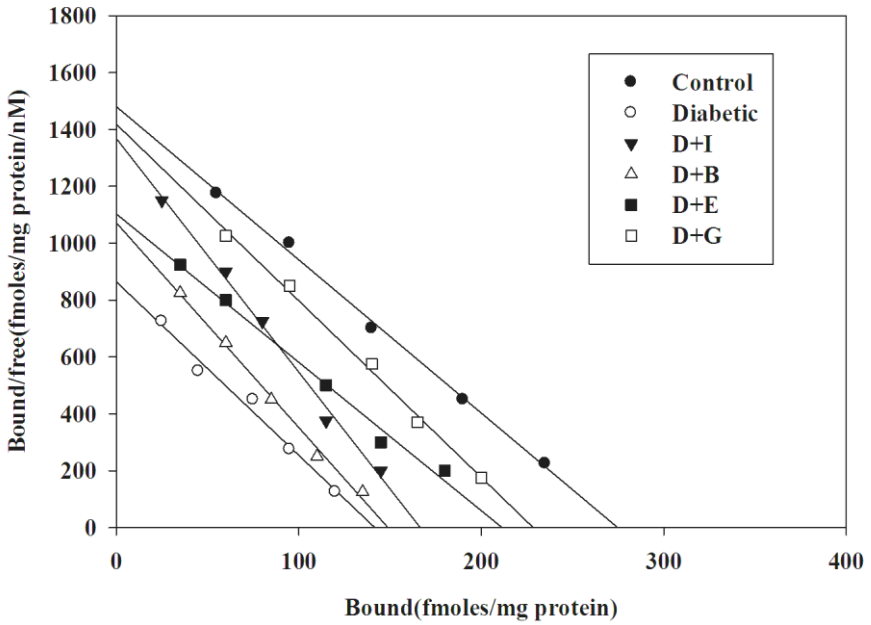


Table-6
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the cerebral cortex of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	275.04± 11.61	0.19 ± 0.05
Diabetic	140.88± 5.52[a, g]	0.16 ± 0.04[a, h]
D + I	166.72± 7.15[a, d]	0.12 ± 0.02[a, d]
D + B	149.28 ± 6.41[a, d, g]	0.13 ± 0.02[a, e]
D+E	210.37±8.39[a, d, g]	0.19± 0.05[d, g]
D+G	227.52±10.72[b, d, g]	0.16±0.04[b, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-7
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the cerebral cortex of experimental rats

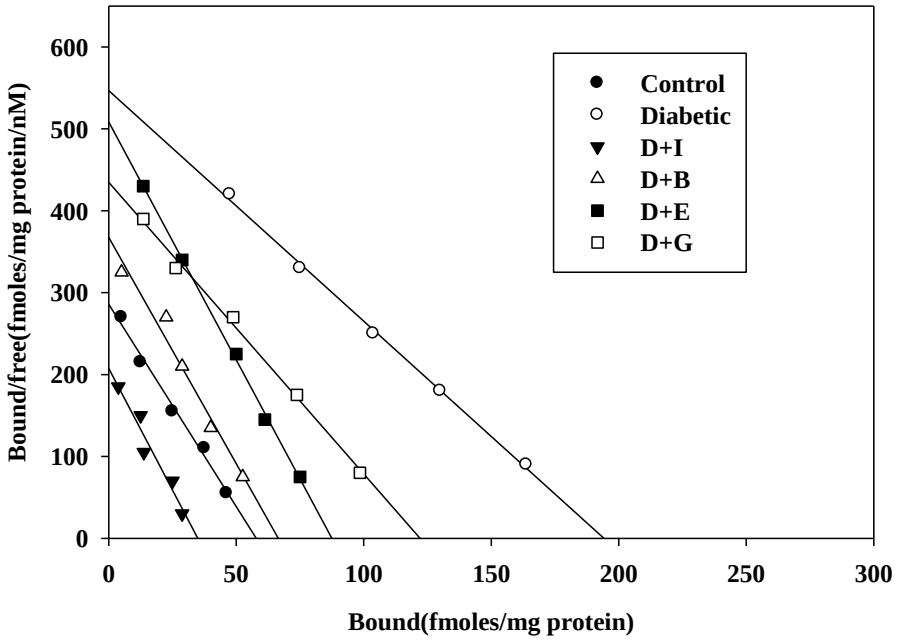


Table-7
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the cerebral cortex of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	58.14± 2.83	0.20 ± 0.07
Diabetic	194.48± 5.78[a, g]	0.35 ± 0.09[a, g]
D + I	35.04± 1.73[a, d]	0.17 ± 0.05[a, d]
D + B	66.21 ± 3.25[a, d, g]	0.18 ± 0.06[a, d]
D+E	88.14±3.89[a, d, g]	0.17± 0.05[a, e]
D+G	121.92±4.62[b, d, g]	0.28±0.08[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-8
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the cerebral cortex of experimental rats

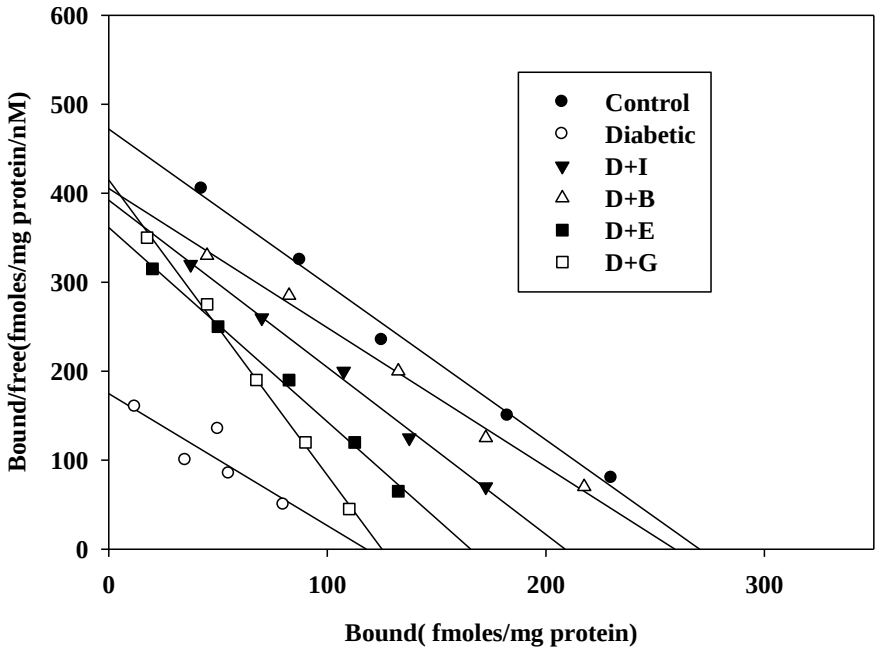


Table-8
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the cerebral cortex of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	269.78± 12.17	0.57 ± 0.07
Diabetic	116.69± 4.38[a, g]	0.66± 0.08[a, g]
D + I	208.53± 6.14[a, d]	0.53 ± 0.07[a, d]
D + B	258.86 ± 7.82[a, d, g]	0.64 ± 0.08[a, h]
D+E	164.78±5.11[a, d, g]	0.45± 0.05[a, d, g]
D+G	124.74±4.80[a, d, g]	0.30±0.04[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-9
Real Time PCR amplification of acetylcholine esterase mRNA in the cerebral cortex of experimental rats

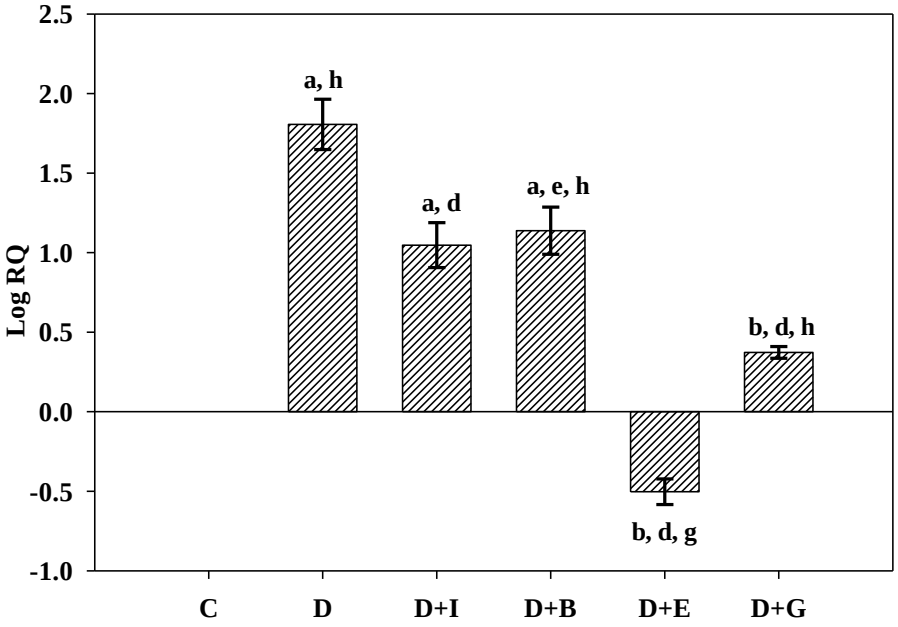


Table-9
Real Time PCR amplification of acetylcholine esterase mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.80± 0.16[a, h]
D+I	1.05±0.14[a, d]
D+B	1.14±0.15[a, e, h]
D+E	-0.51±0.08[b, d, g]
D+G	0.37±0.05[b, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-10
Real Time PCR amplification of choline acetyltransferase mRNA in the cerebral cortex of experimental rats

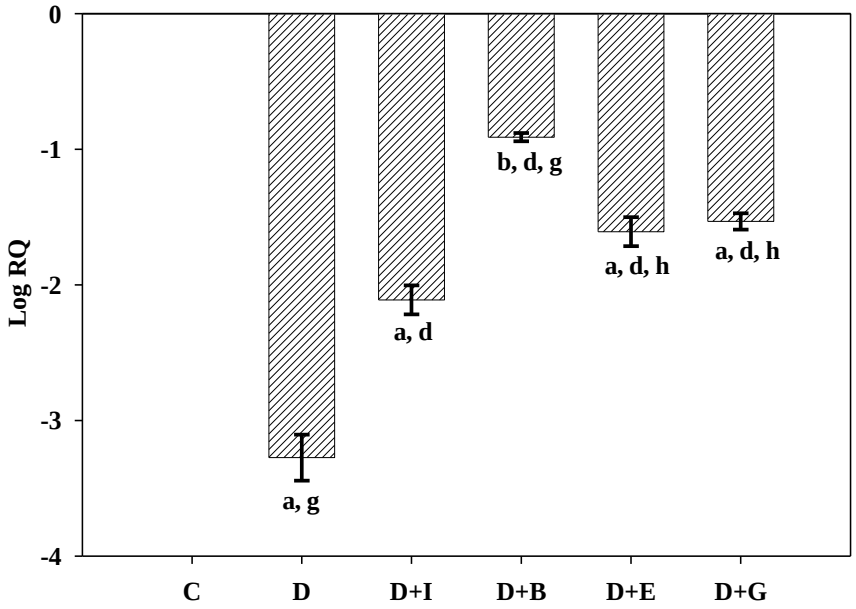


Table-10
Real Time PCR amplification of choline acetyl transferase mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.27± 0.17[a, g]
D+I	-2.11±0.11[a, d]
D+B	-0.91±0.05[b, d, g]
D+E	-1.61±0.10[a, d, h]
D+G	-1.53±0.06[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-11
Real Time PCR amplification of muscarinic M1 receptor mRNA in the cerebral cortex of experimental rats

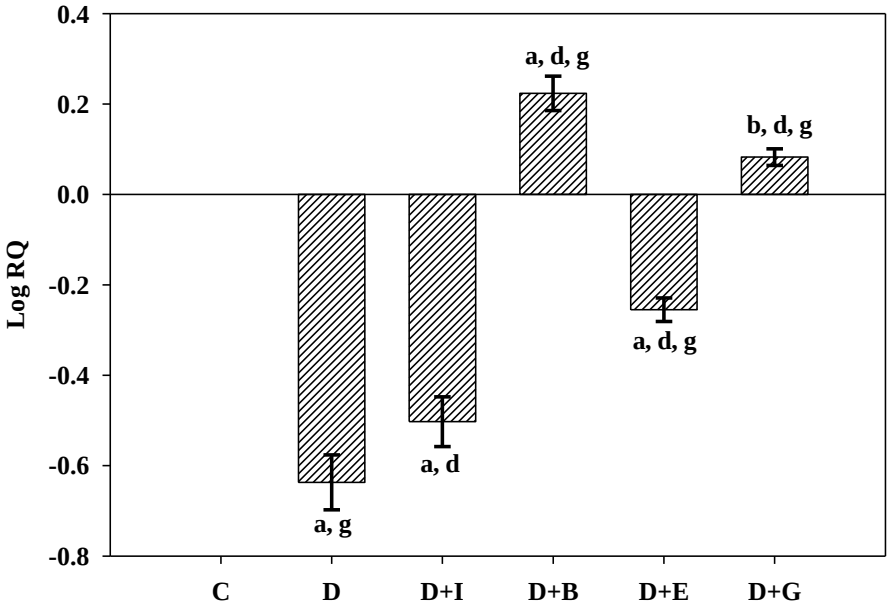


Table-11
Real Time PCR amplification of muscarinic M1 receptor mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-0.64± 0.06[a, g]
D+I	-0.50±0.06[a, d]
D+B	0.23±0.04[a, d, g]
D+E	-0.25±0.03[a, d, g]
D+G	0.08±0.02[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-12
Real Time PCR amplification of muscarinic M3 receptor mRNA in the cerebral cortex of experimental rats

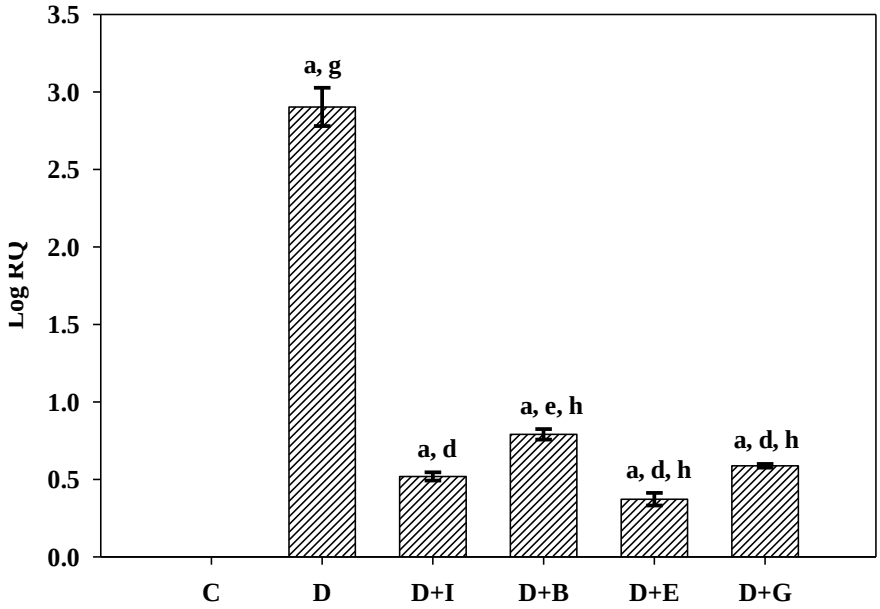


Table-12
Real Time PCR amplification of muscarinic M3 receptor mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	2.90± 0.07[a, g]
D+I	0.52±0.03[a, d]
D+B	0.79±0.04[a, e, h]
D+E	0.37±0.03[a, d, h]
D+G	0.59±0.02[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-13
Real Time PCR amplification of $GABA_B$ receptor mRNA in the cerebral cortex of experimental rats

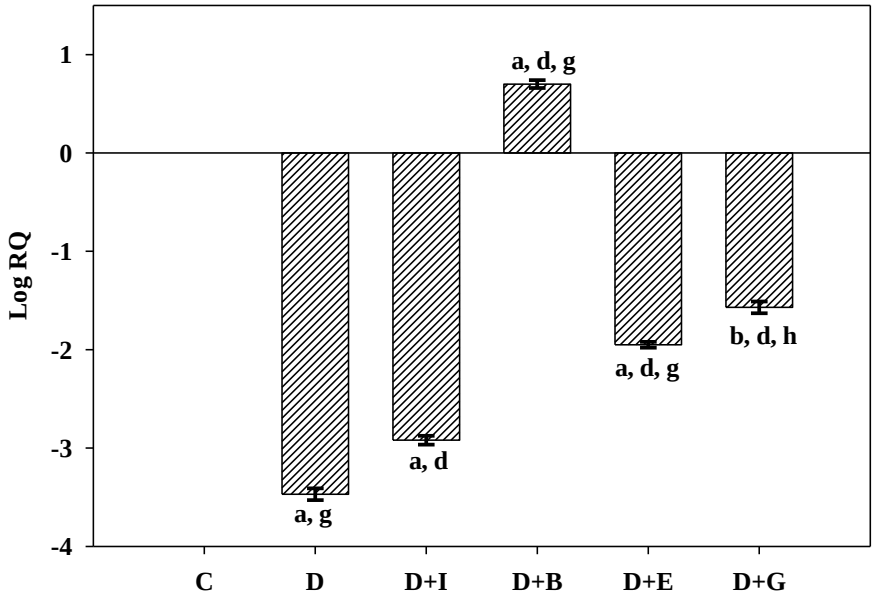


Table-13
Real Time PCR amplification of $GABA_B$ receptor mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.49± 0.05[a, g]
D+I	-2.92±0.05[a, d]
D+B	0.70±0.04[a, d, g]
D+E	-1.95±0.04[a, d, g]
D+G	-1.57±0.07[b, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-14
Real Time PCR amplification of insulin receptor mRNA in the cerebral cortex of experimental rats

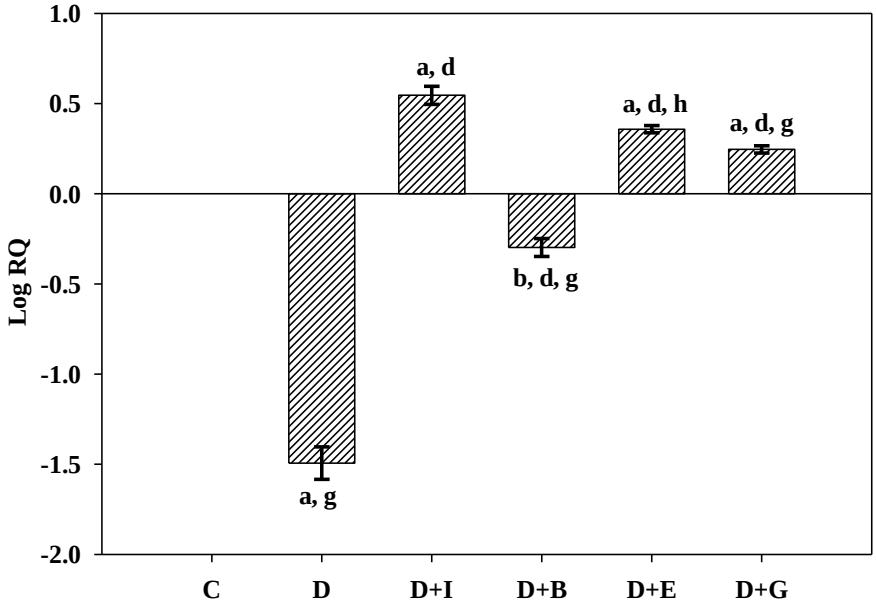


Table-14
Real Time PCR amplification of insulin receptor mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.49± 0.09[a, g]
D+I	0.55±0.05[a, d]
D+B	-0.29±0.05[b, d, g]
D+E	0.36±0.02[a, d, h]
D+G	0.25 ±0.02[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-15
Real Time PCR amplification of Akt-1 mRNA in the cerebral cortex of experimental rats

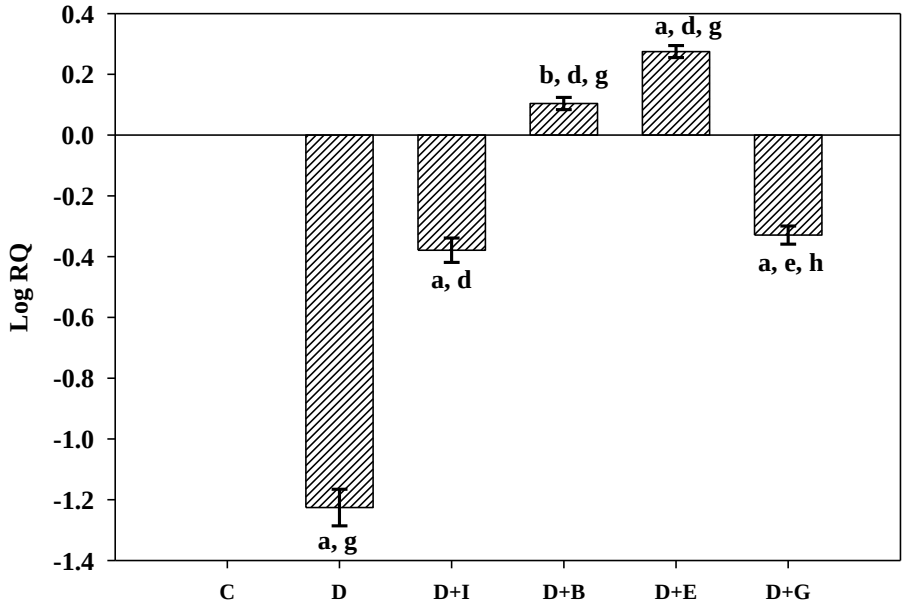


Table-15
Real Time PCR amplification of Akt-1 mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.23± 0.06[a, g]
D+I	-0.38±0.04[a, d]
D+B	0.11±0.02[b, d, g]
D+E	0.28±0.02[a, d, g]
D+G	-0.33 ±0.03[a, e, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-16
Real Time PCR amplification of Bax mRNA in the cerebral cortex of experimental rats

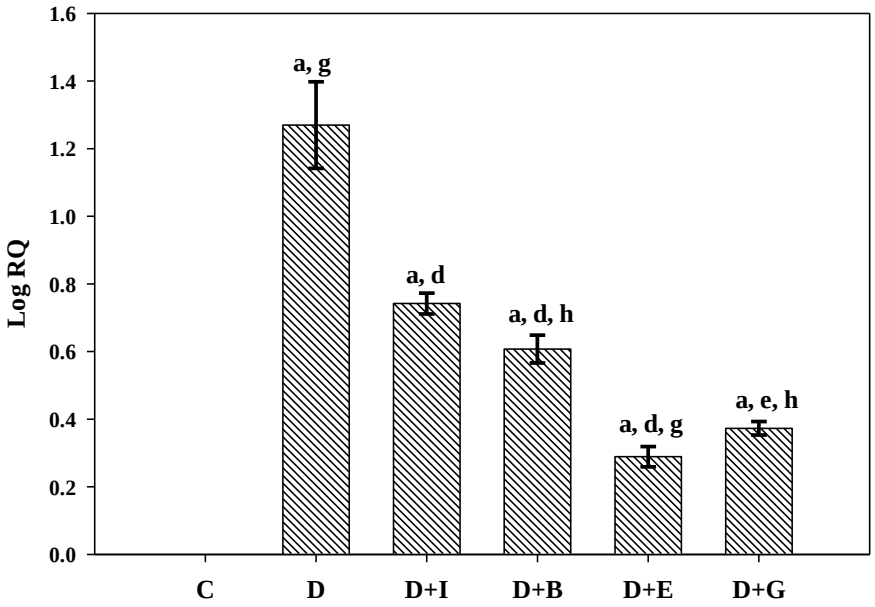


Table-16
Real Time PCR amplification of Bax mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.27± 0.09[a, g]
D+I	0.74±0.03[a, d]
D+B	0.61±0.04[a, d, h]
D+E	0.29±0.03[a, d, g]
D+G	0.37±0.02[a, e, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-17

Real Time PCR amplification of caspase 8 mRNA in the cerebral cortex of experimental rats

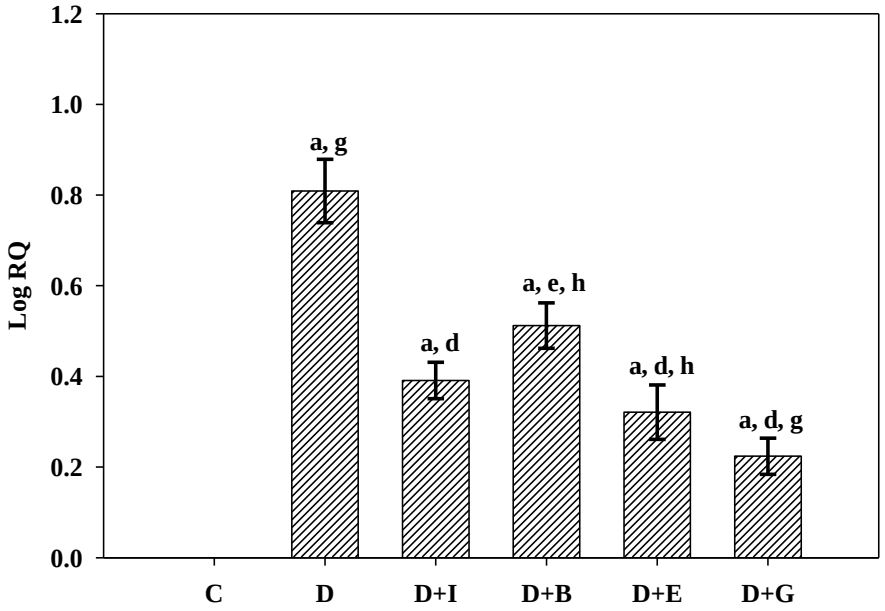


Table-17
Real Time PCR amplification of Caspase 8 mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	0.81± 0.07[a, g]
D+I	0.39±0.04[a, d]
D+B	0.51±0.05[a, e, h]
D+E	0.32±0.06[a, d, h]
D+G	0.22 ±0.04[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-18
Real Time PCR amplification of Gpx mRNA in the cerebral cortex of experimental rats

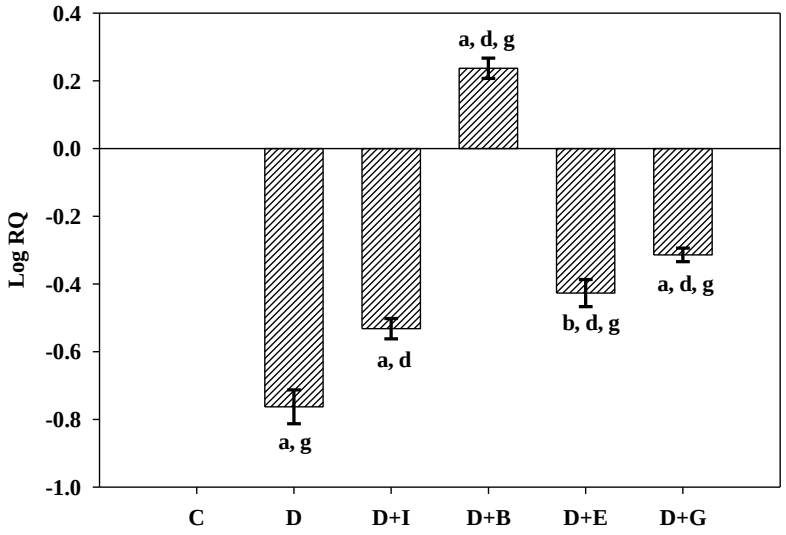


Table-18
Real Time PCR amplification of Gpx mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-0.76± 0.05[a, g]
D+I	-0.53±0.03[a, d]
D+B	0.24±0.03[a, d, g]
D+E	-0.43±0.04[b, d, g]
D+G	-0.31±0.02[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-19
Real Time PCR amplification of Superoxide dismutase mRNA in the cerebral cortex of experimental rats

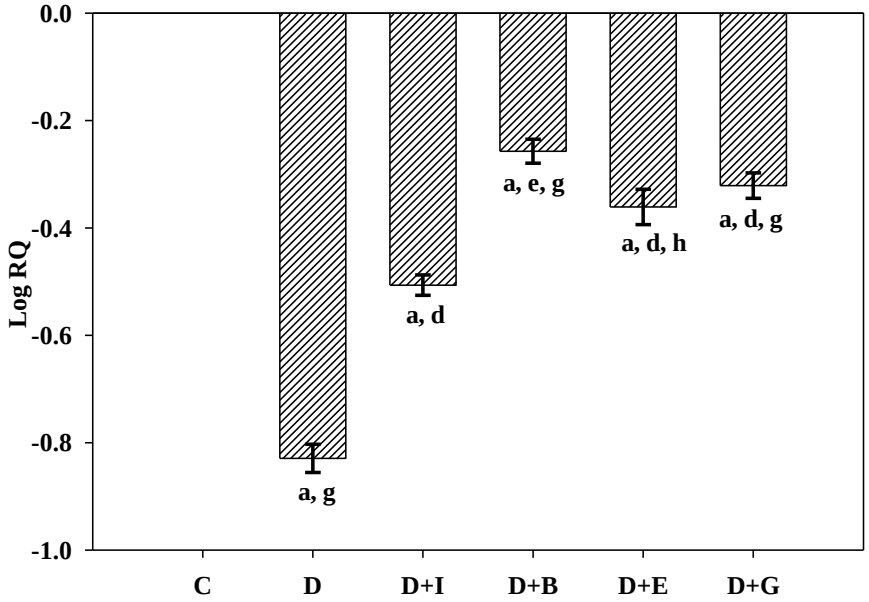


Table-19
Real Time PCR amplification of Superoxide dismutase mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	**0**
Diabetic	**-0.83± 0.03[a, g]**
D+I	**-0.51±0.02[a, d]**
D+B	**-0.26±0.02[a, e, g]**
D+E	**-0.36±0.03[a, d, h]**
D+G	**-0.32±0.02[a, d, g]**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-20
Real Time PCR amplification of GLUT 3 mRNA in the cerebral cortex of experimental rats

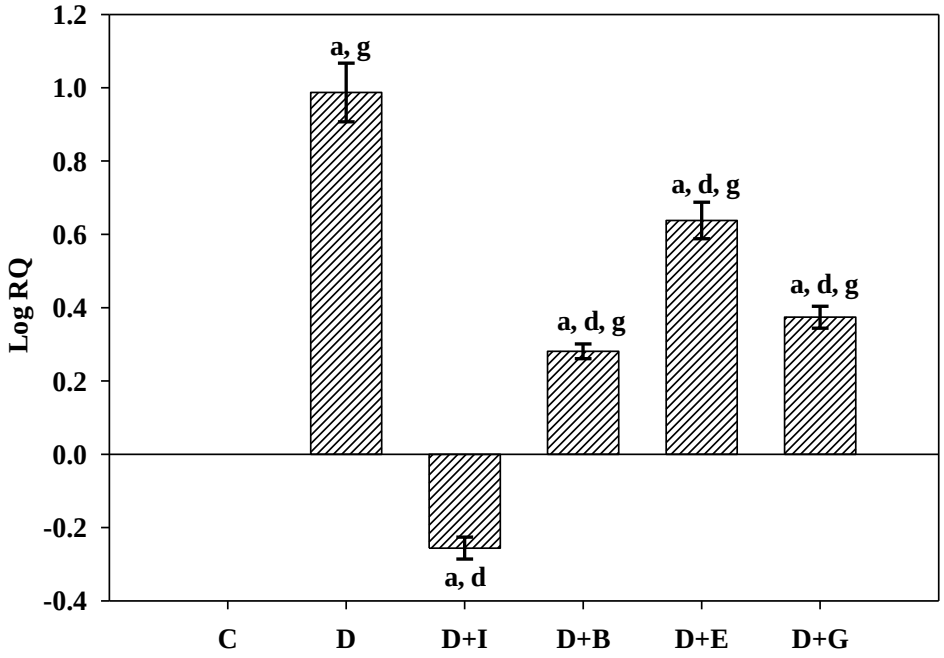


Table-20
Real Time PCR amplification of GLUT 3 mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	0.98± 0.08[a, g]
D+I	-0.256±0.03[a, d]
D+B	0.28±0.02[a, d, g]
D+E	0.64±0.05[a, d, g]
D+G	0.37 ±0.02[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-21
Real Time PCR amplification of phospholipase C mRNA in the cerebral cortex of experimental rats

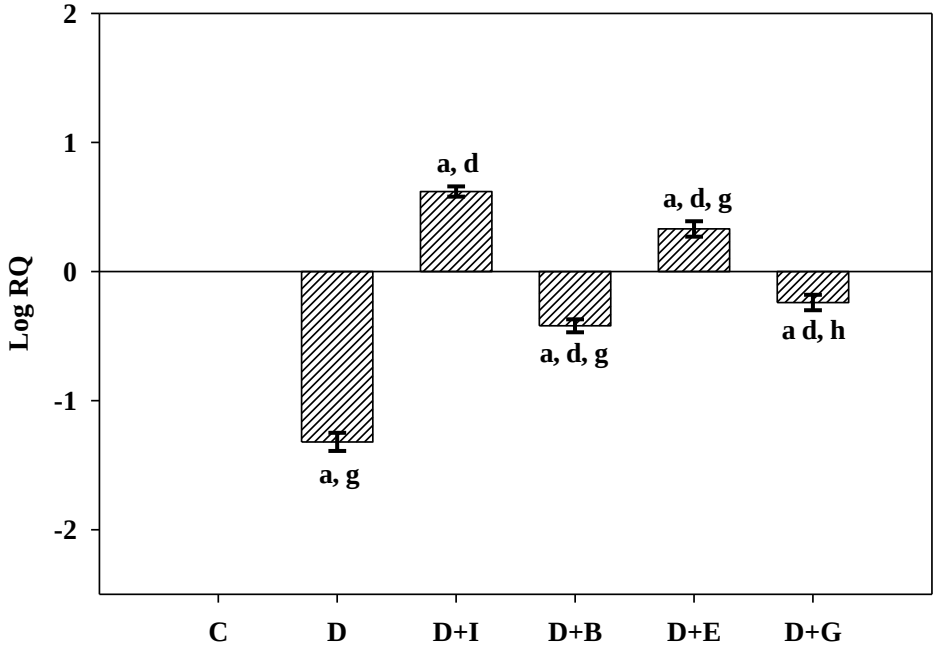


Table-21
Real Time PCR amplification of phospholipase C mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.33± 0.05[a, g]
D+I	0.62±0.04[a, d]
D+B	-0.42±0.05[a, d, g]
D+E	0.33±0.07[a, d, g]
D+G	-0.24±0.06[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-22
Real Time PCR amplification of CREB mRNA in the cerebral cortex of experimental rats

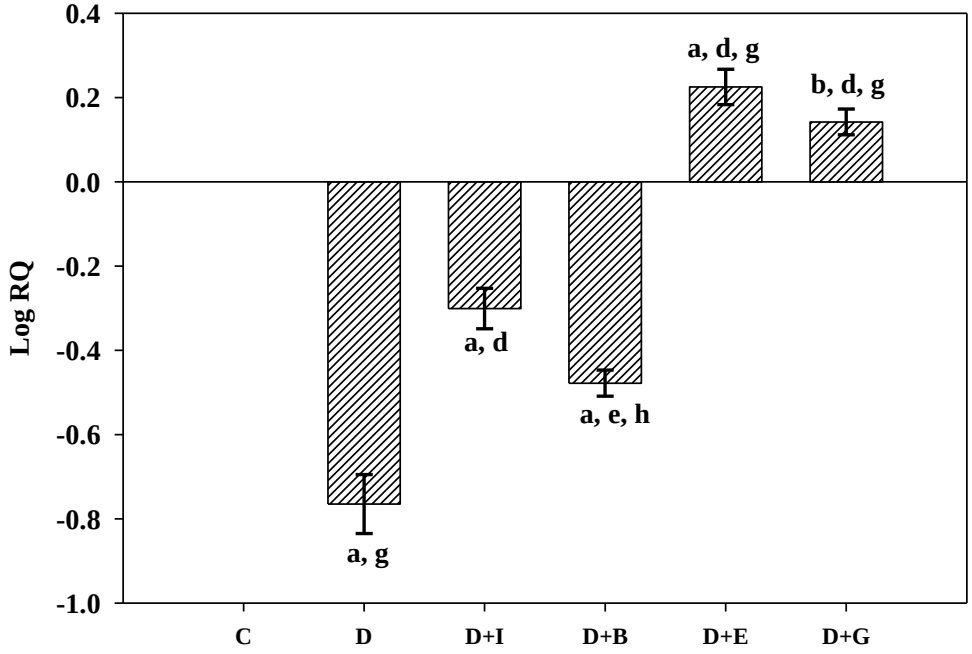


Table-22
Real Time PCR amplification of CREB mRNA in the cerebral cortex of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-0.76± 0.07[a, g]
D+I	-0.30±0.05[a, d]
D+B	-0.47±0.03[a, e, h]
D+E	0.23±0.04[a, d, g]
D+G	0.15±0.03[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-23
Total antioxidant level in the cerebral cortex of experimental rats

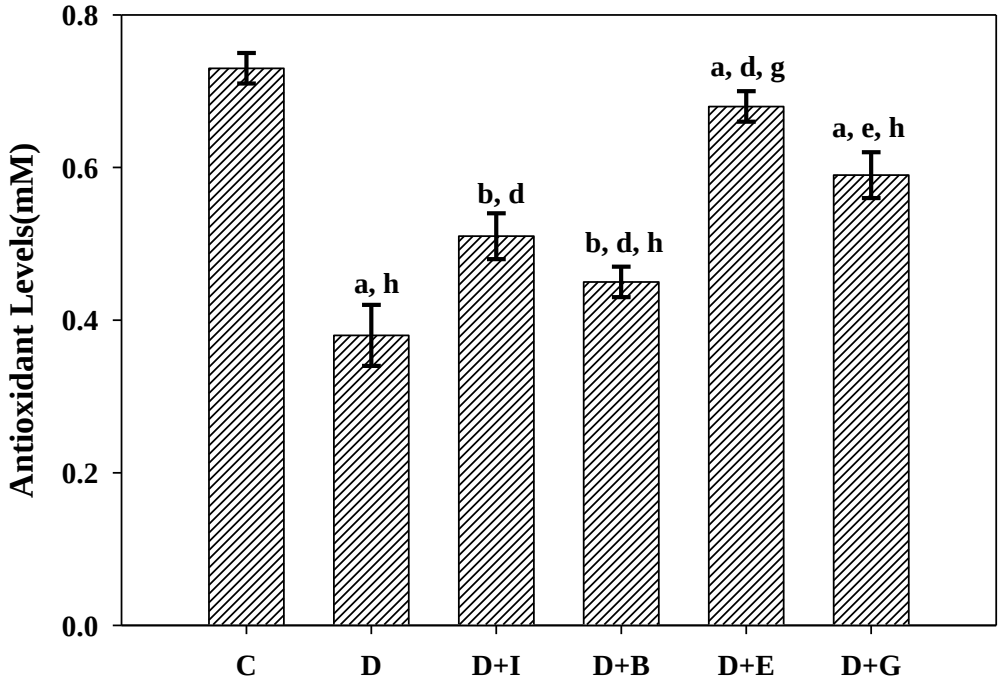


Table-23
Total antioxidant level in the cerebral cortex of experimental rats

Experimental Groups	Antioxidant Level(mM)
Control	0.73±0.02
Diabetic	0.38± 0.04[a, h]
D+I	0.51±0.03[b, d]
D+B	0.45±0.02[b, d, h]
D+E	0.68±0.02[a, d, g]
D+G	0.59±0.03[a, e, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-24
Total aqueous peroxide level in the cerebral cortex of experimental rats

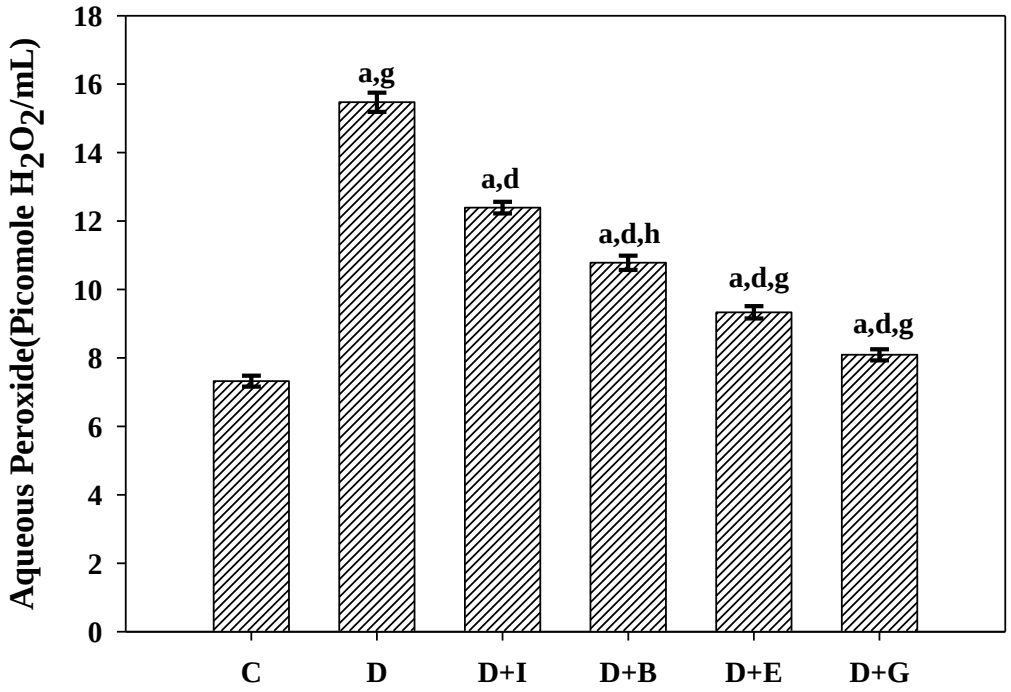


Table-24
Total aqueous peroxide level in the cerebral cortex of experimental rats

Experimental Groups	Aqueous peroxide (Picomole H_2O_2/mL)
Control	7.32±0.16
Diabetic	15.47± 0.28[a, g]
D+I	12.39±0.17[a, d]
D+B	10.78±0.21[a, d, h]
D+E	9.33±0.18[a, d, g]
D+G	8.09±0.16[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure & Table -25

TO-PRO®-3 iodide staining in the cerebral cortex of experimental rats

Figure & Table -26

Muscarinic M1 receptor antibody staining in the cerebral cortex of experimental rats

Figure & Table -27

Muscarinic M3 receptor antibody staining in the cerebral cortex of experimental rats

CEREBELLUM

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the cerebellum of experimental rats

The total muscarinic receptor status was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist atropine. The Scatchard analysis showed that the significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) in diabetic rats compared to control group. In D+B, showed that significantly increased B_{max} ($p<0.05$) and decreased K_d ($p<0.05$) when compared to control, but significantly decreased B_{max} ($p<0.05$) and K_d ($p<0.05$) when compared to diabetic and significantly increased B_{max} ($p<0.05$) and decreased K_d ($p<0.05$) when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly increased when compared to control, but significantly decreased B_{max} ($p<0.001$) and increased K_d ($p<0.05$) when compared to diabetic and significantly increased B_{max} ($p<0.001$) and K_d ($p<0.05$) when compared to) D+I group. D+G group showed that significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.05$) when compared to control, but significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.05$) when compared to D+I group **(**Figure-28, Table-28).

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the cerebellum of experimental rats

Binding analysis of muscarinic M1 receptor was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The Scatchard analysis showed that the B_{max}($p<0.001$) and K_d ($p<0.001$) increased significantly in

diabetic group when compared to control group. In D+B, showed that significantly increased B_{max} ($p<0.001$) and no significant change in K_d when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to diabetic and significantly increased B_{max} ($p<0.05$) and decreased K_d ($p<0.001$) when compared to D+I group. D+E group showed that significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to diabetic and significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to D+I group. D+G group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly increased when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to diabetic and significantly increased B_{max} ($p<0.05$) and decreased K_d ($p<0.001$) when compared to D+I group **(**Figure-29, Table-29).

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor, antagonist, 4-DAMP mustard in the cerebellum of experimental rats

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The Scatchard analysis showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) was increased significantly ($p<0.001$) in diabetic group when compared to control. In D+B, showed that significantly increased B_{max} ($p<0.001$) and no significant change in K_d when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to diabetic and significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to D+I group. D+E group showed that B_{max} ($p<0.05$) and K_d ($p<0.001$) significantly increased when compared to control, but significantly decreased B_{max} ($p<0.05$) and increased K_d ($p<0.001$) when

compared to diabetic and significantly increased B_{max} ($p<0.001$) and K_d ($p<0.05$) when compared to D+I group. D+G group showed that B_{max} ($p<0.001$) and K_d ($p<0.05$) significantly increased when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.05$) significantly decreased when compared to diabetic and significantly increased B_{max} ($p<0.05$) and no significant change in K_d when compared to D+I group **(**Figure-30, Table-30).

$GABA_B$ receptor analysis

Scatchard analysis of [^{3}H] baclofen binding against baclofen to $GABA_B$ receptors in the cerebellum of experimental rats

Binding analysis of $GABA_B$ receptor was done using [^{3}H] baclofen. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) of $GABA_B$ receptor was decreased significantly in diabetic group when compared to control. In D+B, showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly increased when compared to diabetic and significantly increased B_{max} ($p<0.05$) and K_d ($p<0.001$) when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to diabetic and significantly increased B_{max} ($p<0.05$) and K_d ($p<0.001$) when compared to D+I group. D+G group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and there is no significant change in K_d when compared to diabetic and significantly increased B_{max} ($p<0.05$) and K_d ($p<0.001$) when compared to D+I group **(**Figure-31, Table-31).

REAL TIME-PCR ANALYSIS

Real Time-PCR analysis of acetylcholine esterase in the cerebellum of experimental rats

Gene expression of acetylcholine esterase mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant up regulation of AchE mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and up regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant decrease in the mRNA levels when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant down regulation when compared to control ($p<0.05$), diabetic ($p<0.001$) and D+I group ($p<0.001$) **(**Figure-32, Table-32).

Real Time-PCR analysis of choline acetyltransferase in the cerebellum of experimental rats

Gene expression of choline acetyltransferase mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant decrease of ChAT mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant down regulation when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure- 33, Table- 33).

Real Time-PCR analysis of muscarinic M1 receptor mRNA in the cerebellum of experimental rats

Gene expression of muscarinic M1 receptor mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant increase of muscarinic M1 receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and significant up regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group showed a significant increase of muscarinic M1 receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. **(**Figure-34, Table-34).

Real Time-PCR analysis of muscarinic M3 receptor mRNA in the cerebellum of experimental rats

Gene expression of muscarinic M3 receptor mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant decrease of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant up regulation of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-35, Table-35).

Real Time-PCR analysis of $GABA_B$ receptor mRNA in the cerebellum of experimental rats

Gene expression of $GABA_B$ receptor mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant down regulation of $GABA_B$ receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+G group showed a significant down regulation of $GABA_B$ receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-36, Table- 36).

Real Time-PCR analysis of insulin receptor mRNA in the cerebellum of experimental rats

Gene expression of insulin receptor mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant increase of insulin receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and there is no significant change in D+I group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant ($p<0.001$) up regulation of insulin receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.05$) group **(**Figure-37, Table- 37).

Real Time-PCR analysis of Akt-1 mRNA in the cerebellum of experimental rats

Gene expression of Akt-1 mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant increase of Akt-1 mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant decrease of Akt-1 mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-38, Table-38).

Real Time-PCR analysis of Bax mRNA in the cerebellum of experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant increase of Bax mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of Bax mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-39, Table-39).

Real Time-PCR analysis of caspase 8 mRNA in the cerebellum of experimental rats

Gene expression of caspase 8 mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant increase of caspase 8 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of caspase 8 mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-40, Table-40).

Real Time-PCR analysis of Gpx (glutathione peroxidase) mRNA in the cerebellum of experimental rats

Gene expression of Gpx mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant decrease of Gpx mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of Gpx mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-41, Table-41).

Real Time-PCR analysis of SOD (superoxide dismutase) mRNA in the cerebellum of experimental rats

Gene expression of SOD mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant decrease of SOD mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant decrease of SOD mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-42, Table-42).

Real Time-PCR analysis of GLUT 3 mRNA in the cerebellum of experimental rats

Gene expression of GLUT 3 mRNA showed significant up regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant increase of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+G group showed a significant increase of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in ($p<0.05$) D+I group **(**Figure-43, Table- 43).

Real Time-PCR analysis of phospholipase C mRNA in the cerebellum of experimental rats

Gene expression of phospholipase C mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant decrease of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-44, Table-44).

Real Time-PCR analysis of CREB mRNA in the cerebellum of experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significant decrease of CREB mRNA gene expression when to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group showed a significant decrease of CREB mRNA gene expression when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-45, Table-45).

Total antioxidant activity in the cerebellum of experimental rats

Total antioxidant activity showed significantly decreased ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significantly decreased antioxidant level when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group. D+E group showed a significantly decreased antioxidant level when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group showed a significantly decreased antioxidant level when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-46, Table-46).

Total aqueous peroxide activity in the cerebellum of experimental rats

Total aqueous peroxide activity showed significantly increased ($p<0.001$) in the cerebellum of diabetic rats compared to control. In D+B, there was a significantly increased aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.05$) and there is no significant change in D+I group. D+E group showed a significantly increased aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly increased aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-47, Table-47).

CONFOCAL STUDIES

TO-PRO®-3 iodide staining in the cerebellum of experimental rats

TO-PRO®-3 staining showed significantly decreased ($p<0.001$) nuclear density in the cerebellum of diabetic rats compared to control. In D + B, there was a significantly decreased nuclear density when compared to control ($p<0.05$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significantly decreased nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly decreased nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-48, Table-48).

Muscarinic M1 receptor antibody staining in the cerebellum of experimental rats

Muscarinic M1 receptor subunit antibody staining in the cerebellum showed a significantly increased ($p<0.001$) in the mean pixel value of diabetic rats compared to control. In D + B, there was a significantly increased muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.05$) group. D+E group showed a significantly increased in the muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.05$) group. D+G group showed a significantly increased muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.001$) group **(**Figure-49, Table-49).

Muscarinic M3 receptor antibody staining in the cerebellum of experimental rats

Muscarinic M3 receptor subunit antibody staining in the cerebellum showed a significantly increased ($p<0.001$) in the mean pixel value of diabetic rats compared to control. In D+B, there was a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.05$) and significant increase when compared to D+I ($p<0.05$) group. D+E group showed a significantly increased in the muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.001$) group. D+G group showed a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.001$) group **(**Figure-50, Table- 50).

Figure-28
Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the Cerebellum of experimental rats

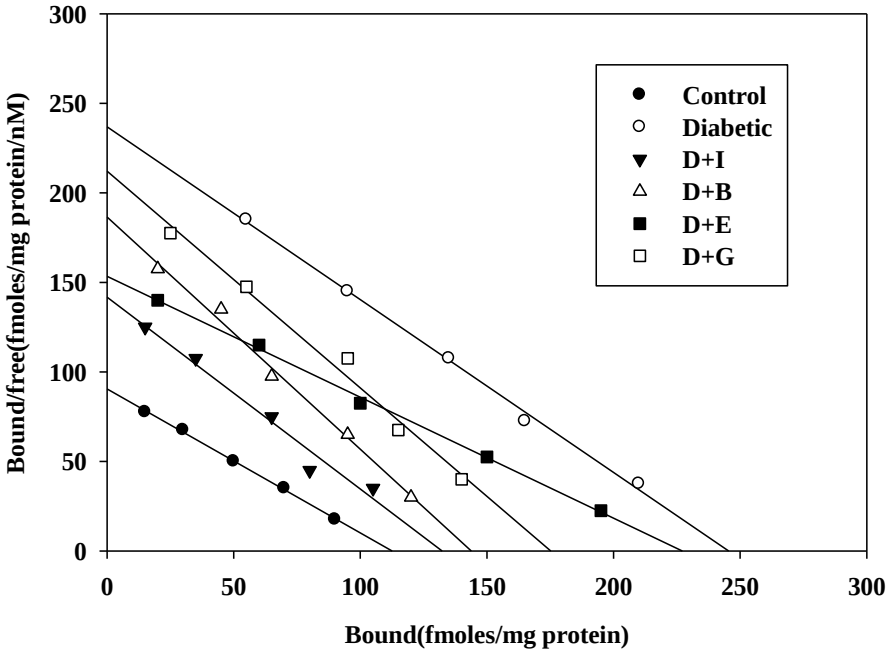


Table-28
Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the Cerebellum of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	111.92± 2.58	1.29 ± 0.15
Diabetic	245.04± 8.37[a, g]	1.03 ± 0.14[a, g]
D + I	132.54± 3.65[a, d]	0.94 ± 0.11[a, d]
D + B	143.76 ± 4.17[b, e, h]	0.77 ± 0.10[b, e, h]
D+E	225.66±6.03[a, d, g]	1.46± 0.17[a, e, h]
D+G	175.02±6.81[a, d, g]	0.83±0.10[b, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-29
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the Cerebellum of experimental rats

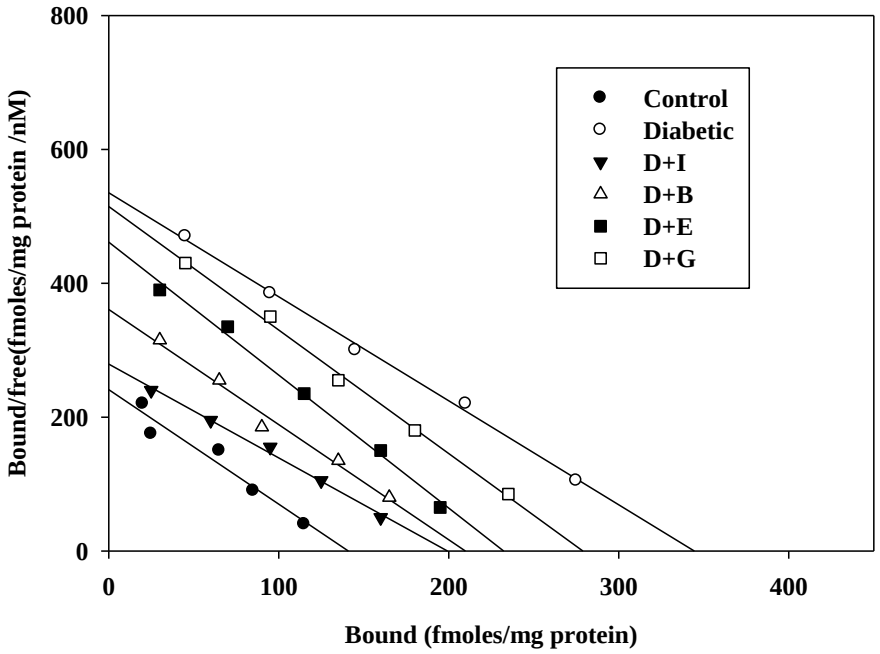


Table-29
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the Cerebellum of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	139.68± 4.39	0.57 ± 0.08
Diabetic	342.18± 10.35[a, g]	0.64 ± 0.09[a, g]
D + I	201.51± 6.83[a, d]	0.72 ± 0.10[a, d]
D + B	209.07 ± 7.21[a, d, h]	0.59 ± 0.08[a, d, g]
D+E	229.68±7.80[a, d, g]	0.50 ± 0.07[a, d, g]
D+G	277.56±9.62[a, d, h]	0.54±0.08[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-30
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the Cerebellum of experimental rats

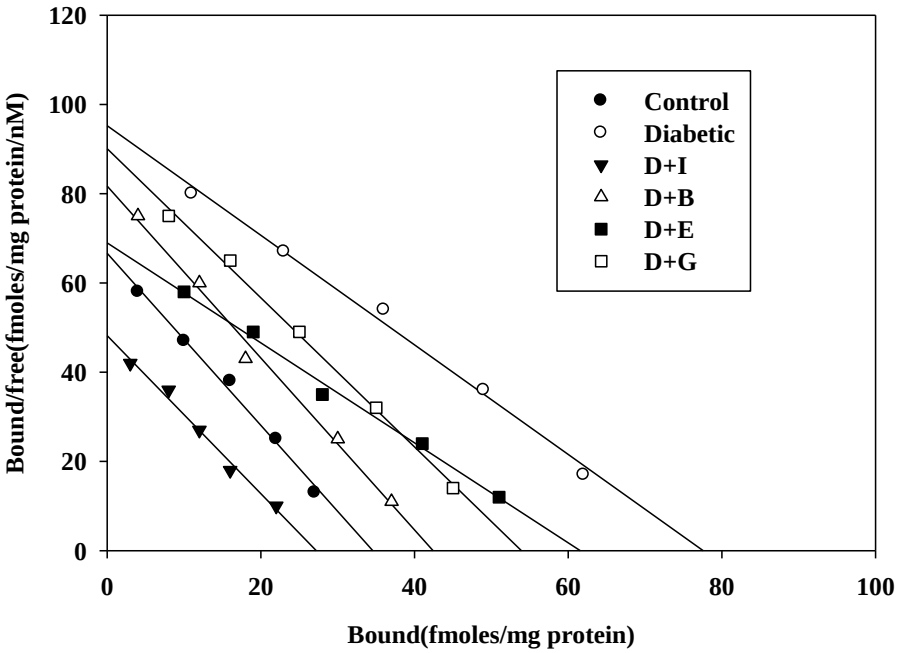


Table-30
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the Cerebellum of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	34.18± 4.05	0.52 ± 0.08
Diabetic	77.30± 5.61[a, g]	0.81 ± 0.11[a, g]
D + I	27.51± 2.79[a, d]	0.57 ± 0.08[a, d]
D + B	42.35 ± 7.24[a, d, g]	0.52 ± 0.07 [d, g]
D+E	61.46±5.13[b, d, g]	0.89 ± 0.11[a, d, h]
D+G	53.75±4.80[a, d, h]	0.59±0.08[b, d]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-31
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the Cerebellum of experimental rats

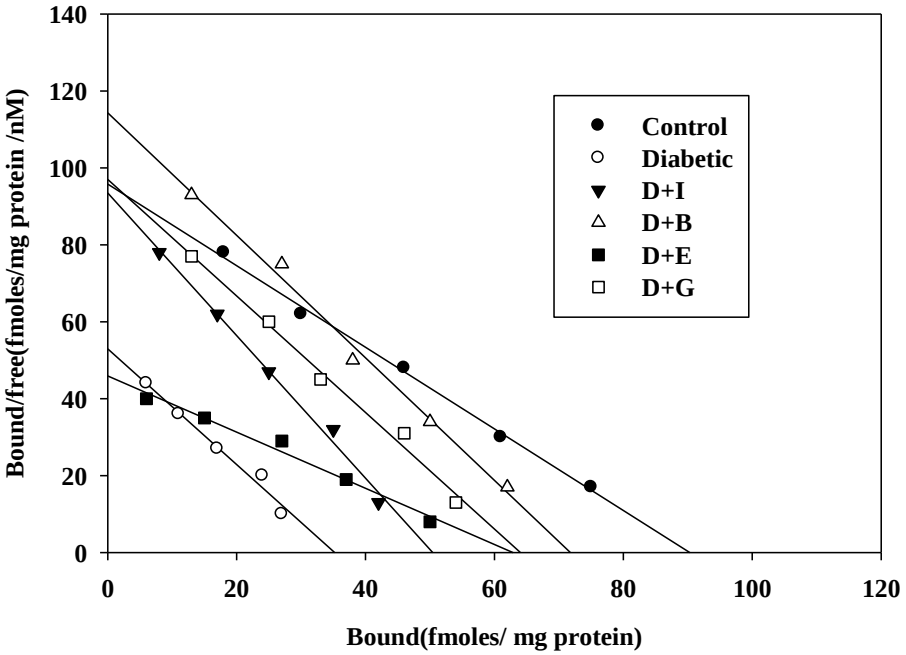


Table-31
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the Cerebellum of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	89.76± 3.96	0.94 ± 12
Diabetic	35.52± 1.85[a, g]	0.68± 0.08[a, g]
D + I	50.25± 2.61[a, d]	0.54 ± 0.07[a, d]
D + B	71.49 ± 3.80[a, d, h]	0.72 ± 0.08[a, d, g]
D+E	62.04±3.27[a, d, h]	0.61± 0.07[a, d, g]
D+G	64.27±3.45[a, d, h]	0.68±0.08[a, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-32
Real Time PCR amplification of acetylcholine esterase mRNA in the Cerebellum of experimental rats

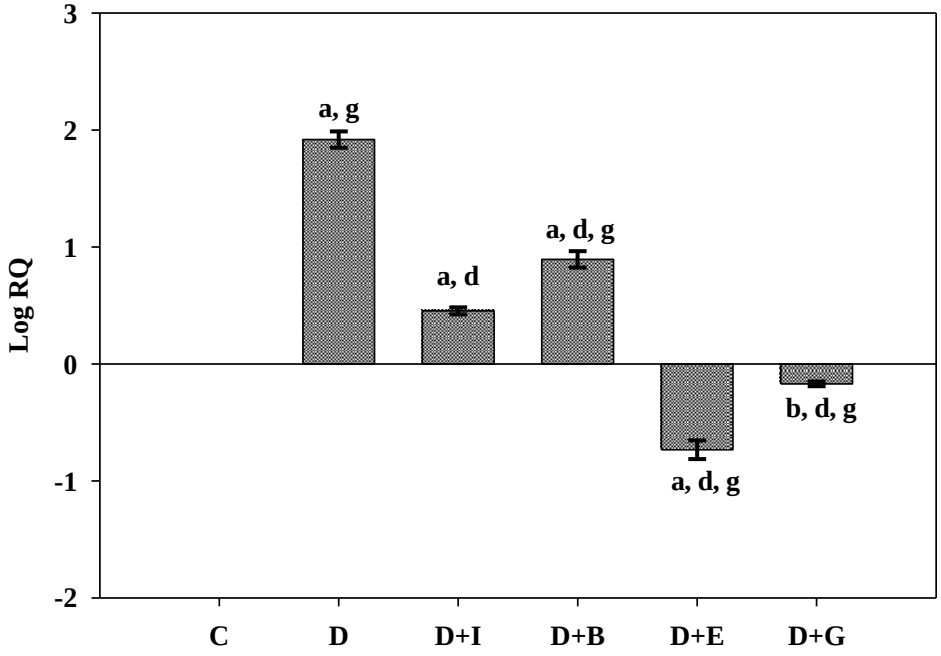


Table-32
Real Time PCR amplification of acetylcholine esterase mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.89± 0.10[a, g]
D+I	0.45±0.04[a, d]
D+B	0.95±0.04[a, d, g]
D+E	-0.67±0.06[a, d, g]
D+G	-0.18±0.04[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-33
Real Time PCR amplification of choline acetyltransferase mRNA in the Cerebellum of experimental rats

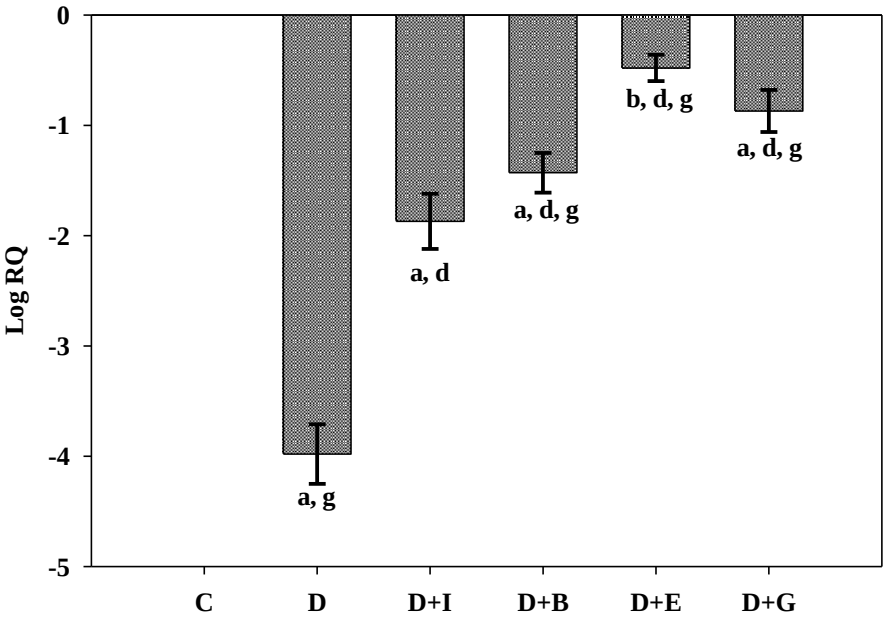


Table-33
Real Time PCR amplification of choline acetyltransferase mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.72± 0.17[a, g]
D+I	-2.61±0.21[a, d]
D+B	-1.54±0.19[a, d, g]
D+E	-0.46±0.06[b, d, g]
D+G	-0.86±0.05[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-34
Real Time PCR amplification of muscarinic M1 receptor mRNA in the Cerebellum of experimental rats

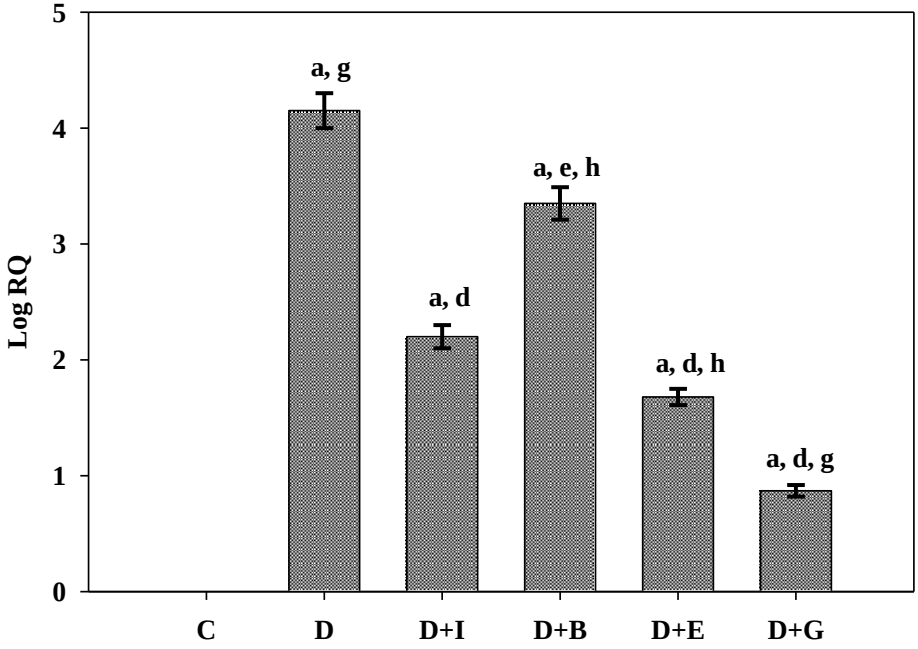


Table-34
Real Time PCR amplification of muscarinic M1 receptor mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	4.15± 0.15[a, g]
D+I	2.20±0.10[a, d]
D+B	3.35±0.14[a, e, h]
D+E	1.68±0.06[a, d, h]
D+G	0.87±0.05[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-35
Real Time PCR amplification of muscarinic M3 receptor mRNA in the Cerebellum of experimental rats

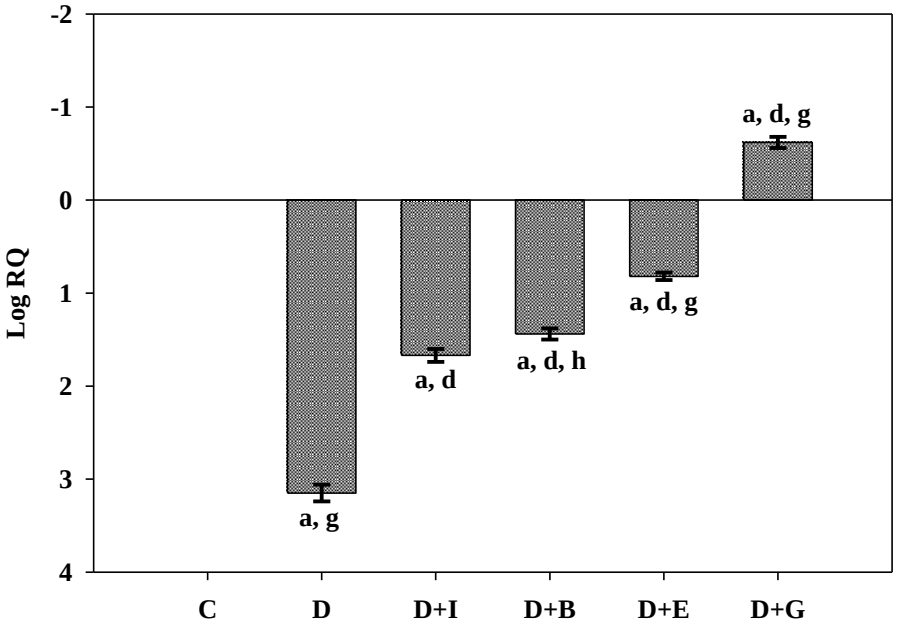


Table-35
Real Time PCR amplification of muscarinic M3 receptor mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.15± 0.09[a, g]
D+I	-1.67±0.07[a, d]
D+B	-1.45±0.06[a, d, h]
D+E	-0.82±0.04[a, d, g]
D+G	0.62±0.06[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-36
Real Time PCR amplification of $GABA_B$ receptor mRNA in the Cerebellum of experimental rats

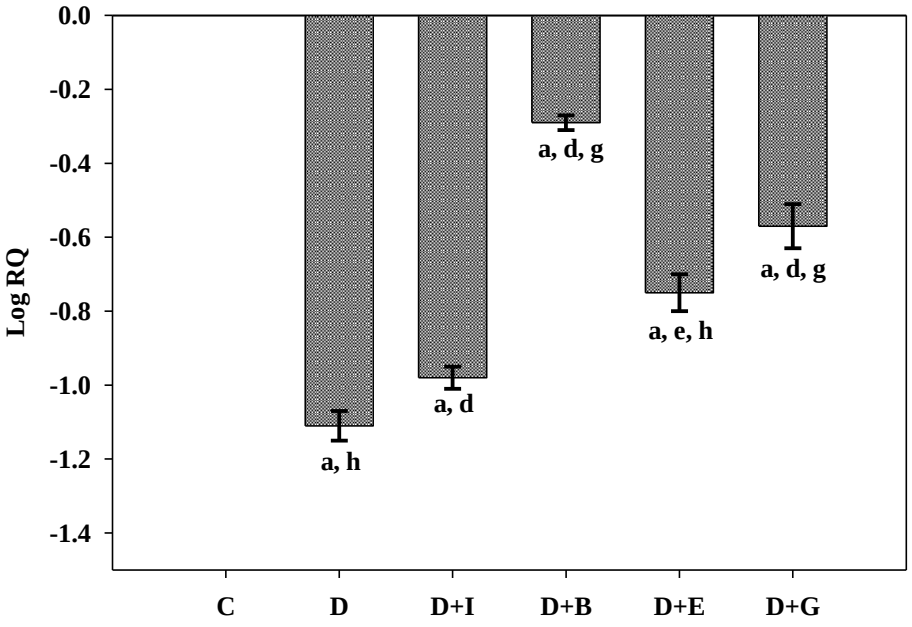


Table-36
Real Time PCR amplification of $GABA_B$ receptor mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.11± 0.04[a, h]
D+I	-0.97±0.04[a, d]
D+B	-0.29±0.03[a, d, g]
D+E	-0.75±0.05[a, e, h]
D+G	-0.57±0.06[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-37
Real Time PCR amplification of insulin receptor mRNA in the Cerebellum of experimental rats

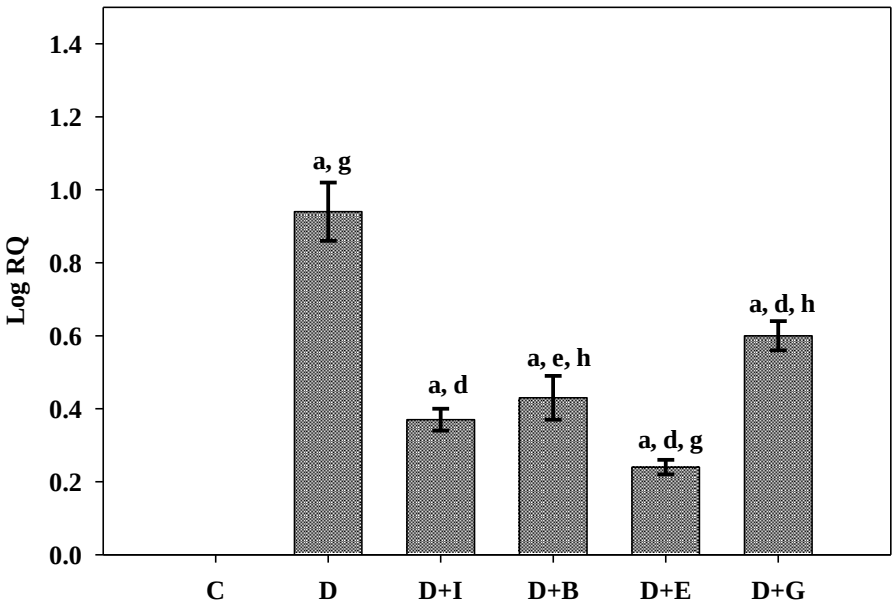


Table-37
Real Time PCR amplification of insulin receptor mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	0.94± 0.08[a, g]
D+I	0.39±0.03[a, d]
D+B	0.43±0.06[a, e, h]
D+E	0.24±0.02[a, d, g]
D+G	0.60±0.04[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-38
Real Time PCR amplification of Akt-1 mRNA in the Cerebellum of experimental rats

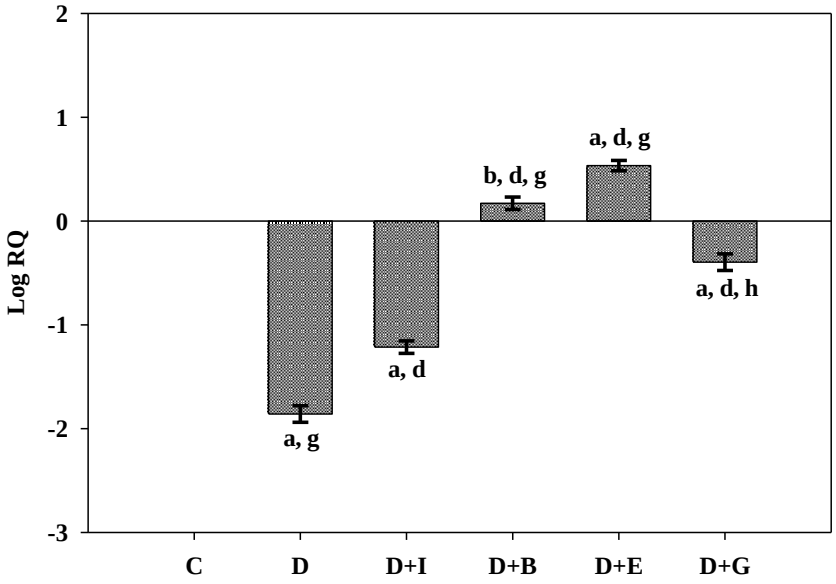


Table-38
Real Time PCR amplification of Akt-1 mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.86± 0.08[a, g]
D+I	-1.22±0.06[a, d]
D+B	0.17±0.07[b, d, g]
D+E	0.53±0.05[a, d, g]
D+G	-0.40 ±0.06[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-39
Real Time PCR amplification of Bax mRNA in the Cerebellum of experimental rats

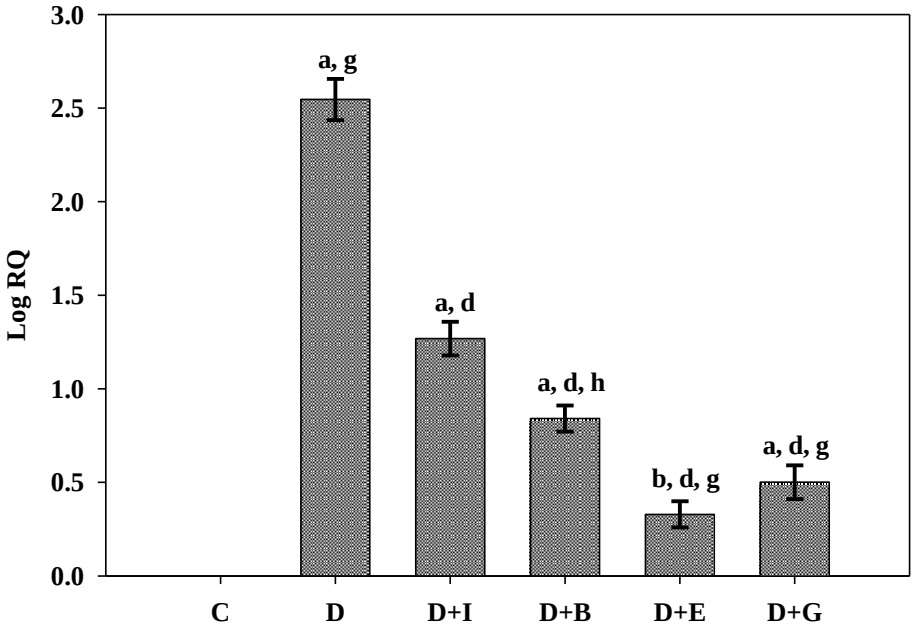


Table-39
Real Time PCR amplification of Bax mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	2.55± 0.11[a, g]
D+I	1.26±0.09[a, d]
D+B	0.84±0.07[a, d, h]
D+E	0.33±0.06[b, d, g]
D+G	0.50 ±0.09[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-40
Real Time PCR amplification of caspase 8 mRNA in the Cerebellum of experimental rats

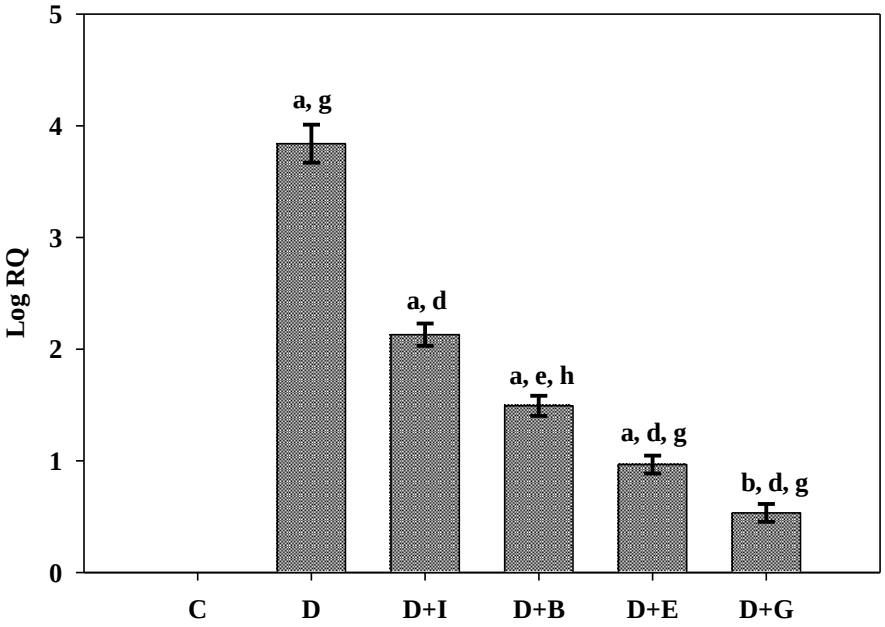


Table-40
Real Time PCR amplification of Caspase 8 mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	3.84± 0.17[a, g]
D+I	2.13±0.09[a, d]
D+B	1.49±0.09[a, e, h]
D+E	0.97±0.08[a, d, g]
D+G	0.53 ±0.08[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-41
Real Time PCR amplification of Gpx mRNA in the Cerebellum of experimental rats

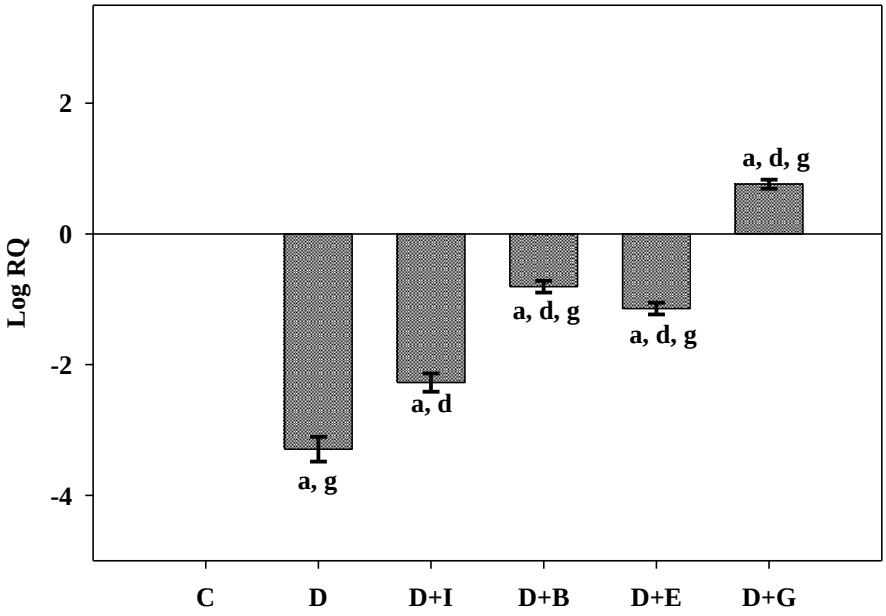


Table-41
Real Time PCR amplification of Gpx mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.29± 0.19[a, g]
D+I	-2.28±0.14[a, d]
D+B	-0.81±0.09[a, d, g]
D+E	-1.14±0.09[a, d, g]
D+G	0.76 ±0.07[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-42
Real Time PCR amplification of Superoxide dismutase mRNA in the Cerebellum of experimental rats

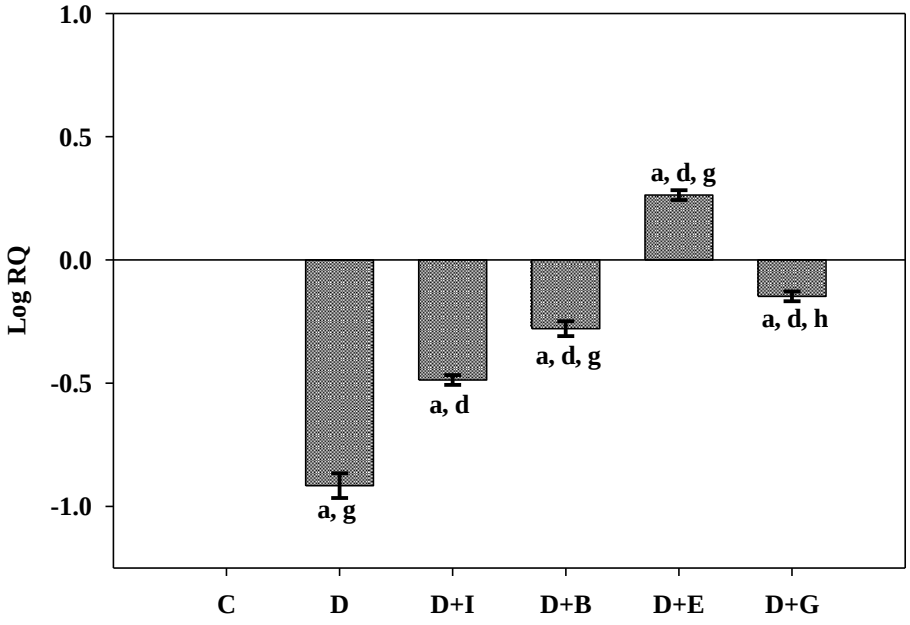


Table-42
Real Time PCR amplification of Superoxide dismutase mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-0.92± 0.05[a, g]
D+I	-0.49±0.02[a, d]
D+B	-0.28±0.03[a, d, g]
D+E	0.26±0.03[a, d, g]
D+G	-0.15±0.02[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-43
Real Time PCR amplification of GLUT 3 mRNA in the Cerebellum of experimental rats

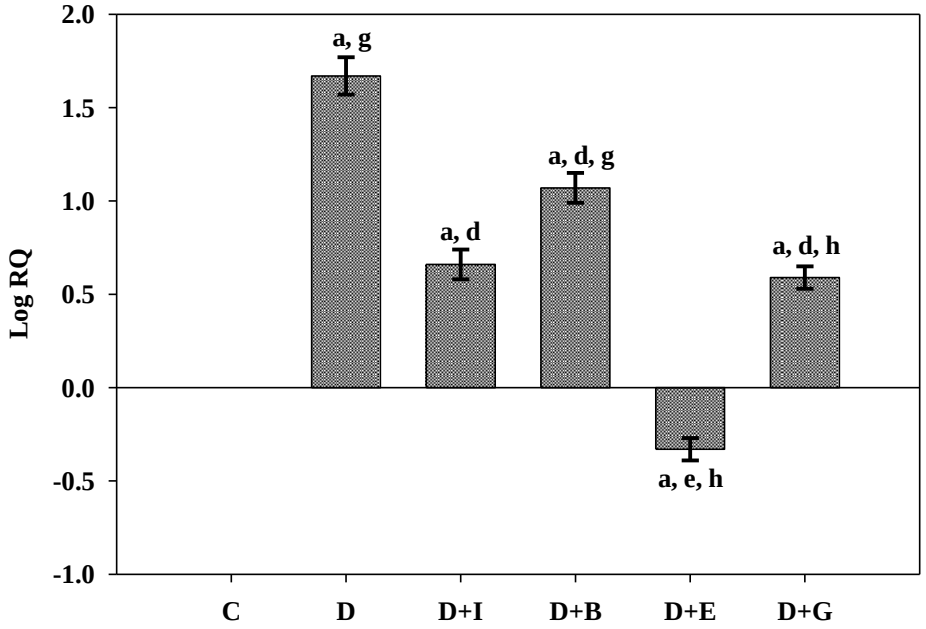


Table-43
Real Time PCR amplification of GLUT 3 mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.66± 0.10[a, g]
D+I	0.66±0.08[a, d]
D+B	1.07±0.08[a, d, g]
D+E	-0.33±0.06[a, e, h]
D+G	0.59±0.06[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-44
Real Time PCR amplification of phospholipase C mRNA in the Cerebellum of experimental rats

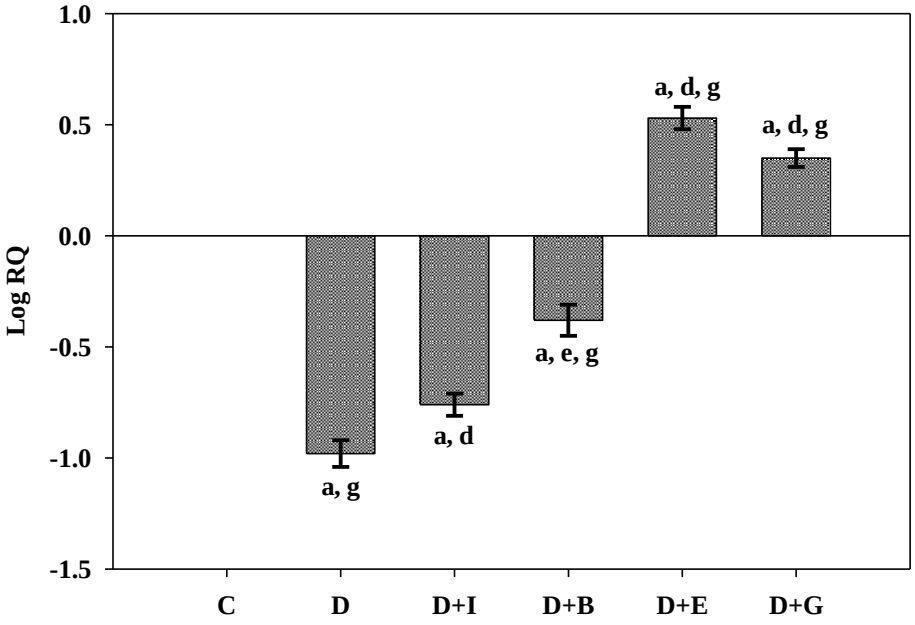


Table-44
Real Time PCR amplification of phospholipase C mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-0.98± 0.06[a, g]
D+I	-0.76±0.05[a, d]
D+B	-0.38±0.06[a, e, g]
D+E	0.52±0.05[a, d, g]
D+G	0.35±0.04[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-45
Real Time PCR amplification of CREB mRNA in the Cerebellum of experimental rats

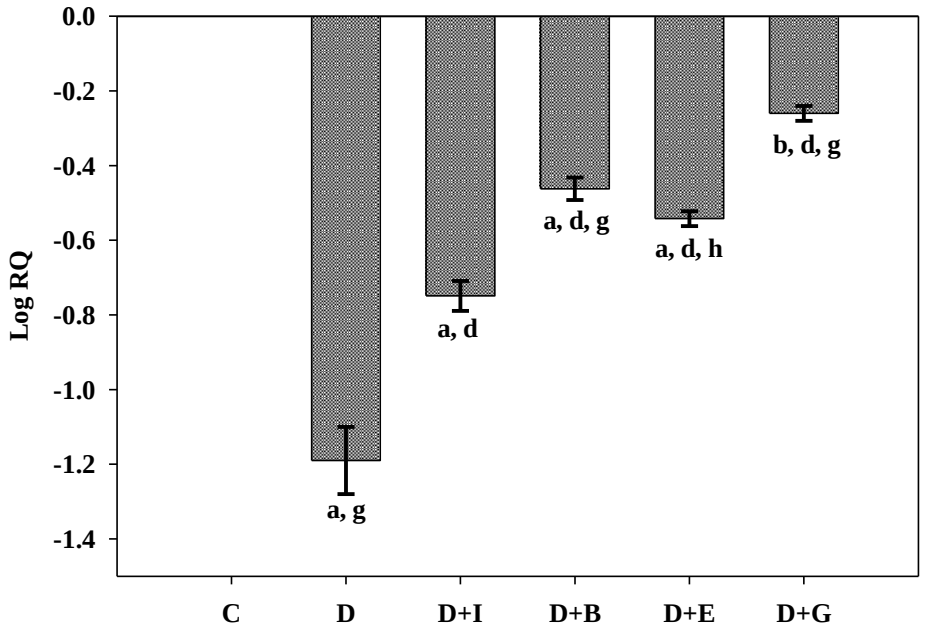


Table-45
Real Time PCR amplification of CREB mRNA in the Cerebellum of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.19± 0.09[a, g]
D+I	-0.75±0.04[a, d]
D+B	-0.46±0.03[a, d, g]
D+E	-0.54±0.03[a, d, h]
D+G	-0.26±0.03[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E-Vitamin E treated Diabetic rats. D+G-Gymnemic acid treated diabetic rats.

Figure-46
Total antioxidant level in the Cerebellum of experimental rats

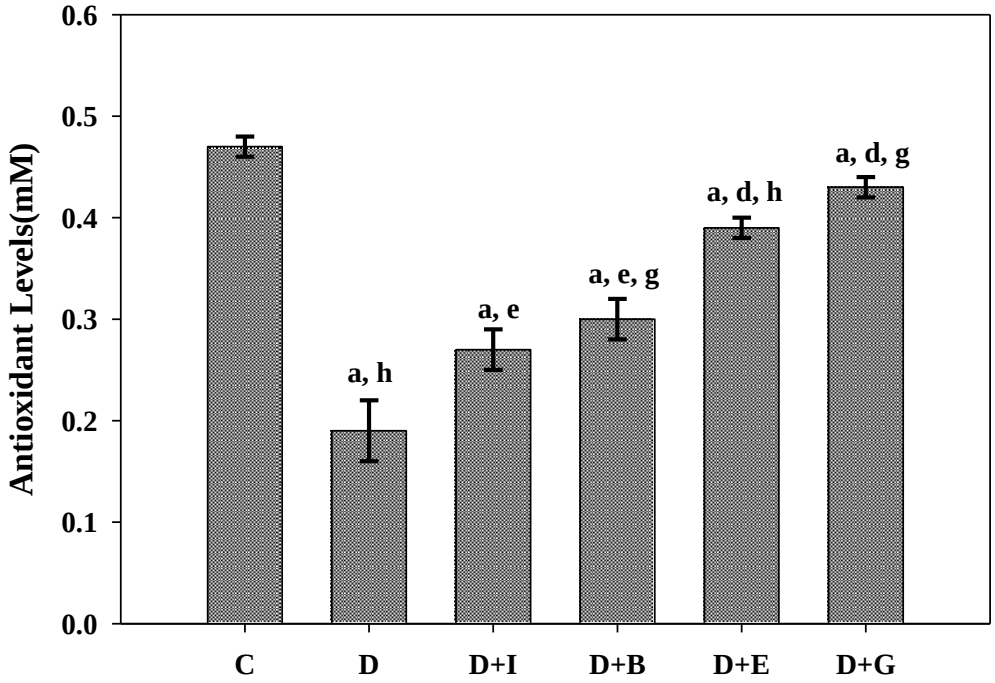


Table-46
Total antioxidant level in the Cerebellum of experimental rats

Experimental Groups	Antioxidant Level(mM)
Control	0.47±0.01
Diabetic	0.19± 0.03[a, h]
D+I	0.27±0.02[a, e]
D+B	0.30±0.02[a, e, g]
D+E	0.39±0.01[a, d, h]
D+G	0.43±0.01[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E-Vitamin E treated Diabetic rats. D+G-Gymnemic acid treated diabetic rats.

Figure-47
Total aqueous peroxide level in the Cerebellum of experimental rats

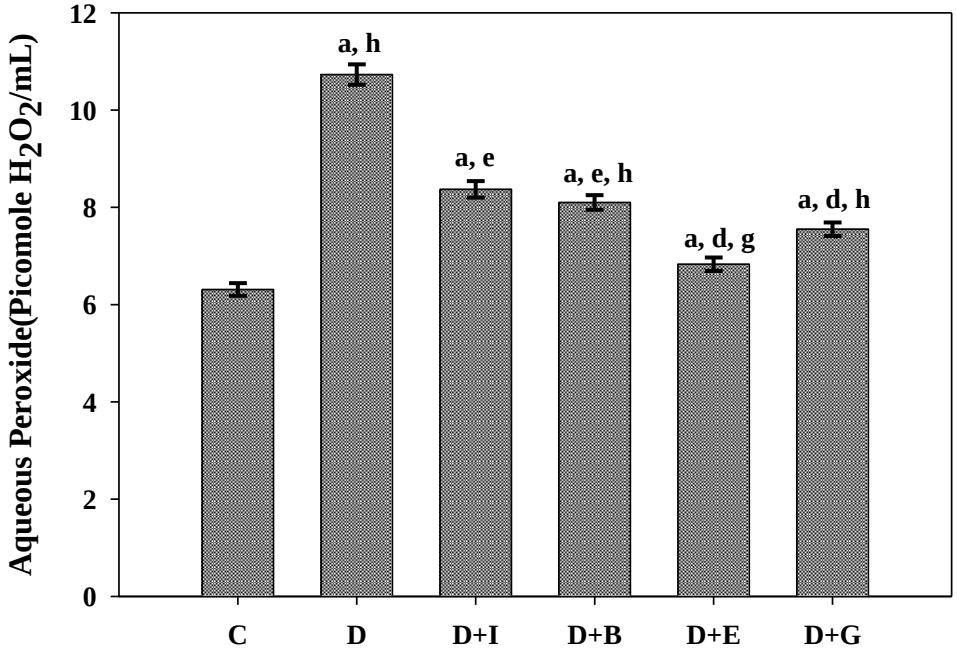


Table-47
Total aqueous peroxide level in the Cerebellum of experimental rats

Experimental Groups	Aqueous peroxide (Picomole H_2O_2/mL)
Control	6.31±0.13
Diabetic	10.73± 0.21[a, h]
D+I	8.37±0.17[a, e]
D+B	8.10±0.15[a, e, h]
D+E	6.83±0.14[a, d, g]
D+G	7.55±0.14[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E-Vitamin E treated Diabetic rats. D+G-Gymnemic acid treated diabetic rats.

Figure-48

Histochemistry of TO-PRO®-3 iodide staining in the cerebellum of experimental Rats

Figure-49

Confocal imaging of muscarinic M1 receptor in the cerebellum of experimental rats

Figure-50

Confocal imaging of muscarinic M3 receptor in the cerebellum of experimental rats

BRAIN STEM

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the brain stem of experimental rats

The total muscarinic receptor status was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist atropine. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) increased significantly in diabetic rats compared to control group. In D+B, showed that significantly decreased B_{max} ($p<0.001$) and there is no significant change in K_d when compared to control, but significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly decreased B_{max} ($p<0.05$) and increased K_d ($p<0.05$) when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly increased when compared to control, but significantly decreased B_{max} ($p<0.001$) and there is no significant change in K_d when compared to diabetic and significantly increased B_{max} ($p<0.05$) and K_d ($p<0.05$) when compared to D+I group. D+G group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, diabetic {B_{max} ($p<0.001$) and K_d ($p<0.05$)} and D+I group **{**B_{max} ($p<0.05$) and K_d ($p<0.05$)} **(**Figure-51, Table-51).

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the brain stem of experimental rats

Binding analysis of muscarinic M1 receptor was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) decreased significantly in diabetic group when compared to control group. In D+B, showed that significantly decreased B_{max} ($p<0.05$) and increased K_d ($p<0.001$) when

compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group {B_{max} ($p<0.001$) and K_d ($p<0.001$)}. D+E group showed that significantly decreased B_{max} ($p<0.001$) and increased K_d ($p<0.05$) when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group {B_{max} ($p<0.001$) and K_d ($p<0.001$)}. D+G group showed that significantly decreased B_{max} ($p<0.001$) and increased K_d ($p<0.001$) when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group **{**B_{max} ($p<0.05$) and K_d ($p<0.05$)} **(**Figure-52, Table-52).

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor, antagonist, 4-DAMP mustard in the brain stem of experimental rats

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The Scatchard analysis showed that the significantly increased B_{max} ($p<0.001$) and no significant change in K_d of diabetic group when compared to control. In D+B, showed that significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to control, but significantly decreased B_{max} ($p<0.05$) and K_d ($p<0.001$) when compared to diabetic and D+I group {B_{max} ($p<0.05$) and K_d ($p<0.05$)}. D+E group showed that significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.05$) when compared to control, but significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group {B_{max} ($p<0.001$) and K_d ($p<0.001$)}. D+G group showed that significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to control, but significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to

diabetic and D+I group {B_{max} ($p<0.001$) and K_d ($p<0.001$)} **(**Figure-53, Table-53).

$GABA_B$ receptor analysis

Scatchard analysis of [^{3}H] baclofen binding against baclofen to $GABA_B$ receptors in the brain stem of experimental rats

Binding analysis of [^{3}H] baclofen binding against baclofen. The Scatchard analysis showed that the significantly decreased B_{max} ($p<0.001$) and no significant change in K_d of diabetic group when compared to control. In D+B, showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly increased when compared to control, diabetic {B_{max} ($p<0.001$) and K_d ($p<0.05$)} and D+I group {B_{max} ($p<0.05$) and K_d ($p<0.001$)}. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, diabetic {B_{max} ($p<0.001$) and K_d ($p<0.05$)} and D+I group {B_{max} ($p<0.05$) and K_d ($p<0.001$)}. D+G group showed that significantly decreased B_{max} ($p<0.001$) and there is no significant change in K_d when compared to control, significantly decreased B_{max} ($p<0.001$) and there is no significant change in K_d in diabetic and B_{max} ($p<0.001$) D+I group **(**Figure-54, Table-54).

REAL TIME-PCR ANALYSIS

Real Time-PCR analysis of acetylcholine esterase in the brain stem of experimental rats

Gene expression of acetylcholine esterase mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant up regulation of AchE mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+E group showed a significant increase in the

mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and significant up regulation when compared to D+I ($p<0.05$) group. D+G group showed a significant up regulation when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-55, Table-55).

Real Time-PCR analysis of choline acetyltransferase in the brain stem of experimental rats

Gene expression of choline acetyltransferase mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D + B, there was a significant increase of ChAT mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant down regulation when compared to control ($p<0.05$), diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure- 56, Table-56).

Real Time-PCR analysis of muscarinic M1 receptor mRNA in the brain stem of experimental rats

Gene expression of muscarinic M1 receptor mRNA showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant decrease of muscarinic M1 receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group.

D+G group showed a significant increase of muscarinic M1 receptor gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-57, Table-57).

Real Time-PCR analysis of muscarinic M3 receptor mRNA in the brain stem of experimental rats

Gene expression of muscarinic M3 receptor mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant decrease of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant up regulation of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-58, Table-58).

Real Time-PCR analysis of $GABA_B$ receptor mRNA in the brain stem of experimental rats

Gene expression of $GABA_B$ receptor mRNA showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant down regulation of $GABA_B$ receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant down regulation of $GABA_B$ receptor gene

expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and there is no significant change in D+I group **(**Figure-59, Table-59).

Real Time-PCR analysis of insulin receptor mRNA in the brain stem of experimental rats

Gene expression of insulin receptor mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant increase of insulin receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.05$), diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant up regulation of insulin receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-60, Table-60).

Real Time-PCR analysis of Akt-1 mRNA in the brain stem of experimental rats

Gene expression of Akt-1 mRNA showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant decrease of Akt-1 mRNA gene expression when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant decrease of Akt-1 mRNA gene expression when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-61, Table-61).

Real Time-PCR analysis of Bax mRNA in the brain stem of experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant increase of Bax mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of Bax mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group **(**Figure-62, Table-62).

Real Time-PCR analysis of caspase 8 mRNA in the brain stem of experimental rats

Gene expression of caspase 8 mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant increase of caspase 8 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group. D+G group showed a significant) increase of caspase 8 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-63, Table-63).

Real Time-PCR analysis of Gpx (glutathione peroxidase) mRNA in the brain stem of experimental rats

Gene expression of Gpx mRNA showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant increase of Gpx mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant decrease of Gpx mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-64, Table-64).

Real Time-PCR analysis of SOD (superoxide dismutase) mRNA in the brain stem of experimental rats

Gene expression of SOD mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant increase of SOD mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of SOD mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-65, Table-65).

Real Time-PCR analysis of GLUT 3 mRNA in the brain stem of experimental rats

Gene expression of GLUT 3 mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant increase of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.05$), diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-66, Table-66).

Real Time-PCR analysis of phospholipase C mRNA in the cerebellum of experimental rats

Gene expression of phospholipase C mRNA showed significant down regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant decrease of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant decrease of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and significant down regulation when compared to D+I ($p<0.001$) group **(**Figure-67, Table-67).

Real Time-PCR analysis of CREB mRNA in the brain stem of experimental rats

Gene expression of CREB mRNA showed significant up regulation ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant decrease of CREB mRNA gene expression when compared to control ($p<0.05$), diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant increase of CREB mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-68, Table-68).

Total antioxidant activity in the brain stem of experimental rats

Total antioxidant activity showed significant decrease ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was a significant decrease of antioxidant level when compared to control ($p<0.001$), but significant increase when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+E group showed a significant decrease in the antioxidant level when compared to control, but significant increase when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant decrease of antioxidant level when compared to control ($p<0.001$), but significant increase when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group **(**Figure-69, Table-69).

Total aqueous peroxide activity in the brain stem of experimental rats

Total aqueous peroxide activity showed significantly increased ($p<0.001$) in the brain stem of diabetic rats compared to control. In D+B, there was significantly increased aqueous peroxide activity level when compared to control, but significant decrease when compared to diabetic

($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significantly increased aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group showed there was no significant change of aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-70, Table-70).

CONFOCAL STUDIES

TO-PRO®-3 iodide staining in the brain stem of experimental rats

TO-PRO®-3 staining showed significantly decreased ($p<0.001$) nuclear density in the brain stem of diabetic rats compared to control. In D+B, there was a significantly decreased nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and significantly decreased when compared to D+I ($p<0.001$) group. D+E group showed a significantly decreased nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and significantly decreased when compared to D+I ($p<0.05$) group. D+G group showed a significantly decreased nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-71, Table-71).

Muscarinic M1 receptor antibody staining in the brain stem of experimental rats

Muscarinic M1 receptor subunit antibody staining in the brain stem showed a significantly decreased ($p<0.001$) in the mean pixel value of diabetic rats compared to control. In D+B, there was a significantly decreased muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$)

and significantly decreased when compared to D+I ($p<0.001$) group. D+E group showed a significantly decreased muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and significantly decreased when compared to D+I ($p<0.001$) group. D+G group showed a significantly decreased muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure- 72, Table- 72).

Muscarinic M3 receptor antibody staining in the brain stem of experimental rats

Muscarinic M3 receptor subunit antibody staining in the brain stem showed a significantly increased ($p<0.001$) in the mean pixel value in diabetic rats compared to control. In D+B, there was a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.05$) group. D+E group showed a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.05$) group. D+G group showed a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and significantly increased when compared to D+I ($p<0.001$) group **(**Figure-73, Table- 73).

Figure-51
Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the Brain Stem of experimental rats

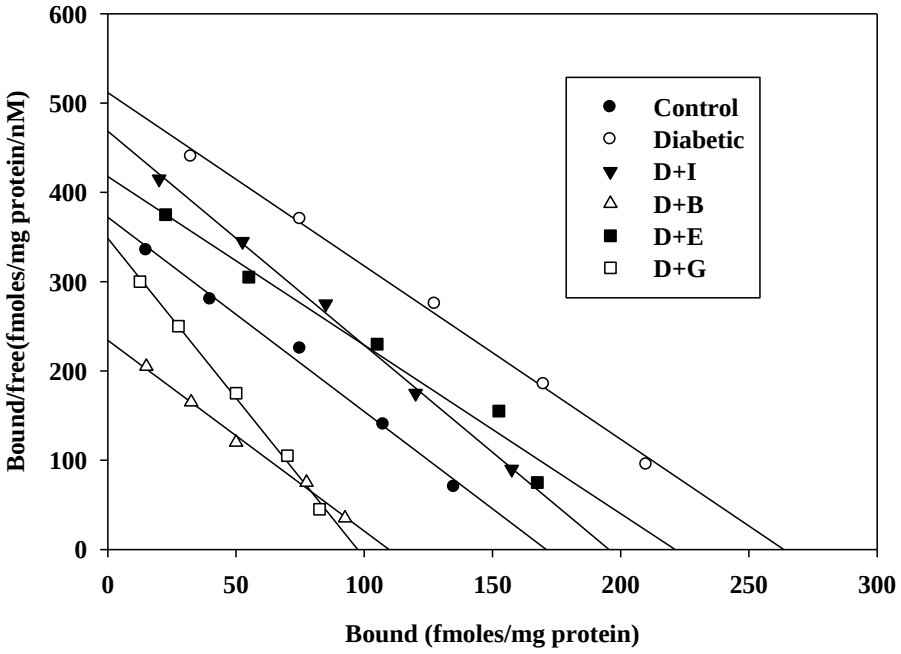


Table-51
Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the Brain Stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	171.24± 6.81	0.46 ± 0.05
Diabetic	263.76± 9.12[a, g]	0.52 ± 0.07[a, g]
D + I	196.26± 8.32[a, d]	0.42 ± 0.05[a, d]
D + B	109.38 ± 5.47[a, d, h]	0.46 ± 0.05[d, h]
D+E	221.28±8.63[a, d, g]	0.53± 0.07[a, h]
D+G	97.5±4.25[a, d, g]	0.28±0.03[a, e, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-52
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the Brain Stem of experimental rats

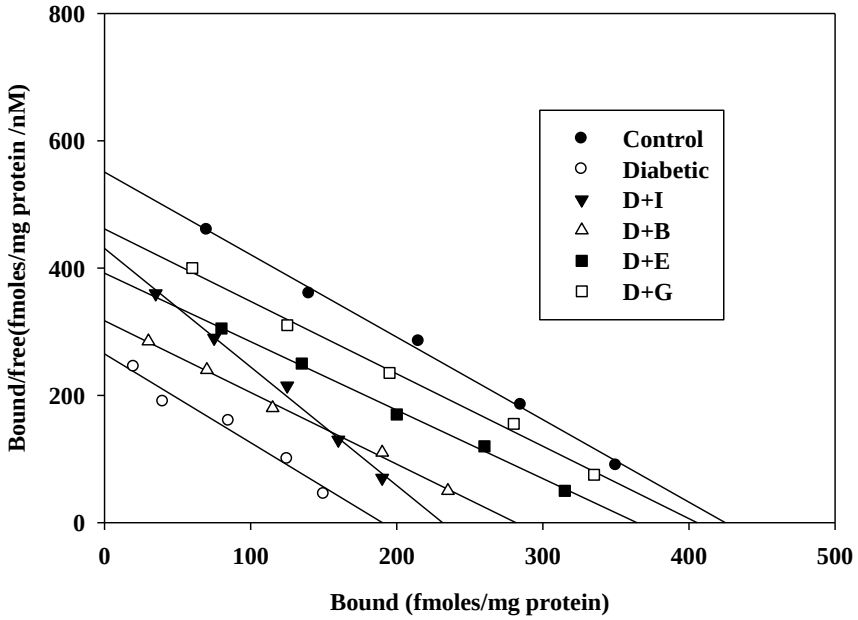


Table-52
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the Brain Stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	422.95± 14.53	0.78 ± 0.10
Diabetic	139.60± 4.41[a, g]	0.53 ± 0.07[a, g]
D + I	231.38± 7.68[a, d]	0.54 ± 0.07[a, d]
D + B	279.21 ± 9.25[b, d, g]	0.88 ± 0.11[a, d, g]
D+E	362.57±12.20[a, d, g]	0.91± 0.12[b, d, g]
D+G	403.13±13.86[a, d, h]	0.87±0.11[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-53
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the Brain Stem of experimental rats

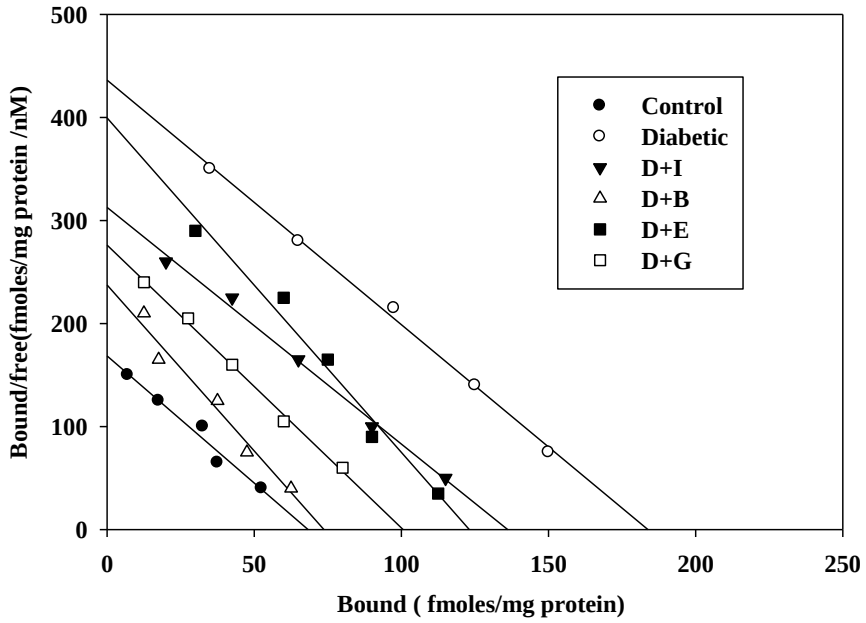


Table-53
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the Brain Stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	68.25± 2.81	0.40 ± 0.05
Diabetic	182.81± 5.63[a, g]	0.42 ± 0.05[a, g]
D + I	135.45± 4.61[a, d]	0.43 ± 0.06[a, d]
D + B	74.62 ± 3.17[a, e, h]	0.32 ± 0.04[a, d, h]
D+E	122.48±4.19[a, d, g]	0.30± 0.03[a, d, g]
D+G	100.35±3.86[a, d, g]	0.35±0.04[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-54
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the Brain Stem of experimental rats

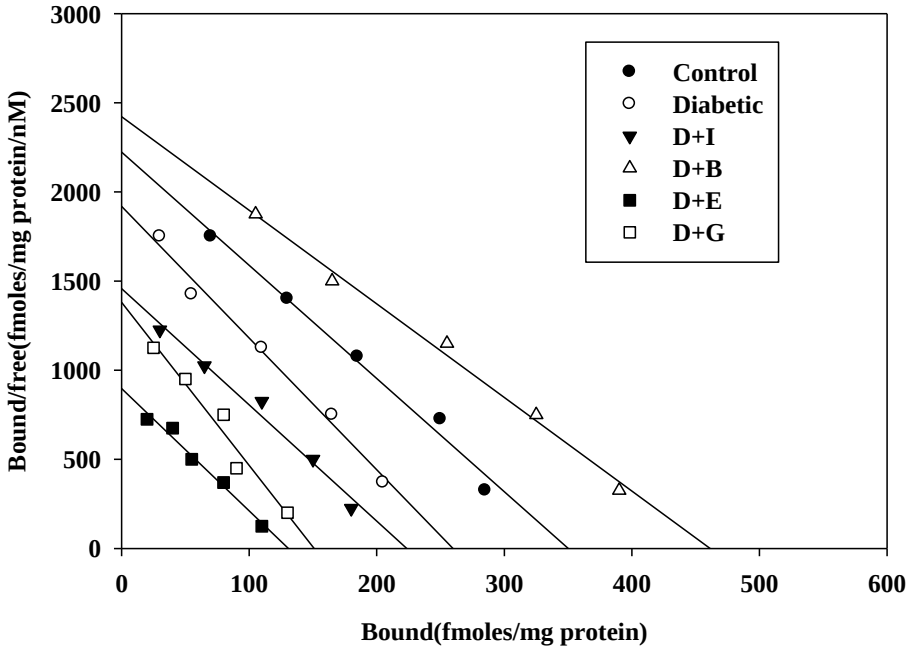


Table-54
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the Brain Stem of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	351.24± 11.80	0.16 ± 0.03
Diabetic	260.04± 9.03[a, g]	0.14± 0.02[a, g]
D + I	223.81± 7.38[a, d]	0.15 ± 0.03[a, d]
D + B	462.48 ± 16.15[a, d, h]	0.19 ± 0.04[a, e, g]
D+E	131.28±4.18[a, d, g]	0.11± 0.02[a, d, g]
D+G	151.32±6.59[a, d, h]	0.15±0.03

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-55
Real Time PCR amplification of acetylcholine esterase mRNA in the Brain Stem of experimental rats

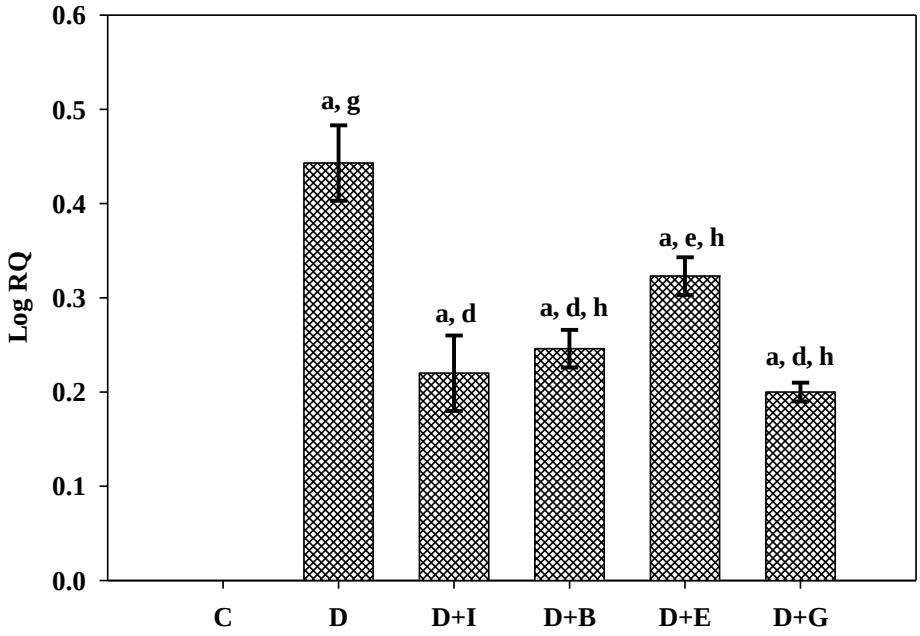


Table-55
Real Time PCR amplification of acetylcholine esterase mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	0.44± 0.04[a, g]
D+I	0.22±0.04[a, d]
D+B	0.25±0.02[a, d, h]
D+E	0.32±0.02[a, e, h]
D+G	0.20±0.01[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-56
Real Time PCR amplification of choline acetyltransferase mRNA in the Brain Stem of experimental rats

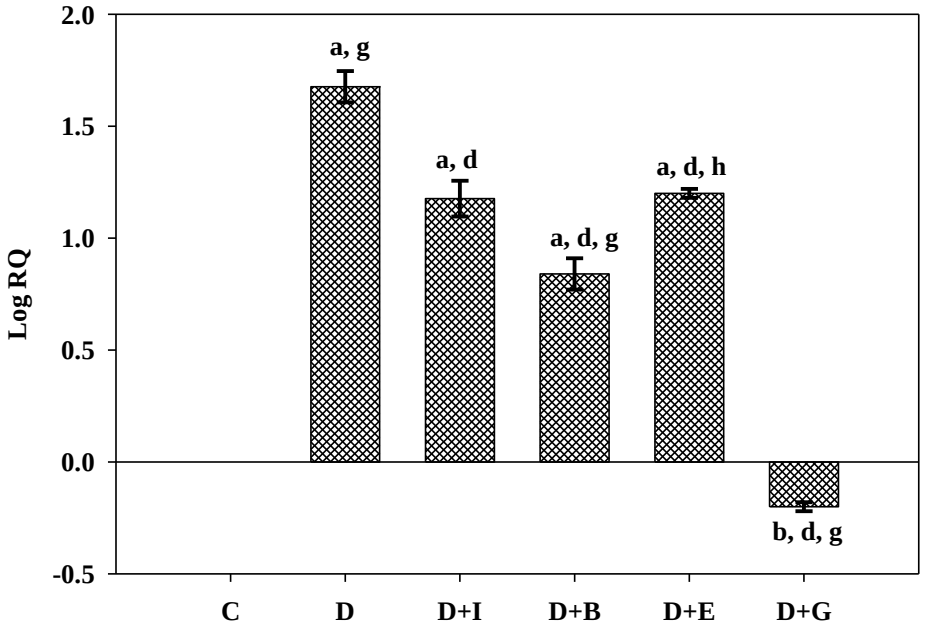


Table-56
Real Time PCR amplification of choline acetyltransferase mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.67± 0.07[a, g]
D+I	1.18±0.08[a, d]
D+B	0.84±0.07[a, d, g]
D+E	1.20±0.02[a, d, h]
D+G	-0.20±0.02[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-57
Real Time PCR amplification of muscarinic M1 receptor mRNA in the Brain Stem of experimental rats

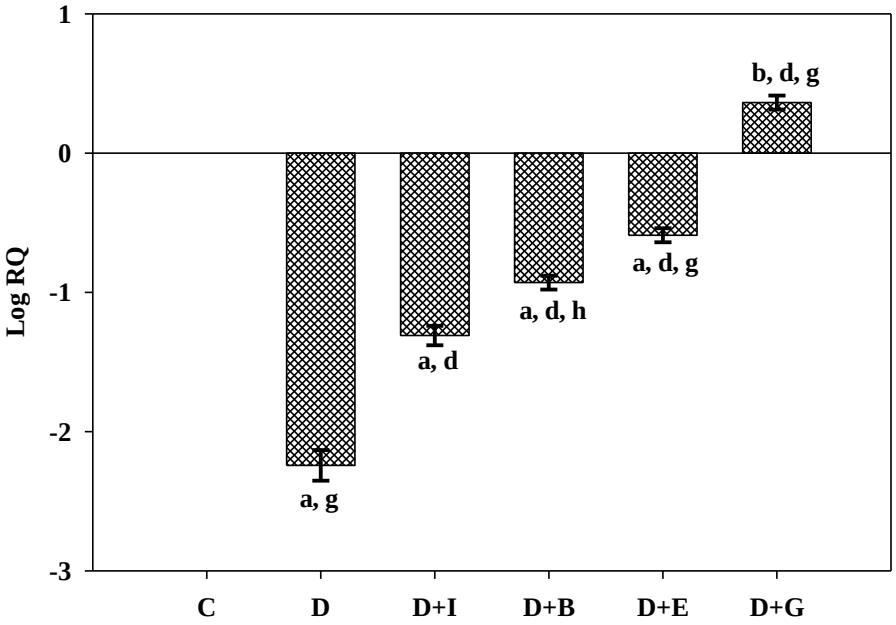


Table-57
Real Time PCR amplification of muscarinic M1 receptor mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-2.24± 0.11[a, g]
D+I	-1.31±0.08[a, d]
D+B	-0.93±0.06[a, d, h]
D+E	-0.59±0.05[a, d, g]
D+G	0.36±0.05[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-58
Real Time PCR amplification of muscarinic M3 receptor mRNA in the Brain Stem of experimental rats

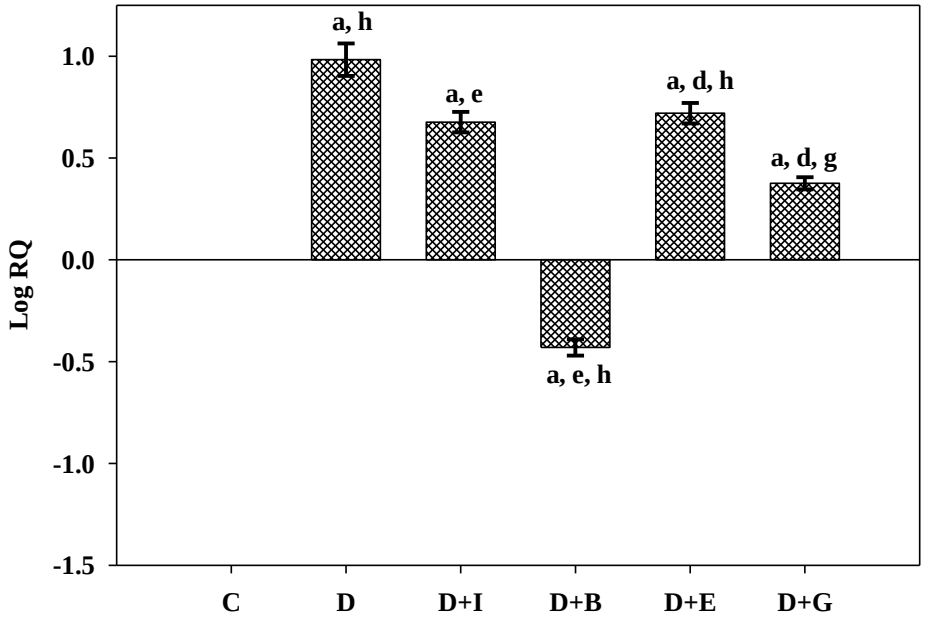


Table-58
Real Time PCR amplification of muscarinic M3 receptor mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	0.98± 0.08[a, h]
D+I	0.67±0.05[a, e]
D+B	-0.43±0.04[a, e, h]
D+E	0.72±0.05[a, d, h]
D+G	0.38±0.04[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-59

Real Time PCR amplification of $GABA_B$ receptor mRNA in the Brain Stem of experimental rats

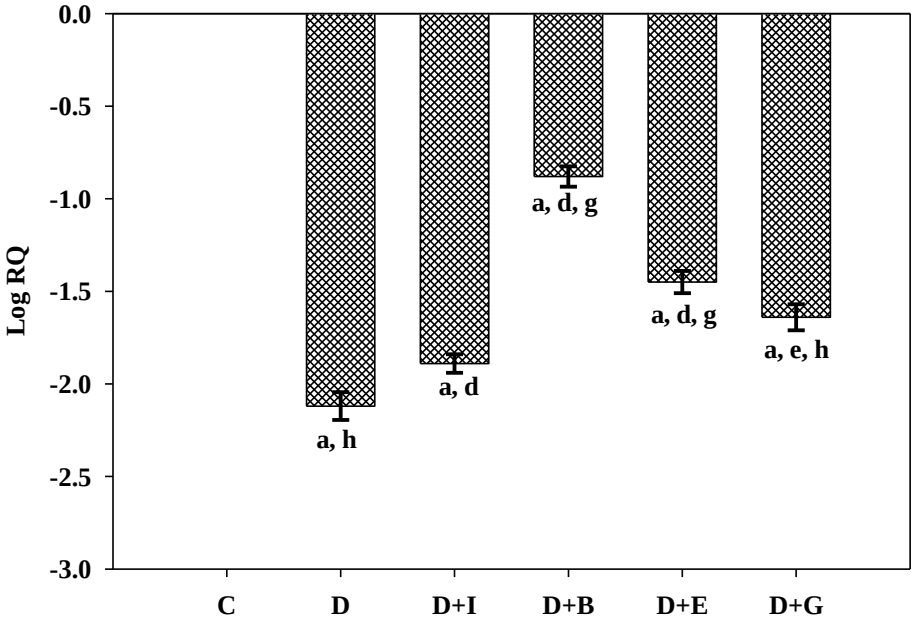


Table-59
Real Time PCR amplification of $GABA_B$ receptor mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-2.12± 0.07[a, h]
D+I	-1.89±0.05[a, d]
D+B	-0.88±0.06[a, d, g]
D+E	-1.45±0.06[a, d, g]
D+G	-1.64±0.07[a, e, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-60
Real Time PCR amplification of insulin receptor mRNA in the Brain Stem of experimental rats

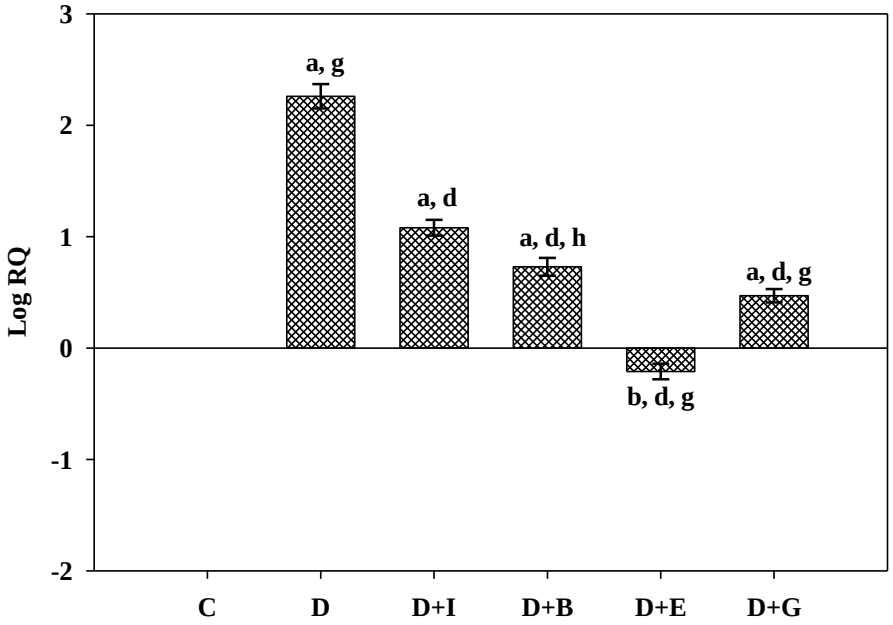


Table-60
Real Time PCR amplification of insulin receptor mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	2.26± 0.11[a, g]
D+I	1.08±0.06[a, d]
D+B	0.73±0.07[a, d, h]
D+E	-0.21±0.07[b, d, g]
D+G	0.46±0.06[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E-Vitamin E treated Diabetic rats. D+G-Gymnemic acid treated diabetic rats.

Figure-61
Real Time PCR amplification of Akt-1 mRNA in the Brain Stem of experimental rats

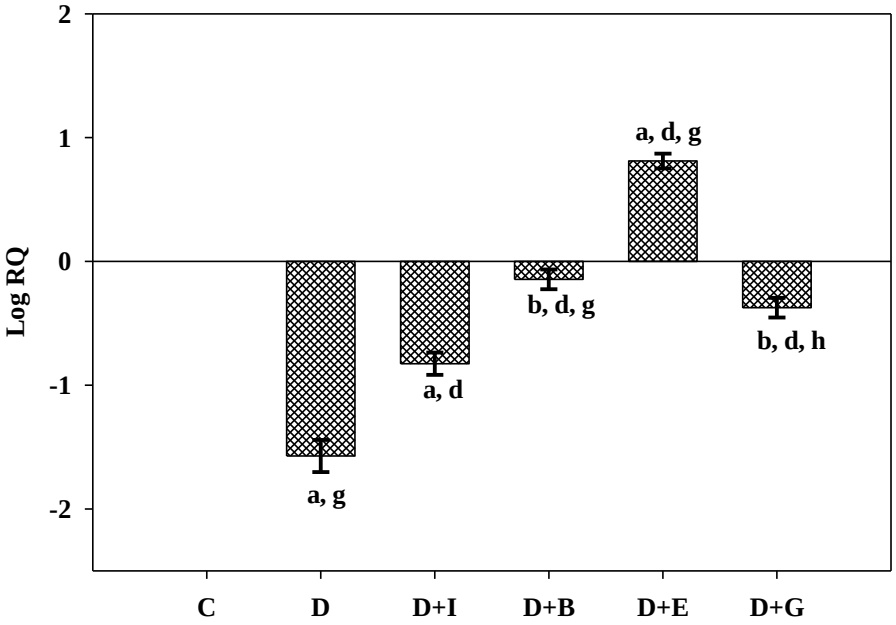


Table-61
Real Time PCR amplification of Akt-1 mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.57± 0.13[a, g]
D+I	-0.83±0.09[a, d]
D+B	-0.15±0.08[b, d, g]
D+E	0.81±0.07[a, d, g]
D+G	-0.37±0.07[b, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-62
Real Time PCR amplification of Bax mRNA in the Brain Stem of experimental rats

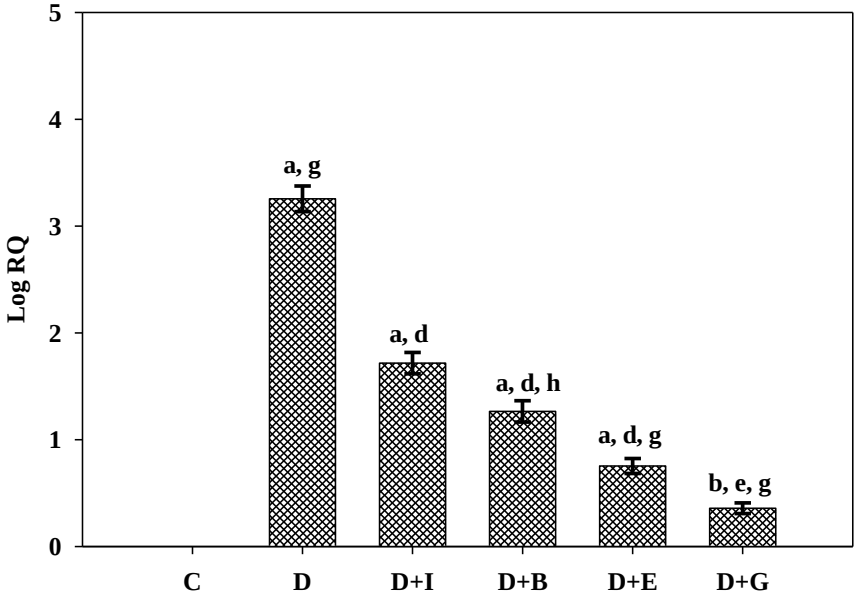


Table-62
Real Time PCR amplification of Bax mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	3.25± 0.12[a, g]
D+I	1.72±0.10[a, d]
D+B	1.27±0.10[a, d, h]
D+E	0.75±0.07[a, d, g]
D+G	0.36 ±0.05[b, e, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-63
Real Time PCR amplification of caspase 8 mRNA in the Brain Stem of experimental rats

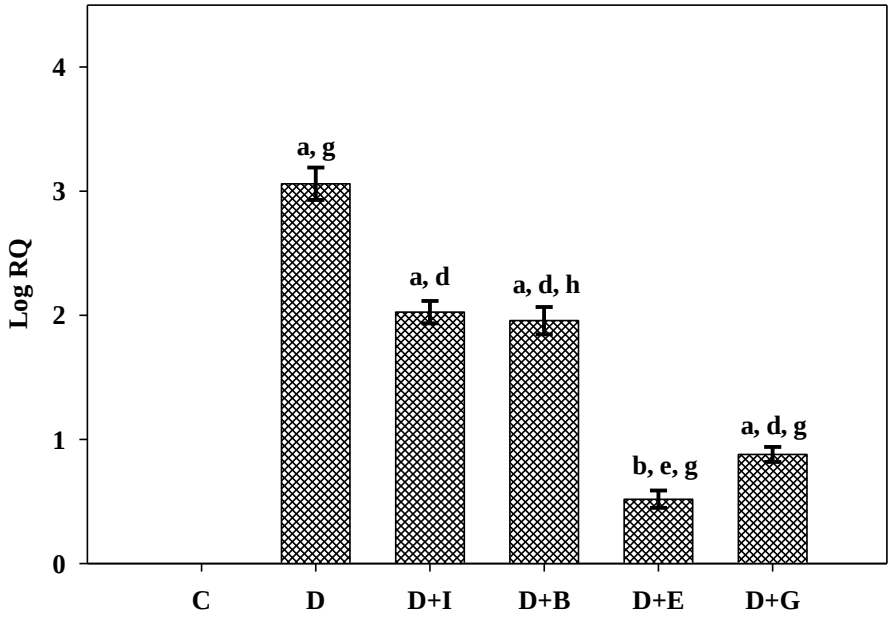


Table-63
Real Time PCR amplification of Caspase 8 mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	3.06± 0.13[a, g]
D+I	2.03±0.09[a, d]
D+B	1.96±0.11[a, d, h]
D+E	0.52±0.07[b, e, g]
D+G	0.87 ±0.06[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E-Vitamin E treated Diabetic rats. D+G-Gymnemic acid treated diabetic rats.

Figure-64
Real Time PCR amplification of Gpx mRNA in the Brain Stem of experimental rats

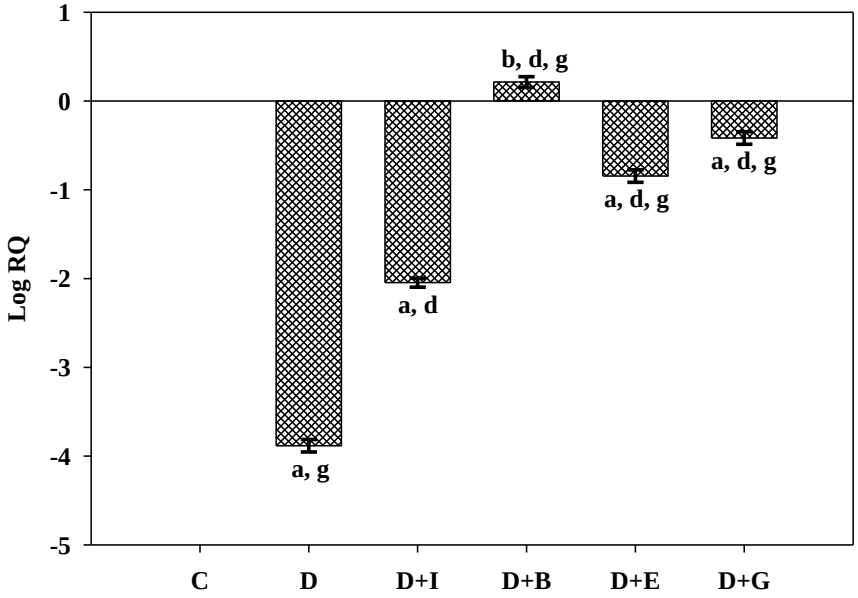


Table-64

Real Time PCR amplification of Gpx mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.88± 0.07[a, g]
D+I	-2.04±0.05[a, d]
D+B	0.21±0.06[b, d, g]
D+E	-0.84±0.07[a, d, g]
D+G	-0.42 ±0.07[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-65
Real Time PCR amplification of Superoxide dismutase mRNA in the Brain Stem of experimental rats

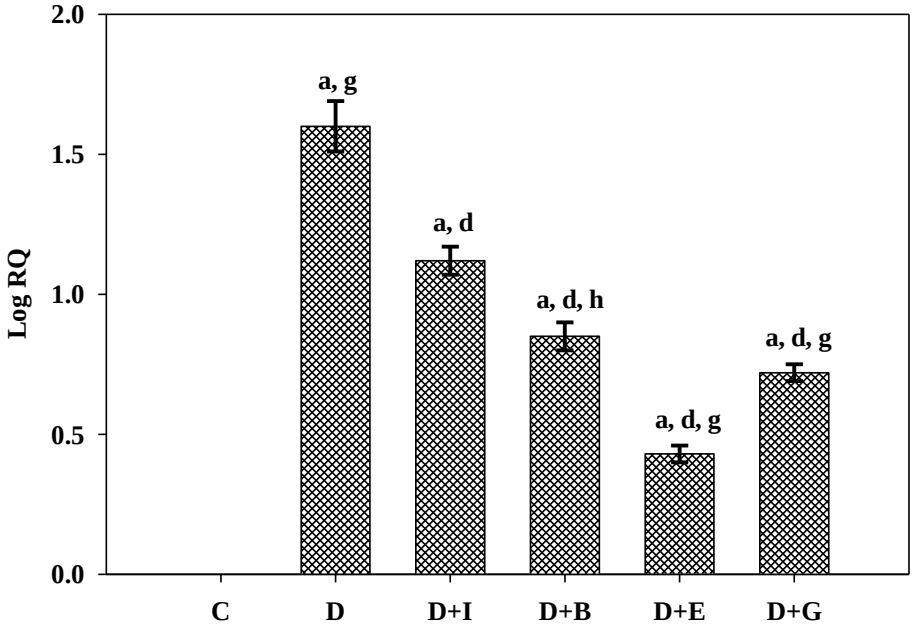


Table-65
Real Time PCR amplification of Superoxide dismutase mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.60± 0.09[a, g]
D+I	1.12±0.05[a, d]
D+B	0.85±0.06[a, d, h]
D+E	0.43±0.03[a, d, g]
D+G	0.73±0.03[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-66
Real Time PCR amplification of GLUT 3 mRNA in the Brain Stem of experimental rats

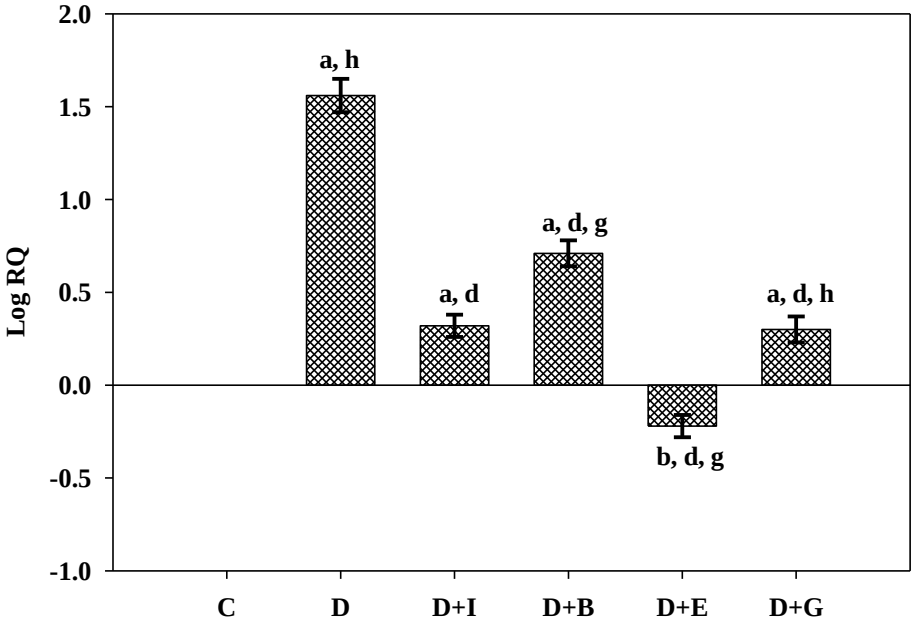


Table-66
Real Time PCR amplification of GLUT 3 mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.56± 0.09[a, h]
D+I	0.32±0.06[a, d]
D+B	0.71±0.07[a, d, g]
D+E	-0.22±0.05[b, d, g]
D+G	0.30±0.06[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-67
Real Time PCR amplification of phospholipase C mRNA in the Brain Stem of experimental rats

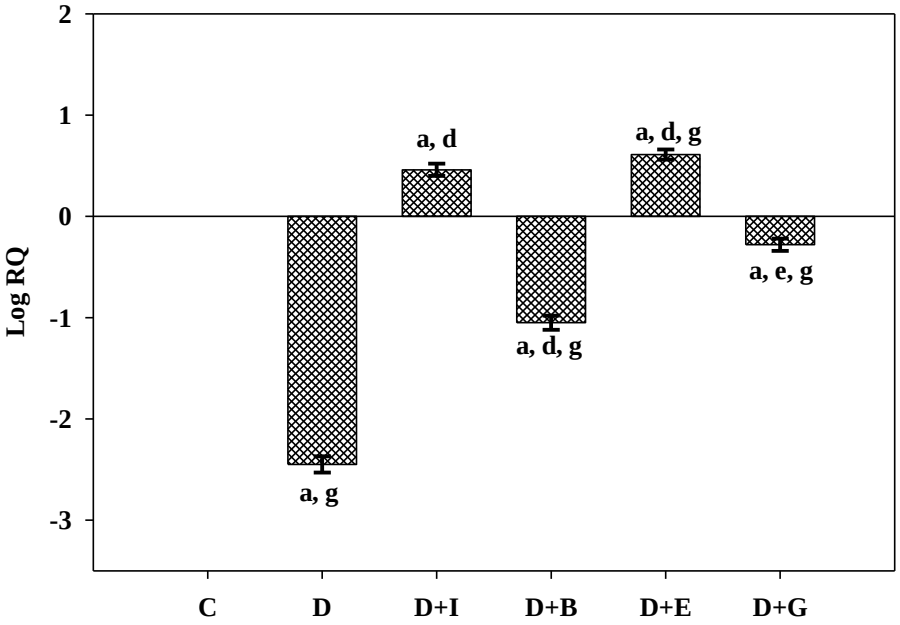


Table-67
Real Time PCR amplification of phospholipase C mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-2.45± 0.08[a, g]
D+I	0.46±0.07[a, d]
D+B	-1.05±0.07[a, d, g]
D+E	0.61±0.06[a, d, g]
D+G	-0.28±0.06[a, e, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-68
Real Time PCR amplification of CREB mRNA in the Brain Stem of experimental rats

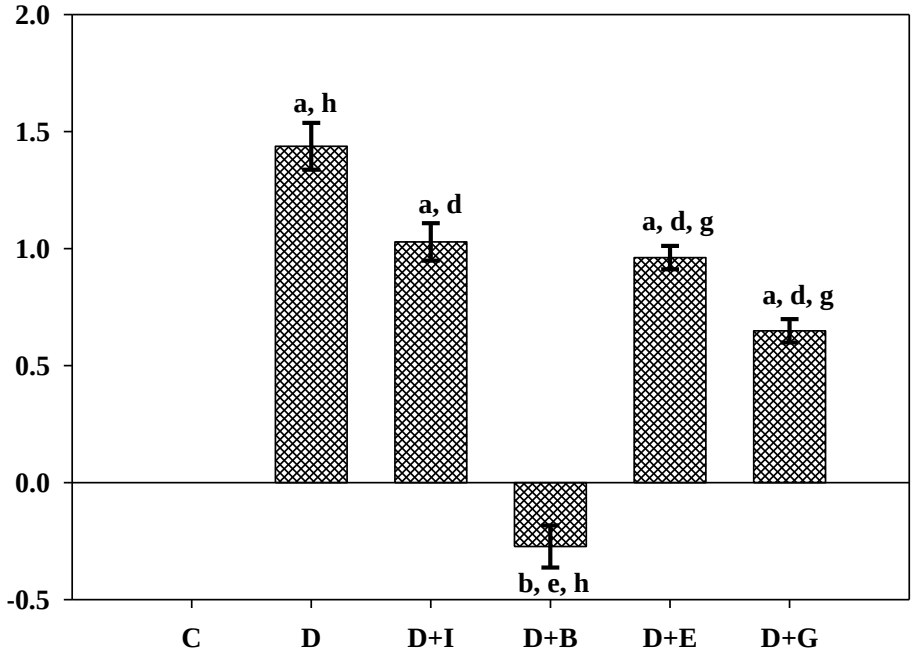


Table-68
Real Time PCR amplification of CREB mRNA in the Brain Stem of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	1.437±0.10[a, h]
D+I	1.028±0.08[a, d]
D+B	-0.273±0.09[b, e, h]
D+E	0.961±0.05[a, d, g]
D+G	0.648±0.05[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-69
Total antioxidant level in the Brain Stem of experimental rats

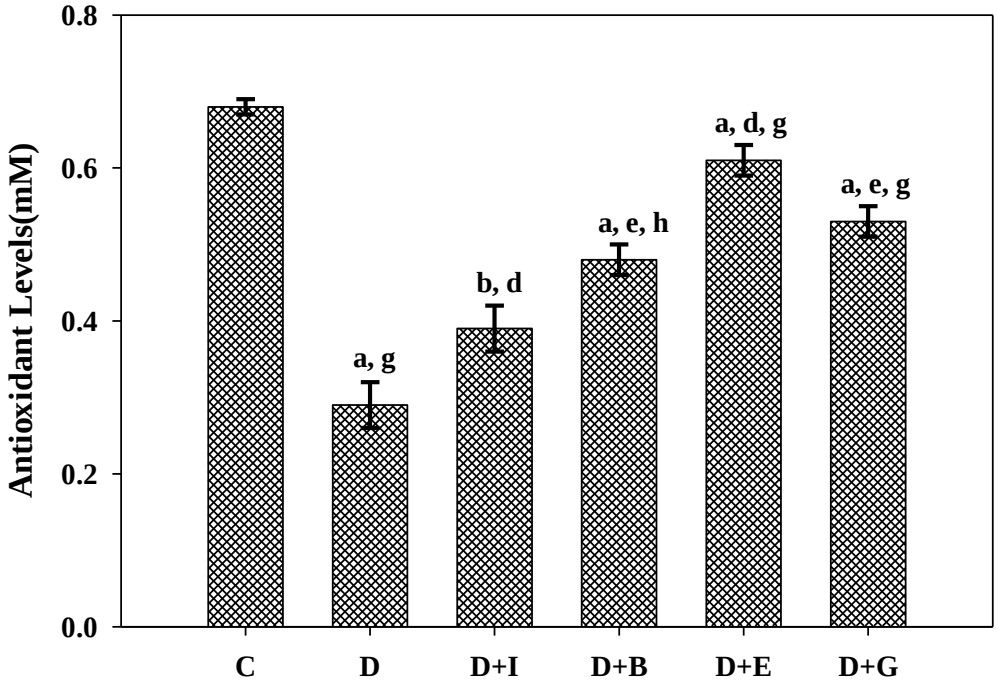


Table-69
Total antioxidant level in the Brain Stem of experimental rats

Experimental Groups	Antioxidant Level(mM)
Control	0.68±0.01
Diabetic	0.29± 0.03[a, g]
D+I	0.39±0.03[b, d]
D+B	0.48±0.02[a, e, h]
D+E	0.61±0.02[a, d, g]
D+G	0.53±0.02[a, e, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-70
Total aqueous peroxide level in the Brain Stem of experimental rats

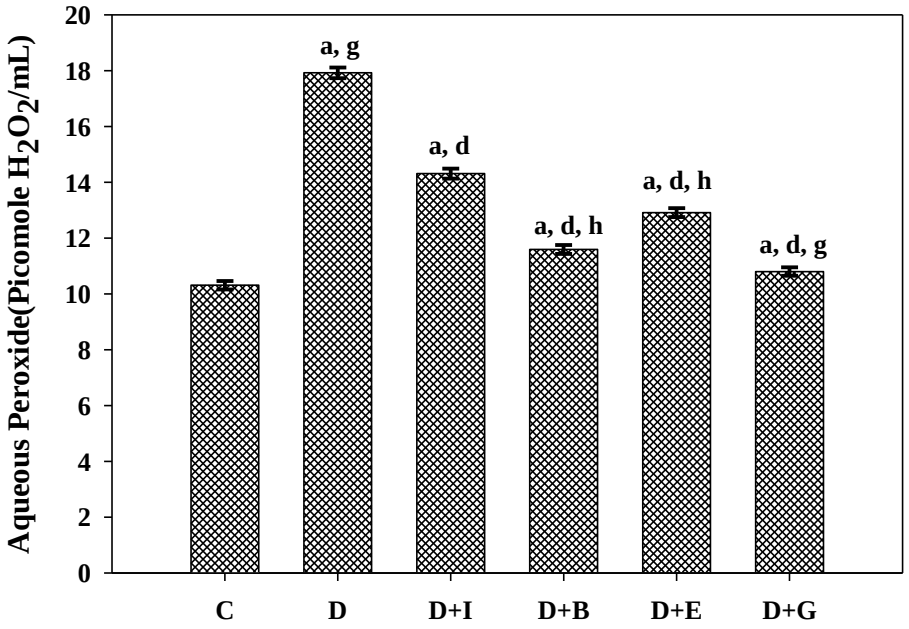


Table-70
Total aqueous peroxide level in the Brain Stem of experimental rats

Experimental Groups	Aqueous peroxide (Picomole H_2O_2/mL)
Control	10.35±0.15
Diabetic	17.92± 0.19[a, g]
D+I	14.31±0.18[a, d]
D+B	11.59±0.16[a, d, h]
D+E	12.91±0.16[a, d, h]
D+G	10.80±0.15[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E-Vitamin E treated Diabetic rats. D+G-Gymnemic acid treated diabetic rats.

Figure -71

TO-PRO®-3 iodide staining in the brain stem of experimental rats

Figure-72

Muscarinic M1 receptor antibody staining in the brain stem of experimental rats

Figure-73

Muscarinic M3 receptor antibody staining in the brain stem of experimental rats

PANCREAS

DNA & PROTEIN INCORPORATION STUDIES

[^{3}H] Thymidine incorporation in the pancreatic β-cells of experimental rats

[^{3}H]Thymidine incorporation showed significantly decreased disintegration /minute/mg protein ($p<0.001$) in the pancreas β-cells of diabetic rats compared to control. In D+B, there was a significantly increased the disintegration /minute/mg protein when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significantly increased the disintegration /minute/mg protein when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly increased the disintegration /minute/mg protein when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-74, Table-74).

[^{3}H] Leucine incorporation in the pancreatic β-cells of experimental rats

[^{3}H] Leucine incorporation showed significantly decreased disintegration /minute/mg protein ($p<0.001$) in the pancreas β-cells of diabetic rats compared to control. In D+B, there was a significantly increased disintegration /minute/mg protein when compared to control ($p<0.05$), diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significantly increased disintegration /minute/mg protein when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group

showed a significantly increased disintegration /minute/mg protein when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-75, Table-75).

Total muscarinic receptor analysis

Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the pancreas of experimental rats

The total muscarinic receptor status was assayed using the specific ligand, [^{3}H] QNB and muscarinic general antagonist atropine. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) decreased significantly in diabetic rats compared to control group. In D+B, showed that B_{max} ($p<0.001$) and K_d ($p<0.05$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.05$) when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly decreased B_{max} ($p<0.001$) and no significant change in K_d when compared to D+I group. D+G group showed that B_{max} ($p<0.05$) and K_d ($p<0.05$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly decreased B_{max} ($p<0.05$) and K_d ($p<0.001$) D+I group **(**Figure-76, Table-76).

Muscarinic M1 receptor analysis

Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the pancreas of experimental rats

Binding analysis of muscarinic M1 receptor was done using [^{3}H] QNB and M1 subtype specific antagonist pirenzepine. The Scatchard analysis

showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) decreased significantly in diabetic group when compared to control group. In D+B, showed that there is no significant change in B_{max} and decreased K_d ($p<0.05$) when compared to control, but B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly increased when compared to diabetic and D+I group {B_{max} ($p<0.001$) and K_d ($p<0.001$)}. D+E group showed that significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.05$) when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) diabetic and D+I group {B_{max} ($p<0.001$) and K_d ($p<0.001$)}. D+G group showed that there is no significant change in B_{max} and decreased K_d ($p<0.001$) when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group {B_{max} ($p<0.05$) and K_d ($p<0.001$)} **(**Figure-77, Table-77).

Muscarinic M3 receptor analysis

Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor, antagonist, 4-DAMP mustard in the pancreas of experimental rats

Binding analysis of muscarinic M3 receptors was done using [^{3}H] DAMP and M3 subtype specific antagonist 4-DAMP mustard. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) was decreased significantly in diabetic group when compared to control. In D+B, showed that significantly decreased B_{max} ($p<0.001$) and increased K_d ($p<0.001$) when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly decreased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to diabetic and significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.05$) when

compared to D+I group. D+G group showed that B_{max} ($p<0.001$) and K_d ($p<0.001$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and there is no significant change in K_d when compared to diabetic and significantly increased B_{max} ($p<0.05$) decreased K_d when compared to D+I group **(**Figure-78, Table-78).

$GABA_B$ receptor analysis

Scatchard analysis of [^{3}H] baclofen binding against baclofen to $GABA_B$ receptors in the pancreas of experimental rats

Binding analysis of [^{3}H] baclofen binding against baclofen. The Scatchard analysis showed that the B_{max} ($p<0.001$) and K_d ($p<0.001$) of $GABA_B$ receptor was decreased significantly in diabetic group when compared to control. In D+B, showed that significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.001$) when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly increased B_{max} ($p<0.001$) and decreased K_d ($p<0.05$) when compared to D+I group. D+E group showed that B_{max} ($p<0.001$) and K_d ($p<0.05$) significantly decreased when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and significantly increased B_{max} ($p<0.05$) and decreased K_d ($p<0.001$) when compared to D+I group. D+G group showed that significantly decreased B_{max} ($p<0.001$) and increased K_d ($p<0.05$) when compared to control, but significantly increased B_{max} ($p<0.001$) and K_d ($p<0.001$) when compared to diabetic and D+I group {B_{max} ($p<0.001$) and K_d ($p<0.001$)} **(**Figure-79, Table-79).

REAL TIME-PCR ANALYSIS

Real Time-PCR analysis of acetylcholine esterase in the pancreas of experimental rats

Gene expression of acetylcholine esterase mRNA showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant up regulation of AchE mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant increase in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+G group showed a significant ($p<0.001$) up regulation when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group **(**Figure-80, Table-80).

Real Time-PCR analysis of choline acetyltransferase in the pancreas of experimental rats

Gene expression of choline acetyltransferase mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of ChAT mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant down regulation when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group **(**Figure-81, Table-81).

Real Time-PCR analysis of muscarinic M1 receptor mRNA in the pancreas of experimental rats

Gene expression of muscarinic M1 receptor mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant increase of muscarinic M1 receptor gene expression when compared to control ($p<0.001$), but significant down regulation when compared to ($p<0.001$) diabetic and there is no significant change in D+I group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.001$) group. D+G group showed a significant decrease of muscarinic M1 receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.001$) group. **(**Figure-82, Table-82).

Real Time-PCR analysis of muscarinic M3 receptor mRNA in the pancreas of experimental rats

Gene expression of muscarinic M3 receptor mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant down regulation of muscarinic M3 receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-83, Table-83).

Real Time-PCR analysis of $GABA_B$ receptor mRNA in the pancreas of experimental rats

Gene expression of $GABA_B$ receptor mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant down regulation of $GABA_B$ receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group. D+G group showed a significant down regulation of $GABA_B$ receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-84, Table-84).

Real Time-PCR analysis of insulin receptor mRNA in the pancreas of experimental rats

Gene expression of insulin receptor mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of insulin receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and there is no significant change in D+I group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic and D+I ($p<0.001$) group. D+G group showed a significant down regulation of insulin receptor gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-85, Table-85).

Real Time-PCR analysis of Pdx-1 mRNA in the pancreas of experimental rats

Gene expression of Pdx-1 mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of Pdx-1mRNA expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and significant down regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+G group showed a significant increase of Pdx-1mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group **(**Figure-86, Table-86).

Real Time-PCR analysis of Akt-1 mRNA in the pancreas of experimental rats

Gene expression of Akt-1 mRNA showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant increase of AKt-1 mRNA expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+G group showed a significant increase of Akt-1 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-87, Table-87).

Real Time-PCR analysis of Bax mRNA in the pancreas of experimental rats

Gene expression of Bax mRNA showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant increase of Bax mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of Bax mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-88, Table-88).

Real Time-PCR analysis of caspase 8 mRNA in the pancreas of experimental rats

Gene expression of caspase 8 mRNA showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant increase of caspase 8 mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to ($p<0.001$) diabetic ($p<0.05$) and there is no significant change in D+I group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of caspase 8 mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-89, Table-89).

Real Time-PCR analysis of Gpx (glutathione peroxidase) mRNA in the pancreas of experimental rats

Gene expression of Gpx mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant increase of Gpx mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of Gpx mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-90, Table-90).

Real Time-PCR analysis of SOD (superoxide dismutase) mRNA in the pancreas of experimental rats

Gene expression of SOD mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of SOD mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and there is no significant change in D+I group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant decrease of SOD mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-91, Table-91).

Real Time-PCR analysis of GLUT 3 mRNA in the pancreas of experimental rats

Gene expression of GLUT 3 mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.05$), but significant up regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed a significant decrease of GLUT 3 mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-92, Table-92).

Real Time-PCR analysis of phospholipase C mRNA in the pancreas of experimental rats

Gene expression of phospholipase C mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and significant down regulation when compared to D+I ($p<0.001$) group. D+E group showed a significant down regulation in the mRNA levels when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and significant down regulation when compared to D+I ($p<0.001$) group. D+G group showed a significant decrease of phospholipase C mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-93, Table-93).

Real Time-PCR analysis of CREB mRNA in the pancreas of experimental rats

Gene expression of CREB mRNA showed significant down regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant decrease of CREB mRNA gene expression when compared to control ($p<0.001$), but significant up regulation when compared to diabetic ($p<0.05$) and down regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.001$) group. D+G group showed a significant increase of CREB mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-94, Table-94).

Real Time PCR amplification of TNF-α mRNA in the pancreas of experimental rats

Gene expression of TNF-α mRNA showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant increase of TNF-α mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of TNF-α mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.05$) and D+I ($p<0.001$) group **(**Figure-95, Table-95).

Real Time PCR amplification of NF-κB mRNA in the pancreas of experimental rats

Gene expression of NF-κB mRNA showed significant up regulation ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significant increase of NF-κB mRNA gene expression when compared to control ($p<0.05$), but significant down regulation when compared to diabetic ($p<0.001$) and significant up regulation when compared to D+I ($p<0.05$) group. D+E group showed a significant up regulation in the mRNA levels when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significant increase of NF-κB mRNA gene expression when compared to control ($p<0.001$), but significant down regulation when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-96, Table-96).

Total antioxidant activity in the pancreas of experimental rats

Total antioxidant activity showed significantly decreased $p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significantly decreased antioxidant level when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+E group showed a significantly decreased in the antioxidant level to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly decreased antioxidant level when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group **(**Figure-97, Table-97).

Total aqueous peroxide activity in the pancreas of experimental rats

Total aqueous peroxide activity showed significantly increased ($p<0.001$) in the pancreas of diabetic rats compared to control. In D+B, there was a significantly increased aqueous peroxide activity level when compared

to control ($p<0.05$), but significantly decreased when compared to diabetic ($p<0.05$) and significantly increased when compared to D+I ($p<0.05$) group. D+E group showed a significantly increased in the aqueous peroxide activity level when compared to control ($p<0.001$), but significantly decreased when compared to diabetic ($p<0.001$) and there is no significant change in D+I group. D+G group showed no significant change of aqueous peroxide activity level when compared to control, but significant decrease when compared to diabetic ($p<0.001$) and significant decrease in D+I ($p<0.001$) group **(**Figure-98, Table-98).

CONFOCAL STUDIES

TO-PRO®-3 iodide staining in the pancreas of experimental rats

TO-PRO®-3 staining showed significantly decreased ($p<0.001$) nuclear density in the pancreas of diabetic rats compared to control. In D+B, there was a significantly decreased nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and significantly decreased when compared to D+I ($p<0.05$) group. D+E group showed a significantly decreased in the nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and significantly decreased when compared to D+I ($p<0.001$) group. D+G group showed a significantly decreased nuclear density when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and there is no significant change in D+I group **(**Figure-99, Table-99).

Muscarinic M1 receptor antibody staining in the pancreas of experimental rats

Muscarinic M1 receptor subunit antibody staining in the pancreas showed a significantly decreased ($p<0.001$) in the mean pixel value in diabetic rats compared to control. In D+B, there was a significantly

decreased muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.05$) and D+I ($p<0.05$) group. D+E group showed a significantly decreased in the muscarinic M1 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.001$) group. D+G group showed a significantly increased muscarinic M1 receptor expression when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-100, Table-100).

Muscarinic M3 receptor antibody staining in the pancreas of experimental rats

Muscarinic M3 receptor subunit antibody staining in the pancreas showed a significantly decreased ($p<0.001$) in the mean pixel value in diabetic rats compared to control. In D+B, there was a significantly decreased muscarinic M3 receptor expression when compared to control ($p<0.001$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+E group showed a significantly decreased in the muscarinic M3 receptor expression when compared to control ($p<0.05$), but significantly increased when compared to diabetic ($p<0.001$) and D+I ($p<0.05$) group. D+G group showed a significantly increased muscarinic M3 receptor expression when compared to control ($p<0.001$), diabetic ($p<0.001$) and D+I ($p<0.001$) group **(**Figure-101, Table- 101).

Figure-74
[^{3}H] Thymidine incorporation in the Pancreatic β-cells of experimental rats

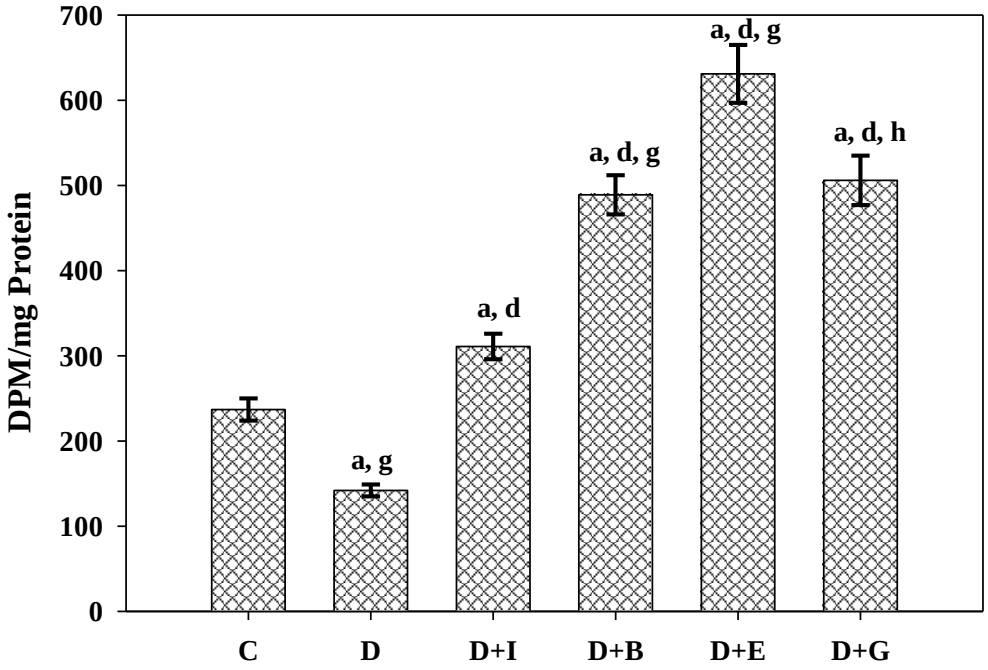


Table-74
[^{3}H] Thymidine incorporation in the Pancreatic β-cells of experimental rats

Experimental Groups	DPM/mg protein
Control	237±13
Diabetic	142± 7[a, g]
D+I	311±15[a, d]
D+B	489±23[a, d, g]
D+E	631±34[a, d, g]
D+G	506±29[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-75
[^{3}H] Leucine incorporation in the Pancreatic β-cells of experimental rats

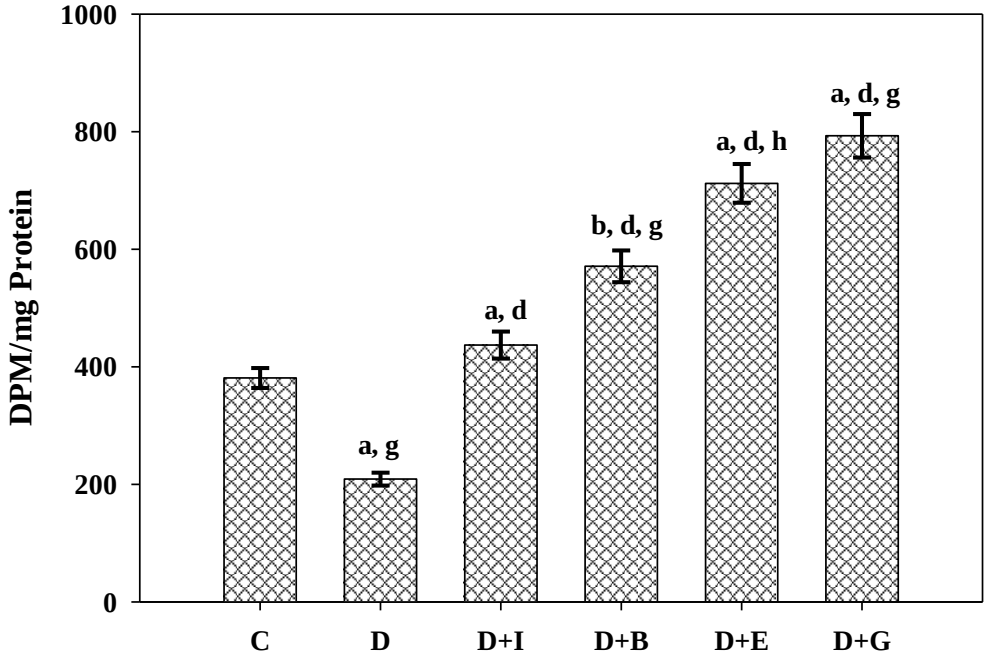


Table-75
[^{3}H] Leucine incorporation in the Pancreatic β-cells of experimental rats

Experimental Groups	DPM/mg protein
Control	381±17
Diabetic	209± 11[a, g]
D+I	437±23[a, d]
D+B	571±27[b, d, g]
D+E	712±33[a, d, h]
D+G	793±37[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-76
Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the Pancreas of experimental rats

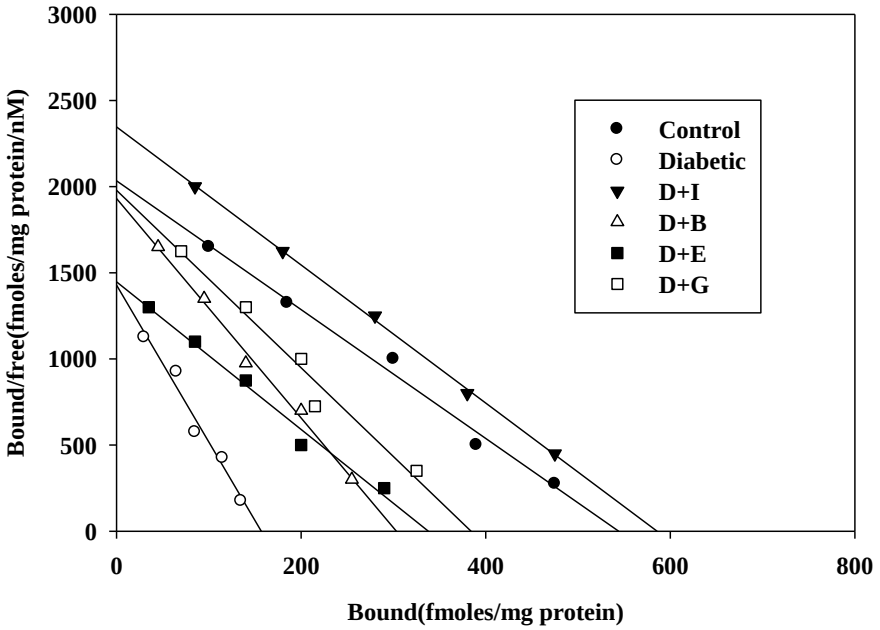


Table-76
Scatchard analysis of [^{3}H] QNB binding against total muscarinic receptor antagonist, atropine in the Pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	545.12± 15.43	0.27 ± 0.07
Diabetic	155.04± 6.78[a, g]	0.11± 0.02[a, g]
D + I	583.36± 16.05[a, d]	0.25 ± 0.06[a, d]
D + B	303.61 ± 10.31[a, d, g]	0.16 ± 0.03[b, d, h]
D+E	335.04±10.58[b, d, g]	0.23± 0.05[a, d, g]
D+G	381.76±11.24[b, d, h]	0.19±0.04[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-77
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the Pancreas of experimental rats

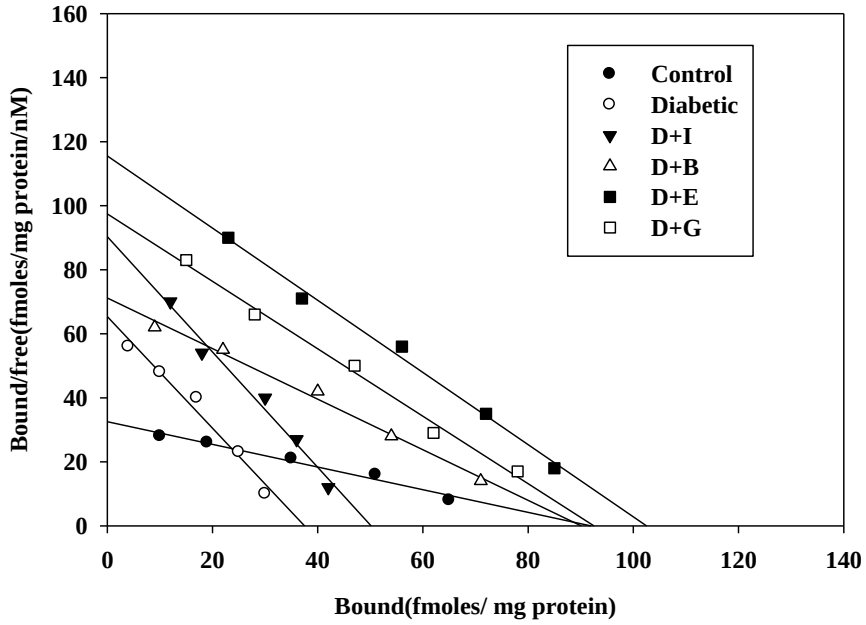


Table-77
Scatchard analysis of [^{3}H] QNB binding against muscarinic M1 receptor antagonist, pirenzepine in the Pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	91.55± 4.07	2.76 ± 0.21
Diabetic	37.63± 1.98[a, g]	0.59± 0.07[a, g]
D + I	49.89± 2.25[a, d]	0.55 ± 0.06[a, d]
D + B	91.30 ± 4.02 [d, g]	1.28 ± 0.15[a, d, g]
D+E	102.67±4.58[a, d, g]	0.89± 0.11[b, d, g]
D+G	92.45±4.31 [d, h]	0.95±0.12[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-78
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the Pancreas of experimental rats

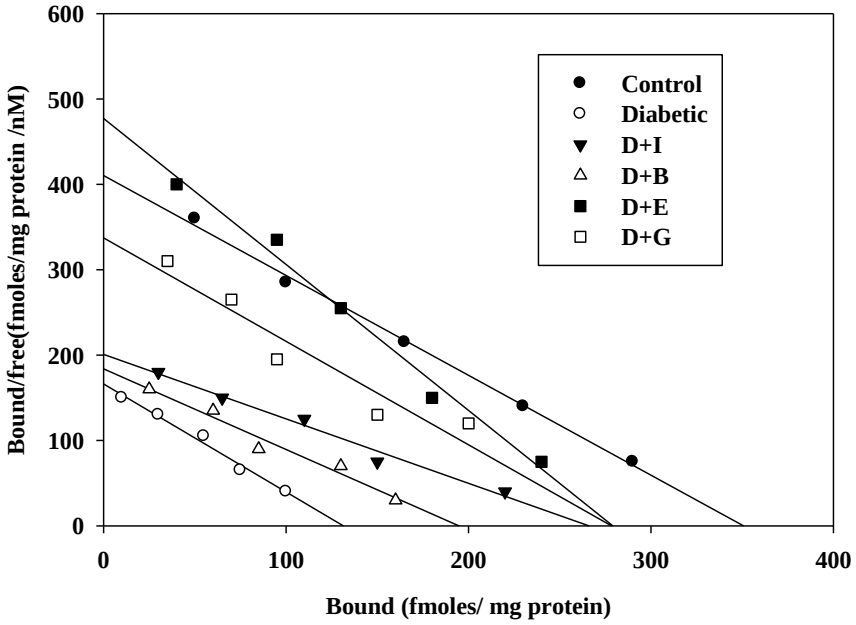


Table-78
Scatchard analysis of [^{3}H] DAMP binding against muscarinic M3 receptor antagonist, 4-DAMP mustard in the Pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	350.38± 11.89	0.86 ± 0.11
Diabetic	130.88± 4.20[a, g]	0.80± 0.10[a, g]
D + I	265.04± 8.95[a, d]	1.31 ± 0.16[a, d]
D + B	193.44 ± 6.21[a, d, g]	1.05 ± 0.14[a, d, g]
D+E	279.25±9.18[a, d, g]	0.59± 0.07[a, d, h]
D+G	277.65±8.93[a, d, h]	0.81±0.10[a, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-79
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the Pancreas of experimental rats

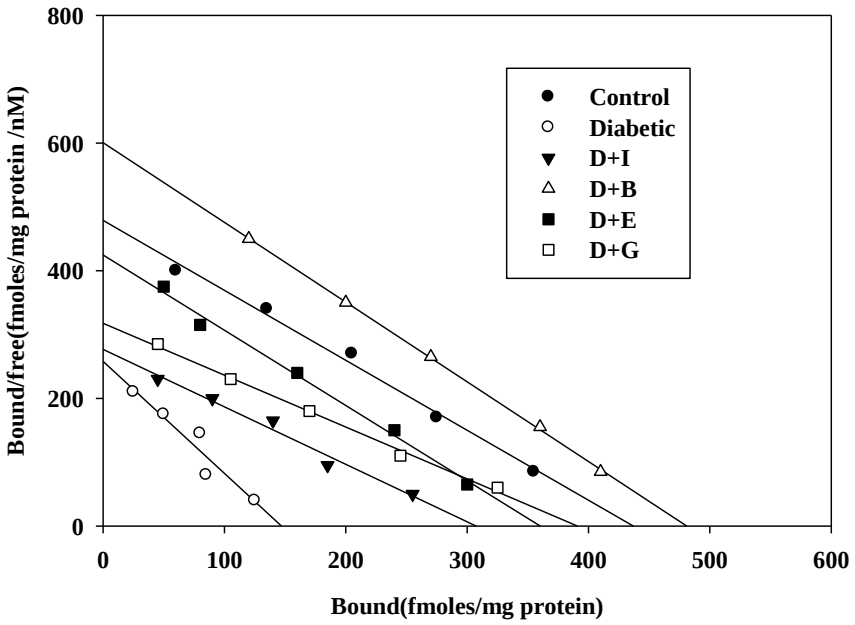


Table-79
Scatchard analysis of [^{3}H] Baclofen binding against Baclofen to $GABA_B$ receptors in the Pancreas of experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	436.32± 11.80	0.92 ± 0.03
Diabetic	148.80± 9.03[a, g]	0.58± 0.02[a, g]
D + I	307.56± 7.38[a, d]	1.10 ± 0.03[a, d]
D + B	481.32 ± 16.15[a, d, g]	0.80 ± 0.04[a, d, h]
D+E	358.83±4.18[a, d, h]	0.85± 0.02[b, d, g]
D+G	390.47±6.59[a, d, g]	1.22±0.03[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-80
Real Time PCR amplification of acetylcholine esterase mRNA in the Pancreas of experimental rats

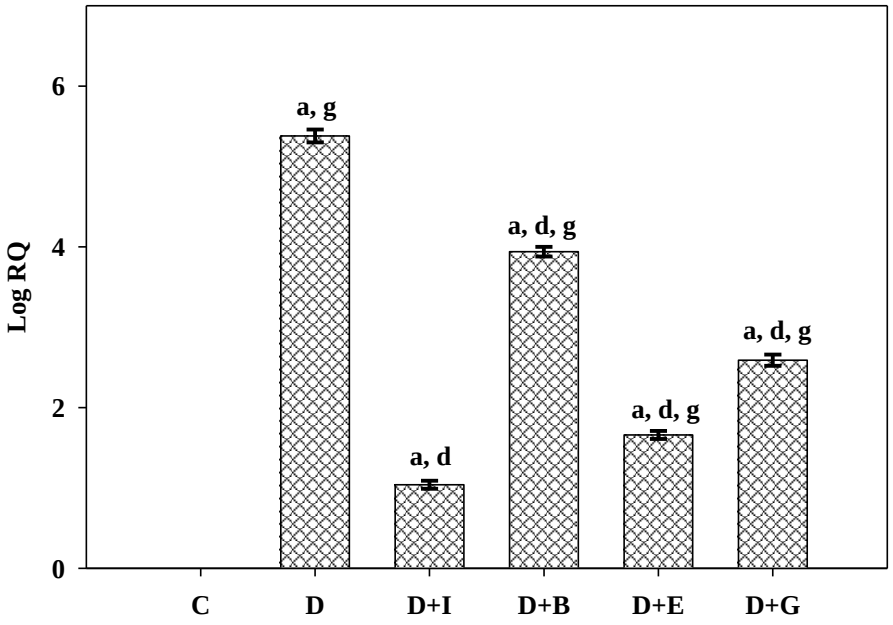


Table-80
Real Time PCR amplification of acetylcholine esterase mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	5.38± 0.08[a, g]
D+I	1.04±0.05[a, d]
D+B	3.94±0.06[a, d, g]
D+E	1.65±0.05[a, d, g]
D+G	2.59±0.08[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-81
Real Time PCR amplification of choline acetyltransferase mRNA in the Pancreas of experimental rats

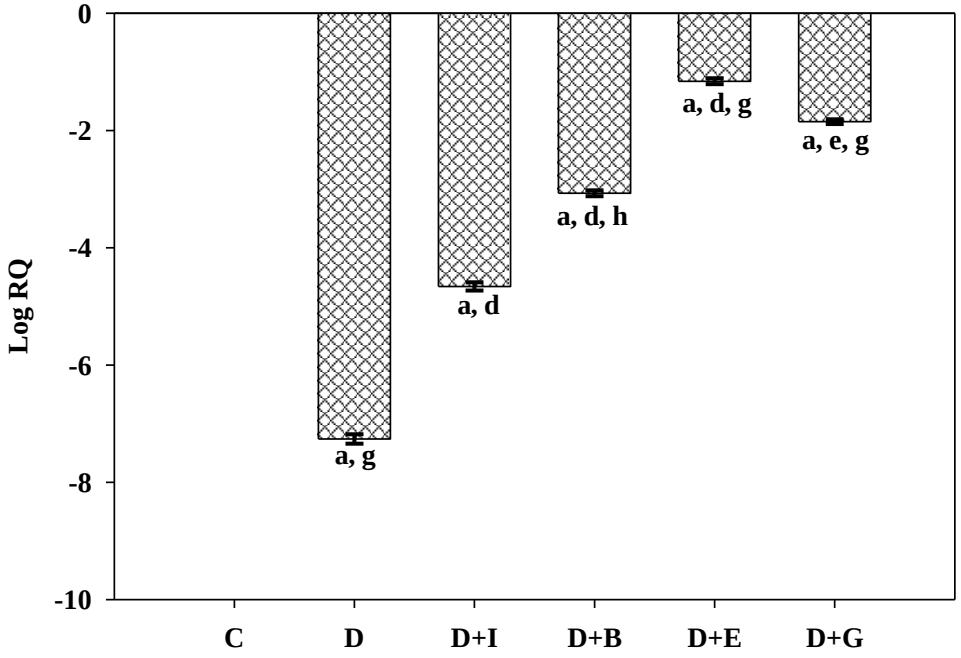


Table-81
Real Time PCR amplification of choline acetyltransferase mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-7.26± 0.08[a, g]
D+I	-4.66±0.07[a, d]
D+B	-3.07±0.05[a, d, h]
D+E	-1.16±0.04[a, d, g]
D+G	-1.85±0.04[a, e, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-82
Real Time PCR amplification of muscarinic M1 receptor mRNA in the Pancreas of experimental rats

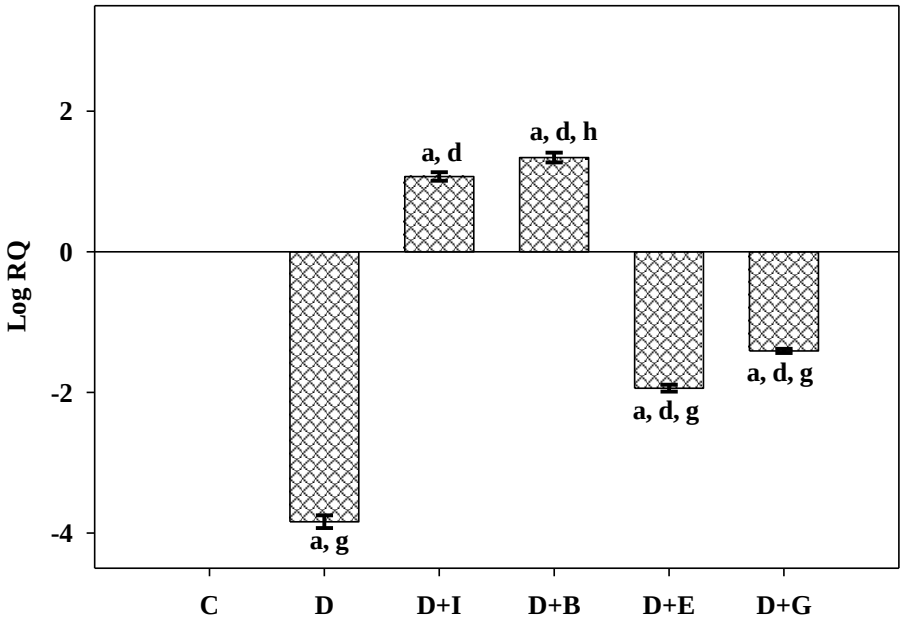


Table-82
Real Time PCR amplification of muscarinic M1 receptor mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.84± 0.09[a, g]
D+I	1.07±0.06[a, d]
D+B	1.34±0.07[a, d, h]
D+E	-1.93±0.05[a, d, g]
D+G	-1.41±0.03[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-83
Real Time PCR amplification of muscarinic M3 receptor mRNA in the Pancreas of experimental rats

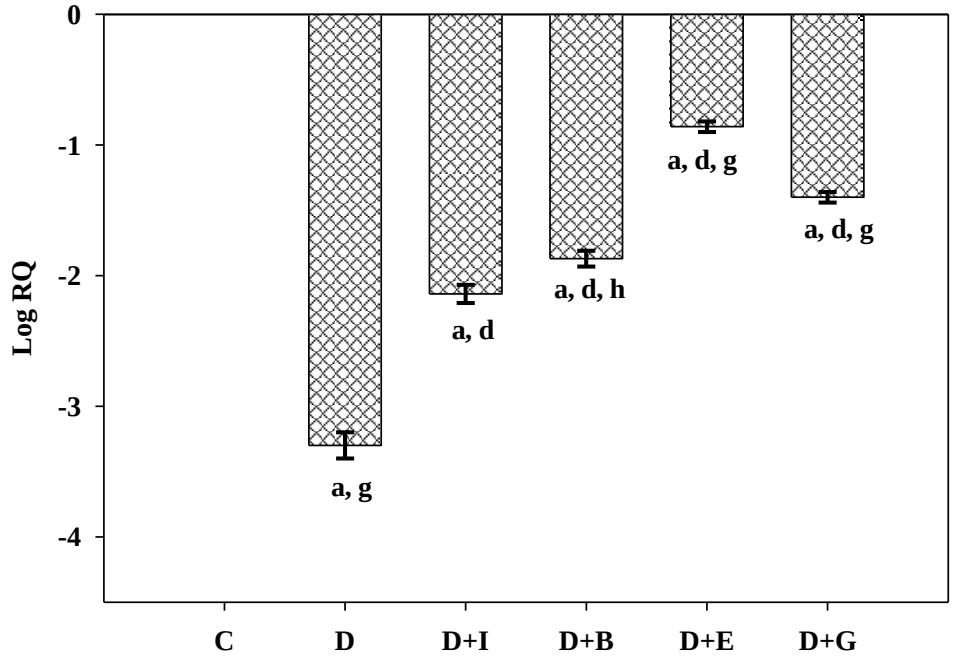


Table-83
Real Time PCR amplification of muscarinic M3 receptor mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.3± 0.10[a, g]
D+I	-2.14±0.07[a, d]
D+B	-1.87±0.06[a, d, h]
D+E	-0.86±0.04[a, d, g]
D+G	-1.39±0.04[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-84
Real Time PCR amplification of $GABA_B$ receptor mRNA in the Pancreas of experimental rats

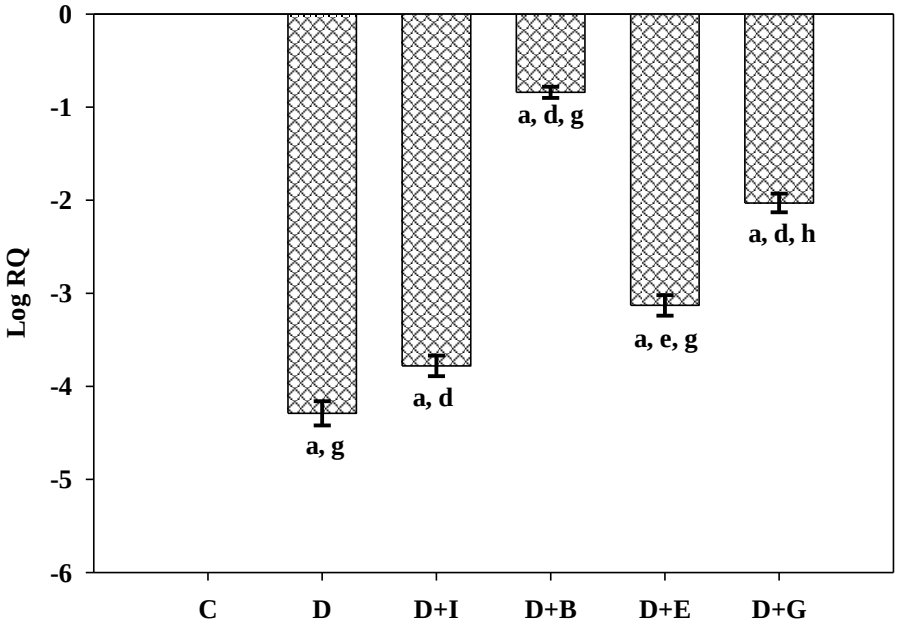


Table-84
Real Time PCR amplification of $GABA_B$ receptor mRNA in the pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-4.29± 0.14[a, g]
D+I	-3.78±0.11[a, d]
D+B	-0.84±0.06[a, d, g]
D+E	-3.13±0.11[a, e, g]
D+G	-2.03±0.10[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-85
Real Time PCR amplification of insulin receptor mRNA in the Pancreas of experimental rats

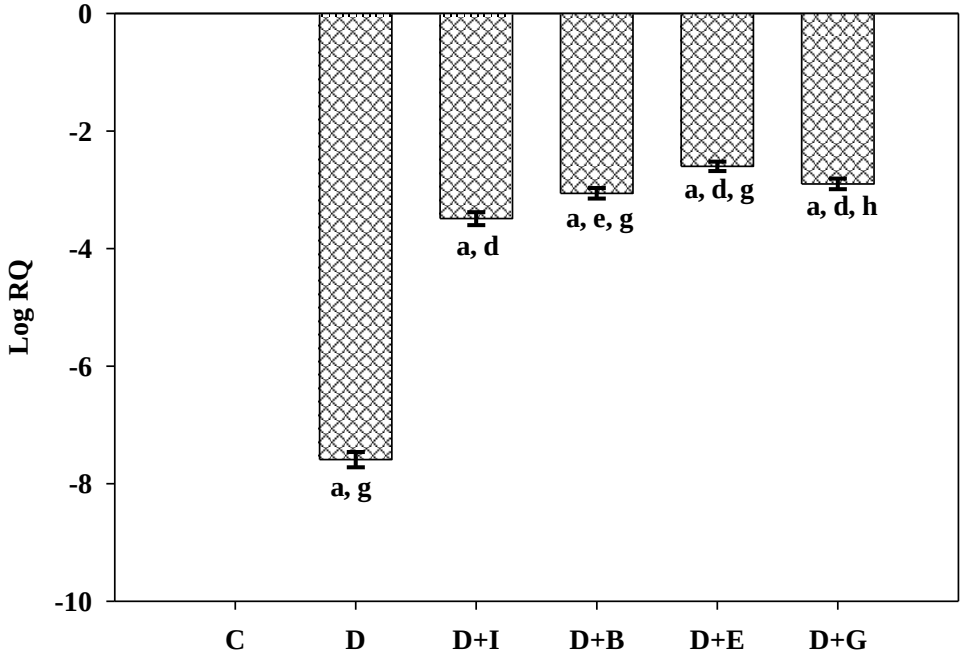


Table-85
Real Time amplification of insulin receptor mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-7.59± 0.14[a, g]
D+I	-3.49±0.11[a, d]
D+B	-3.06±0.09[a, e, g]
D+E	-2.60±0.08[a, d, g]
D+G	-2.90±0.09[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-86
Real Time PCR amplification of Pdx-1 mRNA in the pancreas of experimental rats

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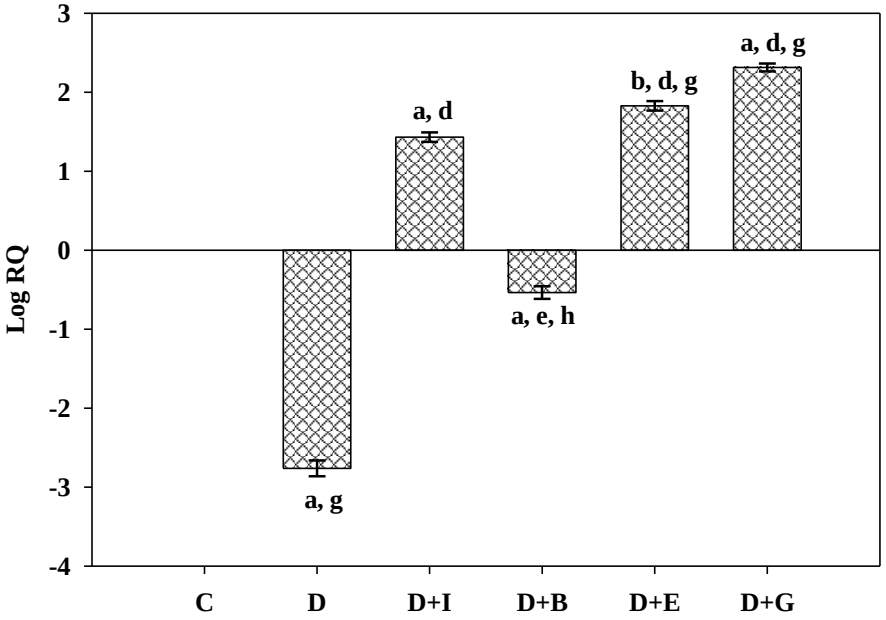

Table-86
Real Time PCR amplification of Pdx-1 mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-2.76± 0.10[a, g]
D+I	1.43±0.06[a, d]
D+B	-0.53±0.08[a, e, h]
D+E	1.82±0.06[b, d, g]
D+G	2.31±0.05[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-87
Real Time PCR amplification of Akt-1 mRNA in the Pancreas of experimental rats

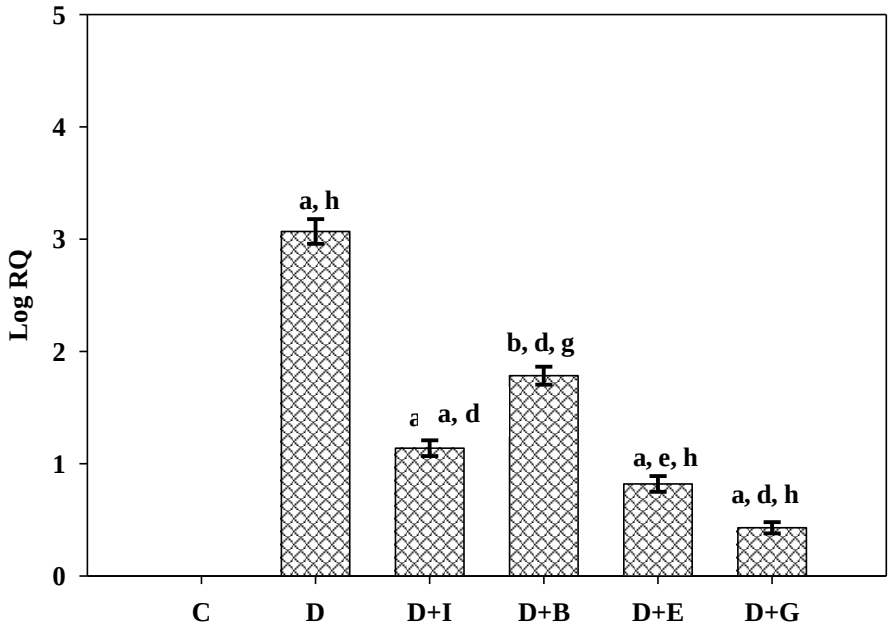

Table-87
Real Time PCR amplification of Akt-1 mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	3.068±0.11[a, h]
D+I	1.138±0.07[a, d]
D+B	1.783±0.08[b, d, g]
D+E	0.819±0.07[a, e, h]
D+G	0.430±0.05[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-88
Real Time PCR amplification of Bax mRNA in the Pancreas of experimental rats

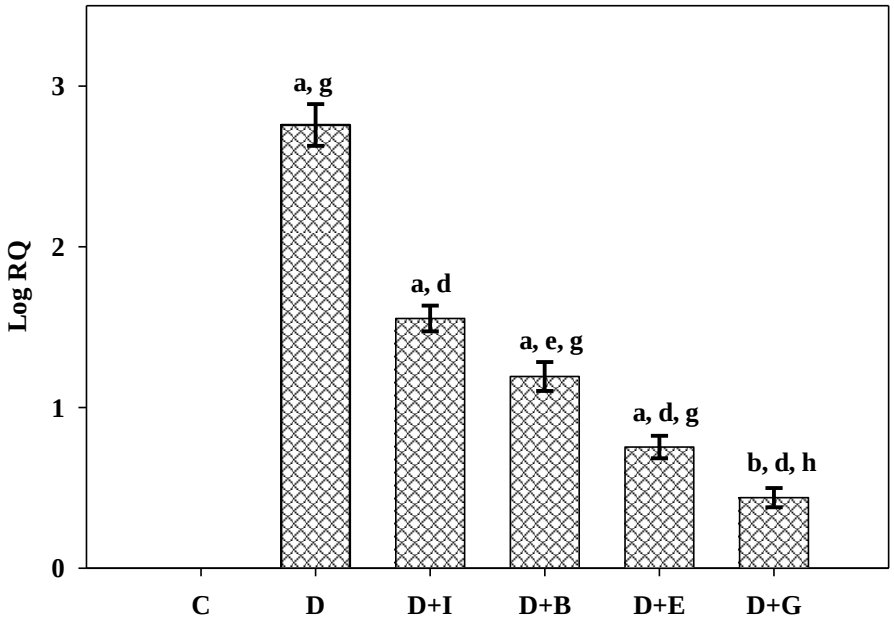

Table-88
Real Time PCR amplification of Bax mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	2.76± 0.13[a, g]
D+I	1.55±0.08[a, d]
D+B	1.19±0.09[a, e, g]
D+E	0.75±0.07[a, d, g]
D+G	0.44 ±0.06[b, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-89
Real Time PCR amplification of caspase 8 mRNA in the Pancreas of experimental rats

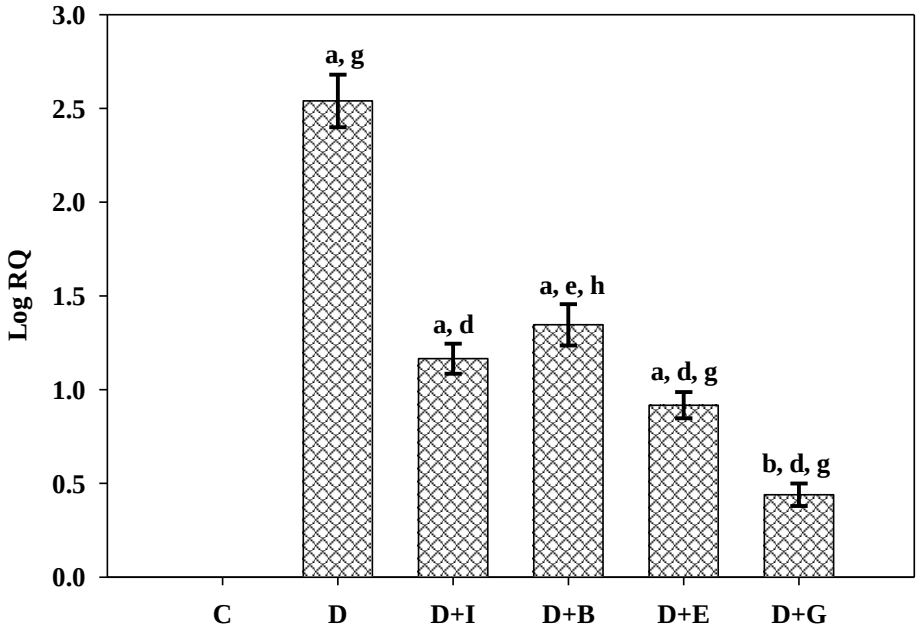

Table-89
Real Time PCR amplification of caspase 8 mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	2.54± 0.13[a, g]
D+I	1.35±0.11[a, d]
D+B	1.17±0.08[a, e, h]
D+E	0.92±0.07[a, d, g]
D+G	0.43 ±0.06[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-90
Real Time PCR amplification of Gpx mRNA in the Pancreas of experimental rats

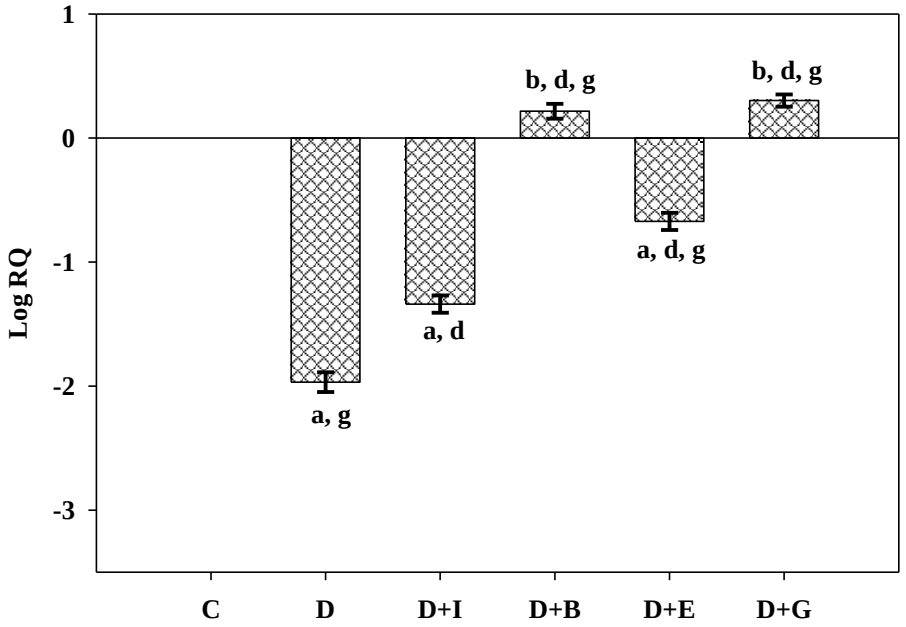

Table-90
Real Time PCR amplification of Gpx mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-1.97± 0.08[a, g]
D+I	-1.34±0.07[a, d]
D+B	0.22±0.07[b, d, g]
D+E	-0.67±0.07[a, d, g]
D+G	0.30 ±0.05[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-91
Real Time PCR amplification of superoxide dismutase mRNA in the Pancreas of experimental rats

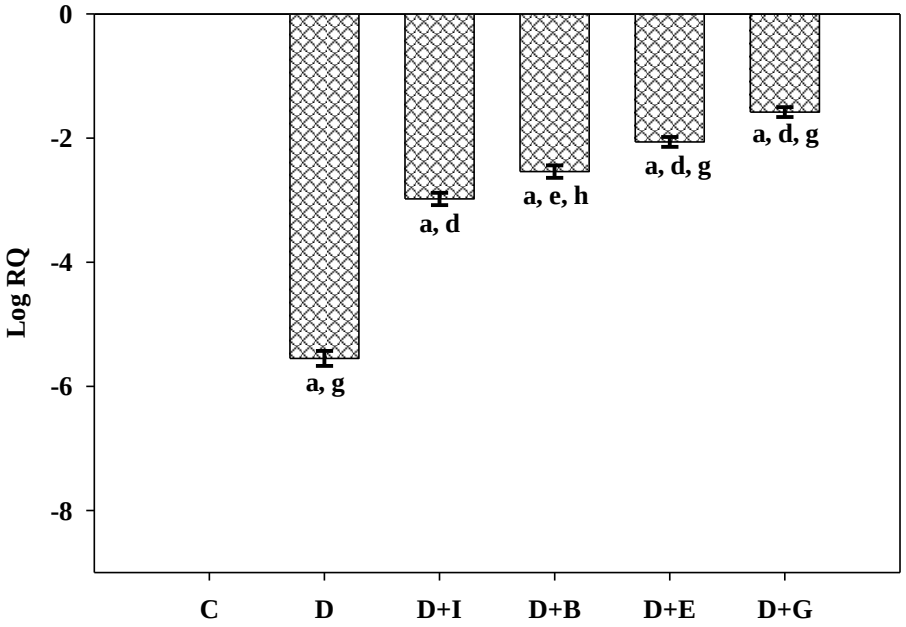

Table-91
Real Time PCR amplification of superoxide dismutase mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-5.55± 0.13[a, g]
D+I	-2.98±0.10[a, d]
D+B	-2.54±0.10[a, e, h]
D+E	-2.04±0.08[a, d, g]
D+G	-1.59±0.09[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-92
Real Time PCR amplification of GLUT 3 mRNA in the Pancreas of experimental rats

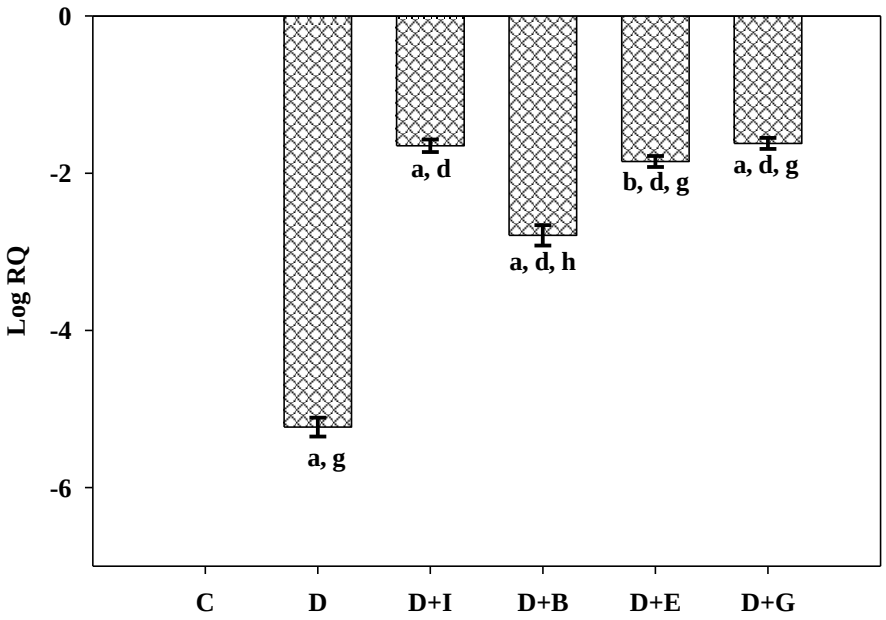

Table-92
Real Time PCR amplification of GLUT 3 mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-5.23± 0.12[a, g]
D+I	-1.65±0.08[a, d]
D+B	-2.79±0.12[a, d, h]
D+E	-1.85±0.07[b, d, g]
D+G	-1.62±0.07[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-93
Real Time PCR amplification of phospholipase C mRNA in the Pancreas of experimental rats

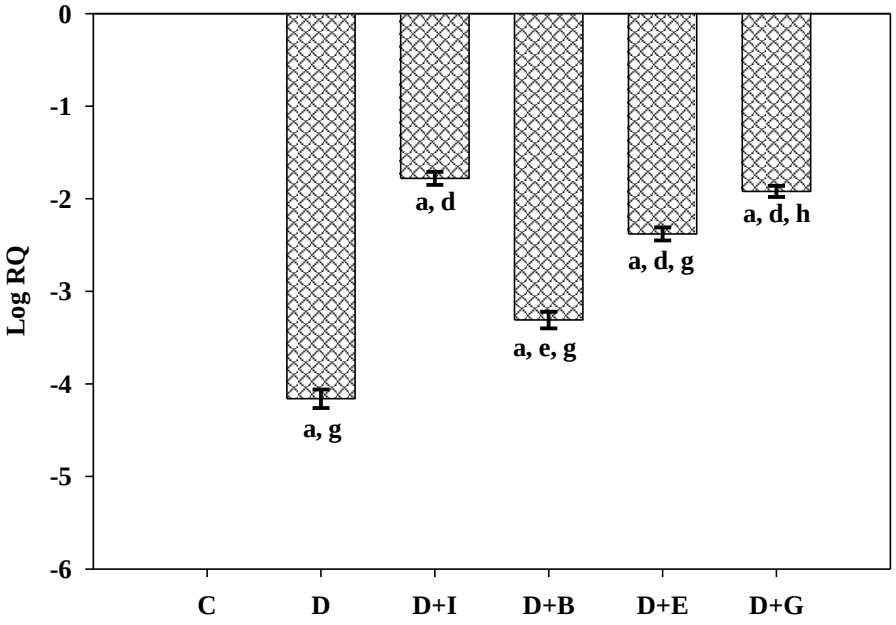

Table-93
Real Time PCR amplification of phospholipase C mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-4.17± 0.10[a, g]
D+I	-1.78±0.08[a, d]
D+B	-3.31±0.09[a, e, g]
D+E	-2.38±0.08[a, d, g]
D+G	-1.92±0.06[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-94
Real Time PCR amplification of CREB mRNA in the Pancreas of experimental rats

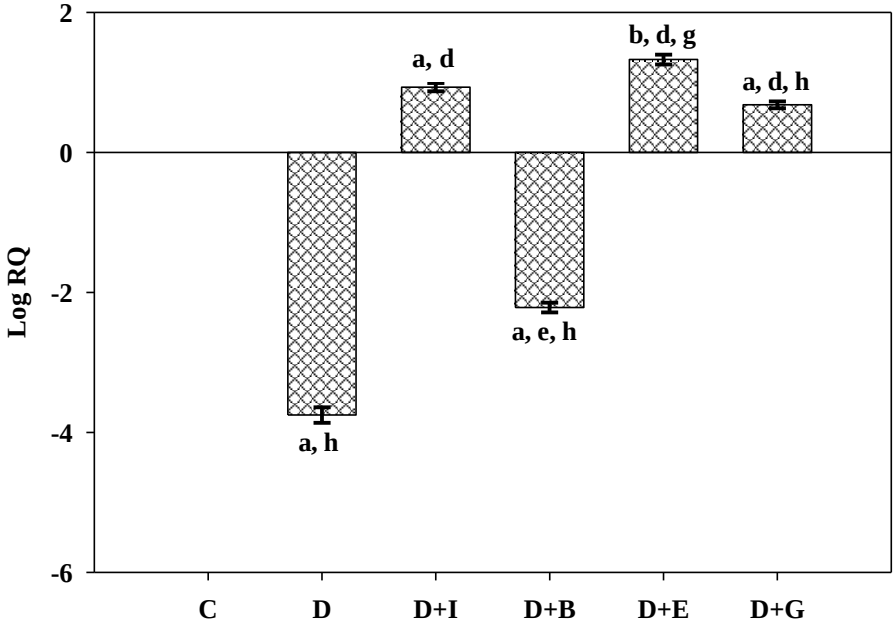

Table-94
Real Time PCR amplification of CREB mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	-3.753±0.11[a, h]
D+I	0.932±0.06[a, d]
D+B	-2.217±0.07[a, e, h]
D+E	1.326±0.07[b, d, g]
D+G	0.679±0.05[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-95
Real Time PCR amplification of TNF-α mRNA in the Pancreas of experimental rats

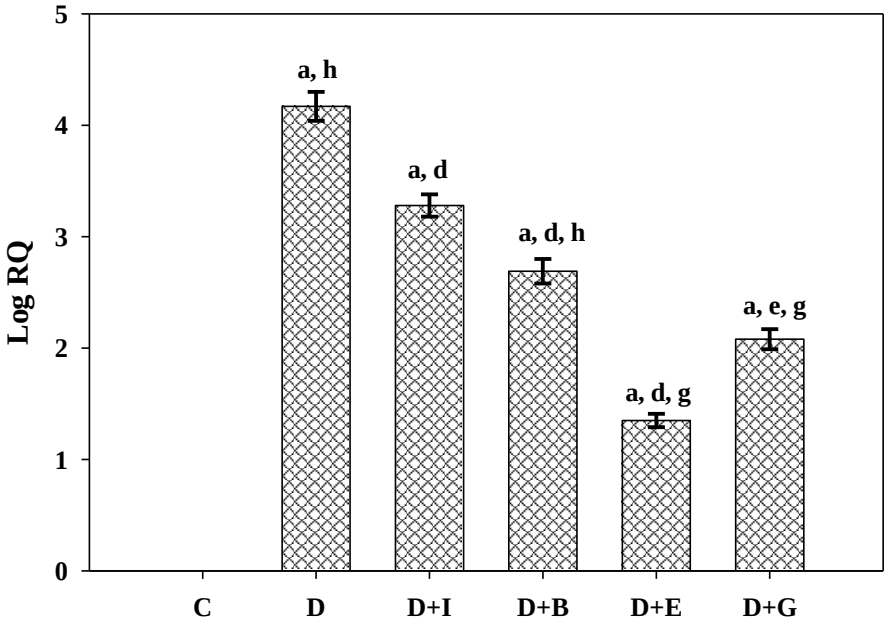

Table-95
Real Time PCR amplification of TNF-α mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	0
Diabetic	4.17± 0.13[a, h]
D+I	3.28±0.10[a, d]
D+B	2.69±0.11[a, d, h]
D+E	1.35±0.06[a, d, g]
D+G	2.08 ±0.09[a, e, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-96

Real Time PCR amplification of NF-κB mRNA in the Pancreas of experimental rats

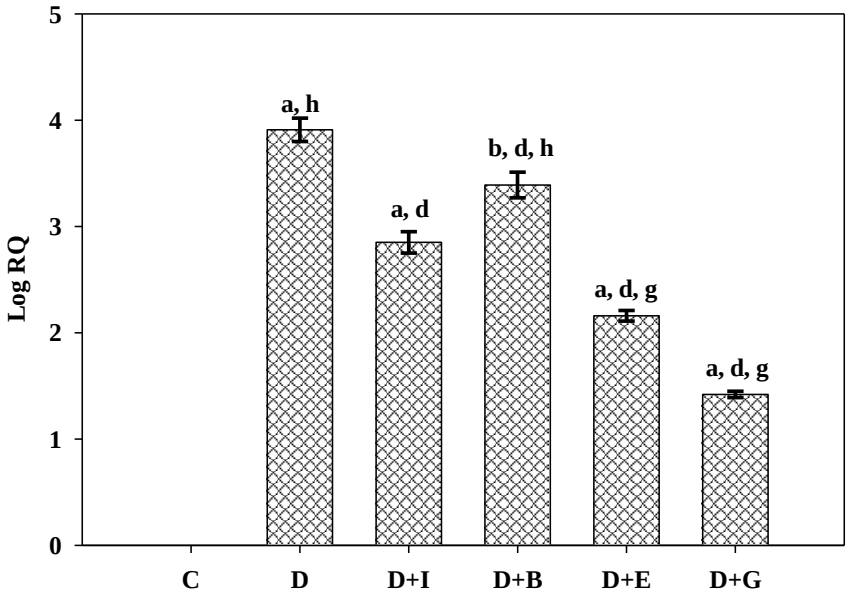

Table-96
Real Time PCR amplification of NF-κB mRNA in the Pancreas of experimental rats

Experimental Groups	Log RQ
Control	**0**
Diabetic	**3.91± 0.11[a, h]**
D+I	**2.85±0.10[a, d]**
D+B	**3.39±0.12[b, d, h]**
D+E	**2.16±0.05[a, d, g]**
D+G	**1.42 ±0.03[a, d, g]**

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-97
Total antioxidant level in the Pancreas of experimental rats

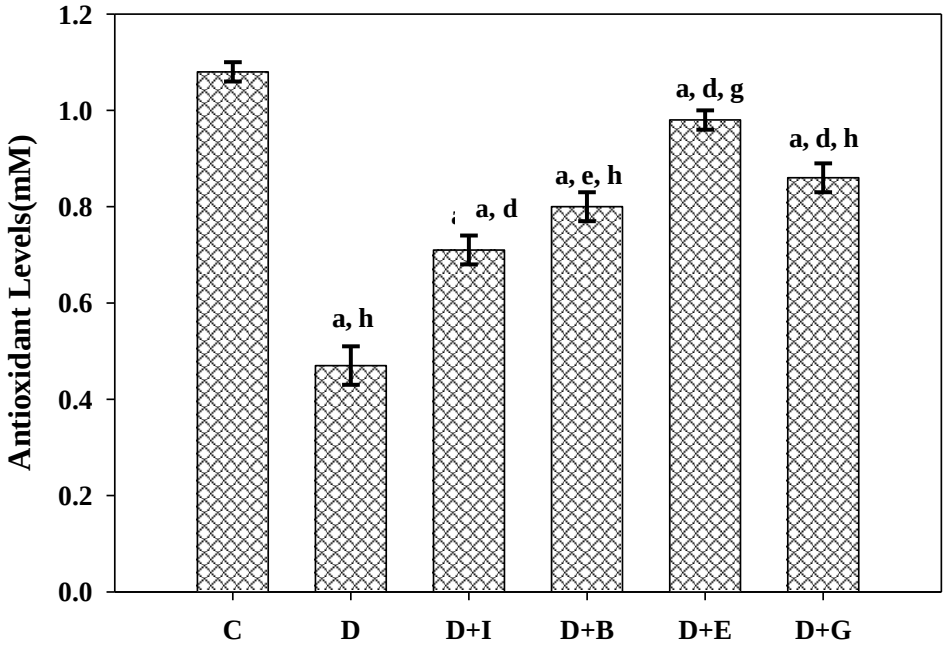

Table-97
Total antioxidant level in the Pancreas of experimental rats

Experimental Groups	Antioxidant Level(mM)
Control	1.08±0.02
Diabetic	0.49± 0.04[a, h]
D+I	0.71±0.03[a, d]
D+B	0.80±0.03[a, e, h]
D+E	0.98±0.02[a, d, g]
D+G	0.86±0.03[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-98
Total aqueous peroxide level in the Pancreas of experimental rats

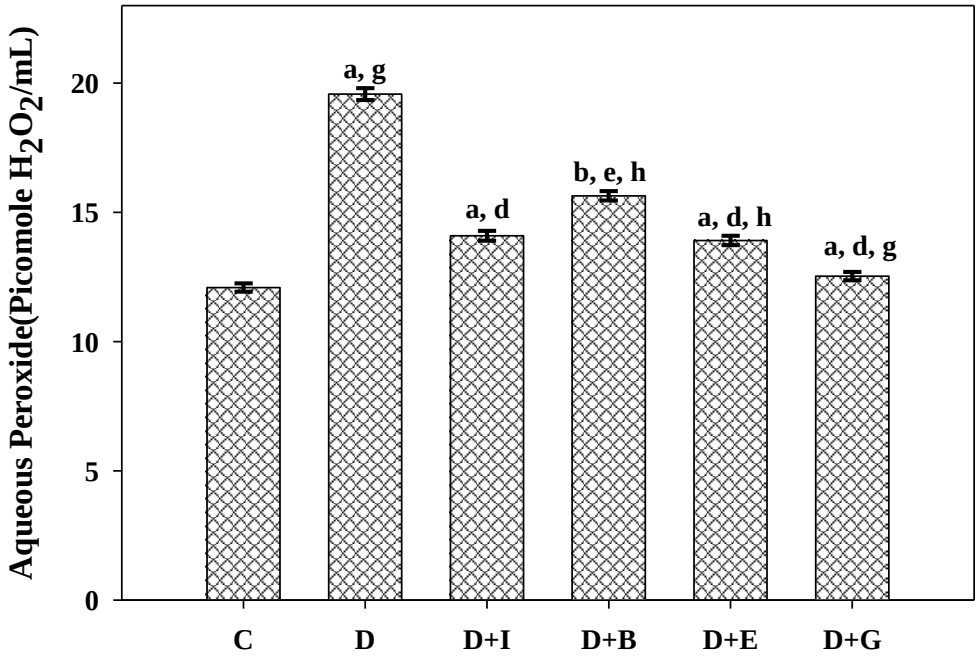

Table-98
Total aqueous peroxide level in the Pancreas of experimental rats

Experimental Groups	Aqueous peroxide (Picomole H_2O_2/mL)
Control	12.09±0.16
Diabetic	19.57± 0.23[a, g]
D+I	14.09±0.19[a, d]
D+B	15.64±0.18[b, e, h]
D+E	13.97±0.18[a, d, h]
D+G	12.53±0.16[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I- Insulin treated diabetic rats. D+B- Baclofen treated diabetic Rats. D+E- Vitamin E treated Diabetic rats. D+G- Gymnemic acid treated diabetic rats.

Figure-99
Histochemistry of TO-PRO®-3 iodide staining in the pancreas experimental rats

Figure-100
Confocal imaging of muscarinic M1 receptor in the pancreas of experimental rats

Figure-101
Confocal imaging of muscarinic M3 receptor in the pancreas of experimental rats

Discussion

Diabetes is characterized by hyperglycemia and associated with long-term vascular complications such as retinopathy, nephropathy, cardiopathy, and neuropathy (Auslander *et al.*, 2002). Besides symptoms associated with hyperglycemia such as thirst, polyuria and weight loss may also cause potentially life-threatening severe hyperglycemic emergencies. Hyperglycemia increases oxidative stress, which contributes to the impairment of the main processes that fail during diabetes, insulin action. In addition, antioxidant mechanisms are diminished in diabetic patients, which may further augment oxidative stress (Rains & Jain, 2011).

BLOOD GLUCOSE & BODY WEIGHT

Streptozotocin (STZ) is known for its selective pancreatic islet cell toxicity and has been widely used for the induction of diabetes mellitus in animals. STZ-induced type 1 diabetes is a well-documented model of experimental diabetes. STZ is a pancreatic β-cell toxin that induces rapid and irreversible necrosis of β-cells (Rerup, 1970). After intraperitoneal or intravenal administration, STZ enters pancreatic β-cells through the GLUT 2 transporter and causes alkylation of DNA (Szkudelski, 2001). Previous reports indicate that the type of diabetes and characteristics differ with the employed dose of STZ and animal species used (Low *et al.*, 1997). STZ-induced type 1 diabetes provides a relevant example of endogenous chronic oxidative stress due to the resulting hyperglycemia.

The augmented glucose level might be due to the fact that STZ caused a prominent decrease in insulin release by the destruction of pancreatic β-cells. Numerous studies have demonstrated that a variety of plant extracts effectively lowered the glucose level in STZ-induced type 1 diabetic rats (Rajasekaran *et al.*, 2005). The possible mechanism of hypoglycemic action may be through

potentiation of pancreatic secretion of insulin from β-cells of islets or due to enhanced transport of blood glucose to the peripheral tissue (Doda, 1996). Insulin and C-peptide are the products of the enzymatic cleavage of pro-insulin and secreted into the circulation in equimolar concentrations. The measurement of both insulin and C-peptide levels has been reported to be a valuable index of insulin secretion rather than insulin alone (Doda, 1996). C-peptide and insulin levels were significantly decreased in STZ-induced type 1 diabetic rats due to the destruction of β-cells of pancreas thereby inhibiting insulin release. STZ-induces experimental insulin-dependent diabetes mellitus (type 1) in animals through its cytotoxic effects on β-cells of the pancreas, *via* a mechanism associated with the generation of ROS (Punitha *et al*., 2005; Evelson *et al*., 2005). It leads to a deficiency of insulin, which acts as a diabetogenic agent (Szkudeslski, 2001).

The present study substantiates the role of GABA agonist Baclofen on glucose concentrations of STZ-induced type 1 diabetic rats. The importance of the GABA system for the function of hormonal secretion still needs to be elucidated in greater detail. GABA is decreased in endocrine pancreatic tissue in experimental and human diabetes. Also, the role of the GABA system in the endocrine pancreas should not be underestimated since there is a connection of autoimmune mechanisms related to GAD and the origin of diabetes mellitus (Junod, 1969). The treatment of healthy human volunteers with oral GABA or Baclofen increased plasma insulin concentration (Passariello *et al*., 1982). The GABA receptors may induce insulin release from isolated islets and are suited to bind positive modulators such as benzodiazepines (Marchetti *et al*., 1996).

In STZ-induced type 1 diabetes mellitus, the effect of vitamin E supplementation on lipid peroxidation and antioxidant defense were investigated in the blood. STZ causes generation of oxygen free radicals; the increase in oxidative stress over the 4-week period may be due to diabetes because, STZ acts selectively on pancreatic β-cells and it is readily metabolized with a half-life of 6 hours (Loven *et al*., 1986). A high dose of 400 mg/kg body weight of vitamin E acetate had been shown to be effective in inhibiting lipid peroxidation in diabetic

rats. The reactive oxygen species and the oxidative stress, which not only induce an alteration in the cellular redox state in the presence of chronic hyperglycemia, but also reduce the ability of tissues to use carbohydrates, which finally results in disturbances in the metabolism of protein and fat (Je *et al*., 2001).

Treatments with antioxidant vitamins mainly vitamin E have potential protective activity. So vitamin E used may prevent the development of diabetes mellitus (Bonnefont-Rousselot *et al*., 2000). The protective efficacy of vitamin E against the body weight loss could be attributed to its ability to reduce hyperglycemia. This may be achieved *via* the inhibition of hepatic gluconeogenesis and glucose output from the liver, which are accompanied by the suppression of lipolysis in adipose tissue (Postic *et al*., 2001). This is consistent with the fact that vitamin E causes a lowering of the circulating glucagon level in diabetic rats (Shami *et al*., 2006). The augmented levels of SOD and GSH-Px and reduced hydroperoxide levels could be due to the improvement of glycemia promoted by vitamin E. Thus, the imbalance between the generation of ROS and enzyme activity is controlled in diabetic rats (Abdella emam *et al*., 2014).

The direct association between vitamin E and a disease like diabetes, particularly because of the multiple mechanisms by which ROS are generated by elevated blood glucose, which shown that plasma α-tocopherol concentrations are lower in diabetes (Nourooz-Zadeh *et al*., 1997), and appear to be even lower in diabetics with complications like microangiopathy than diabetics without complications (Martin-Gallan *et al*., 2003). During diabetes mellitus, the excess glucose present in the blood reacts with hemoglobin to form HbA1c. HbA1c is used as a marker for estimating the degree of protein glycation in diabetes mellitus (Kaleem *et al*., 2006).

The treatment of diabetes mellitus is based on insulin and/or oral hypoglycemic drugs (Daisy *et al*., 2009). These drugs act by various mechanisms to control blood glucose level, but many side-effects have been reported (Patel *et al*., 2012). Therefore, there is considerable interest in the field of medicinal plants due to their natural origin and low side effects (Jarald *et al*., 2008). One of these medicinal plants is *Gymnema sylvestre* (Gurmar), which means sugar killer

(Najafi & Deokule, 2011). It is a wild plant that grows in the open forest in India, China, Indonesia, Japan, Malaysia, Sri Lanka, Vietnam, and South Africa (Gurav *et al*., 2007 & Spasov *et al*., 2008). The leaves of the plant in particular are used as antiviral, diuretic, antiallergic, hypoglycemic, hypolipidemic, antibiotic, and in stomach pains and rheumatism (Saneja *et al*., 2010). *Gymnema sylvestre* is an important medicinal plant used in different systems of medicine as a remedy for the treatment of diabetes (Jarald *et al*., 2008). The present study showed a significant decrease in blood glucose level in diabetic rats treated with gymnemic acid compared to that of untreated diabetic rats.

The results of this study have demonstrated that Insulin, Baclofen, Vitamin E and Gymnemic acid treatments in STZ-induced type 1 diabetic rats have beneficial effects in reducing blood glucose levels to near the control. The decreased body weight in the diabetic rats may be due to excessive breakdown of tissue proteins. The treatment of diabetic rats with Insulin, Baclofen, Vitamin E and Gymnemic acid significantly improved the body weight which indicates prevention of muscle tissue damage due to hyperglycemic condition. The detection of glucose in the urine can also be measured as an indication of diabetes. In models of type 1 diabetes, it is essential that the animals are also weighed to ensure that decreased blood glucose concentrations are not linked with weight loss. This indicates that decreases in blood glucose concentrations are not due to toxic effects of the therapy but possibly due to cessation of eating. However, it should be noted that in models of diabetes, the mechanism of the drugs to lower blood glucose levels may include weight loss (Knudsen, 2010).

ANTI-DIABETIC EFFECTS OF BACLOFEN, VITAMIN E AND GYMNEMIC ACID

Role of Baclofen

The main diabetes complication today in the world is painful neuropathy. The mechanism of neuropathic pain and other physiologic normal pains are different (Valizade & Zarrindast, 2000). GABAergic system, a pain relief mechanism, produces inhibitory neurotransmitter in CNS. Baclofen has excitatory synaptic effect on GABA receptors so that it is used as an anti-nociceptive drug in the cure of trigeminal neuralgia. Baclofen essentially manages pain by central mechanisms compared to diabetic neuropathy which acts peripherally (Terrence, 1985). It is also used as an anti-inflammatory drug (Tjolsen *et al*., 1992). Exogenic administration of GABA agonist reverses spinal nerve ligation-induced hyperalgesia (Gomez *et al*., 1999).

Alternative animal models of neuropathic pain conditions may involve selection of an animal that naturally possesses a painful disease condition providing neuropathic pain and its symptoms such as HIV, Herpes, cancer and diabetes neuropathy (Courteix *et al*., 1993). GABA, Baclofen, and muscimol treatments promoted significant human β-cell replication, as suggested by the increased percentage of total insulin$^+$ cells within islet grafts (Fiorina *et al*., 2008). The activation of $GABA_A$ or $GABA_B$ receptors is inhibited in diabetes murine and human β-cell apoptosis (Tian *et al*., 2013). The treatment with either a $GABA_AR$- or $GABA_BR$-specific agonist promoted mouse and human β-cell proliferation in mice. Lastly, GABA's anti-inflammatory activity may partially explain the observed results. The GABA-ergic system, a well-known target of autoimmunity, appears to be a promising tool for β-cell regeneration (Fiorina, 2013).

Baclofen and other GABA drugs act on the endocrine pancreas *in vivo*, ultimately increasing plasma insulin and decreasing high blood glucose levels of diabetic rats (Moulin, 1996). So Baclofen may be effective in delaying diabetes onset in mice by stimulating GABA activity, as this neurotransmitter, localized in the *islets*, may regulate insulin secretion and the antigen expression linked with it.

Orally administered GABA is safe for humans, and acts on peripheral GABA receptors but does not affect CNS functions, since it does not cross the blood–brain barrier, and thus it represents a promising new therapeutic agent for diabetes (Yun wan *et al*., 2015). In the present study, the oral administration of baclofen reduced blood glucose level in STZ-induced type 1 diabetic rats when compared to the control.

Role of Vitamin E

The increased free radical production and reduced activity of antioxidant defense systems in diabetes facilitate tissue damage (Zimmet *et al*., 2005). It is well known that vitamin E, a lipid-soluble antioxidant vitamin protects unsaturated fatty acid, a main component of cell membranes, from attack by oxygen-derived free radicals. There have been a few reports concerning the effect of vitamin E on experimental diabetes. Vitamin E inhibited the oxidation of proteins in organs such as liver and kidney in STZ-induced type 1 diabetic rats (Je *et al*., 2001). There is also evidence that hyperglycemia may cause damage to islet β-cells, an event which may play a secondary pathogenic role in type 1 diabetes. The free radicals are involved in this effect and that antioxidants such as vitamin E could be beneficial in preventing islet damage (Tanaka *et al*., 1999). The increased lipid peroxidation can be detected in the early stages of diabetes, well before the development of any diabetic complications (Nourooz-zadeh *et al*., 1997). Since lipid peroxides are well-known to cause tissue damage, the possibility arises that nephropathy, retinopathy, endothelial dysfunction and peripheral neuropathy associated with poor diabetic control involve free radical damage (Rosen *et al*., 2001).

It is also possible that elevated lipid peroxidation precedes the development of diabetes. There is a considerable variation in levels of lipid peroxides even in healthy subjects, and it has been proposed that persons with elevated lipid peroxidation may be more prone to develop diabetes and cardiovascular disease (Knekt *et al*., 1999). When highly-reactive species attack lipids within membranes or lipoproteins, they set off the chain reaction of lipid

peroxidation (Halliwell & Gutteridge, 1999). Vitamin E halts this chain reaction, e.g. it acts as a chain breaking inhibitor of lipid peroxidation (Parks & Traber, 2000). The weight of evidence from animal, epidemiological and human studies supports the view that a high dose of vitamin E, possibly in combination with vitamin C could be beneficial in the treatment of diabetes. The studies on the obese Zucker rats and epidemiological studies suggest that vitamin E may help in delaying the development of diabetes in insulin-resistant subjects and it may delay or prevent the onset of complications in subjects with diabetes (Kaikkonen, 2001).

The diabetes is associated with elevated oxidative stress (Giugliano *et al*., 1995), which is associated with an imbalance in the activity of cardiac autonomic nervous system (Bellavere, 1992; Giugliano *et al*., 1995). The potent antioxidant, such as vitamin E, may have beneficial effects on the cardiac autonomic nervous system. Such effects might be particularly evident in persons with diabetes, who have a higher degree of oxidative stress (Giugliano *et al*., 1996) and are more susceptible to increases in the plasma vitamin E concentration. Vitamin E seems the most appropriate antioxidant to investigate because it is widely used and because several studies showed it to be useful in lowering the risk of cardiovascular disease in diabetic and non-diabetic subjects (Stephens *et al*., 1996; Klipstein-grobusch *et al*., 1999). Vitamin E was found to be excellent for strengthening the antioxidative defense system, reducing the generation of ROS and damaging oxidative substances, and maintaining membrane fluidity in the brain of diabetes-induced rats (Hong *et al*., 2004).

Vitamin E administration improves diabetes-induced oxidative stress *via* decreasing lipid peroxidation and protein oxidation as a free radical generation source and elevating antioxidant defense system enzymes like SOD and catalase activities (Shirpoor *et al*., 2007). Diabetes leads to long-term complications in the brain, such as increased risk of stroke and small vessel disease (Makar *et al*., 1995). On the basis of above studies, suggests that the use of Vitamin E in diabetic patients is suggested in order to protect from the cerebrovascular complications associated with diabetes (Sibel *et al*., 2009). In fact, vitamin E administration can reduce plasma catecholamine concentrations and improve autonomic nervous

system balance in diabetic animals (Behrens, 1986). In diabetic rats, treatment with antioxidants lowers markers of oxidative damage in the pancreas (Jin *et al*., 2008).

The dosage of 1,800 units/day of α-tocopherol for four months improved retinal blood flow and renal dysfunction in patients with type-1 diabetes without changing glycated haemoglobin levels (Bursell *et al*., 1999). Vitamin E at 900 mg/day for four months improved insulin responses in diabetic patients. In elderly diabetes 900 mg/day of vitamin E produced a modest improvement in metabolic control (Paolisso *et al*., 1993). Some studies suggest that vitamin E improves nerve function in diabetes; 600 mg/day for four months appeared to improve cardiac autonomic nerve function whereas 900 mg/day for six months improved peripheral motor nerve conduction velocity (Manzella *et al*., 2001). The antioxidant treatment was revealed by immunohistochemical analysis to protect the number of insulin-positive β-cells. Suitable maintenance of antioxidant defenses might be useful for reducing the development of diabetes itself by sustaining functional pancreatic β-cells. In the present study, the subcutaneous injection of Vitamin E reduces blood glucose level in STZ-induced type 1 diabetic rats when compared to the control group.

Role of Gymnemic acid (Bioactive compound from *Gymnema sylvestre* herbal plant*)*

The *Gymnema sylvestre*, a plant native to the tropical forests of India has long been used as a treatment for diabetes. It is a traditional herb that helps to promote weight loss possibly through its ability to reduce cravings for sweets and control blood sugar levels. When *Gymnema* leaf is placed directly on the tongue, it eliminates the sensation of sweetness, even if sugar is put in the mouth immediately. When taken internally, it helps to control blood-sugar levels in diabetes (Lucy *et al*., 2002). The main constituent of *Gymnema* is supposed to be gymnemic acid, a mixture of at least 17 different saponins. The Gymnemic acid formulations have been found useful against obesity (Yoshikawa *et al*., 1993). This is attributed to the capability of gymnemic acid to delay the glucose

absorption in the blood. The atomic arrangement of gymnemic acid molecules is similar to that of glucose molecules and used as a treatment for diabetes (Nakamura *et al.,* 1999; Murray, 1999).

When *Gymnema* leaf extract is administered to a diabetic patient, there is stimulation of the pancreas by virtue of which there is an increase in insulin release. These compounds have also been found to increase fecal excretion of cholesterol (Defronzo, 1999). A number of studies have evaluated the effects of *Gymnema sylvestre* on blood sugar in animals (Rahman *et al.,* 1989). Similarly, Gymnemic acid molecules fill the G-protein coupled taste receptor location in the absorptive external layers of the intestine thereby preventing the sugar molecules absorption by the intestine, which results in low blood sugar level (Sahu *et al*., 1996). The leaves and especially Gymnemic acids from *Gymnema sylvestre* exert its hypoglycemic effects which enhances secretion of insulin and promotes regeneration of pancreatic *islet* cells. It increases utilization of glucose and augment the activities of enzymes responsible for utilization of glucose by insulin-dependent pathways, an increase in phosphorylase activity, and reduction in gluconeogenic enzymes and sorbitol dehydrogenase (Nakamura *et al*., 1999). The effects of an alcoholic extract of *Gymnema sylvestre* (GS4) on insulin secretion from *islets of Langerhans* and several pancreatic β-cell lines were examined (Persaud *et al*., 2009).

The active principles present in medicinal plants have been reported to possess properties such as pancreatic β-cells regenerating, insulin releasing and fighting the problem of insulin resistance (Welihinda *et al*., 1982). Hyperglycemia is involved in the etiology of development of diabetic complications. Hypoglycemic herbs increase insulin secretion, enhance glucose uptake by adipose or muscle tissues and inhibit glucose absorption from intestine and glucose production from liver (Hongxiang *et al*., 2009). Streptozoztocin causes selective destruction of β-cells, accounting for hypoinsulinemia and the finding was well supported by histopathology (Sarkar *et al*., 1996; Ahmed *et al*., 2000).

Gymnema sylvestre exerts its hypoglycemic effects through increasing secretion of insulin, promoting regeneration of *islets* cells, increasing utilization of glucose through increased activities of enzymes responsible for utilization of glucose by insulin-dependent pathways and by inhibition of glucose absorption from intestine (Kanetker *et al*., 2007). Gymnemic acid maintains the blood glucose to normoglycemia during diabetes, which acts as an essential trigger for both liver and kidney to revert to their normal metabolic homeostasis (Thirunavukkarasu & Anuradha, 2003). The low concentrations of the *Gymnema sylvestre* isolate (novel *Gymnema sylvestre* extract) stimulate insulin secretion *in vitro*, at least in part as a consequence of Ca^{2+} influx, without compromising β-cell viability. Identification of the component of the novel *Gymnema sylvestre* extract that stimulates regulated insulin exocytosis, and further investigation of its mode of action, may provide promising lead targets for diabetes therapy (Liu *et al*., 2009). The *Gymnema sylvestre* crude extracts and its isolated compound dihydroxy gymnemic triacetate shows hypoglycemic effect against STZ-induced type 1 diabetic rats in dose-and time-dependent manner (Pitchai Daisy *et al*., 2009).

The reduced glucose levels are exerted by the crude extract due to the presence of dihydroxy gymnemic triacetate that has the ability to release insulin by the stimulation of a regeneration process and revitalization of the remaining β-cells (Rokeya *et al*., 1999; Bolkent *et al*., 2000). Gymnemic acid has been shown to have multiple benefits in patients with diabetes such as reduction of blood sugar and its complications. Many earlier studies whether using the whole seeds or extracts showed that Gymnemic acid decreased fasting blood sugar levels in animals (Nahar *et al*.,2010). At present, the treatment of diabetes mainly involves a sustained reduction in hyperglycemia by the use of biguanides, thiazolidinediones, sulphonylureas in addition to insulin. However, due to unwanted side effects there is a demand for new compounds for the treatment of diabetes (Chattopadhayay, 1999). In the present study, the oral administration of gymnemic acid reduces blood glucose level in STZ-induced type 1 diabetic rats when compared to control.

ALTERATIONS OF CHOLINERGIC ENZYMES IN BRAIN AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS

The various neurotransmitter systems including dopaminergic, serotonergic, cholinergic, glutamatergic and GABAergic undergo a significant change in diabetes mellitus (Jackson & Paulose, 1999; Gireesh *et al*., 2008; Antony *et al*., 2010a; Anu *et al*., 2010; Kumar *et al*., 2010). In the brain acetylcholine functions either as a neuromodulator or as a neutotransmitter, activated *via* metabotropic muscarinic acetylcholine receptors (mAChRs),triggering a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of acetylcholine release and, thus, controlling the functional, behavioral and pathological states of the central nervous system (Dani, 2001). Acetylcholine also activates ionotropic nicotinic acetylcholine receptors that form ligand-gated ion channels in the plasma membrane of neurons and on the postsynaptic side of the neuromuscular junction. The activation of nicotinic receptors in the central nervous system induces depolarization of plasma membrane, culminating in an excitatory postsynaptic potential in the neuron, the activation of voltage gated ion channels and increase of calcium permeability. The changes in the number and activity of the metabotropic and ionotropic acetylcholine receptors have been implicated in the pathophysiology of many diseases of the central nervous system.

It was shown that in the cerebral cortex, hypothalamus and brain stem of STZ-induced type 1 diabetic rats, the number of Gq-coupled M1-mAChRs and the expression of genes encoding M1-mAChR were decreased with an increase in affinity of the receptor to agonists, and the binding parameters of the M1- mAChR were reversed to near control by the treatment with insulin (Gireesh *et al*., 2008; Peeyush *et al*., 2011). In the cerebral cortex of the diabetic and control rats with insulin induced long-term hypoglycemia the maximal binding of M1-mAChRs and their expression were reduced to a greater extent compared with diabetic animals with hyperglycemia (Sherin *et al*., 2011). At the same time, in the cerebellum and corpus striatum of diabetic rats and hypoglycemic diabetic and

control rats the binding parameters and gene expression of M1-mAChRs was increased (Antony *et al*., 2010b). This indicates that the alterations in the initial steps of M1-mAChR signaling in the diabetic brain are area-specific. The STZ-induced type 1 diabetes mellitus leads to a significant increase of the binding of another Gq-coupled M3-mAChR in the cerebral cortex and cerebellum but the extent of changes induced by hypoglycemia was significantly higher compared to diabetes mellitus, which indicates the detrimental effect of recurrent hypoglycemia on cholinergic system in the brain (Antony *et al*., 2010b; Peeyush *et al*., 2011; Sherin *et al*., 2011). This leads to the conclusion that the imbalance in glucose homeostasis affects acetylcholine metabolism and cholinergic muscarinic neurotransmission in the brain, and changes the expression and function of cholinergic receptors.

The study of 7-week- and 90-week-old STZ-induced type 1 diabetic rats showed that in the brain stem of both groups of animals the number of M1-mAChRs was significantly decreased whereas the number of M3-mAChRs greatly increased compared to their respective controls, and the insulin treatment reversed the binding parameters of M1- and M3-mAChRs to near control level (Balakrishnan *et al*., 2009). In the cerebral cortex of 7- week-old STZ-induced type 1 diabetic rats, the number of M1-mAChRs decreased by 28 %, while the number of M3- mAChRs increased by 30%. In the cerebral cortex of 90-week-old diabetic rats the number of M1- and M3-mAChRs increased by 43 and 23 %, respectively, and the level of acetyl choline was significantly increased compared to control (Savitha *et al*., 2010). These alterations of M1- and M3-mAChRs expression correlate with cholinergic hypo function in short-term and prolonged STZ-induced type 1 diabetes mellitus. It should be noted that M1- and M3-mAChRs are abundantly expressed in the brain regions involved in cognition, including the cerebral cortex, hippocampus and striatum (Porter *et al*., 2002). As a rule, most animal models of obesity and hyper insulinemia are associated with increased vagal cholinergic activity that is strongly associated with the M3-mAChR expressed in the brain and the peripheral tissues (Gautam *et al*., 2008). The absence of M3-mAChR protects the animals against experimentally or

genetically induced obesity and obesity-associated metabolic deficit and greatly ameliorates the impairments in glucose homeostasis and insulin sensitivity. The M3-mAChR-deficient mice are largely protected against obesity-associated glucose intolerance, insulin resistance, hyperinsulinemia, and hyperglycemia triggered by a high-fat diet, chemical disruption of hypothalamic neurons by gold-thioglucose, and genetic disruption of the leptin gene (Kumar *et al*., 2011).

Central cholinergic activity was studied in experimental rats after using choline acetyl transferase (ChAT) and acetylcholine esterase (AChE) as markers. Our results showed an increased expression of AChE in cerebral cortex, cerebellum, and brain stem of diabetic rats when compared to the control group. ChAT shows a decreased expression in cerebral cortex and cerebellum. In brain stem ChAT expression was increased. These results are in accordance with that of (Kuhad *et al*., 2007) where a significant elevation in AChE activity was observed in cerebral cortex from STZ-induced type 1 diabetic rats. These data favor the fact that the M3-mAChR and other subtypes of mAChRs can represent a potential pharmacologic target for the treatment of diabetes mellitus, obesity and associated neurological disorders. Along with insulin, vitamin D3 and curcumin in particular, which differ in the chemical nature and the mechanism of action, are also capable of restoring the functions of cholinergic system in the diabetic brain. Vitamin D3, as well as insulin, markedly recovered the altered gene expression of M1- and M3-mAChRs in the cerebral cortex and cerebellum of STZ-induced type 1 diabetic rats and binding parameters of these receptors to the near the control (Kumar *et al*., 2011).

Vitamin D3-induced improvement of the cholinergic system and glucose homeostasis in the diabetic brain is due to the influence of vitamin D3 on activity of pancreatic M3-mAChR followed by enhanced synthesis and secretion of insulin and reduction of the neuronal disorders in diabetes mellitus (Kumar *et al*., 2011). It was found, in addition, that vitamin D3 restored the disrupted expression of IR in the cerebral cortex of diabetic rats. Curcumin possesses powerful anti-diabetic properties and has the ability to modulate mAChRs thereby ameliorating the impaired cognitive functions in diabetes mellitus (Peeyush et *al*., 2011).

Ionotropic nicotine acetylcholine receptors are also involved in the pathogenesis of neurodegenerative processes in diabetes mellitus. The stimulation of nicotinic acetylcholine receptors and mAChRs provokes opposing physiological and behavioral responses, which is due to the existence of multiple nicotinic and muscarinic receptor subtypes and their different anatomical distributions in the central nervous system. Nicotine administration inhibits food intake, increases metabolic rate, and leads to reduced adiposity (Li *et al*., 2003), while the activation of M3-mAChRs induces hyperphagia and obesity (Gautam *et al*., 2008). α7-Nicotinic receptors highly expressed in the course of brain development are implicated in memory, attention and information processing (Picciotto *et al*., 2000). In the cerebral cortex of STZ-induced type 1 diabetic rats, the expression of α7-nicotinic receptors was markedly increased (Peeyush *et al*., 2011).

The receptors significantly influenced the activity within the cerebral cortex circuitry, and diabetes mellitus-associated deregulation of this activity could contribute to disorders involving the cerebral cortex (Peeyush *et al*., 2011). Alongside with the increase in α7-nicotinic receptors expression, in the cerebral cortex of diabetic rats, was revealed the increased AChE and decreased ChAT mRNA levels, which indicate fast acetylcholine degradation and a subsequent down stimulation of acetylcholine receptors causing undesirable effects on cognitive functions (Sherin *et al*., 2012). These changes in the expression of AChE and ChAT in diabetes mellitus leads to a reduction of cholinergic neurotransmission efficiency due to a decrease in acetylcholine levels in the synaptic cleft, thus contributing to neurological dysfunctions in diabetes mellitus (Antony *et al*., 2010b). Tocotrienol partially normalized the increase in AChE activity in the cerebral cortex of diabetic rats (Kuhad *et al*., 2009). Vitamin E reduced oxidative stress in the hippocampus and frontal cortex in diabetic rats (Tuzcu & Baydas, 2006). Also, it has been proposed that tocotrienols are especially potent as neuroprotective agents (Sen *et al*., 2004).

Insulin therapy and curcumin substantially regularized the increased expression of AChE and ChAT, and significantly reverted up regulation of α7-nicotinic receptor in the cortex of STZ-induced type 1 diabetic rats (Peeyush *et*

al., 2011). Our result showed that diabetic condition influenced the expression of both AChE and ChAT enzymes and the reversal of altered expression near to the control, in the Insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rat brain regions, and is a compensatory mechanism to maintain the normoglycemic level. The antidiabetic compounds are suggestive of diverse mechanisms including increasing cholinergic activity by inhibiting acetylcholine esterase activity.

Pancreas

Acetylcholine is crucial for pancreatic β-cell function. It stimulates insulin secretion by increasing the cytoplasmic free Ca^{2+} concentration, *via* inositol phosphate production and enhancing the effects of Ca^{2+} on exocytosis *via* protein kinase C in β-cells (Gilon & Henquin, 2001). Muscarinic receptors found in pancreatic β-cells are essential for maintaining proper insulin secretion and glucose homeostasis in mice (Gautam *et al*., 2006). Cholinergic agonists have been reported to restore defective glucose-stimulated insulin secretion (Doliba *et al*., 2004). In humans, variations in the gene that encodes the muscarinic receptor M3 are associated with increased risk for early-onset of diabetes (Guo *et al*., 2006). It is generally believed that acetylcholine is released during feeding from parasympathetic nerve endings in pancreatic *islets* (Ahrén, 2000). The consensus is that the endocrine pancreas is richly innervated by the autonomic nervous system (Conn *et al*., 1998), with studies based on the cholinesterase technique revealing dense parasympathetic innervation in cat, rat, rabbit and human islets (Brunicardi *et al*.,1995). Human pancreatic islets, however, have not been examined for the presence of prototypical cholinergic markers such as vesicular acetylcholine transporter (vAChT) or ChAT.

Our results showed an increased expression of AChE and decreased expression of ChAT in the pancreas of diabetic rats when compared to the control. The treatment with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed these altered expression near to the control level. Confocal studies using M1 and M3 specific antibodies in isolated pancreatic islets confirmed the results of gene

expression studies. The present study highlighted the involvement of cholinergic enzyme dysfunction in the pancreas of diabetic animals by the treatments with Baclofen, Vitamin E and Gymnemic acid.

CENTRAL MUSCARINIC RECEPTOR ALTERATIONS

Acetylcholine, a classical neurotransmitter that also functions as a non-neuronal paracrine signal, activates muscarinic receptors that play a key role in maintaining many metabolic functions, including glucose homeostasis. There is strong evidence that cholinergic mechanisms are important for function and survival of the endocrine pancreas, the *islet of Langerhans* (Gilon & Henquin, 2001). Activation of muscarinic receptors leads to improved insulin secretion from pancreatic *islets* (Zawalich *et al*., 2004). Because the muscarinic M3 receptor has been shown to play a critical role in maintaining blood glucose homeostasis in mouse models, approaches aimed at enhancing signaling through β-cell M3 receptors have been proposed as selective pharmacologic intervention points in the treatment of diabetes (Gautam *et al*., 2007). The multiple sources for acetylcholine and the various muscarinic receptors, the net effects of cholinergic signaling in the *islets* will be the sum of many activities that may be constantly fine-tuned under different physiological conditions.

The different muscarinic receptors have different desensitization, internalization, and down regulation properties that may affect the net effects of acetylcholine in the *islets* (Nathanson, 2008). A selective down regulation of M1 receptors in β-cells after prolonged exposure to acetylcholine, for instance, could shift the balance to a point at which enhancing the cholinergic signaling. It is also likely that changes in glucose concentration or diabetic conditions affect cholinergic signaling. Because the circumstances in the human *islets* will be very different from those in rodent models and because experiments are more difficult to conduct in human beings, addressing these possibilities experimentally may require establishing research models in which mice are transplanted with human *islets* (Thangaraju & Sawyer, 2011). The effects of endogenous acetylcholine on insulin secretion likely depend on the spatial and temporal patterns of

acetylcholine secretion from neural or paracrine sources as well as on the biophysical properties of the different muscarinic receptors. The presence of multiple muscarinic receptors on different endocrine cells in the human *islets* has important implications for the use of cholinergic agents to intervene in diabetes (Molina *et al*., 2014).

The muscarinic receptors, a member of class I, seven transmembrane. G-protein-coupled receptors (GPCRs), comprise five distinct subtypes, denoted as muscarinic M1, M2, M3, M4 and M5 receptors (Hammer *et al*., 1980; Bonner *et al*., 1987; Caulfield 1993; Caulfield & Birdsall, 1998). Acetylcholine exerts physiological control by both hormonal and neuronal mechanisms, *via* activation of all five muscarinic receptor subtypes. Amongst a wide range of effects, the auto/paracrine actions of acetylcholine include regulation of cell proliferation and cancer, skin cell signaling, and immune responsiveness (Sastry & Sadavongvivad, 1978; Eglen, 2006; Grando *et al*., 2007; Wessler & Kirkpatrick 2008; Nirish *et al*., 2009; Shah *et al*., 2009). Each muscarinic receptor subtype also has a unique distribution throughout the central and peripheral nervous systems, being expressed both pre- and post junctionally. Prominent actions of muscarinic receptors in the peripheral nervous system include slowing of the heart rate and stimulation of glandular secretion and smooth muscle contraction (Wess *et al*., 2007).

At most stages of development five muscarinic receptors mediate the actions of acetylcholine in almost all tissues, and *via* hormonal and neuronal effector systems. Given this key physiological role, it is unsurprising that extensive efforts have been made to develop therapeutics that selectively agonize, modulate or antagonize each receptor subtype. Initially, several naturally occurring compounds were found to mimic the actions of the endogenous agonist, acetylcholine, including the agonists, muscarine (a toxin from the mushroom *Amanita muscat* and from which the receptor family derives its name), pilocarpine (from the *ruraceae* plant family), or antagonists such as atropine or (-)-hyoscine (from the *solanaceae* plant family). These were also used with limited clinical benefits (Felder *et al*., 2000; Langmead *et al*., 2008a). Over the succeeding

decades, many derivatives, while selective for muscarinic receptors over other GPCRs, lacked intra subtype selectivity, and exhibited several side effects restricting their therapeutic use. Recently, however, selective compounds, including those that allosterically modulate muscarinic receptors, have been reported that display authentic intra subtype selectivity, and consequently have opened new avenues for therapeutic interventions (Conn *et al*., 2009a, b).

Recombinant receptor expression of these subtypes lead subsequently to the unambiguous delineation of muscarinic receptor pharmacology (Dorjc *et al*., 1990; Wang & El-fakahany, 1993). These properties were in good agreement with the pharmacology of endogenously expressed receptors and provided robust tools to characterize agonist and antagonist pharmacology. Over the succeeding decades, concerted medicinal chemistry efforts identified compounds with some degree of selectivity for muscarinic receptor subtypes, resulting in several compounds evaluated clinically. These compounds were augmented by the identification and purification of naturally occurring toxins with exquisite subtype specificity (Karlsson *et al*., 2000; Servent & Fruchan-gaillard, 2009). Collectively providing important pharmacological agents to study the receptor family, *in vitro* and *in vivo*. The knowledge generated with these and other compounds was confirmed and extended by phenotypic studies in transgenic mice (generated by homologous recombination methods) lacking muscarinic receptors (Wess *et al*., 2003, 2007; Wess, 2004).

Cerebral cortex

Muscarinic M1 receptors are abundantly expressed in all major forebrain areas including the cerebral cortex, hippocampus, and striatum (Levey, 1993). Consistent with this distribution, activation of muscarinic M1 receptors is implicated in learning and memory processes (Volpicelli & Levey, 2004; Fisher, 2008). A complex role of muscarinic M1 receptors in cognition or the participation of more than one subtype. Conceivably, muscarinic M1receptors are not critical for memory formation, but are important for memory processes involving interactions between the cerebral cortex and hippocampus (Wess *et al*.,

2007). The M1-muscarinic receptor–positive allosteric modulator, BQCA, have suggested that M1-muscarinic receptors can mediate learning and memory through an indirect mechanism by stimulating the prefrontal cortex (Shirey, 2009).

The binding studies with [^{3}H] QNB and atropine, a muscarinic general antagonist shown that the total muscarinic receptors are decreased in the cerebral cortex during diabetic condition. The binding parameters were reversed to near control in the case of Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats. At this stage the treatment groups maintained glucose and circulating insulin levels near to the control. The central cholinergic neurons take part in the complex neural events responsible for the hyperglycemic response to neurocytoglucopenia and to stressful situations. The hyperglycemic effect of carbachol and neostigmine has been revealed to be suppressed by co-administration of small amounts of atropine intraventricularly, or by adrenalectomy (Iguchi *et al*., 1986). The decreased expression of muscarinic receptors during diabetes is a regulatory mechanism to help insulin action and maintenance of normoglycemia in diabetic rats.

The muscarinic M1 receptor changes for the period of diabetes were studied using subtype specific antagonist, pirenzepine and [^{3}H] QNB. The specific muscarinic M1 receptors were decreased in diabetic rats with a decrease in Kd representing an increase in the affinity of receptors during diabetic condition. In the STZ-induced type 1 diabetes, the mRNA expression level and binding parameter of muscarinic M3 receptors showed an increase in the cerebral cortex when compared to control. In Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats, these binding parameters were reversed to near the control level. The fact that a deficiency in insulin release and glucose homeostasis was observed for the G protein-biased mutant receptor demonstrates that mechanisms other than G protein-dependent signaling play an important role in mediating muscarinic-receptor augmentation of insulin release. This finding is set in the context of earlier studies that have pointed to a role of G protein-signaling in the action of the M3-muscarinic receptor in insulin release (Gilon & Henquin, 2001)

The Real Time-PCR analysis has also shown a down regulation of the muscarinic M1 receptor mRNA expression level during diabetic condition. This study is concordant with our M1 receptor binding assays. TO-PRO®-3 iodide staining studies showed that the nuclear density is decreased during diabetic condition. Treatment with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the nuclear density level near to the control group. Immunohistochemistry studies using laser confocal microscope confirmed a comparable expression pattern in the localization of muscarinic M1 receptor in the cerebral cortex of control and experimental rats.

Cerebellum

The organization of the cerebellum reflects its involvement in motor control. The mossy fibers send excitatory glutamatergic outputs to granule neurons, which in turn transmit excitatory synaptic information to Purkinje cells. Cerebellar long-term depression (LTD) is a type of synaptic plasticity and has been considered as a critical cellular mechanism for motor learning (Hirano, 2013). Cholinergic signaling in the cerebellum which may result from long-term depression (LTD)-related dis inhibition of cholinergic neurons in the vestibular nuclei, suppresses presynaptic long-term potentiation to prevent an up regulation of transmitter release that opposes the reduction of postsynaptic responsiveness. This modulatory capacity of muscarinic acetyl choline receptor signaling could promote the functional penetrance of LTD (Rinaldo & Hansel, 2013). The developing central nervous system (CNS) is a target of the environmental toxicant methyl mercury (MeHg), and the cerebellum seems the most susceptible tissue in response to this neuro-toxicant. The cholinergic system is essential for brain development, acting as a modulator of neuronal proliferation, migration and differentiation processes; its muscarinic receptors (MRs) play pivotal roles in regulating important basic physiologic functions (Roda *et al.*,2008). The dysfunction of cerebellar cholinergic receptor is due to impaired neuronal glucose transport in the cerebellum during recurrent hypoglycemia in diabetic rats.

The receptor analysis and gene expression studies along with muscle AChE activity implicate a role for acetylcholine and cholinergic receptors in the modulation of neuronal network excitability and neuromuscular dysfunction associated with hypoglycemia (Sherin *et al*., 2010). The gene expression studies showed a substantial increase muscarinic M1 and a decrease in M3 receptor numbers in the cerebellum of STZ-induced type 1 diabetic rats when compared to the control. The binding parameters Bmax of total muscarinic, muscarinic M1 and M3 receptors were increased in STZ-induced type1 diabetic rats compared to the control in different treatment groups. The radio ligand binding, in situ hybridization, and immunohistochemical studies have demonstrated the existence of several subtypes of acetylcholine (ACh) receptors, such as α3, α4, α6, α7, β2 in the cerebellum (Tayebati *et al*., 2001). These cholinergic receptors have been reported to have significant roles in cerebellar neuronal functions, such as the modulation of neuronal firing and synaptic plasticity (Prestori *et al*., 2013).

The current study showed the modulatory function of Insulin, Baclofen, vitamin E and gymnemic acid on total muscarinic, muscarinic M1 and M3 receptors by minimizing the altered receptor gene expression and binding parameters to near control. TO-PRO®-3 iodide staining showed that the nuclear density was decreased during diabetic condition. Treatment with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the changes in nuclear density level near to the control group. The immunohistochemical analysis by confocal microscope and binding parameters of various treated groups confirmed the results of mRNA expression. In the present study, we revealed the neuroprotective effect of Baclofen, Vitamin E and Gymnemic acid on muscarinic receptor subtypes in cerebellum.

Brain stem

The brain stem is a part of the brain located beneath the cerebrum and in front of the cerebellum. It connects the spinal cord to the rest of the brain. The brain stem controls involuntary muscles such as the stomach and heart. The brain stem also acts as a relay station between the brain and the rest of the body. All five

subtypes of the muscarinic receptors are found in the human CNS, the basal ganglia and cortex predominantly express M1 and M4 receptors, whereas M2 receptors predominate in the thalamus and brain stem (Vilaro et *al*., 1991; Flynn & Mash, 1993). Overall, the M1, M3 and M4 subtypes are found abundantly in the brain whereas the M5- subtype is the least abundant (Vilaro *et al*., 1990). However, the M5- subtype may be relevant to schizophrenia as it is located in the brain stem and midbrain, where it has an effect on dopamine release (Miller & Blaha, 2005). The ontogeny of mAChR in the human fetal brain shows two distinct phases during *in utero* development: first, they appear between 16 and 18 weeks and gradually increase up to 20 weeks. Second, there is a lag period between 20 and 24 weeks, at which time the receptor density does not change perceptibly. The mAChR decreases in all regions of the human brain after birth (Bar-peled *et al*., 1991). During middle and late gestation, M2 receptors are abundant in the cerebellum of human fetus and M3 and M4 receptors appear to predominate in the brain stem (Aubert *et al*., 1996). High mAChR densities are noted in certain brain stem nuclei that are important for the development of fetal and neonatal behaviors (Berger-Sweeney, 2003).

The binding analysis of total muscarinic receptor is increased during diabetic condition in brain stem. The muscarinic M1 receptor is decreased and muscarinic M3 receptor is increased during diabetic state. In Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats, binding parameters were reversed near to the control levels. The gene expression studies by RT-PCR analysis also revealed a down regulation of the muscarinic M1 receptor mRNA expression level during diabetic condition. This is consistent with our receptor binding studies. Also confocal studies by specific antibodies of muscarinic M1 and M3 receptors in the brain stem confirmed the Real time PCR and Scatchard analysis data. TO-PRO®-3 iodide staining studies showed that the nuclear density is decreased during diabetic condition. Treatment with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the nuclear density level to near the control group value. The brain stem provides the major motor and sensory innervation through the cranial nerves. Our results showed that Baclofen and Vitamin E and

Gymnemic acid treatment on STZ-induced type 1 diabetic rats restored the altered muscarinic functions related to brain stem.

Pancreas

It is essential to identify the muscarinic receptor subtype(s), mediating stimulation of insulin release. Classic pharmacological studies using different "subtype-preferring" muscarinic antagonists have suggested that the M3 receptor subtype plays a key role in the control of insulin secretion (Verspohl *et al*., 1990). However, the proper interpretation of these experiments is complicated by the limited subtype selectivity of the muscarinic antagonists used in these studies. For example, virtually all antagonists that have a high affinity for M3 receptors (e.g., 4-DAMP, derivatives of sila-hexocyclium) also exhibit a high affinity for M1 and M5 receptors (Caulfield & Birdsall, 1998). Moreover, it is especially difficult to predict the simultaneous involvement of two or more muscarinic receptor subtypes (e.g., M1 and M3 receptors) in a specific functional response by using the currently available subtype-preferring muscarinic antagonists.

Insulin secretion by β cells of the *islets of Langerhans* in the pancreas is a process strongly regulated by glucose and other circulating nutrients. It is also modulated by many other factors, including hormones and neurotransmitters. One of the most prominent of these regulatory mechanisms is mediated by acetylcholine (Ach) originating from the parasympathetic cholinergic input (Gilon & Henquin, 2001). Although cholinergic regulation of insulin release has been known for many years, the mechanism of regulation and in particular the subtype of cholinergic receptor responsible for this regulation has only recently been established. The work of Gautam and colleagues using transgenic and gene knockout technology Ach has shown that the M3-muscarinic receptor is the bonafide Ach receptor that is responsible for enhancing glucose-dependent insulin release in β-cells (Gautam *et al*., 2006).

The M3-muscarinic receptor plays an important mechanistic role in facilitating insulin release from pancreatic islets, using a knock-in mouse strain that expresses a phosphorylation-deficient M3-muscarinic receptor mutant. The

M3-muscarinic receptor is able to activate protein kinase D1 (PKD1) in pancreatic islets and that this activity is essential for muscarinic receptor-augmentation of insulin release from *islets* (Kong *et al*., 2010). In the diabetic condition, total muscarinic, muscarinic M1 and M3 receptor binding parameters decreased when compared to the control. Gene expression studies also showed the down regulation of muscarinic receptors in diabetic rats. Insulin, Baclofen, Vitamin E and Gymnemic acid treatment reversed the binding parameters to the near control levels. The gene expression studies by RT-PCR analysis also revealed a down regulation of the muscarinic M3 receptor mRNA expression level during diabetic condition.

In particular, sustained insulin release associated with the enteric phase appears to be mediated by a process that is independent of G-protein signaling. This is evidenced by studies from our laboratory and others which demonstrated that PKD1 as one of the key components by which M3R regulates glucose-dependent insulin release (Kong *et al*., 2010). PKD1 is activated by the phosphorylated form of the M3R *via,* a G-protein-independent, β-arrestin-dependent process that results in secretary vesicle priming (Kong *et al*., 2010). In addition, M3R has also been shown to stimulate insulin release by inhibiting the mitogen activated protein kinase p38δ activity, which has inhibitory effects on PKD1 in β-cells (Sumara *et al*., 2009). The effectiveness of β cell M3-muscarinic receptor and/or downstream signaling components as drug targets for the treatment of diabetes and in particular it will be important to extend the studies that have been largely conducted in rodent models to humans (Kong & Tobin, 2011). Specific knock-out of the muscarinic M3 receptor (M3R) in β-cells leads to glucose intolerance (Gautam *et al*., 2006). The localization of muscarinic M1 and M3 receptors using confocal laser scanning microscopy showed a decreased mean pixel value in pancreatic *islets* of diabetic rats when compared to the control level.

Over expression of M3R also appeared to protect against high-fat-diet-induced hyperglycemia and glucose intolerance suggesting the therapeutic potential of manipulating M3R expression (Gautam *et al*., 2010). Our study substantiates that the Baclofen, Vitamin E and Gymnemic acid supplementation

ameliorated the down regulated muscarinic receptor function in pancreatic *islets* of diabetic rats. TO-PRO®-3 iodide staining showed that nuclear density is decreased in the diabetic condition. The treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the nuclear density level to near the control levels. In Insulin, Baclofen, Vitamin E and Gymnemic acid treated diabetic rats, muscarinic M1 and M3 receptor function was reversed to the control level. The present results indicate the potential anti-diabetic role of Baclofen, Vitamin E and Gymnemic acid.

CENTRAL GABA-ERGIC RECEPTOR ALTERATIONS IN CEREBRAL CORTEX, CEREBELLUM AND BRAIN STEM

The GABA-ergic inhibitory function in the cerebral cortex is of great significance in the regulation of excitability and responsiveness of cortical neurons. The GABA inhibition is facilitated by $GABA_A$ receptors, which open membrane chloride channels and stabilize the membrane potential below firing threshold, and $GABA_B$ receptors, which act *via*, G proteins to decrease transmitter release from presynaptic terminals. The GABA-releasing inter neurons mediate the function of excitatory glutamatergic neurons in the brain regions, which contributes extensively to the control of glutamate content in brain regions and inhibits glutamate toxicity induced in the brain of hypo- and hyperglycemic diabetic rats. The disruption of GABA-ergic inhibition induces seizures leading to neuronal damage and, therefore, pathophysiology of many seizure disorders is the result of alteration of GABA receptor function (Antony *et al.*, 2010a).

It was shown that the synaptic level of GABA and its release in the diabetic brain were slightly changed or remain unchanged. The extracellular basal level of GABA at dentate gyrus of STZ-induced type 1 rats, 12 weeks after the induction of diabetes mellitus, showed no changes (Reisi *et al*., 2009). The content of vesicular GABA transporter was significantly decreased in hippocampal synaptosomal membranes in two week diabetes mellitus, although only minor changes in the release of GABA and in the loading capacity of GABA transporters were found (Baptista *et al.*, 2011). This envisages that the alterations of GABA

signaling, typical of the diabetic brain, are due to the alteration in the level and functional activity of GABA receptors and downstream signal components of GABA-regulated intracellular cascades.

GABA binding and the gene expression of the subunits of $GABA_A$ and $GABA_B$ receptors were decreased in the cerebral cortex of STZ- induced type 1 diabetic rats compared to control animals. In the diabetic hypoglycemic rats having two sequences of insulin-induced hypoglycemia in the course of 10 days GABA binding and expression of GABA receptor subunits were decreased to a greater extent with reference to diabetic hyper/euglycemic animals. This indicates that hypoglycemia amplifies the unfavourable effects of hyperglycemia on GABA-ergic system and the impairments of functions of GABA-ergic neurons in the cerebral cortex of diabetic animals are intensified in hypoglycemia.

The expression of glutamate decarboxylase, the rate-limiting enzyme of GABA synthesis, which is used as a marker of GABA-ergic activity, was also significantly down regulated in diabetes mellitus and hypoglycemia exacerbated the altered expression (Antony *et al*., 2010a). The same picture was found in the cerebellum where GABA receptors played a critical role in neuronal excitability and modulation of synaptic neurotransmission (Luján, 2007). In the cerebellum of STZ-induced type I diabetes rats with hyperglycemia, the gene expression of $GABA_{A\alpha1}$ subunit and glutamate decarboxylase was decreased and these molecular alterations were exacerbated by recurrent hypoglycemia (Sherin *et al*., 2010). The gene expression of CREB, a stimulus-inducible transcription activator implicated in the activation of protein synthesis required for long-term memory and seizure formation, was significantly down regulated in diabetes mellitus and recurrent hypoglycemia. Since CREB up regulates endogenous $GABA_{A\alpha1}$ transcription, the decreased expression of CREB in the cerebellum of hypoglycemic and hyperglycemic rats leads to the attenuation of GABA-ergic system and, as a result, to excitotoxic damage of neuronal cells (Sherin *et al*., 2010).

A single oral dose of GABA (5 or 10 mg) significantly increased plasma insulin and C-peptide levels in 12 healthy subjects (Cavagnini *et al*., 1982). In

another study, 20 mg $GABA_BR$ agonist baclofen was administered orally to ten healthy subjects 1 hour prior to glucose challenge and post treatment test, which resulted in significantly increased insulin responses to glucose challenge and increased basal glucagon levels. It was shown that intravenous administration of GABA (2-4 mg) significantly reduced blood glucose levels in the majority of diabetic individuals (Passariello *et al*., 1982). It follows that both hypo - and hyperglycemia in diabetes mellitus reduced GABA-ergic neuroprotective function in the cerebral cortex and cerebellum, which accounts for increased susceptibility of these brain areas to subsequent neuronal damage.

Thus, Baclofen, Vitamin E and Gymnemic Acid treatment exerted antidepressant-like effect and decreased stress by normalizing the augmented expression of $GABA_B$ receptors in cerebral cortex and cerebelum. Our study showed that diabetes decreases the expression of $GABA_B$ receptor which reduces the cerebellar function. In our study, Insulin, Baclofen, Vitamin E and Gymnemic acid decreased $GABA_B$ receptor expression levels in the cerebellum, which suggests that the Baclofen supplementation modulated the functional regulation of this receptor to maintain normal GABA-ergic function and this is involved as a mechanism for preventing cerebellar dysfunctions.We observed a down regulation of $GABA_B$ receptor in the brain stem of diabetic rats. The results indicate that the GABAergic activity in the brain stem was altered in hyperglycemic rats, impairing GABA-related functions of brain stem. The Insulin, Baclofen, Vitamin E and Gymnemic acid treatments reversed the increased expression to near control level. So the $GABA_B$ agonist, Baclofen has a significant role in diabetic pain-related neuropathic complications and improve the GABAergic system in brain regions.

CENTRAL GABAERGIC RECEPTOR ALTERATIONS IN PANCREAS

GABA is also present in peripheral organs, such as the testes, gastrointestinal tract, ovaries, placenta, uterus, and adrenal medulla, as well as the pancreas, where its concentration is the highest and comparable to that in the central nervous system (Gladkevich *et al*., 2006). In accord with this, high levels of GAD have been detected in the *islets of Langerhans* (Sorenson *et al*., 1991).

Moreover, it has been reported that both pancreatic α- and β-cells express a vesicular GABA transporter, which transports GABA into the intracellular vesicles for packaging before it is released, and GAT3, which mediates cellular uptake of GABA (Saeter *et al*., 2004). The abundance of GABA and the presence of molecular machinery for GABA synthesis and release suggest an important role in pancreatic physiology.

The signaling differs sharply in β-cells, where GABA induces membrane depolarization (Braun *et al*., 2010). In isolated rodent and human islets, GABA was shown to stimulate Akt activation, promoting β-cell proliferation and survival in a $GABA_AR$ antagonist- and/or Ca^{2+} channel blocker-sensitive fashion (Soltani *et al*., 2011). This suggests that the $GABA_AR$-mediated Ca^{2+}-dependent PI3K/Akt pathway is a major mediator in conveying the trophic effects of GABA on β-cells. $GABA_BR$ is a G-protein-coupled receptor that initiates cyclic adenosine monophosphate signaling and Ca^{2+}-dependent signaling. Previous studies demonstrated that in neurons $GABA_AR$ activation induced VGCC-dependent extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores, whereas $GABA_BR$ evoked intracellular Ca^{2+} only (Schwirtlich *et al*., 2010). This is consistent with the observation that GABA-mediated elevation of intracellular Ca^{2+} in human β-cells was blocked by the type A receptor antagonist (picrotoxin), while it was only partially attenuated by the type B receptor antagonist (saclofen) (Purwana *et al*., 2014). The GABA stimulated CREB activation in a cyclic adenosine monophosphate/PKA-dependent signaling pathway mediated by $GABA_BR$ (Purwana *et al*., 2014).

CREB plays a key role in regulating β-cell mass homeostasis, as mice lacking CREB in their β-cells have diminished expression of IRS249 and display excessive β-cell loss (Withers *et al*., 1998). CREB is also a target gene of Akt signaling (Montminy, 1998). The GABA-$GABA_BR$-induced CREB activation is independent of the PI3K/Akt pathway, because upon inhibition of PI3K/Akt, activation of CREB was not suppressed, whereas blockade of PKA-dependent CREB did not affect GABA-stimulated Akt activation (Purwana *et al*., 2014).

In severely diabetic mice, GABA therapy regenerated β-cell mass and completely reversed hyperglycemia (Soltani *et al*., 2011). This was associated with anti-inflammatory and immunoregulatory events, which also appear to contribute to the success of therapy. The activation of both $GABA_AR$ and $GABA_BR$ are important in mediating GABA trophic effects to promote β-cell replication and survival in both rodents and humans (Tian *et al*., 2013). To analyze whether GABA could exert therapeutic effects on human *islet* cells, a suboptimal (marginal mass) *islet*-xeno transplantation model was used. This suboptimal mass of human islets was transplanted into immunodeficient NOD-severe combined immunodeficiency-γ mice after induction of diabetes with streptozotocin (Purwana *et al*., 2014). This *in vivo* approach revealed that oral GABA treatment increased graft-cell proliferation and decreased apoptosis, leading to a significantly enhanced β-cell mass. Furthermore, GABA lowered blood glucose levels and ameliorated glucose tolerance. The Ca^{2+}-dependent PI3K/Akt and CREB/IRS2 are two synergistic and independent signaling pathways that mediate the trophic effect of GABA in human *islet* cells (Purwana *et al*., 2014). GABA has multiple beneficial effects on β-cells, which include the stimulation of cell proliferation and anti-apoptotic activities, making it an attractive agent for diabetes treatment (Purwana *et al*., 2014; Tian *et al*., 2013). In addition, GABA appears to be beneficial to diabetes mellitus. The oral treatment with GABA improves glucose tolerance and insulin sensitivity in high-fat diet-fed mice (Tian *et al*., 2013).

Here, we investigated the molecular mechanisms established in β-cell responses to $GABA_B$ receptors action in diabetes and Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats. Baclofen proved a novel therapeutic role in modulating $GABA_B$ receptor in the pancreas of diabetic rats. Our data indicated that a down regulation of $GABA_B$ receptor in the pancreas of diabetic rats compared to the control. $GABA_B$ receptor ($GABA_B$-R)-specific agonist baclofen to culture media has been shown to inhibit β-cell apoptosis in cultured rodent cell lines and *islets* (Ligon *et al*., 2007). It remains to be determined whether GABA treatment can inhibit mouse β-cell apoptosis *in vivo* or, more importantly, whether

it can protect human β-cells from stress-induced apoptosis. If GABA can inhibit human β-cell apoptosis, elucidating whether this effect is mediated through the G-protein–coupled $GABA_B$-Rs, and/or the chloride channel $GABA_A$-Rs will enable more specific drug targeting (Soltani *et al*., 2011). The activation of $GABA_A$-R or $GABA_B$-R inhibited oxidative stress–related β-cell apoptosis and preserved pancreatic β-cells in hyperglycemic mice (Tian *et al*., 2011). The treatment with either a $GABA_A$-R– or $GABA_B$-R–specific agonist promoted mouse and human β-cell replication in mice. Hence, GABA acts as a growth factor that regulates the survival and replication of *islet* β-cells (Tian *et al*., 2013).

GABA-stimulated insulin secretion in the β-cells is partially contributed by activation of the B-type GABA receptor (Ligon *et al*., 2007). These observations suggest that the autocrine GABA-$GABA_AR$ system in β-cells constitutes an effective signaling component of the glucose-sensing machinery. The physiological relevance of GABA signaling in the regulation of islet β-cell function has yet to be fully identified. The depolarizing effects of GABA may lead to activation of PI3-K/Akt dependent cell growth and survival pathways in the β-cells (Soltani *et al*., 2011). Insulin is an important positive autocrine regulator of β-cell growth and survival (Bansal & Wang, 2008). GABA, when co-released with insulin (Braun *et al*., 2010), synergistically enhances insulin-stimulated cell growth and survival pathways in the β-cells (Soltani *et al*., 2011).

GABA also has beneficial effects on β-cell survival and regeneration, which results in enlarged β-cell mass. Furthermore, GABA suppresses insulitis and systemic inflammatory cytokine production. GABA therapy is useful in regulating islet cell function, glucose homeostasis, and autoimmunity (Wan *et al*., 2015). The treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the decreased expression of $GABA_B$ receptor to the near control. Therefore, the potential of Baclofen in modulating $GABA_B$ receptor action on β-cells have significant implications for the better management of diabetes.

1.1 INSULIN RECEPTOR ALTERATIONS IN BRAIN

Insulin is a polypeptide hormone synthesized in humans and other mammals within the β-cells of the *islets of Langerhans* in the pancreas. *The islets of Langerhans* form the endocrine part of pancreas, accounting for 2% of the total mass of the pancreas, with β-cells constituting 60-80% of all the cells of *islets of Langerhans* (Anon, 2004). Insulin exhibits a multitude of effects in many tissues, with liver, muscle, and adipose tissue being the most important target organs for insulin action. The basic physiological function of insulin is promoting the synthesis of carbohydrates, proteins, lipids, and nucleic acids. The effects of insulin on carbohydrate metabolism include stimulation of glucose transport across muscle and adipocyte cell membranes, regulation of hepatic glycogen synthesis, and inhibition of glycogenolysis and gluconeogenesis (Piero, 2006). Insulin starts its action by binding to insulin receptor, a transmembrane protein belonging to protein tyrosine kinase receptor super family, which can autophosphorylate. This initiates a series of events involving protein and membrane lipid phosphorylation, coupling proteins and cytoskeleton activity (Bjornholm & Zierath, 2005). Many diabetic patients have normal levels of insulin in the blood. Thus, diabetes is not caused by the destruction of β-cells in the pancreas but by other mechanisms, such as insulin resistance related to down regulation of insulin receptors, and other changes to the glucose transporter system (Cho *et al*., 2002).

Brain insulin receptors have similar kinetics and pharmacological properties to those described in peripheral tissues, although they differ in molecular size (as indicated, the subunits of brain IR, named IR-A, are smaller than the subunits of peripheral ones, called IR-B), degree of glycosylation (being higher in peripheral than in brain IR), and antigenicity (Zahniser *et al*., 1984). In addition, regulation by insulin also occurs in a different way, thus, while peripheral IRs are down- regulated in response to insulin excess, their counterparts in the brain do not record such down regulation (Heidenreich *et al*.,1983). Receptor heterogeneity is a powerful principle that allows the independent and

specific regulation of cellular functions *via*, identical hormones or second messengers.

Furthermore, the presence of different receptor isoforms allows an independent regulation of their expression by different mechanisms (Joost, 1995). Some regions show marked difference in IR density between the embryonic and adult brain, which may play a developmental role. Thus, high concentrations of IR are found in the thalamus, caudate–putamen, and some mesencephalic and brain stem nuclei during neurogenesis, but these same areas have a low IR density in adult rat brains (Kar *et al*., 1993). Insulin receptors are also widely distributed in the human brain, with the highest specific binding of [^{125}I] labeled human insulin in homogenates prepared from hypothalamus, cerebral cortex, and cerebellum obtained post-mortem from non-diabetic subjects (Hopkins & William, 1997). Iodinated insulin-binding to synaptosomal membranes within the human cortex was found to be a function of age. Binding to IR was observed as early as week 14 of gestation, with a slight decrease around week 30, and a marked decrease after birth (Potau *et al*., 1991).

A wide but uneven distribution of insulin receptor (IR) in the central nervous system has been reported and it was shown that membrane preparations from the hypothalamus specifically bound greater [^{125}I] insulin than membranes from the cortex and thalamus, and that this binding was higher for preparations from the anterior rather than the posterior portions of the hypothalamus (Landau *et al*., 1983). Likewise, the binding of [^{125}I] insulin was high not only in all olfactory areas and in closely related limbic regions, but also in the neocortex and accessory motor areas of the basal ganglia, hippocampus, cerebellum and choroid plexus, which suggested a neuro-modulatory function for insulin in the brain (Hill *et al*., 1986).

When IRs were quantified by autoradiography and computerized densitometry, the highest concentrations were detected in regions concerned with olfaction, appetite and autonomic functions, all of which contain dendritic fields receiving rich synaptic input (Werther *et al*.,1987). *In situ* hybridization showed that IR mRNA was the most abundant in the granule cell layers of the olfactory

bulb, cerebellum, dentate gyrus, in the pyramidal cell body layers of the piriform cortex hippocampus, in the choroid plexus, and in the arcuate nucleus of the hypothalamus; these findings were consistent with the distribution of IR binding (Marks *et al*., 1990). Interestingly, the expression of IR mRNA seems to be higher in the brain from obese (fa/fa) Zucker rats as compared with lean (Fa/) age-matched controls (Amessou *et al*., 2010). However, brain homogenates from normal and STZ-induced type 1 diabetic rats showed similar specific insulin-binding, which indicated the absence of the up regulation of these receptors.

Our results showed that insulin receptor expression was down regulated in cerebral cortex and up regulated in cerebellum and brain stem of diabetic rats when compared to the control. In this study, the altered expression of insulin receptor in the brain regions of diabetic rat was reversed near to the control level by the treatment with Insulin, Baclofen, Vitamin E and Gymnemic acid. These results provide a confirmatory indication for the hindrance of insulin receptor dysfunction in brain with Insulin, Baclofen, Vitamin E and Gymnemic acid treatments and correspond to a novel option for the better management of diabetic related neurological complications.

INSULIN RECEPTOR ALTERATIONS IN PANCREAS

As insulin enters the circulation, it reacts with target cells that have insulin receptors on the plasma membrane (Katzung, 1995). The most important target organs, which have insulin receptors, are liver, muscle and fat. The number of insulin receptors on individual cells and the affinity of the receptor to insulin are varied. The receptors bind insulin with high specificity and affinity in the picomole range. Insulin action is initiated through the binding to and activation of its cell-surface receptor, which consists of two α subunits and two β-subunits that are disulfide linked into a α2, β2 heterotetrameric complex. Insulin binds to the extracellular α subunits, transmitting a signal across the plasma membrane that activates the intracellular tyrosine kinase domain of the β-subunit. Although PI 3-kinase activity is clearly necessary for insulin-stimulated glucose uptake, additional signals are also required for the stimulation of GLUT 4 translocation

(Pessin & Saltiel, 2000). Upon binding with insulin, the protein tyrosine kinase phosphorylates itself as well as target substances, such as the insulin receptor proteins (IRS-1 and IRS-2), Cbl and p52Sho (Galic *et al*., 2005). IRS-1 plays a more prominent role in stimulating glucose uptake by muscle and fat, whereas IRS-2 functions mainly in the liver. It has been discovered that IRS-2 boosts insulin production by the pancreas (Alper, 2000).

It has been suggested that hyperglycemia can generate chronic oxidative stress by the glucose oxidation pathway leading to an excess in mitochondrial superoxide production, which further activates uncoupling protein-2 (UCP-2) (Robertson *et al*., 2003). This protein lowers ATP/ADP relationship through proton leak in the β-cell, which reduces insulin secretion (Brownlee *et al*., 2003). ROS also increases the stress signaling pathways in the β-cells, such as NF-κB activity, which potentially leads to β-cell apoptosis, and the JNK pathway which has been related to suppression of insulin gene expression, possibly by reduction of Pdx-1 DNA binding activity, a major regulator of insulin expression (Kaneto *et al*., 2005). It has also been shown that the activation of the hexosamine pathway in β-cells leads to suppression of Pdx-1 binding to the insulin and other genes involved in insulin expression, perhaps contributing to the β-cell dysfunction present in diabetes mellitus (Kaneto *et al*., 2001).

Our results indicated a decreased expression of mRNA of Insulin receptor in the pancreatic islets of diabetic rats. The treatment with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the decreased gene expression near to the control level. Our findings suggest that insulin receptor-dependent relative mechanisms are necessary for normal growth and function of β-cell and recommend a novel role of treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid in STZ-induced type 1 diabetic rats for modulating insulin receptors in pancreatic *islets*.

GLUT EXPRESSION IN BRAIN AND PANCREAS

The non-insulin sensitive glucose transporters GLUT 1 (astrocytes), GLUT 3 (neurons), and GLUT 5 (microglia) account for the majority of glucose uptake by the central nervous system (McEwen & Reagan, 2004). Insulin-insensitive GLUT 2, the glucose transporter used by the pancreas to sense blood glucose levels, is located on some cells in the hypothalamus, raising the possibility that this transporter is critical to the CNS reaction to hypoglycemia (Hendry & Reagan, 2009). Characterization of placental GLUT 3 gene expression will help to shed light on severe pregnancy-associated disorders such as those encountered in diabetes (macrosomia, malformations) and intra-uterine growth retardation (hypoglycemia, low birth weight) (Boileau *et al*.,1995). If the GLUT proteins present in class I, GLUT 3 has the highest apparent affinity and highest maximum turnover number of glucose. The main glucose transporter expressed at the blood-nerve and blood-brain barrier is GLUT 1 while GLUT 3 is responsible for uptake of glucose into the neurons. GLUT 3 acts in tandem with GLUT 1 to meet the high energy demand of these tissues (Gould & Holman, 1993).

In addition, 64% sequence similarity has been reported between GLUT 1 and GLUT 3 proteins (Mueckler & Thorens, 2013). GLUT 3 has relevant role in gestation diabetes and alterations of placental function in diabetic pregnancies. There is insufficient information on the expression pattern of GLUT 3 and the role of flavonoids in modulating this glucose transporter isoform. Reduction in blood glucose levels below that for which GLUT 1 can compensate results immediately in the signs and symptoms of hypoglycemia. The brain responds by increasing sympathetic outflow and releasing hypothalamic regulatory factors, all of which directly or indirectly result in the release of counter-regulatory hormones that oppose the action of insulin (Frizzell et *al*., 1993). Our results indicated that in brain regions the treatments with Baclofen, Vitamin E and Gymnemic acid reversed the increased expression of GLUT 3 in diabetes near to the control level. The Vitamin E and Gymnemic acid supplementation significantly modulated the elevated gene expression of CREB in the brain regions of diabetic rats near to the control level. Insulin treatment did not show any significant effect in the GLUT 3

expression of diabetic rats in cerebral cortex, and cerebellum. In pancreas, the down regulated GLUT 3 expression is reversed by treatments with these anti-diabetic compounds. Our findings suggest a modulation of GLUT 3 expression in the brain with Baclofen, Vitamin E and Gymnemic acid supplementation which successively regulate the glucose transport in central nervous system.

CREB EXPRESSION IN BRAIN AND PANCREAS

The protein kinases in the liver that can potentially phosphorylate CREB on serine 133 are PKA and calcium/calmodulin-dependent protein kinase II, indicating that CREB is a potential target for the convergence of the cAMP pathways in the regulation of transcription (Sun *et al*., 1994). The activity of nuclear PKA, and while no significant differences were observed between control and diabetic nuclear extracts, in the diabetic samples tended to be lower, suggesting that a decrease in this kinase activity might contribute to the decreased phosphorylation state of CREB (Gerald *et al*.,1995). A decrease in hepatic protein kinase activity measured both in the absence and presence of cyclic AMP was made in STZ-induced type 1 diabetic animals (Pugazenthi & Khandelwal, 1990). The protein kinase activity was also restored after insulin administration to diabetic animals. The fact that insulin treatment of diabetic animals augmented the phosphorylation state back to the control levels. It suggests that insulin treatment has an indirect effect, such as reversing some hormonal or metabolic abnormality present in diabetes which impacts on the pathway leading to the phosphorylation of CREB.

Mice transgenically expressing a dominant-negative peptide termed 'A-CREB' under the control of the rat insulin promoter, and thus in islet β-cells, developed a diabetic phenotype at about 8 weeks of age due to an increase in apoptosis causing a progressive decrease in β-cell mass (Jhala, 2003). This defect in β-cell survival was attributed to down regulation of insulin receptor substrate-2 (IRS-2), which was proposed as a direct target of CREB activation (Jhala, 2003). A second model employing β-cell-restricted, transgenic over expression of the inducible cAMP early repressor (ICER), a splice variant of the cAMP response

element modulator (CREM) gene that can inhibit CREB and related proteins, also elicited a diabetic phenotype, but by an entirely different mechanism (Inada, 2004). The chronic inflammation in obese mouse models results in cytokine-mediated β-cell dysfunction, and the cytokine-induced β-cell loss is thought to be a major contributor to diabetes (Donath, 2009; Dobrian, 2011). Furthermore, it has been postulated that exendin-4 protects β-cells from cytokine-induced apoptosis, while previous overexpression and dominant-negative approaches have indicated that CREB might be required for this effect on β-cell survival (Natalicchio, 2010; Velmurugan, 2012; Wang *et al*., 2002).

Our results indicated that Baclofen, Vitamin E and Gymnemic acid treatment reversed the decreased expression of CREB in cerebral cortex and cerebellum and increased expression in brain stem of experimental groups near to the control level. The Vitamin E and Gymnemic acid supplementation significantly modulated the elevated gene expression of CREB in the brain regions of diabetic rats near to the control level. The Insulin treatment did not show any significant effect in the CREB expression of diabetic rats in cerebral cortex and cerebellum whereas pancreas showed a significant reversal. This study demonstrated that Baclofen, Vitamin E and Gymnemic acid possess regulatory effect in the transcription factor CREB expression, which is crucial in regulating the normal neuronal function and improved management in diabetes. The effect of Baclofen, Vitamin E and Gymnemic acid in interrelating with the cholinergic, GABAergic receptor and CREB in STZ-induced type 1 diabetes established its potential in managing CNS disorders in diabetes.

OXIDATIVE STRESS AND INSULIN RESISTANCE IN PANCREAS

Pancreatic β-cells produce a large amount of GABA (Adeghate & Ponery, 2002) whereas $GABA_AR$s are expressed in both β-cells (Braun *et al*., 2010) and α-cells. In α-cells, GABA hyperpolarizes the membrane potential and suppresses glucagon secretion *via* a mechanism involving PI3-K/Akt signaling dependent $GABA_AR$ plasma membrane translocation (Xu *et al*., 2006). GABA depolarizes β-cells and stimulates insulin action from these *islet* cells (Dong *et al*., 2006).

These observations suggest that GABA, as a paracrine or autocrine factor plays an important role within pancreatic islets in the regulation of islet cells secretion and function. Vitamin E increased liver GSH as well but had no effect on Gpx activity. This effect may depend on the tissue being studied. Vitamin E administered alone increased Gpx activity in red blood cells (RBCs) of diabetic rats and decreased GSH in the same cells (Naziroglu & Cay, 2001). The antioxidant capabilities of brain are of interest due to the enrichment of this tissue with omega-3 polyunsaturated fatty acids which tend to serve as susceptible targets of ROS attack.

Diabetes decreased activity of SOD and Gpx in the brain of rats (Hong *et al*., 2004). Over time and with progression of diabetes, capabilities for proper insulin secretion are often diminished. This appears to be because the disease state induces significant oxidative stress in the pancreas of rat models for diabetes (Ihara *et al*., 1999). The oxidative stress is accompanied by a substantial decrease in the number of insulin-secreting β-cells in the pancreas (Jin *et al*., 2008). The mechanisms by which oxidative stress appears to be through increased β-cell death and dysfunction in the transcriptional regulation of insulin *via*, Pdx-1 (pancreatic and duodenal homeobox 1) and MafA (Tanaka *et al*., 2002; Harmon *et al*., 2005). Interestingly, low glucose also promotes oxidative stress and consequential apoptosis, a pathway suppressed by vitamin E (Cai *et al*., 2007). In diabetic rats, treatment with antioxidants lowers markers of oxidative damage in the pancreas (Jin *et al*., 2008).

The lipid peroxidation studies substantiated those STZ-induced type 1 experimental rats is a reason for the considerable reduction in the functional pancreatic β-cell population. As the β-cells are fundamentally susceptible to oxidative stress, STZ-induced free radical formation and DNA damage lead to stable dysfunction and death of β-cells. These rats are also incapable to augment an effective β-cell compensatory response against the subsequent hyperglycemia. Insulin, Baclofen, Vitamin E and Gymnemic acid treatments protect β-cells against Streptozotocin harmful effect by their endogenous antioxidant properties and by their capacity to stimulate the antioxidant defense system of cell. These

anti-diabetic compounds initiate effective β-cell compensatory response by increasing β-cell growth and differentiation. The variation in the pattern of blood glucose levels in treated rats indicate that hyperglycemia induced activation of β-cells could manage the Streptozotocin deleterious effects.

SUPEROXIDE DISMUTASE EXPRESSION IN BRAIN AND PANCREAS

SOD converts superoxide (O^{2-}) to hydrogen peroxide (H_2O_2) and three isoforms have been identified. SOD 1 (Cu Zn-SOD) is present in red blood cells (RBCs), SOD 2 (Mn-SOD) is primarily mitochondrial and SOD 3 is extracellular (Kinnula *et al*., 2004; Whitin *et al*., 2002; Zelko *et al*., 2002). Diabetic hyperglycemia has been reported to promote the overproduction of reactive oxygen species such as superoxide, causing hyperglycemic vascular damage *in vivo* and *in vitro*. Superoxide is secreted from various cells in the vascular system and is implicated in the insulin-resistant state. The increase in superoxide may contribute to oxidative processes in the vessel wall, such as induction and enhancement of cell membrane lipid per oxidation and oxidation of low-density lipoprotein (LDL). The activity of superoxide dismutase was also found to be decreased during diabetes. This might be due to the fact that there is excessive formation of free radicals during diabetes. These enzymes might be used up in scavenging these radicals, thus leading to their decreased activity during diabetes.

Moreover, superoxide reacts extremely rapidly with nitric oxide (NO), which modulates vasomotor tone, inhibits platelet-and leukocyte-aggregation, and produces peroxynitrite. An increase in superoxide dismutase (SOD) has been reported to attenuate diabetic vascular dysfunction (Adachi, 2004). SOD 2 has been shown to play a major role in promoting cellular differentiation, protecting against hyperoxic-induced pulmonary toxicity, and providing cellular resistance to cytotoxicity by tumor necrosis factor (TNF). EC-SOD has been found in serum, cerebrospinal, ascetic, and synovial fluids. In some human tissues such as uterus, umbilical cord, placenta, and arteries, EC-SOD enzyme activity equals or exceeds that of CuZn-SOD and Mn-SOD EC-S. The EC-SOD gene is one of candidate genes for diabetes mellitus (Abou-Seif & Youssef, 2004). The mechanism through

which the decreased SOD 1-expression and activity leads to the development of nephropathy, but the results suggest that this polymorphism (+35 A/C) with functional role in antioxidant defense is associated with diabetic nephropathy in Bangladeshi diabetic subjects (Kilpatrick *et al*., 2007; Pambianco *et al*., 2007).

The decrease of SOD activity might be attributed to the following reasons: (1) Hyperglycemia activates various biochemical pathways such as glucose autoxidation, nonenzymatic glycation of proteins and activation of protein kinase C, which, in turn, overproduce oxidants like superoxide and hydroxyl radicals as well as hydrogen peroxide. (2) The increase of glycosylated SOD that leads to the inactivation of this enzyme. (3) Loss of its two factors, Zn^{2+} and Cu^{2+} (Abou-Seif & Youssef, 2004). The SOD deficiency may leads to retinal capillary cell death and involved in the pathogenesis of retinopathy in diabetes. Our results showed that in cerebral cortex, cerebellum and pancreas the treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the decreased expression of SOD in diabetes near to the control level, whereas in brain stem SOD activity is increased and a significant reversal by treatments with anti-diabetic compounds. The Vitamin E supplementation significantly augmented the altered gene expression of SOD in the brain regions and pancreas of diabetic rats near to the control level. In enzymatic antioxidant system, SOD enzyme in target organs which are susceptible to diabetic complications such as heart and kidney can prove in the prevention and treatment of dysfunctions related to diabetic complications.

GLUTATHIONE PEROXIDASE, BAX AND CASPASE EXPRESSION IN BRAIN AND PANCREAS

The major antioxidant enzyme glutathione peroxidase-1 (Gpx1), which is responsible for the removal of hydrogen and lipid peroxides as well as peroxynitrites, has been shown to be highly expressed in the developing eye at embryonic day (Haan & Cooper, 2011; Esposito *et al*., 2000). The development of insulin resistance in mammals with elevated expression of an antioxidant enzyme suggest that increased Gpx-1 activity may interfere with insulin function by over quenching intracellular reactive oxygen species required

for insulin sensitizing (McClung *et al*.,2004). Intracellular and tissue levels of Gpx-1 activity affect apoptotic signalling pathway, protein kinase phosphorylation, and oxidant-mediated activation of NF-κB and abnormality of Gpx-1 expression to etiology of cardiovascular disease and diabetes (Schnabel *et al*., 2005). GPx-1 is a key enzyme for the protection of vessels against oxidative stress and atherogenesis and that GPx-1 may be of particular importance in the highly pro-oxidant diabetic environment.

The protective role of GPx-1 may be of clinical relevance in (retinopathy of prematurity) ROP since retinal tissue has the highest level of polyunsaturated fatty acids of any known tissue, and the aforementioned molecules are known to cause damaging peroxidation reactions. Lipid peroxidation of cell membranes results in loss of structural integrity and function, and retinal endothelial cells are particularly susceptible to peroxidation-induced injury (Sapieha *et al*., 2010). Gpx-1 in the protection against ROP and provides evidence that a deficiency in this antioxidant enzyme leads to retinal vascular damage, most likely as a consequence of increased oxidative injury. The reduced antioxidant capacity of preterm retinas is an important factor in the development of ROP (Sih *et al*., 2013). Our results substantiated that in cerebral cortex, cerebellum, brain stem and pancreas, the treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the decreased expression of Gpx in diabetes near to the control level. The Baclofen and Gymnemic acid supplementation significantly augmented the altered gene expression of Gpx in the brain regions and pancreas of diabetic rats to the control level.

High Bax activity, induced through various apoptotic stimuli, has been considered a critical event in the process of β-cell apoptosis (Grunnet *et al*., 2009; Tonnesen *et al*., 2009). The formation of heterodimers between Bax and Bcl-2 homologues with death repressor function (Bcl-2 and Bcl-Xl) leads to the inhibition of the death-promoting effects of Bax (Reed, 1994). The up regulation of cleaved caspase-3 occurs in addition to the increase in Bax/Bcl-2 ratios *in vitro* in high glucose-stimulated podocytes and *in vivo* in glomeruli of Streptozotocin - induced diabetic rats (Lee *et al*., 2015). Bax over expression in diabetic subjects

indicates that there is endothelial cell dysfunction, which in turn triggers endothelial cell death (Hasnan *et al*., 2010).

Bax levels were increased in human diabetic retinas that had high levels of glucose. The expression of Bax was increased in the cortex of the kidney of diabetic animal models (Podesta *et al*., 2000; Li *et al*., 2004). The treatment of diabetic rats with desferoxamine or tempol (antioxidants/ hydroxyl radical scavengers) significantly attenuated the increase in both hydroxyl radical production and in lipid peroxidation produced by hyperglycemia, preventing apoptosis by reduction of mitochondrial Bax and cytosolic cytochrome c levels (Daniel *et al* ., 2010). Our results substantiated that in cerebral cortex, cerebellum, brain stem and pancreas, the treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the increased expression of Bax in diabetes near to the control level. The Vitamin E and Gymnemic acid supplementation significantly augmented the altered gene expression of Bax in the brain regions and pancreas of diabetic rats near to the control level.

It is known that high D-glucose induces endothelial apoptosis through activation of the Bax–Caspase proteases pathway. The effectors of apoptosis are now well known to be represented by a family of intracellular cysteine proteases known as Caspases (Green & Reed, 1998). Caspases is the key inducer of apoptosis. These activated Caspases destroy numerous cellular structures, leading to cell death (Wang et *al*., 2007). Induction of Bax protein and its translocation from the cytosol to the mitochondria lead to the release of cytochrome *c*, which results in Caspase activation inducing apoptotic cell death (Zimmermann *et al*., 2001). The intracellular signaling in maternal diabetes-induced embryonic apoptosis is a complex network and Caspases have been found to be essential in diabetic embryopathy (Reece *et al*., 2006; Yang *et al*., 2008a; Zhao *et al*., 2008).

Caspase-8 may control diabetic embryopathy-associated apoptosis *via* regulation of the Bid-stimulated mitochondrion/caspase-9 pathway (Zhiyong *et al*., 2009). Our results substantiated that in cerebral cortex, cerebellum, brain stem and pancreas the treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid reversed the increased expression of Caspase 8 in diabetes near to the control

level. The Baclofen and Gymnemic acid supplementation significantly augmented the altered gene expression of Caspase 8 in the brain regions and pancreas of diabetic rats near to the control level.

NEURONAL SURVIVAL FACTORS AND DIABETES

NF-κB, TNF-α, Akt-1, Pdx-1

Diabetes and insulin resistance are accompanied by varying degrees of hyper insulinemia. Before the discovery of NF-κB, it was discovered that high doses of salicylates ameliorate hyperglycemia in diabetes (Yuan *et al*., 2001; Shoelson *et al*., 2003). NF-κB first became a chief suspect in the development of insulin resistance and type-2 diabetes after the milestone discovery that the anti-inflammatory agent, aspirin inhibits NF-κB and prevents degradation of the NF-κB inhibitor, IκB (Kopp & Ghosh, 1994; Yin *et al*., 1998). It is possible that NF-κB may be involved in a compensatory mechanism that develops in β-cells during the loss of insulin sensitivity. NF-κB has been shown to be important for the expression of GLUT 2, which contributes to glucose stimulated insulin secretion by β-cells (Norlin *et al*., 2005). Inhibition of this transcription factor, therefore, may have deleterious effects leading to the development of insulin resistance and diabetes. Prolonged hyperglycemia is believed to be one of the major causes of vascular complications associated with diabetes (Ruderman *et al*., 1992).

NF-κB is a pleiotropic oxidant-sensitive transcription factor and hyperglycemia- induced oxidative stress may play a key role in the pathogenesis of diabetic vascular disease. Prolonged hyperglycemia can also lead to the formation of AGEs, which act through specific receptors on vascular cells, leading to oxidant stress and cellular dysfunction in the pathology of atherosclerosis and diabetic complications (Schmidt *et al*., 1994; Vlassara, 1996). Indeed, studies have shown that AGEs directly activate NF-κB in vascular smooth muscle cells (VSMCs) (Lander *et al*., 1997). Persistent hyperglycemia *in vivo* and *in vitro* can lead to the activation of protein kinase C (PKC), which has been associated with diabetic complications (Koya & King, 1998).

Activation of the NF-kB pathway induces the production of vascular endothelial growth factor and inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (Kiriakidis *et al*., 2003). Vascular endothelial cell damage is aggravated by increased levels of AGEs and ROS. Endothelial dysfunction leads to vascular complications of diabetic mellitus (Karasu, 2010). Severe glucose toxicity occurs in diabetic rat ovaries. This injury depends on the activation of the NF-kB pathway in diabetic rats (Pala *et al*., 2013). NF-kB immunoexpression is significantly higher in non-treated diabetic rats. Diabetes mellitus had several negative effects on ovaries such as stromal fibrosis, and stromal and follicle degeneration in rat models. The resveratrol treatment seems to ameliorate the negative ovarian effects of oxidative stress through the NF-kB pathway (Oytun *et al*., 2014). Our present study substantiates up regulation of NF-kB expression in STZ-induced type 1 diabetic rats. The treatment with Insulin, Baclofen, Vitamin E and Gymenmic acid reversed the expression of NF-kB to its control level in pancreatic islets.

Akt is an important mediator of biological functions of insulin. One of the major effects of this hormone is the enhancement of glucose uptake in muscle, adipocytes, liver, and other tissues. Therefore, it is not surprising that Akt signaling has major impact on glucose metabolism. Earlier studies recognized that PI3K is responsible, at least in part, for insulin stimulation of GLUT 4, the major insulin-regulated glucose transporter, from intracellular vesicles to the plasma membrane in insulin-sensitive cells (Frevert *et al*., 1998; Okada *et al*., 1994). Therefore, the role of Akt in this process has also been evaluated. It has been suggested that Akt involvement in glucose metabolism occurs on two principal levels. Upon insulin stimulation Akt associates with GLUT 4 transporters (Calera *et al*., 1998) and glucose uptake is mediated by Akt-induced translocation of vesicles containing GLUT 4 from intracellular stores to the plasma membrane (Kohn *et al*., 1996; Hajduch *et al*., 1998).

Akt appears to positively regulate at least two pro-survival transcription factors. Transcription factor NF-κB is involved in the regulation of cell proliferation, apoptosis, and survival by a wide range of cytokines and growth

factors (Orlowski & Baldwin, 2002). Insulin-stimulated Akt phosphorylation was impaired in the skeletal muscle of insulin-resistant Goto-Kakizaki rats and in muscle biopsies from diabetic patients. Impaired activation of Akt in response to insulin has been then described in insulin-resistant human (Rondinone *et al*., 1999) and rodent adipocytes (Carvalho *et al*., 2000), as well as in rodent skeletal myocytes (Song *et al*., 1999). Defects in GLUT 4 translocation and expression were associated with the defective Akt phosphorylation (Carvalho *et al*., 2000; Tremblay *et al*., 2001).

Akt is preferentially expressed in non-insulin dependent tissues, reduced Akt activity was reported in skeletal muscle of obese and insulin resistant subjects in diabetes mellitus (Brozinick *et al*., 2003; Tschopp *et al*., 2005; Easton *et al*., 2005). Significant reductions of insulin-stimulated Ser473 or Thr308 phosphorylation occured in skeletal muscle of type 2 diabetic patients , while others showed no alterations of phosphorylation or enzymatic activity of PKB between control subjects and diabetic patients (Nikoulina *et al*.,2001;Meyer *et al*.,2002). The unaltered Akt/PKB phosphorylation in muscle cultures and biopsies from control subjects and diabetic patients was reported by employing phospho-specific antibodies (Nikoulina *et al*., 2001; Kim *et al*., 2003). Our present study substantiates down regulation of Akt expression in STZ-induced type 1 diabetic rats in brain regions. The treatment with Insulin, Baclofen, Vitamin E and Gymenmic acid reversed the expression of NF-kB to its control group. In pancreas, up regulation of Akt expression in STZ-induced type 1 diabetic rats in pancreas was observed. The Baclofen and Vitamin E supplementation significantly augmented the altered gene expression of Akt in the brain regions and pancreas of diabetic rats near to the control level.

Pdx-1 has been shown to be the master regulator of the pancreas development and plays a crucial role during the development and function of pancreatic β-cells (Oliver Krasinski *et al*., 2009). The β-cell–specific overexpression of GPx-1 significantly ameliorated hyperglycemia in diabetic db/db mice and prevented glucotoxicity-induced deterioration of β-cell mass and function (Jana Mahadevan *et al*., 2013). Mutations in (Pdx-1 can cause a

monogenic form of diabetes (maturity onset diabetes of the young 4) in humans, and silencing Pdx-1 in pancreatic β-cells of mice causes diabetes (Beatrice *et al*., 2012). The treatment of prediabetic female NOD mice with recombinant Pdx-1 or with a non-functional mutant Pdx-1 protein prevented the onset of diabetes and also involved in immunoregulation (Koya *et al*., 2008). Our present study showed down regulation of Pdx-1 expression in STZ-induced type 1 diabetic rats in pancreas. The treatment with Insulin, Baclofen, Vitamin E and Gymenmic acid reversed the expression of Pdx-1 to its control group. The Vitamin E and Gymnemic acid supplementation significantly increased the improved gene expression of Pdx-1 in the pancreas of diabetic rats near to the control level.

Molecular mechanisms of TNF-α function have been intensively investigated. Many studies demonstrated increased circulating levels of TNF-α both in animals and humans, as well as in the retina of diabetic rats (Joussen *et al*., 2002; Moller, 2000; Mohamad *et al*., 2013). High levels of TNF-α are involved in diabetic retinopathy and nephropathy associated with both forms of diabetes (Behl *et al*., 2008; Navarro-Gonzalez & Mora-fernandez, 2008). One mechanism through which diabetes may cause enhanced inflammation during the healing process is through the activity of AGEs or elevated levels of TNF-α (Goova *et al*., 2001). The latter is supported by a recent report that blocking TNF-α with an antibody improves healing, which was associated with a decrease in inflammation (Goren *et al*., 2007).

Mice with genetic ablation of TNF-R1 have accelerated wound healing with reduced leucocyte infiltration (Mori *et al*., 2002). Persistently high levels of TNF-α from systemic or local application *in vivo* interfere with dermal wound healing, which is consistent with the effects seen in diabetic wound healing where, TNF-α is elevated (Buck *et al*., 1996; Regan *et al*., 1993). Our present study showed that up regulation of TNF-α expression in STZ-induced type 1 diabetic rats in pancreas. The treatment with Insulin, Baclofen, Vitamin E and Gymenmic acid reversed the expression of TNF-α to its control group. The Vitamin E and Gymnemic acid supplementation significantly increased the improved gene expression of TNF-α in the pancreas of diabetic rats near to the control level.

Thus, our results indicated that uncontrolled hyperglycemia, insulin deficiencies, or both contributes to CNS disorders facilitated through cholinergic, GABA-ergic, insulin and $GABA_B$ receptor. Similarly, gene expression of cholinergic enzymes, glucose transporter GLUT 3, transcription factor CREB, second messenger enzyme phospholipase C and antioxidant and lipid peroxidation enzymes, superoxide dismutase and glutathione peroxidase was found to be elevated in the CNS of diabetic rats. Nutritional therapy using Vitamin E and Gymnemic acid revealed potential effects on improving and restoring the elevated functional regulation of receptors and enzymes of STZ-induced type 1diabetic rats to near normal level. Thus, Baclofen, Vitamin E and Gymnemic acid treatments have been revealed to reduce β-cell mass deterioration related with all types of diabetes. The neuroprotective role of Baclofen, Vitamin E and gymnemic acid represent a novel therapeutic option for the better management of diabetic-mediated neurological complications.

Summary

1. Streptozotocin-induced diabetic rats were used as model to study the alterations of cholinergic, GABAergic, Insulin and $GABA_B$ receptors, Glucose transporter, GLUT 3, second messenger enzyme phospholipase C, transcription factor CREB, antioxidant enzymes superoxide dismutase and glutathione peroxidase, apoptotic markers, Bax, caspase 8 and TNF-α, and their regulation by treatments with Baclofen, Vitamin E and Gymnemic acid in insulin secretion.

2. The anti-diabetic property of Insulin, Baclofen, and Vitamin E and Gymnemic acid-treatments were assessed using STZ-induced induced diabetic Wistar rat models.

3. Antihyperglycemic activity of Insulin, Baclofen, Vitamin E and Gymnemic acid were evaluated by the blood glucose level measurement of experimental rats. Diabetic rats showed increased blood glucose level. Insulin, Baclofen, Vitamin E and Gymnemic acid-supplementations to diabetic rats reversed the blood glucose level near to the control level.

4. DNA and protein synthesis in the pancreatic β-cells isolated from experimental rats were determined using [^{3}H] thymidine and [^{3}H] leucine incorporation studies. There was a significant reduction in DNA and protein biosynthesis in β-cells isolated from diabetic rats when compared to the control. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated rats showed a significant increase in the DNA and protein synthesis when compared to both control and diabetic group.

5. The gene expression of pancreatic regeneration marker, Pdx-1 in the pancreas of diabetic and experimentally treated rats was significantly

down regulated when compared to the control. Pdx-1 mRNA levels were significantly reversed in the Insulin, Baclofen, Vitamin E and Gymnemic acid-treated groups when compared to the diabetic group.

6. The gene expression of protein kinase B/Akt was analyzed in the brain regions and pancreas of experimental rats. The β-cell compensatory response to hyperglycemia was studied by analyzing the gene expression of regeneration marker, Akt. Real Time PCR studies confirmed significant reduction of Akt expression in cerebral cortex, cerebellum and brain stem of diabetic rats when compared to the control group. Pancreatic gene expression of Akt in diabetic rats showed a significant up regulation compared to the control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treatments induced a significant up regulation and reversal of Akt expression when compared to both control and diabetic groups.

7. The gene expression of NF-κB in the pancreas of diabetic and experimentally treated rats was significantly up regulated when compared to the control. NF-κB mRNA levels were significantly reduced in the Insulin, Baclofen, Vitamin E and Gymnemic acid-treated groups when compared to the diabetic group.

8. The gene expression of the apoptotic marker TNF-α in the pancreas of diabetic and experimentally treated rats was significantly up regulated when compared to the control. TNF-α mRNA levels were significantly reduced in the Insulin, Baclofen, Vitamin E and Gymnemic acid-treated groups when compared to the diabetic group.

9. The gene expression of apoptotic markers like Bax, caspase 8 was studied in the brain regions and pancreas of experimental rats. These markers were significantly up regulated in the cerebral cortex, cerebellum and

brain stem of diabetic rats and treated groups when compared to the control. Pancreatic expression of Bax and caspase 8 in diabetic rats was increased compared to the control. The expression of all the apoptotic markers was significant decreased in the treated groups, when compared with diabetic group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated rats showed a significant decrease in these apoptotic markers expressions in brain regions and pancreas when compared to the diabetic group.

10. Acetylcholine esterase gene expression level has been used as a marker for cholinergic activity. Acetylcholine esterase expression was experimentally analyzed in the brain regions and pancreas. During diabetic stage the expression was increased in the cerebral cortex, cerebellum and brain stem. Pancreas showed an up regulation in diabetic rats compared to the control. In insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats, the expression of the enzyme reversed to the control level.

11. Choline acetyltransferase gene expression level was used as a marker for acetylcholine synthesis. Choline acetyltransferase expression was experimentally analyzed in the brain regions and pancreas. During diabetes, the expression was decreased in the cerebral cortex, cerebellum while in brain stem it was increased. Pancreas showed a significant down regulation in diabetic rats compared to the control group. In insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats, the expression of the enzyme reversed to the control level.

12. The muscarinic M1 receptor gene expression was analyzed in the brain regions and pancreas of control and experimental rats. Muscarinic M1 receptor expression was significantly down regulated in cerebral cortex and brain stem while in cerebellum it was increased. Pancreas showed a

significant down regulation in diabetic rats compared to the control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats showed a significant increase in muscarinic M1 receptor expression, which reversed to the control level. Immunohistochemistry studies using specific antibodies confirmed the Real Time PCR data of muscarinic M1 receptor expression at protein level in control and experimental rats.

13. The muscarinic M3 receptor gene expression was analyzed in the brain regions and pancreas of control and experimental rats. Muscarinic M3 receptor expression was significantly up regulated in cerebral cortex and brain stem whereas, it was significantly down regulated in cerebellum. Pancreas showed a significant down regulation in diabetic rats compared to the control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats showed a significant reversal in muscarinic M3 receptor expression to the control level. Immunohistochemistry studies using specific antibodies confirmed the Real Time PCR data of muscarinic M3 receptor expression at protein level in control and experimental rats.

14. Total muscarinic receptor binding by Scatchard analysis was estimated in the brain regions and pancreas of control and experimental rats. Total muscarinic receptor binding was decreased in cerebral cortex while cerebellum and brainstem showed increased expression in diabetic rats when compared to the control level. In pancreas, total muscarinic receptor binding was decreased when compared to the control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats showed a significant increase in total muscarinic receptor numbers. Immunohistochemistry studies using specific antibodies confirmed the Scatchard analysis in control and experimental rats.

15. Bmax and Kd of muscarinic M1 receptor subtype was decreased in cerebral cortex, brain stem whereas it was increased in cerebellum of diabetic rats when compared to the control group. In pancreas, muscarinic receptor M1 binding number was decreased when compared to the control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats showed a significant reversal and increase of Bmax and Kd in muscarinic M1 receptor subtype. Immunohistochemistry studies using specific antibodies confirmed the M1 receptor Scatchard analysis in control and experimental rats.

16. Muscarinic M3 receptor subtype binding by Scatchard analysis was increased in cerebral cortex, cerebellum and brain stem of diabetic rats when compared to the control group. In pancreas, muscarinic receptor M3 binding number was decreased when compared to the control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats showed a significant reversal and increase of Bmax and Kd in muscarinic M3 subtype. Immunohistochemistry studies using specific antibodies confirmed the M3 receptor Scatchard analysis in control and experimental rats.

17. The $GABA_B$ receptor binding by Scatchard analysis was estimated in the brain regions and pancreas of control and experimental rats. $GABA_B$ receptor binding was decreased in cerebral cortex, cerebellum and brain stem of diabetic rats when compared to the control group. In pancreas, $GABA_B$ receptor binding number was decreased when compared to control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treated diabetic rats showed a significant reversal and increase in $GABA_B$ receptor binding numbers. Real time PCR analysis confirmed the $GABA_B$ receptor Scatchard analysis in control and experimental rats.

18. $GABA_B$ receptor mRNA level was studied in the brain regions and pancreas of experimental rats. A decreased expression of $GABA_B$ receptor was observed in cerebral cortex, cerebellum and brain stem in diabetic rats. Pancreatic expression of $GABA_B$ receptor in diabetic rats was decreased compared to the control group. Insulin, Baclofen, Vitamin E and Gymnemic acid-treatments to diabetic rats substantially restored the altered $GABA_B$ receptor expression to near control level. Baclofen treatment showed a significant reversal in $GABA_B$ receptor expression in the brain regions and pancreas.

19. Insulin receptor mRNA level was studied in the brain regions and pancreas of experimental rats. A decreased expression of insulin receptor was observed in cerebral cortex whereas in cerebellum and brain stem there was an increased expression in diabetic rats when compared to the control group. Pancreatic expression of insulin receptor in diabetic rats showed a significant down regulation compared to the control. Insulin, Baclofen, Vitamin E and Gymnemic acid-treatments to diabetic rats considerably ameliorated the altered insulin receptor expression to the control level.

20. Real time PCR analysis of gene expression studies indicated that Insulin, Baclofen, Vitamin E and Gymnemic acid-treatments substantially reversed the increased expression of GLUT 3 in brain regions:- cerebral cortex, cerebellum and brain stem of diabetic rats to the control group. GLUT 3 expression was studied in the pancreas and showed down regulation in diabetic rats when compared to the control group. The treatments reversed the altered expression of GLUT 3 to the control level.

21. Transcription factor, CREB expression in the brain regions:- cerebral cortex and cerebellum showed a significant down regulated expression in diabetic rats whereas in brain stem there was an increased expression in

diabetic rats when compared to the control group. Pancreatic expression of CREB in diabetic rats was decreased compared to the control group. Diabetes-induced altered CREB expression in brain regions was reversed by Insulin, Baclofen, Vitamin E and Gymnemic acid-treatments to the control level.

22. Antioxidant enzyme, superoxide dismutase expression was studied in experimental rats. In diabetic rats, its mRNA level was decreased in cerebral cortex and cerebellum whereas in brain stem, it was up regulated when compared to the control group. Pancreatic expression of superoxide dismutase in diabetic rats was decreased compared to the control level. Oxidative stress seen in diabetic brain regions and pancreas was considerably lowered by reversing the expression of superoxide dismutase near to the control by treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid.

23. Glutathione peroxidase gene expression was studied in brain regions and pancreas of experimental rats. The results showed that in diabetic rats, its mRNA level was showed a significant down regulation in cerebral cortex, cerebellum and brain stem when compared to the control level. Pancreatic expression of glutathione peroxidase in diabetic rats was decreased compared to the control group. Oxidative stress seen in diabetic brain regions and pancreas was considerably reduced by reversing the expression of glutathione peroxidase to the control level by treatments with Insulin, Baclofen, Vitamin E and Gymnemic acid.

24. Second messenger enzyme, phospholipase C showed a decreased expression in diabetic brain regions: - cerebral cortex, cerebellum, brain stem. Diabetic pancreas also showed a decreased phospholipase C expression when compared to the control group. Insulin, Baclofen,

Vitamin E and Gymnemic acid-administrations to diabetic rats reversed the altered phospholipase C expression to near control level.

In summary, the present study substantiated that the functional regulation of muscarinic and GABA-ergic receptor subtypes in cerebral cortex, cerebellum, brain stem and pancreas play an important role in the pathogenesis of diabetics and its related complications in STZ-induced diabetic rat models. The anti-diabetic properties of Baclofen, Vitamin E and Gymnemic acid were modulated due to the augmented glucose-stimulated β-cell insulin release together with increased β-cell mass proliferation and reduced β-cell apoptosis. Baclofen, Vitamin E and Gymnemic acid-treatments facilitate to regulate muscarinic and GABA-ergic receptors function, intracellular signaling, protective effect of hyperglycemia-induced oxidative stress and second messenger levels in cerebral cortex, cerebellum, brain stem and pancreas. The appropriate maintenance of antioxidant defenses might be effective for slowing progression of diabetes itself by sustaining functional pancreatic β-cells. Baclofen, Vitamin E and Gymnemic acid have significant therapeutic role for normalizing CNS dysfunctions and glucose homeostasis through cholinergic and GABA-ergic functional regulation. The present study focuses on the identification of the molecular basis of neuroprotection afforded by muscarinic and GABA-ergic receptors, antioxidant and herbal bioactive compound-based studies leading to the discovery of pharmacological targets for novel therapies to prevent and better management of diabetes.

Conclusion

Diabetes mellitus is a metabolic disorder associated with chronic hyperglycemia. Our results showed that cholinergic and GABA-ergic functional regulations were impaired in the brain and pancreas of STZ-induced type 1 diabetic rat models. STZ-induced type I diabetes with significant changes in the expression profiles of genes were related to Muscarinic M1, M3 receptors, antioxidant and lipid peroxidation, cell survival and apoptotic pathways. Down regulation of Phospholipase C, a second messenger enzyme, in the brain regions of diabetic rats revealed a malfunctioning in signal transduction. Impaired cholinergic and GABA-ergic neurotransmission, up regulated the apoptotic factors Bax and caspase 8 and down regulated the anti-apoptotic factor Akt-1 resulting in neuronal death in the brain regions of diabetic rats.

There was an enhanced state of oxidative stress observed in pancreas of diabetic rats through reduced SOD activity and GPx gene expression. Along with this, the down regulated Pdx-1 and up regulated Bax and caspase 8 in Baclofen, Vitamin E and Gymnemic acid treatments prevented the pancreatic β cell death. Differential expression of antioxidant and lipid peroxidation enzymes, superoxide dismutase and glutathione peroxidase in diabetes imparts increased oxidative stress. Antioxidant therapy could prevent a disturbance in the mechanism of protection against the deleterious cellular and bio molecular effects that leading to alterations in the cell function. Since, diabetes is associated with increased oxidative stress as a consequence of persistent hyperglycemia, the supplementation with vitamin E could have a protective effect against lipid peroxidation in diabetes.

Disrupted cholinergic and GABA-nergic signaling and increased hyperglycemic stress in diabetes contributed to neuronal loss. The neuronal loss in diabetic rats was inferred from the expression of pattern of GLUT 3, CREB, Pdx-1, Akt-1 and NFκB, and activation of apoptotic factors-TNF-α, caspase-8 and defective functioning of neuronal survival factors that modulated neuronal viability in diabetes. Hyperglycemic stress activated the expression of TNF-α,

caspase-8, Bax and differential expression of antioxidant enzymes – SOD and Gpx in brain regions and pancreas, leading to apoptosis. Neuronal stimulation of hyperglycemia-induced β-cell compensatory response is facilitated by accelerated signaling through cholinergic, muscarinic M1 and M3 receptors. Anti-diabetic treatment mediated functional regulation of cholinergic and GABA-ergic receptors, decreased insulin gene expression may enhance cell survival and β-cell mass proliferation in pancreas. The alterations in cholinergic and GABA-ergic receptor function, insulin, $GABA_B$ receptor, GLUT 3, phospholipase C, CREB, superoxide dismutase expression, antioxidant enzyme function and associated neuronal death in brain and pancreas were reversed by Insulin, Baclofen, Vitamin E and Gymnemic acid treatments. Thus, it is suggested that the corrective measures for the brain and pancreas functional damage caused during diabetes and antidiabetic treatment, through cholinergic and GABA-ergic receptors have clinical significance in the therapeutic management of diabetes.

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PAPERS PUBLISHED

1. **Ajayan M S,** Akhilraj P R, Paulose C S. (2015). Effect of vitamin E supplementation on Muscarinic M1 and M3 receptor subtypes in the cerebral cortex of streptozotocin - induced diabetic rats. Journal of Biomedial reports., 1(2):11-24.
2. Anju T R, **Ajayan M S**, Paulose C S. (2013). Disruption of cerebellar cholinergic system in hypoxic neonatal rats and its regulation with glucose, oxygen and epinephrine resuscitations. Neuroscience., 236:253-61.
3. Shilpa J, Anju T R, **Ajayan M S**, Paulose C S. (2014). Increased cortical neuronal survival during liver injury: Effect of GABA and 5-HT chitosan nanoparticles. Journal of Biomedical Nanotechnology., 10 (4): 622-631.
4. Roshni Baby Thomas, Shilpa Joy, **Ajayan M S**, Paulose C S. (2013). Neuroprotective Potential of Bacopa monneieri and Bacopa A against Dopamine receptor Dysfunction in the Cerebral Cortex of Neonatal Hypoglycaemic Rats. Cellular and Molecular Neurobiology.
   Doi 10.1007/s10571-013-9973-0

BOOK PUBLISHED

1. Naijil George, **Ajayan M.S** and Paulose C.S. (2012). Muscarinic Receptor subtypes Functional Regulation in the Pancreas of multiple low dose Streptozotocin Induced Diabetic Rats., Ist Edition, Bloomsbury Publishing India Pvt.Ltd.

2. T.R.Anju, **M.S.Ajayan** and Paulose C.S. (2012). Long Term Effects of Neonatal Hypoxia on Cognition and Memory., Ist Edition, Bloomsbury Publishing India Pvt.Ltd.

ABSTRACTS PRESENTED

1. **Ajayan M S**, Anju T R, Paulose C S. (2013). Differential expression of proteins in the Akt Pathway in the cerebral cortex of experimental rats., 25th Kerala Science congress.
2. **Ajayan M S**, Anju T R, Ancy Abraham, Paulose C S . (2015). Effect of vitamin E supplementation on Muscarinic M1 and M3 Receptor subtypes in the Cerebral Cortex of Streptozotocin–induced Diabetic Rats., (Best paper presentation). 27th Kerala Science congress.
3. **Ajayan M S**, Anju T R, Roshni B T, Paulose C S.(2013). Gymnemic acid mediated neuronal survival in the cerebral cortex of streptozotocin induced diabetic rats-Muscarinic Receptor subtypes functional regulation., National Conference on Current Advances in Biotechnology & Annual Meeting of Society for Biotechnologists (India) organized by Sant Gadge Baba Amravati University, Maharashtra.
4. **Ajayan M S**, Anju T R, Ancy Abraham, C S Paulose. (2014). Effect of Cholinergic receptors and antioxidant activity of vitamin E supplementation on Cerebral Cortex alterations in streptozotocin-induced diabetic rats., International Symposium on Genetic Analysis: Translational and Developmental.
5. **Ajayan M S**, Anju T R, Ancy Abraham, Paulose C S. (2015). Effect of vitamin E supplementation on Muscarinic M1 and M3 Receptor subtypes

in the Cerebral Cortex of Streptozotocin-induced Diabetic Rats.(Best paper presentation)., 27th Kerala Science congress.

6. Ancy A, Lakshmi S, **Ajayan M S**, Anju T R, Paulose C S. (2014). Regulation of Malate Dehydrogenase activity, HIF-α and HGF gene regulation in the liver of Streptozotocin- induced Diabetic rats: Effect of Vitamin E supplementation., National symposium on Emerging Trends in Biotechnology 2014 organized by Department of Biotechnology, Cochin University of Science and Technology, Cochin.

7. Johns D A, **Ajayan M S**, Anju T R, Paulose C S. (2014). Baclofen induced proliferation of pancreatic β-cells and functional regulation of adrenergic β_2 receptor subtype in the cerebral cortex of streptozotocin induced diabetic rats., Organized by Pushpagiri Research Centre and Pushpagiri Centre for Virology, Kerala.
8. Anju T R, **Ajayan M S**, Paulose C S. (2012). Long term effects of neonatal hypoxia on cognition and memory., Annual meeting of Society for Biotechnologists (India) and the national conference on Current Scenario in Biotechnology. K. S. Rangaswamy college of Technology, Tamil Nadu.
9. Anju T R, **Ajayan M S**, Paulose C S. (2013). Long term effects of neonatal hypoglycemia on pancreatic function., National Conference on Current Advances in Biotechnology & Annual Meeting of Society for Biotechnologists (India) organized by Sant Gadge Baba Amravati University, Maharashtra.

No.25/1/99-AWD
Government of India
Ministry of Statistics & Programme Implementation
(Committee for the Purpose of Control and Supervision of Experiments on Animals)
*****

Shastri Bhavan, New Delhi-110001.
Dated the 18th February, 2002.

To

The Principal/Director/Dean
Department of Biotechnology
Cochin University of Science & Technology
Cochin – 682 002 (Kerala)

Subject: Registration of Establishments/ Breeders under Rule 5(a) of the "Breeding of and Experiments on Animals (Control and Supervision) Rules 1998".

Sir,

With reference to your application on the above-mentioned subject, this is to inform that your Establishment is hereby registered for "Research". Your Registration Number is 363/01/a/CPCSEA. The nominee of CPCSEA on the Institutional Animal Ethics Committee (IAEC) of your Establishment will be intimated in due course.

1. You are requested to quote the above Registration Number in all your future correspondence with the Committee.
2. You are also requested to convene IAEC meeting at the earliest.
3. For further correspondence you are requested to contact Office of CPCSEA at Chennai, at the address given below:

Office of the CPCSEA,
Ministry of Statistics & Programme Implementation
3rd Seaward Road, Valmiki Nagar,
Thiruvanmiyur, Chennai-600 041

Yours faithfully,

(R.K.JAIN)
MEMBER SECRETARY, CPCSEA/DIRECTOR (AW)
Tel. No.3381498

Copy to: - Ms. Prema Veeraraghavan, Expert Consultant (CPCSEA), 3rd Seaward Road, Valmiki Nagar, Thiruvanmiyur, Chennai.

F.No. 25/530/2009-AWD
Government of India
Ministry of Environment & Forests
***

8th floor, Jeevan Prakash Building,
25, Kasturba Gandhi Marg, New Delhi-110 001
Dated: 9/5/2013

To,

Dr. C. S. Paulose, Director,
Department of Biotechnology,
Cochin University of Science and Technology,
Cochin - 682 022, Kerala

Subject: - Reconstitution of Institutional Animals Ethics Committee (IAEC) – regarding.

Sir,

With reference of your letter dated 07.06.2012 on the above mentioned subject, enclosing the biodata and consent letter of 7 IAEC members are as per Rules. Therefore, CPCSEA has selected/ accepted the following members recommended by your institute/ establishment:-

Sl. No.	Name of the IAEC Member	Designation
1	Dr. E. Vijayan	Chairperson cum Biological Scientist
2	Dr. Jacob Philip	Scientist from different discipline
3	Dr. A. A. Mohamed Hatha	Scientist from different discipline
4	Dr. M. S. Babu Issac	Veterinarian
5	Dr. C. S. Paulose	Scientist Incharge of Animal Facility
6	Dr. Oommen V.	Scientist from outside the institute
7	Ms. Subbulakshmi P.	Non scientific socially Aware member

2. CPCSEA hereby nominates the following members to the Institutional Animals Ethics Committee (IAEC) of your establishment/ institute:-

S.No.	Name	Designation
(i)	Dr.P.K. Gopalakrishnan Easwar Vilas, Press Road, Near Boat Jetty Alappuzha – 688 011, Kerala Phone: 0484-2625090 / 2393242 Mobile: 9446401198 Email: srgkrishna@yahoo.co.in	Main Nominee
(ii)	Dr. E.M. Elango, Scientist 33/186, Sruthi, Jawan Cross Road Elamakara, Kochin-682 026 Kerala Phone: 0484-2800504, Mobile: 9447190504 Email: emelango@hotmail.com	Link Nominee

Please note that any change in IAEC members can be made only with prior approval of CPCSEA.

3. The establishment is registered only for the purpose of "Research". "Breeding" of animal is permitted only after prior approval from CPCSEA.

Certificate

This is certify that the project title ...Neurotransmitter receptor functional interrelationship in diabetic rats and other animal models... has been approved by the IAEC.

Name of Chairman/ Member Secretary IAEC: E. VIJAYAN

Name of CPCSEA nominee:

Signature with date

Chairman/ Member Secretary of IAEC: **CPCSEA nominee:**

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

ORIGINAL ARTICLE

Effect of vitamin E supplementation on Muscarinic M1 and M3 receptor subtypes in the cerebral cortex of streptozotocin- induced diabetic rats.

Ajayan M S[1], Akhilraj P R [1], Paulose C S[2]

***Abstract*—** The focus of vitamin E pertains to its properties as an important dietary constituent that helps in the defense against cellular damage. At the cellular level, the incorporation of vitamin E is responsible for its potential to confer antioxidant protective properties and prevent both the initiation and propagation of lipid peroxidation was evaluated in the present study. The gene expression studies, antioxidant and peroxidation assays were done in the cerebral cortex to analyze the changes in muscarinic M1, M3 receptors, Bax, cAMP regulatory element binding protein (CREB) ,Caspase and Akt-1 and the enzymes involved in acetylcholine metabolism – choline acetyl transferase and acetyl choline esterase. Its impact on cellular processes is also multipurpose, from its involvement in preventing DNA damage to its influence in intracellular signaling. However; it is also becoming very clear that vitamin E has great potential in being used therapeutically in a number of diseases and conditions. The vitamin E supplementation reduced blood sugar levels in diabetes. The altered activity in primary antioxidant enzyme, superoxide dismutase augment lipid peroxidation and carbonyl protein content, thus ensuring the occurrence of oxidative stress in diabetic rats.

***Keywords*—** Oxidative stress, Muscarinic, Gene expression

1. INTRODUCTION

Diabetes Mellitus is a metabolic disorder associated with insulin deficiency, which not only affects the carbohydrate metabolism but also is associated with various central and peripheral complications.

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Chronic hyperglycemia during diabetes mellitus is a major initiator of diabetic microvascular complications like retinopathy, neuropathy, nephropathy [1].Type 2 diabetes can be linked to be accounting for around 90 per cent of all cases, it is a chronic metabolic disorder, in which the body is unable utilize glucose from food because of the inability of the pancreas to produce insulin or produces insufficient insulin, or the insulin itself is inactive [2].The number of people with diabetes worldwide is expected to rise to well over 366 million by 2030 [3].The treatment of diabetes mellitus is based on different potential compounds that works on different mechanism of actions including stimulation of insulin secretion, decrease of hepatic gluconeogenesis, augment in insulin receptor sensitivity and hindrance of digestion and absorption of carbohydrate, respectively. The identification of substances that mediate or mimic the action of insulin could lead to the development of novel structures which may be of clinical use in the treatment of persons having disorders of glucose metabolism, such as impaired glucose tolerance, elevated blood glucose associated with type II diabetes and insulin resistance [4].

Acetylcholine, a major neurotransmitter from the autonomic nervous system,regulates the cholinergic stimulation of insulin secretion, through interactions with muscarinic receptors [5,6,7].The activation of

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DISRUPTION OF CEREBELLAR CHOLINERGIC SYSTEM IN HYPOXIC NEONATAL RATS AND ITS REGULATION WITH GLUCOSE, OXYGEN AND EPINEPHRINE RESUSCITATIONS

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Abstract—Cholinergic system is important for respiratory control from the first days of life. Disturbances in cholinergic pathway due to early life stress like hypoxic shock can adversely affect the ventilatory response. The present study evaluates neonatal hypoxic insult mediated cholinergic disturbances and the role of glucose, oxygen and epinephrine resuscitation. The changes in total muscarinic, muscarinic M1, M2, M3 receptors and the enzymes involved in acetylcholine metabolism – cholineacetyl transferase and acetylcholine easterase in the cerebellum were analyzed. Hypoxic stress decreased cerebellar muscarinic receptor density with a decreased muscarinic M1, M2 and M3 receptor gene expression. The metabolic shift in the acetylcholine synthesis and release is indicated by the decreased cholineacetyl transferase mRNA expression and increased acetylcholine esterase gene expression. Glucose, acting as a precursor for acetyl choline synthesis and an immediate energy source, helps in reversing the cholinergic disturbances in hypoxic neonates. The limitation of immediate oxygenation and epinephrine administration in ameliorating cholinergic disturbances in hypoxic neonates was also reported. This will help in devising a better resuscitation program for the management of neonatal hypoxia.

Key words: neonatal hypoxia, cholineacetyl transferase, acetylcholine easterase, muscarinic receptor, glucose, oxygen.

INTRODUCTION

The perinatal period is a critical time which determines the fundamental changes in the cardiorespiratory status of the baby. Respiratory gas exchange, formerly a placental function, must be established by the lungs within minutes after birth. Therefore, frequent and serious difficulties in cardiorespiratory adaptation in the perinatal and neonatal periods are not surprising. Respiration is a highly integrated process that involves a complex network of interplay between the brain, spinal cord, cranial and spinal nerves, diaphragm, intercostal muscles, laryngeal and pharyngeal structures, lungs and the vasculature. It also involves diverse sets of neurotransmitters, neuromodulators, receptors, second messengers and transcription factors. Hypoxia occurring before or shortly after birth is a major cause of life-threatening injury and lifelong disability (du Plessis and Volpe, 2002; Schubert et al., 2005). Hypoxia results in multi-organ failure and structural/functional damage especially devastating to the cardiovascular, renal, gastrointestinal and central nervous systems (Shah et al., 2004; Vento et al., 2005). Hypoxic brain injury is very complex and has different neuropathological manifestations depending on the maturity of the newborn. Thus, understanding the diagnosis, pathogenesis, resuscitation and treatment of those infants suffering hypoxic brain injury is paramount to reduce disability, improving survival and enhancing quality of life.

Muscarinic cholinergic mechanisms are important for respiratory control from the first days of life. Respiratory stimulation might be the predominant, although not the exclusive, effect of muscarinic receptor activation in the respiratory neuronal network. Consequently, any alteration in baseline breathing resulting in a respiratory depression can be attributed to muscarinic receptors and originating within this network. The cerebellum significantly differs with respect to ischemia and hypoxia, this response being directly related to the duration and intensity of the injury. The cerebellum could cover the eventual need for nitric oxide during the hypoxia, boosting the nitric oxide synthase activity, but overall ischemia would require de novo protein synthesis, activating the inducible nitric oxide synthase to cope with the new situation (Rodrigo et al., 2004). Further, acetyl choline is released from the cerebellum during hypoxia (Fitzgerald et al., 2000). Hypoxic insult resulted in considerable neurocytological deficits of the Purkinje cells and altered glial fibrillary acid protein immunoreactivity in the fetal cerebellum. Acetylcholine (ACh) modulates neurons involved in the generation of respiratory frequency and pattern (Shao and Feldman, 2005) and influences chemoreceptor responses (Nattie, 1999). Cholinergic stimulation of the pontine reticular formation alters respiratory regulation (Kubin and Fenik, 2004), slows respiratory rate (Lydic and Baghdoyan, 2003) and suppresses ventilatory responses via connections of this cholinoceptive region to respiratory nuclei (Lee et al.,

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Abbreviations: ACh, acetylcholine; AChE, acetylcholine esterase; ANOVA, analysis of variance; ChAT, choline acetyltransferase; CoA, choline *O* acetyltransferase; QNB, quinuclidinylbenzilate; ROS, reactive oxygen species; S.E.M., standard error of the mean.

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ORIGINAL RESEARCH

Neuroprotective Potential of *Bacopa monnieri* and Bacoside A Against Dopamine Receptor Dysfunction in the Cerebral Cortex of Neonatal Hypoglycaemic Rats

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Abstract Neonatal hypoglycaemia initiates a series of events leading to neuronal death, even if glucose and glycogen stores return to normal. Disturbances in the cortical dopaminergic function affect memory and cognition. We recommend *Bacopa monnieri* extract or Bacoside A to treat neonatal hypoglycaemia. We investigated the alterations in dopaminergic functions by studying the Dopamine D1 and D2 receptor subtypes. Receptor-binding studies revealed a significant decrease ($p < 0.001$) in dopamine D1 receptor number in the hypoglycaemic condition, suggesting cognitive dysfunction. cAMP content was significantly ($p < 0.001$) downregulated in hypoglycaemic neonatal rats indicating the reduction in cell signalling of the dopamine D1 receptors. It is attributed to the deficits in spatial learning and memory. Hypoglycaemic neonatal rats treated with Bacopa extract alone and Bacoside A ameliorated the dopaminergic and cAMP imbalance as effectively as the glucose therapy. The upregulated Bax expression in the present study indicates the high cell death in hypoglycaemic neonatal rats. Enzyme assay of SOD confirmed cortical cell death due to free radical accumulation. The gene expression of SOD in the cortex was significantly downregulated ($p < 0.001$). Bacopa treatment showed a significant reversal in the altered gene expression parameters ($p < 0.001$) of Bax and SOD. Our results suggest that in the rat experimental model of neonatal hypoglycaemia, Bacopa extract improved alterations in D1, D2 receptor expression, cAMP signalling and cell death resulting from oxidative stress. This is an important area of study given the significant motor and cognitive impairment that may arise from neonatal hypoglycaemia if proper treatment is not implemented.

Keywords Neonatal hypoglycaemia · Dopamine · *Bacopa monnieri* · Bacoside A · Cerebral cortex

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Introduction

Hypoglycaemia is a common problem in the neonatal period and it frequently reflects difficulties in adapting to extra-uterine life (Blanca and Irene 2011). When severe, it leads to permanent neurological dysfunction including seizures, microcephaly and motor and/or developmental abnormalities (Blattner 1968; Hawdon 1999; Karp 1989; Ryan et al. 1985; Vannucci and Vannucci 2001). Vulnerable areas of the developing brain include the cerebral cortex (Hawdon 1999), striatum (Mina et al. 2005) and hippocampus (Auer and Siesjo 1988). Hyperinsulinism (HI) is the most common cause of both transient and permanent disorders of hypoglycaemia. The initial phase of neuronal damage occurs within minutes of hypoglycaemic shock. This is because more than 99 % of cerebral energy production results from the oxidation of glucose, and the energy failure gives rise to selective neuronal necrosis (Auer and Siesjo 1988). Glucose levels in the newborn decrease in the initial 2 h, but steadily rise afterwards and thereafter remain constant. Hypoglycaemia occurs when this equilibrium fails and is usually transient (Blanca and Irene 2011). In the presence of persistent hypoglycaemia, three main possible scenarios must be considered: depletion of energetic storage (prematurity and intra-uterine growth restriction), increase of tissue energetic consumption and foetal hyperinsulinism (Mitanchez 2008; Wight 2006; Ward and Desphande 2005).

Article

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Increased Cortical Neuronal Survival During Liver Injury: Effect of Gamma Aminobutyric Acid and 5-HT Chitosan Nanoparticles

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Use of nanoparticulate drug delivery system for an effective therapeutic outcome against diseases gains immense hope in the study of drug delivery to partially hepatectomised rats. In the present study, partially hepatectomised rats treated with Gamma aminobutyric acid (GABA) and serotonin (5-HT) chitosan nanoparticles, individually and in combination, were evaluated to analyse their role in $GABA_B$ and $5\text{-}HT_{2A}$ receptors functional regulation, interrelated neuronal survival mechanisms by nuclear factor kappa *B* (NF-κB), tumour necrosis factor-α (TNF-α), Akt-1 and the antioxidant enzyme superoxide dismutase (SOD) in the cerebral cortex. A significant decrease in $GABA_B$ and $5\text{-}HT_{2A}$ receptor numbers and gene expressions denoted a homeostatic adjustment by the cerebral cortex to trigger the sympathetic innervations during elevated DNA synthesis in the liver. $GABA_B$ and $5\text{-}HT_{2A}$ signalling directly influenced the cyclic AMP response element binding protein (CREB) expression, neuronal survival and ROS mediated cell damage which was confirmed from the gene expression of NF-κB, TNF-α, Akt-1 and SOD. In addition to enhanced hepatocyte proliferation, GABA and 5-HT chitosan nanoparticle treatment protected the neurons from ROS mediated cell damage and promoted their survival in the cerebral cortex. This has application in liver based diseases and treatments with nanosized active compounds.

KEYWORDS: *Chitosan Nanoparticles, GABA, Serotonin, Neuronal Survival, Liver Regeneration, Antioxidant System.*

INTRODUCTION

Nanoparticulate drug delivery systems provide new hopes in solving problems in the area of drug delivery.[1] Nanotechnology is the technology that deals with one billionth of a meter scale.[2] To a wide extend, biopolymer conjugated drugs coupled with nanotechnology enhances the stability and pharmacological efficiency of the active compound. Chitosan, deacetylated chitin, is a copolymer of β-(1,4) linked glucosamine (deacetylated unit) and *N*-acetyl glucosamine (acetylated unit).[3] Chitosan is nontoxic, biodegradable and bio compatible. Nanoparticles of chitosan coupled drugs are utilized for drug delivery in eye, brain, liver, cancer tissues, treatment of spinal cord injury and infections. Polymeric drug delivery systems can be used to deliver drugs directly to the intended site of action which results in slow release and minimized side effects elsewhere in the body and decrease the long-term use of many drugs.

The liver is a vital organ and has a wide range of functions, which include synthesis, storage, detoxification, metabolism and redistribution of amino acids, carbohydrates, fats, vitamins and proteins. Damage to liver occurs by over consumption of alcohol death, drugs with analgesic and antipyretic action, attack of parasites like *Entamoeba histolytica* and hepatotoxic chemicals. Patients often develop signs of liver dysfunction in the immediate postoperative period as a result of reduced liver mass, but normal liver function resumes once the removed liver mass is restored[4] but not completely in advanced liver disease. The presence of various ligands in the initiation, propagation and termination of the mitotic stimulus, such as priming factors, co-mitogens, growth factors and their suppressors, is necessary for the successful and complete restoration of hepatic mass.[5] Prolonged liver dysfunction, such as liver cirrhosis leading to hepatocyte damage, can harm the brain, leading to a serious and potentially fatal brain disorders.

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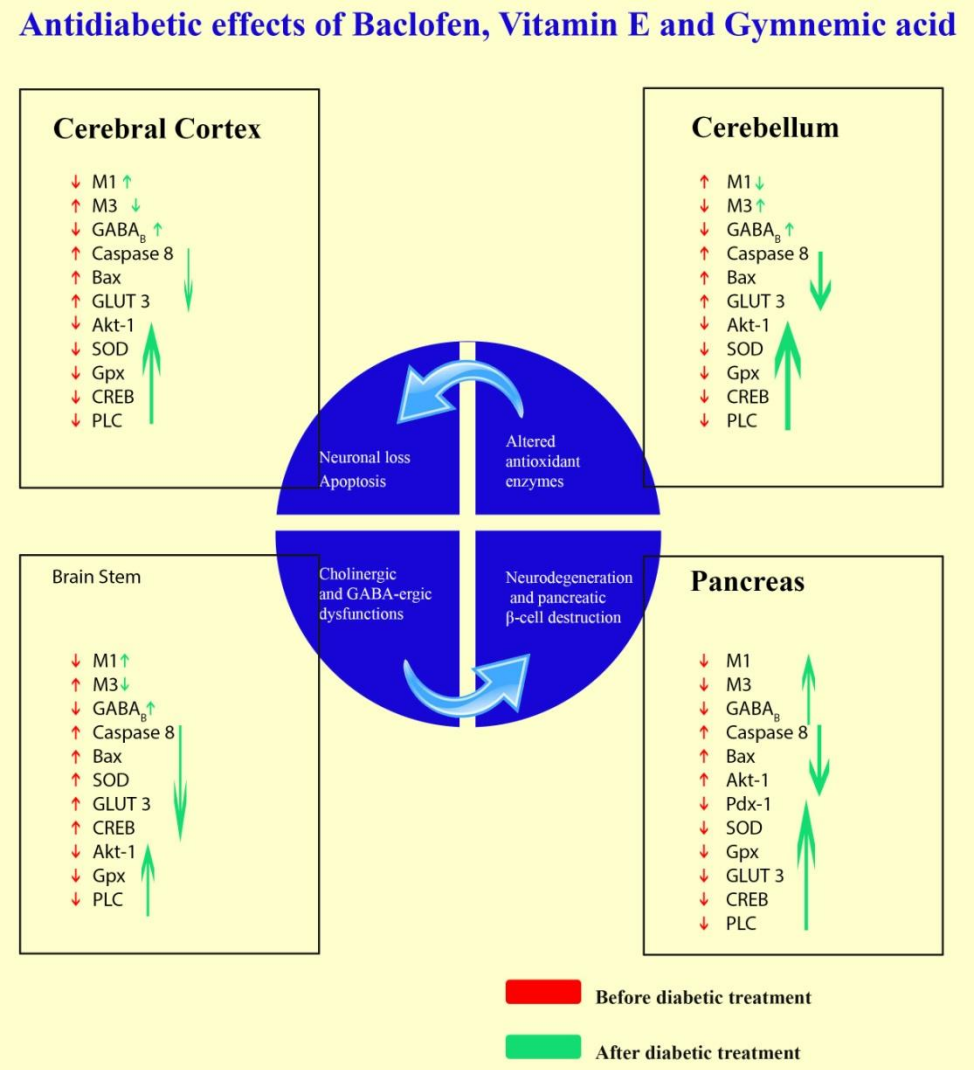
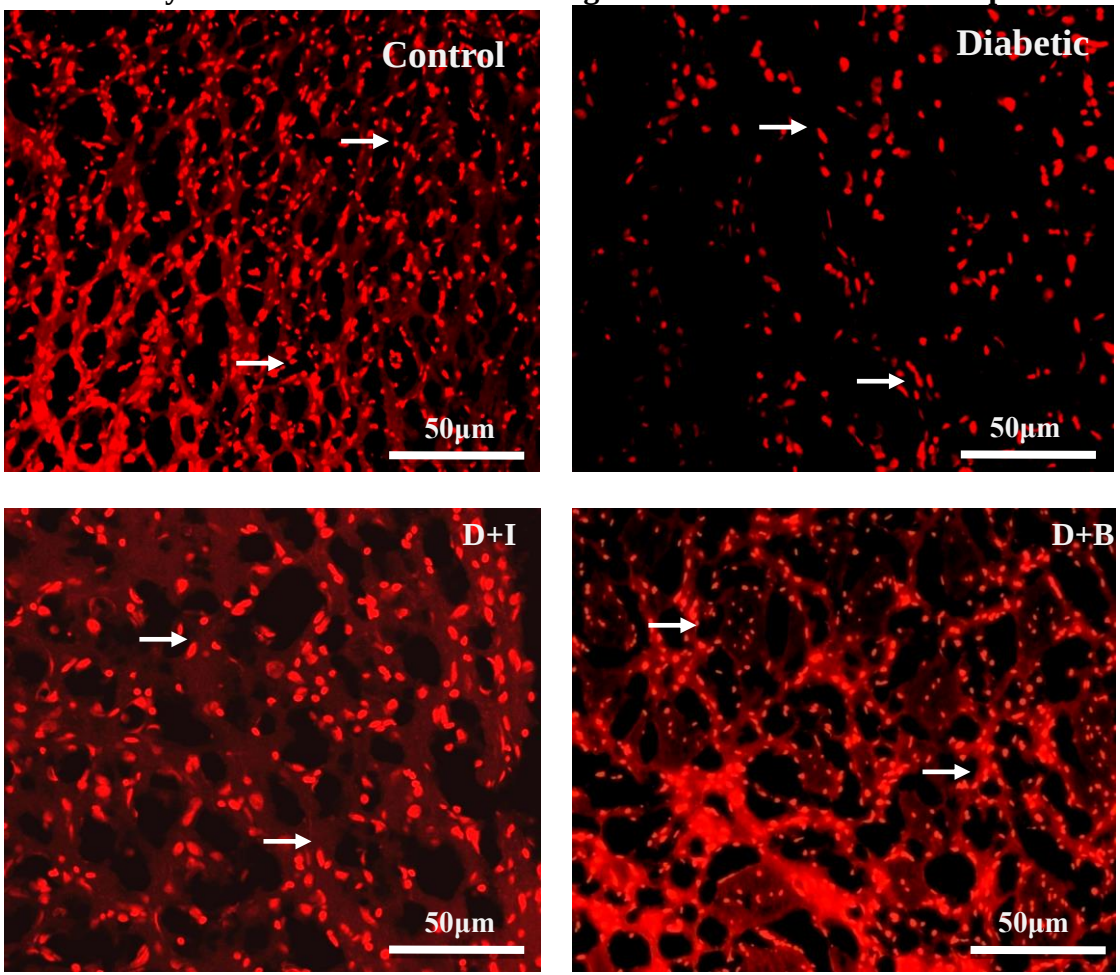

Antidiabetic effects of Baclofen, Vitamin E and Gymnemic acid
Cerebral Cortex
↓ M1 ↑
↑ M3 ↓
↓ GABA$_B$ ↑
↑ Caspase 8
↑ Bax
↑ GLUT 3
↓ Akt-1
↓ SOD
↓ Gpx
↓ CREB
↓ PLC
Cerebellum
↑ M1 ↓
↓ M3 ↑
↓ GABA$_B$ ↑
↑ Caspase 8
↑ Bax
↑ GLUT 3
↓ Akt-1
↓ SOD
↓ Gpx
↓ CREB
↓ PLC
Neuronal loss
Apoptosis
Altered
antioxidant
enzymes
Cholinergic
and GABA-ergic
dysfunctions
Neurodegeneration
and pancreatic
β-cell destruction
Brain Stem
↓ M1 ↑
↑ M3 ↓
↓ GABA$_B$ ↑
↑ Caspase 8
↑ Bax
↑ SOD
↑ GLUT 3
↑ CREB
↓ Akt-1
↓ Gpx
↓ PLC
Pancreas
↓ M1
↓ M3
↓ GABA$_B$
↑ Caspase 8
↑ Bax
↑ Akt-1
↓ Pdx-1
↓ SOD
↓ Gpx
↓ GLUT 3
↓ CREB
↓ PLC
Before diabetic treatment
After diabetic treatment

Figure-25

Histochemistry of TO-PRO®-3 iodide staining in the Cerebral cortex of Experimental Rats



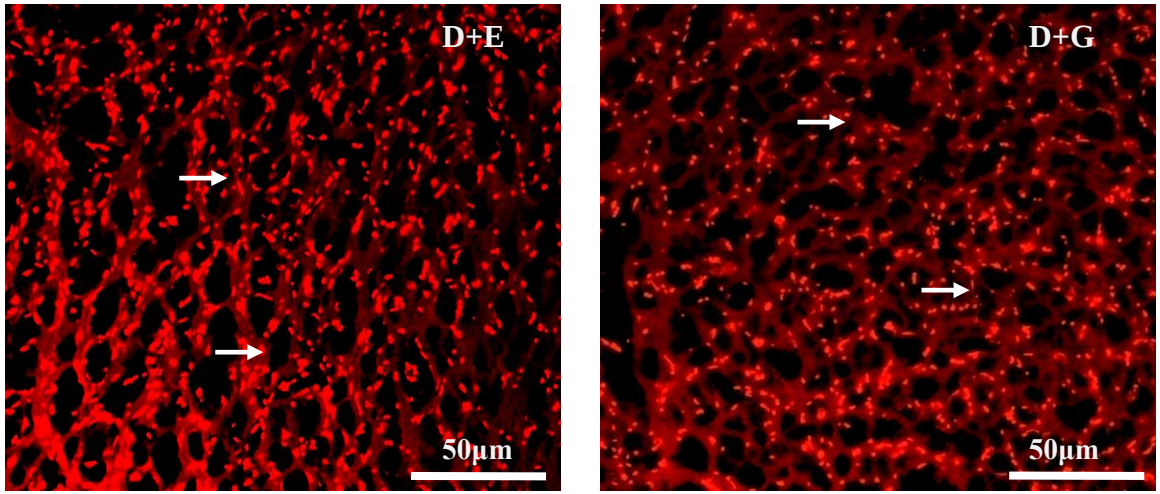


Table-25

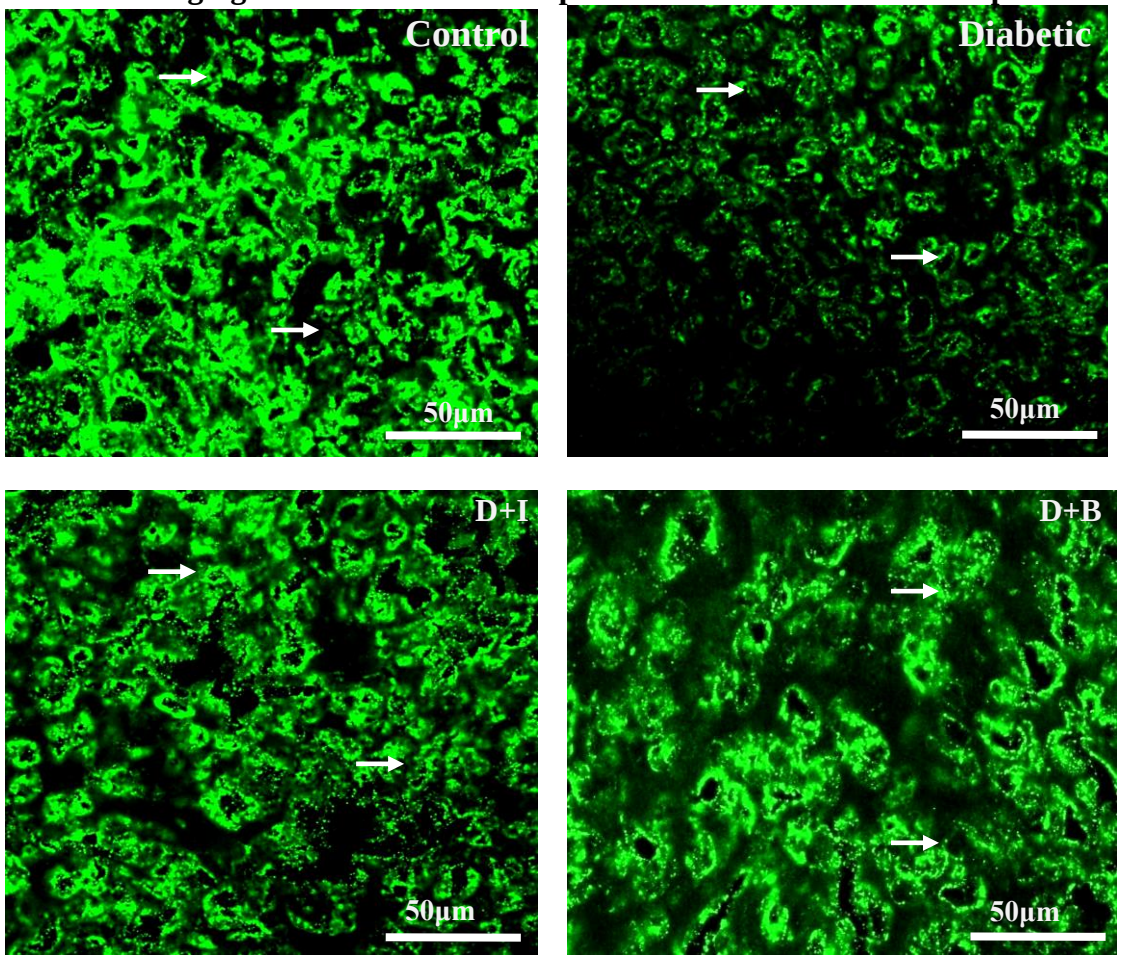
Histochemistry of TO-PRO®-3 iodide staining in the Cerebral cortex of Experimental Rats

Experimental groups	Mean Pixel Intensity
Control	66.23 ± 4.35
Diabetic	20.57 ± 1.28[a, g]
D + I	44.65 ± 2.69[a, d]
D + B	52.81 ± 3.17[b, d, h]
D + E	58.66 ± 3.79[a, e, h]
D +G	56.71 ± 4.08[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. (⟹) in white shows nuclear density. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 50 µm.

Figure-26

Confocal imaging of muscarinic M1 receptor in the cerebral cortex of experimental rats



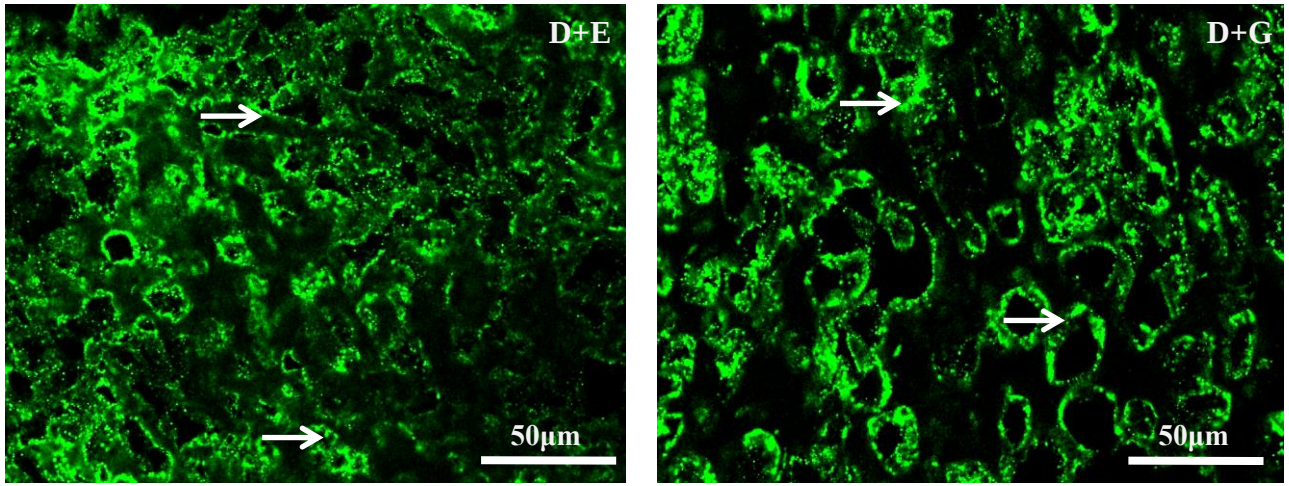


Table-26

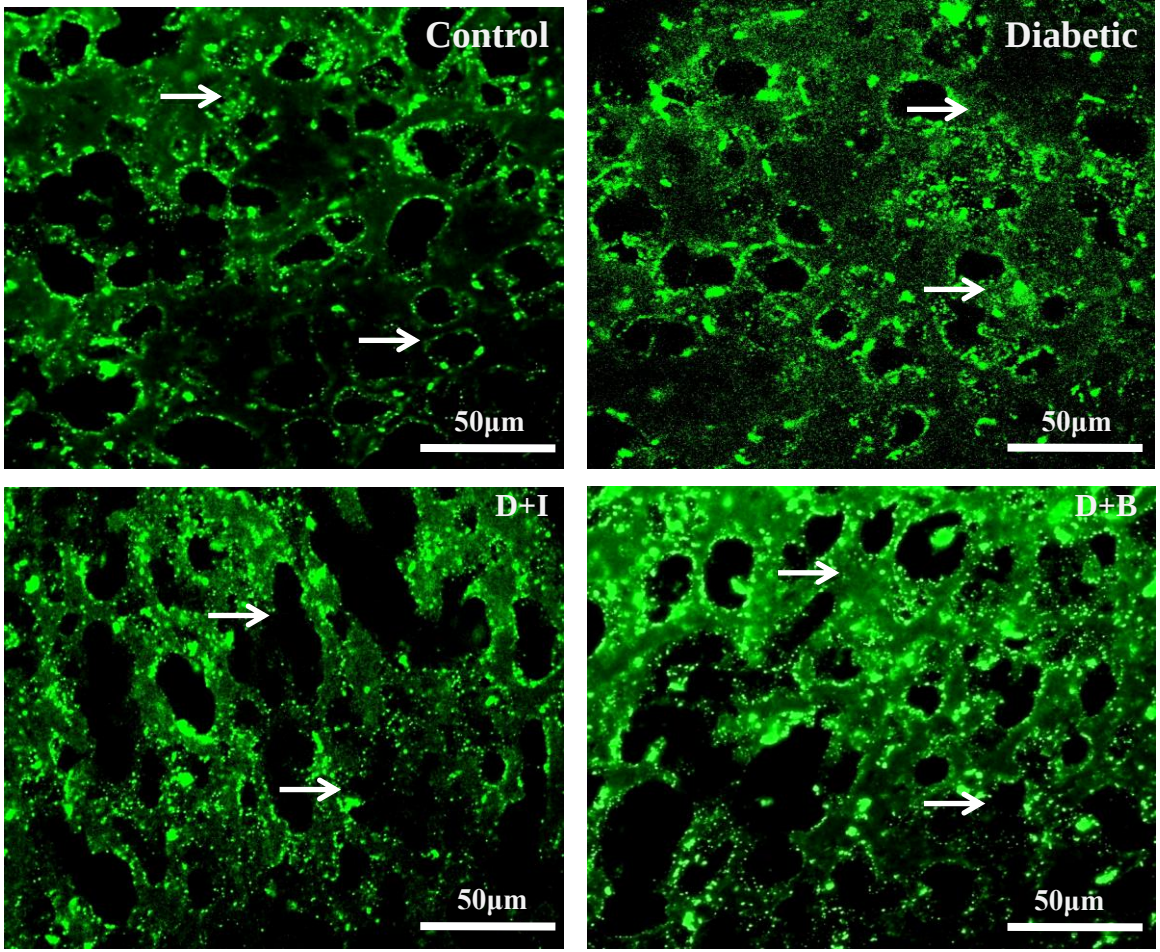
Confocal imaging of muscarinic M1 receptor in the cerebral cortex of experimental rats

Experimental groups	Mean Pixel Intensity
Control	83.99 ± 6.42
Diabetic	26.01 ± 2.81[a, g]
D + I	70.36 ± 3.24[a, d]
D + B	74.63 ± 3.79[a, d, g]
D + E	76.15 ± 3.45[a, d, g]
D +G	79.58 ± 4.11[b, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. Using immunofluorescent muscarinic M receptor specific primary antibody and cy5® as secondary antibody. (⟹) in white shows muscarinic M1 receptors. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 50µm.

Figure-27

Confocal imaging of muscarinic M3 receptor in the cerebral cortex of experimental rats



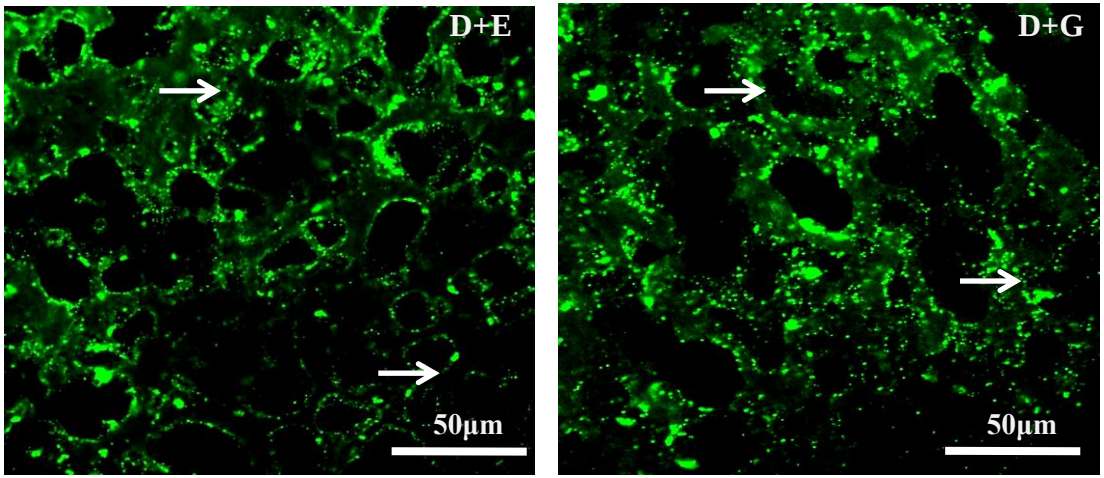


Table-27

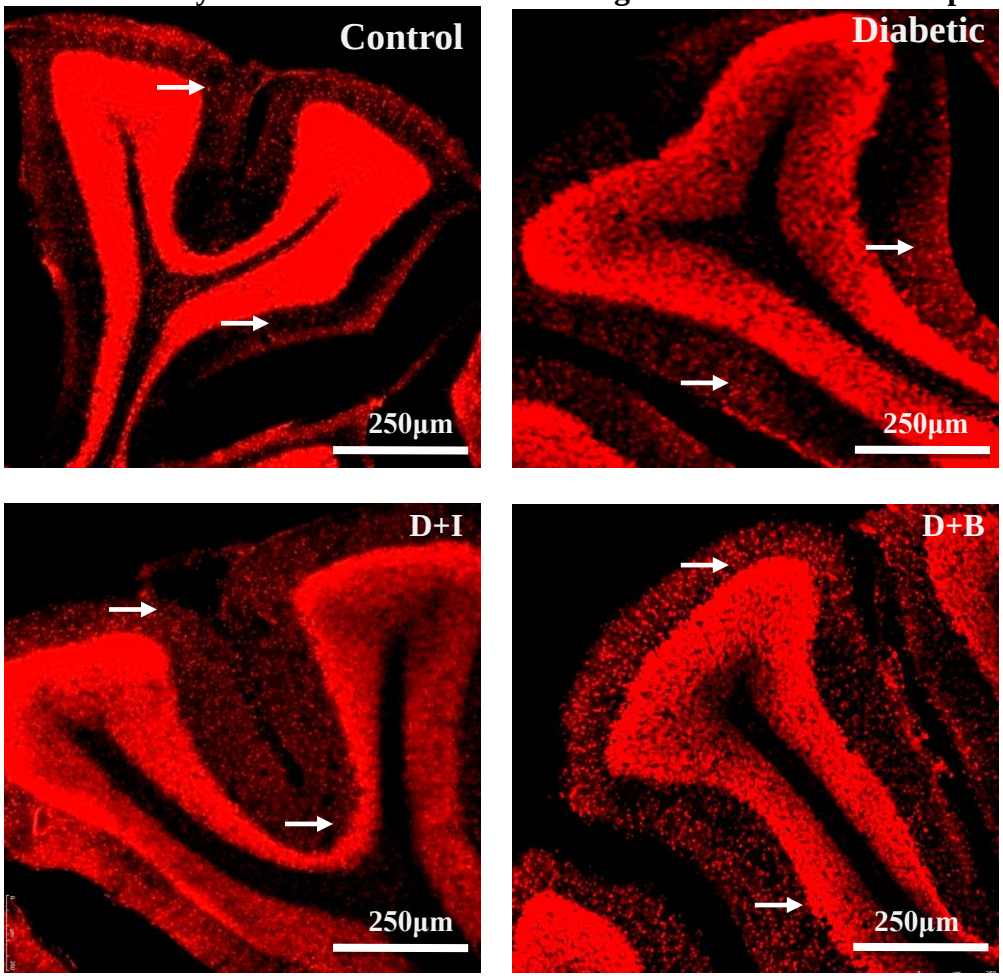
Confocal imaging of muscarinic M3 receptor in the cerebral cortex of experimental rats

Experimental groups	Mean Pixel Intensity
Control	31.87 ± 5.13
Diabetic	75.72 ± 3.21[a, g]
D + I	58.67 ± 4.82[a, d]
D + B	42.61 ± 2.97[a, e, h]
D + E	45.32 ± 5.38[a, d, h]
D +G	46.70 ± 4.59[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. Using immunofluorescent muscarinic M3 receptor specific primary antibody and cy5® as secondary antibody. (⟹) in white shows muscarinic M3 receptors. [a]P<0.001,B[b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 50µm.

Figure-48

Histochemistry of TO-PRO®-3 iodide staining in the cerebellum of experimental Rats



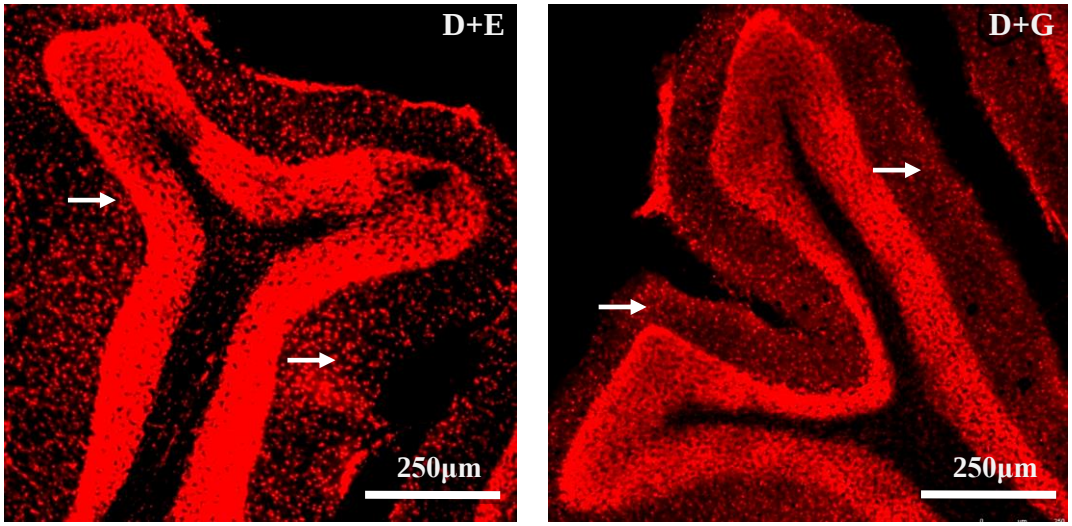


Table-48

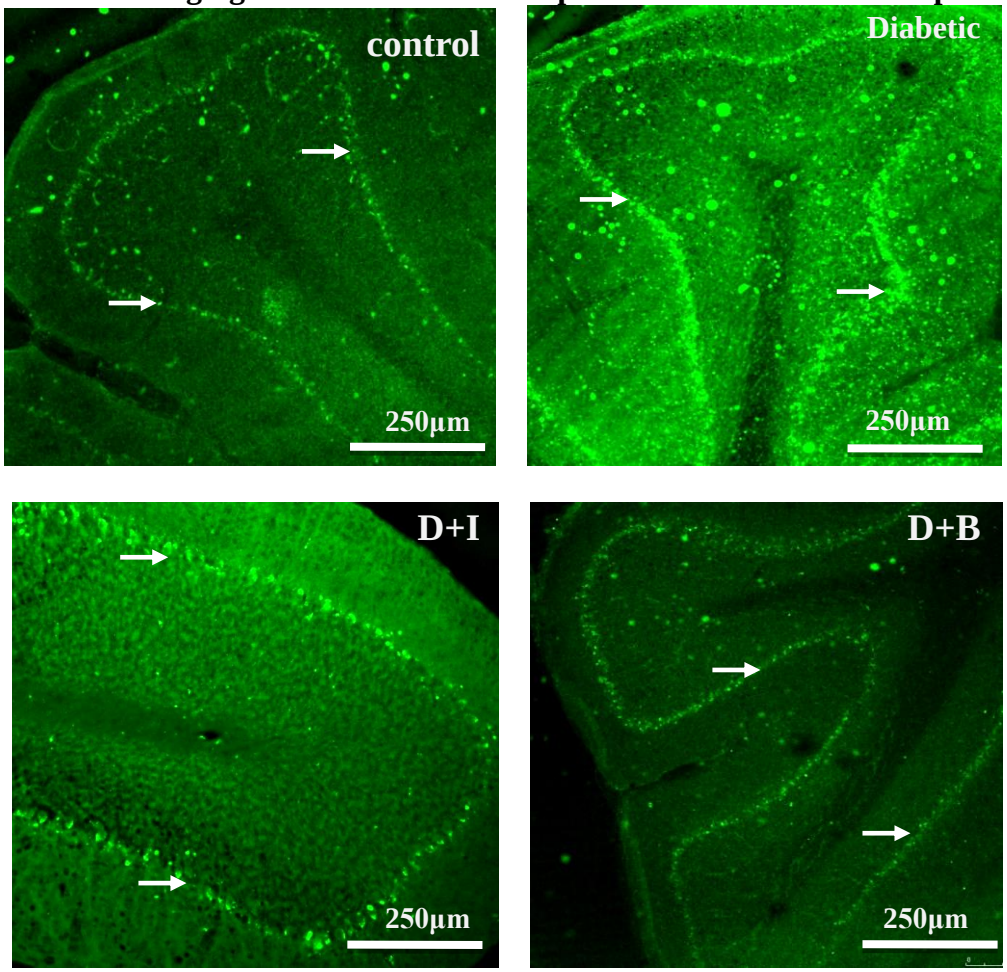
Histochemistry of TO-PRO®-3 iodide staining in the cerebellum of experimental Rats

Experimental groups	Mean Pixel Intensity
Control	81.16 ± 5.02
Diabetic	39.05 ± 2.13[a, g]
D + I	56.53 ± 3.74[a, d]
D + B	71.45 ± 4.63[b, d, g]
D + E	76.35 ± 3.96[a, d, g]
D +G	69.08 ± 3.41[a, d, h]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. (⟹) in white shows nuclear density. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 250 µm.

Figure-49

Confocal imaging of muscarinic M1 receptor in the cerebellum of experimental rats



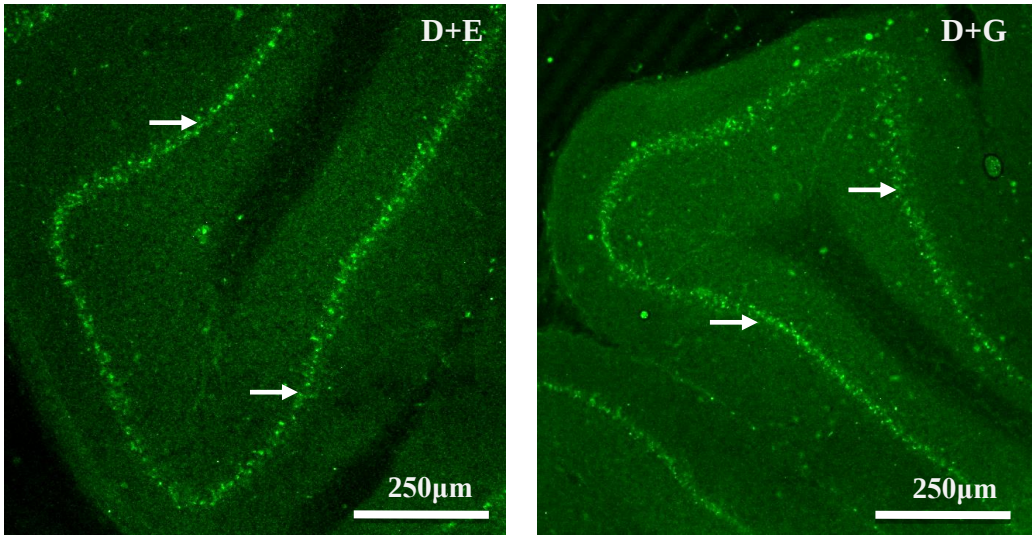


Table-49

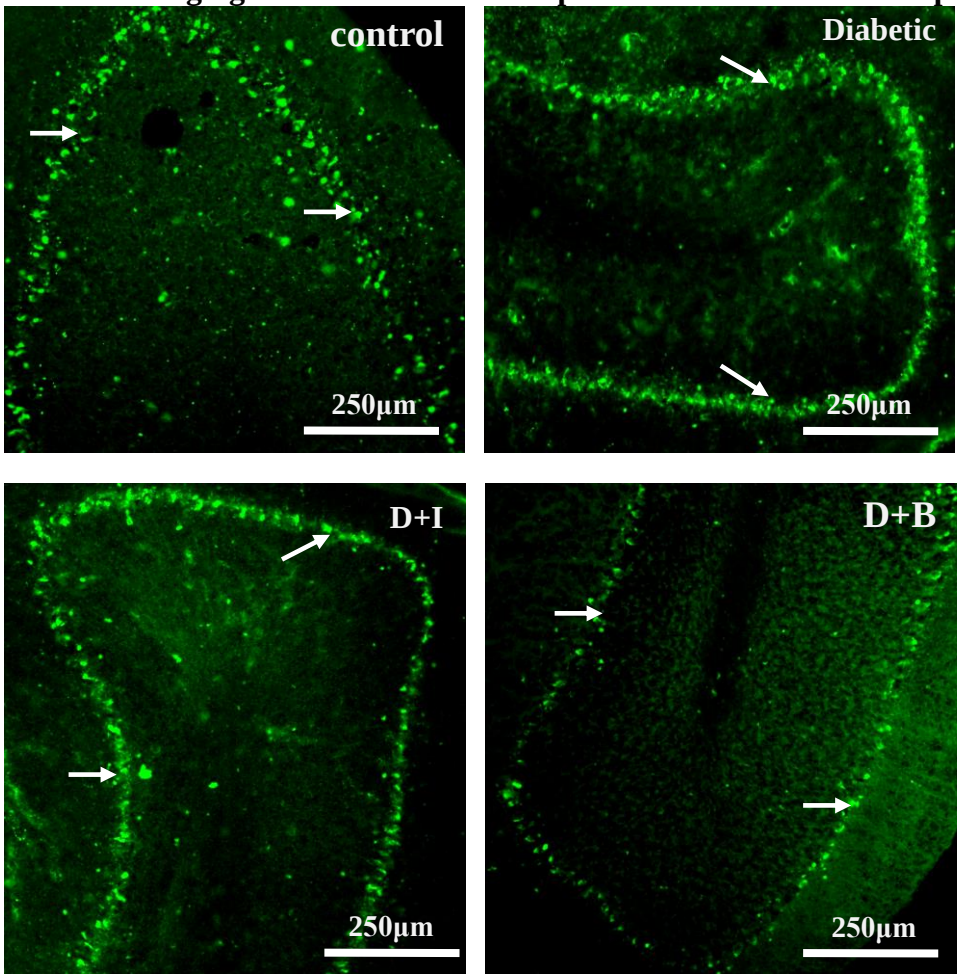
Confocal imaging of muscarinic M1 receptor in the cerebellum of experimental rats

Experimental groups	Mean Pixel Intensity
Control	27.88 ± 2.83
Diabetic	72.59± 4.20[a, g]
D + I	37.43 ± 3.07[a, d]
D + B	54.94 ± 4.71[a, d, h]
D + E	52.42 ± 3.85[a, d, h]
D +G	59.88 ± 3.26[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. Using immunofluorescent muscarinic M1 receptor specific primary antibody and cy5® as secondary antibody. (⟹) in white shows muscarinic M3 receptors. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 250µm.

Figure-50

Confocal imaging of muscarinic M3 receptor in the cerebellum of experimental rats



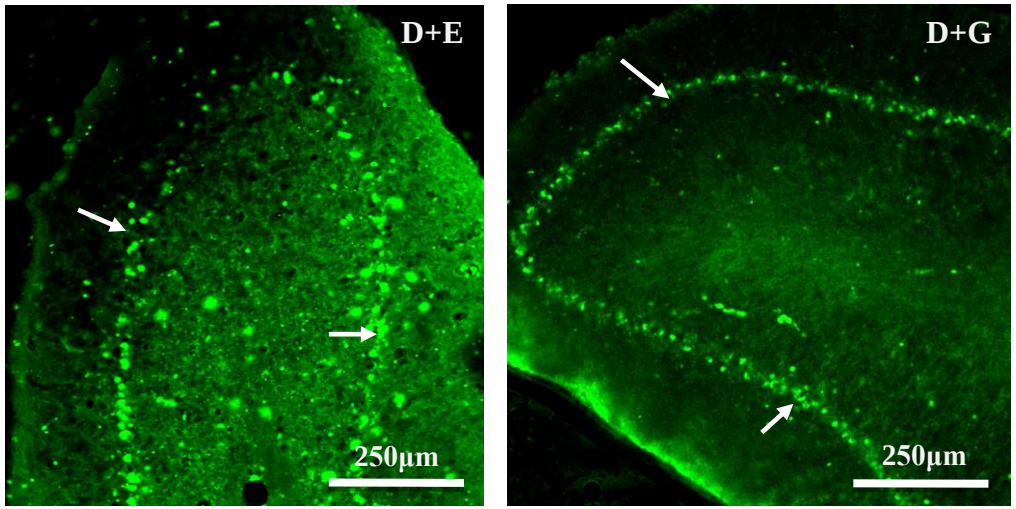


Table-50

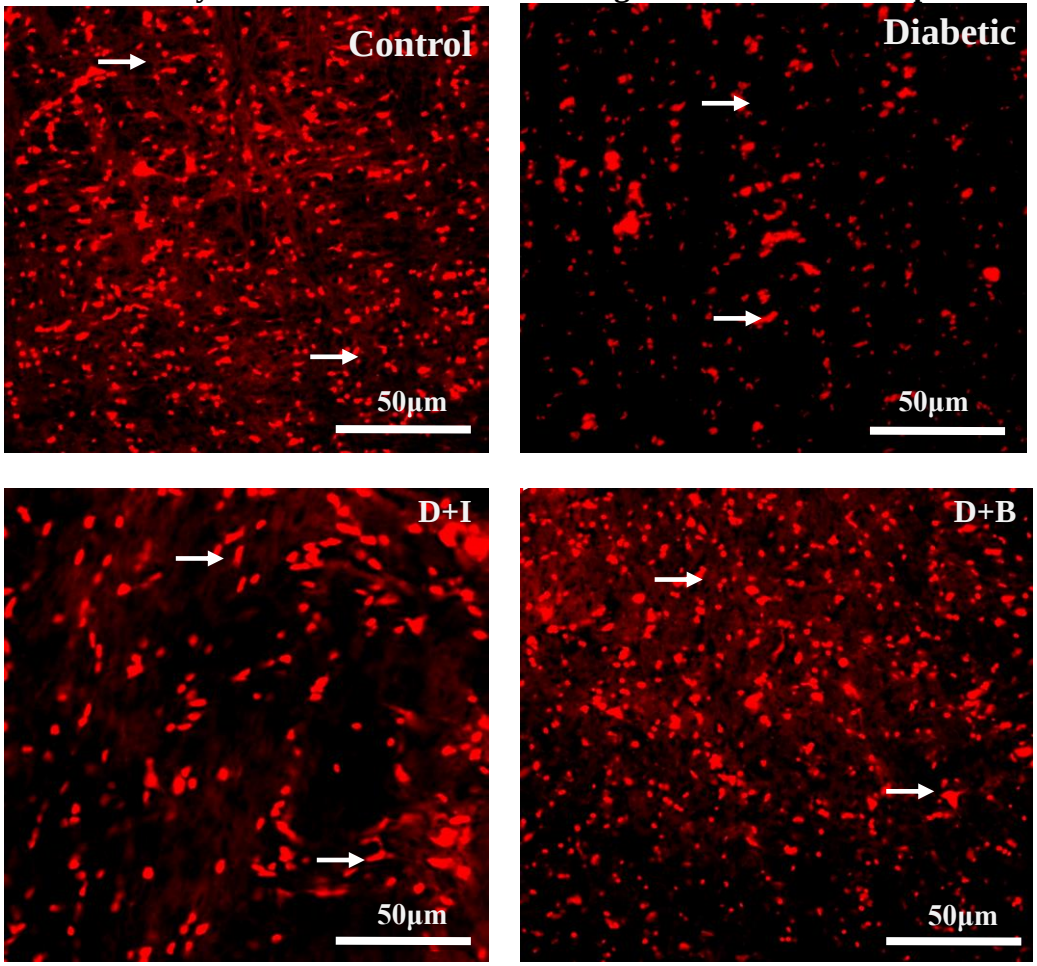
Confocal imaging of muscarinic M3 receptor in the cerebellum of experimental rats

Experimental groups	Mean Pixel Intensity
Control	33.17 ± 2.58
Diabetic	87.35± 5.13[a, g]
D + I	39.11 ± 3.07[a, d]
D + B	57.30 ± 3.38[a, e, h]
D + E	67.92 ± 3.57[a, d, g]
D +G	75.41 ± 3.79[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. Using immunofluorescent muscarinic M3 receptor specific primary antibody and cy5® as secondary antibody. (⟹) in white shows muscarinic M3 receptors. [a]P<0.001,B[b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 250 µm.

Figure-71

Histochemistry of TO-PRO®-3 iodide staining in the brain Stem experimental rats



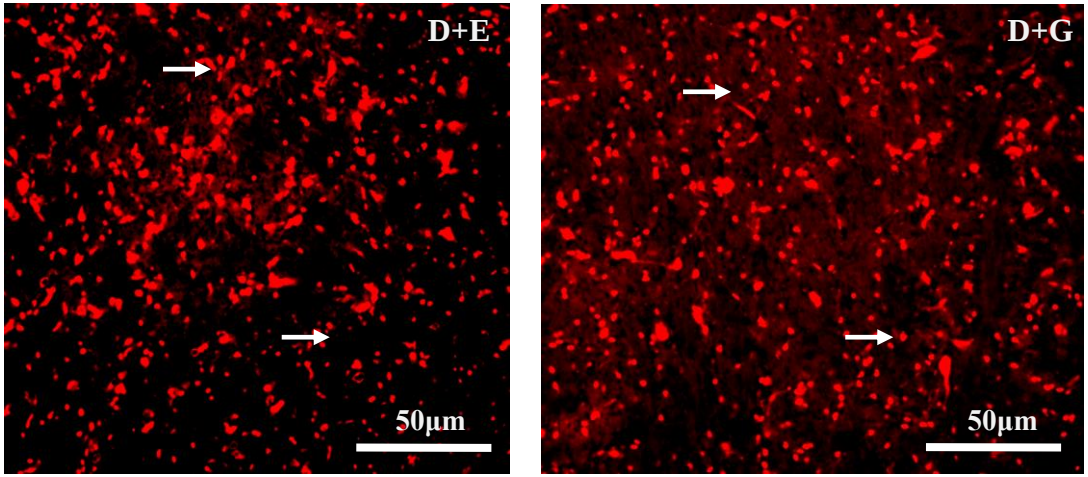


Table-71

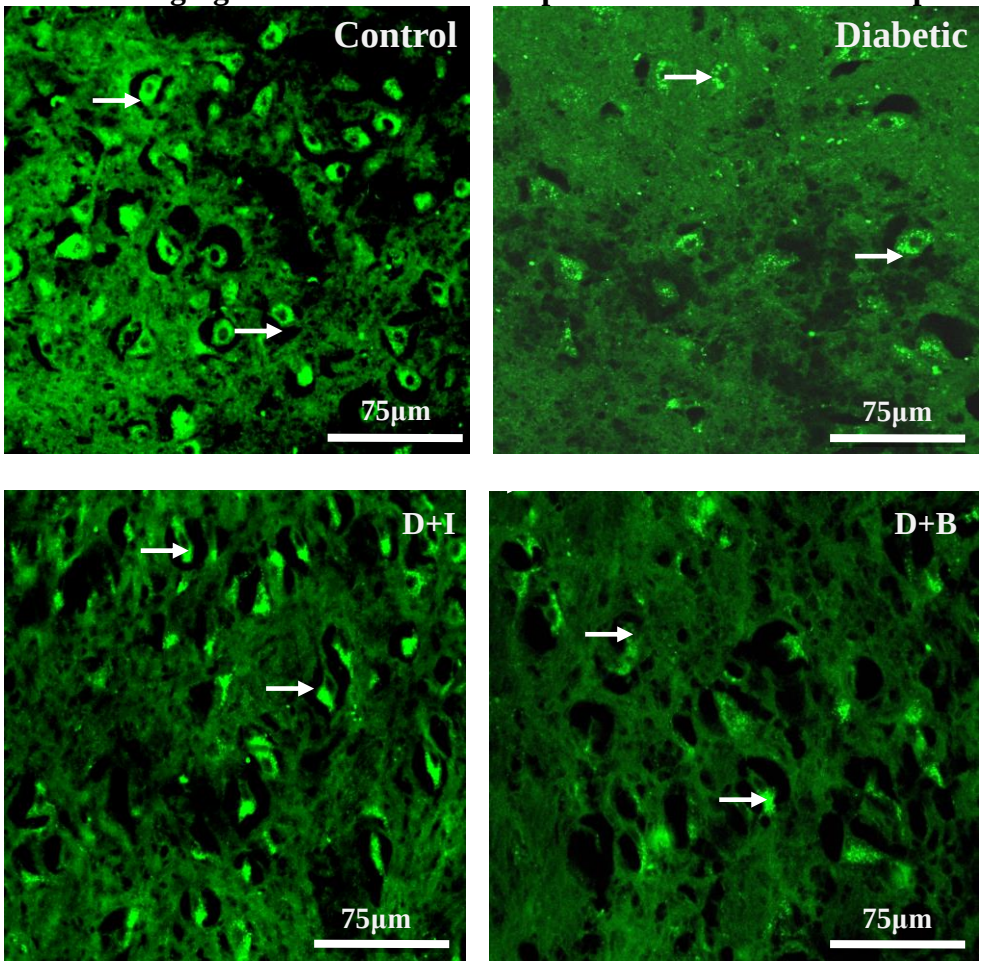
Histochemistry of TO-PRO®-3 iodide staining in the brain Stem experimental rats

Experimental groups	Mean Pixel Intensity
Control	82.14 ± 5.30
Diabetic	32.56 ± 1.97[a, g]
D + I	51.75 ± 3.68[a, d]
D + B	49.17 ± 4.05[a, d, g]
D + E	44.62 ± 4.39[a, d, h]
D +G	61.38 ± 3.92[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. (⟹) in white shows nuclear density. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 50 µm.

Figure-72

Confocal imaging of muscarinic M1 receptor in the brain stem of experimental rats



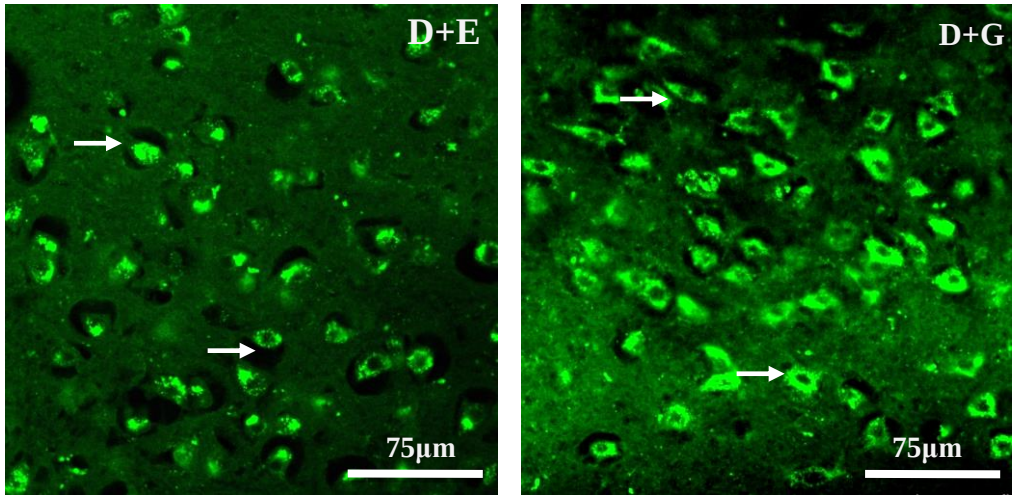


Table-72

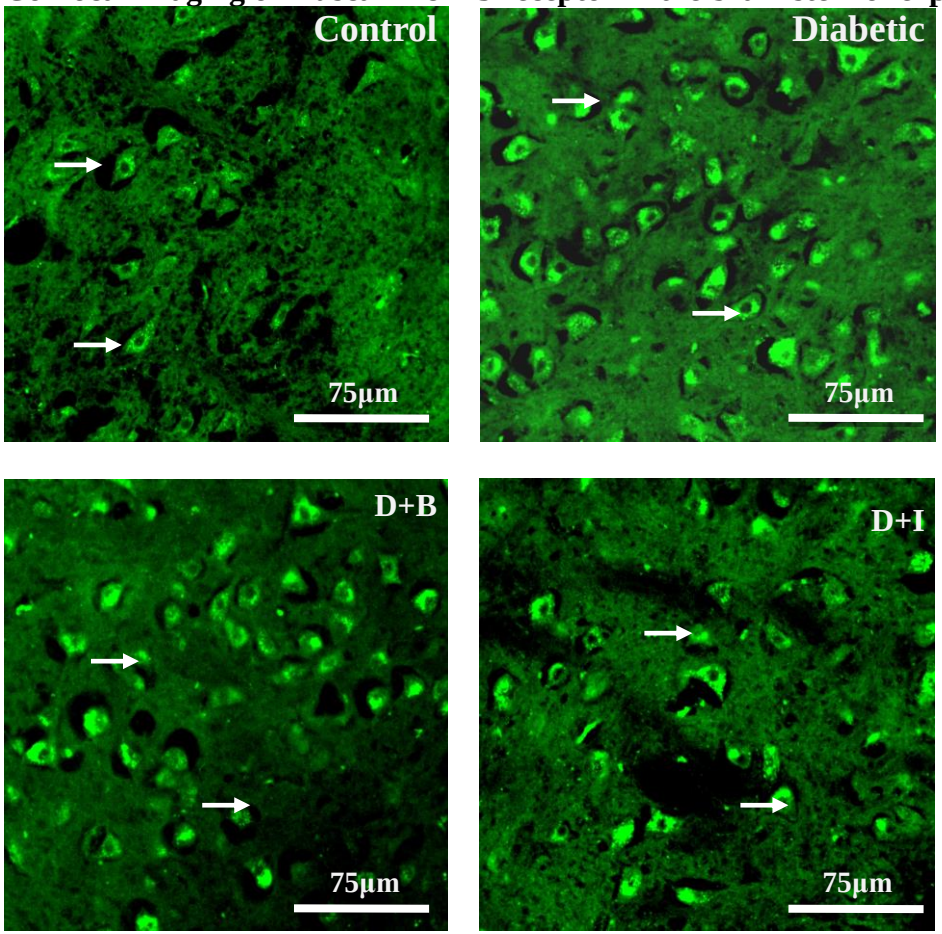
Confocal imaging of muscarinic M1 receptor in the brain stem of experimental rats

Experimental groups	Mean Pixel Intensity
Control	71.18 ± 4.69
Diabetic	29.48 ± 2.57[a, g]
D + I	53.80.75 ± 3.48[a, d]
D + B	35.19 ± 3.81[a, d, g]
D + E	47.32 ± 3.95[a, d, g]
D +G	65.30± 4.21[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats.Using immunofluorescent muscarinic M1 receptor specific primary antibody and cy5® as secondary antibody. (⟹) in white shows muscarinic M1 receptors. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 75µm.

Figure-73

Confocal imaging of muscarinic M3 receptor in the brain stem of experimental rats



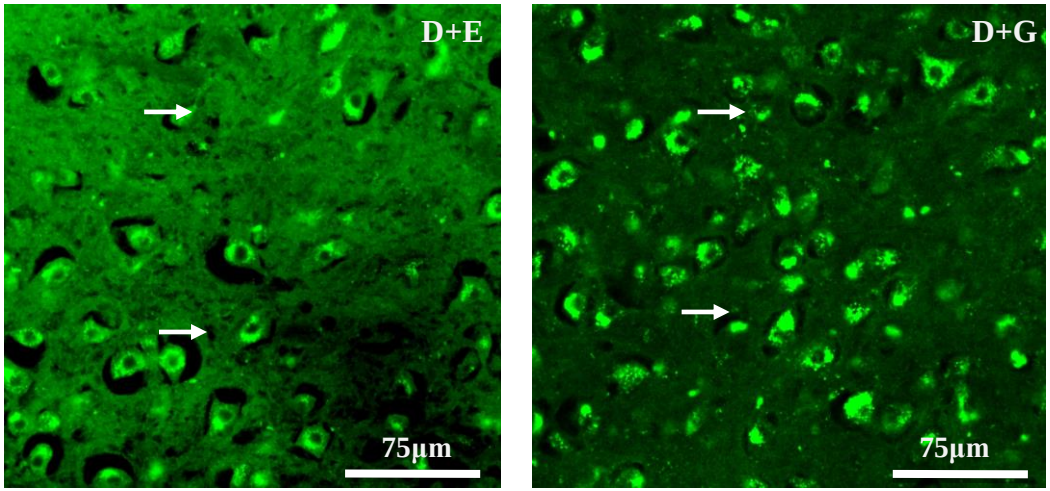


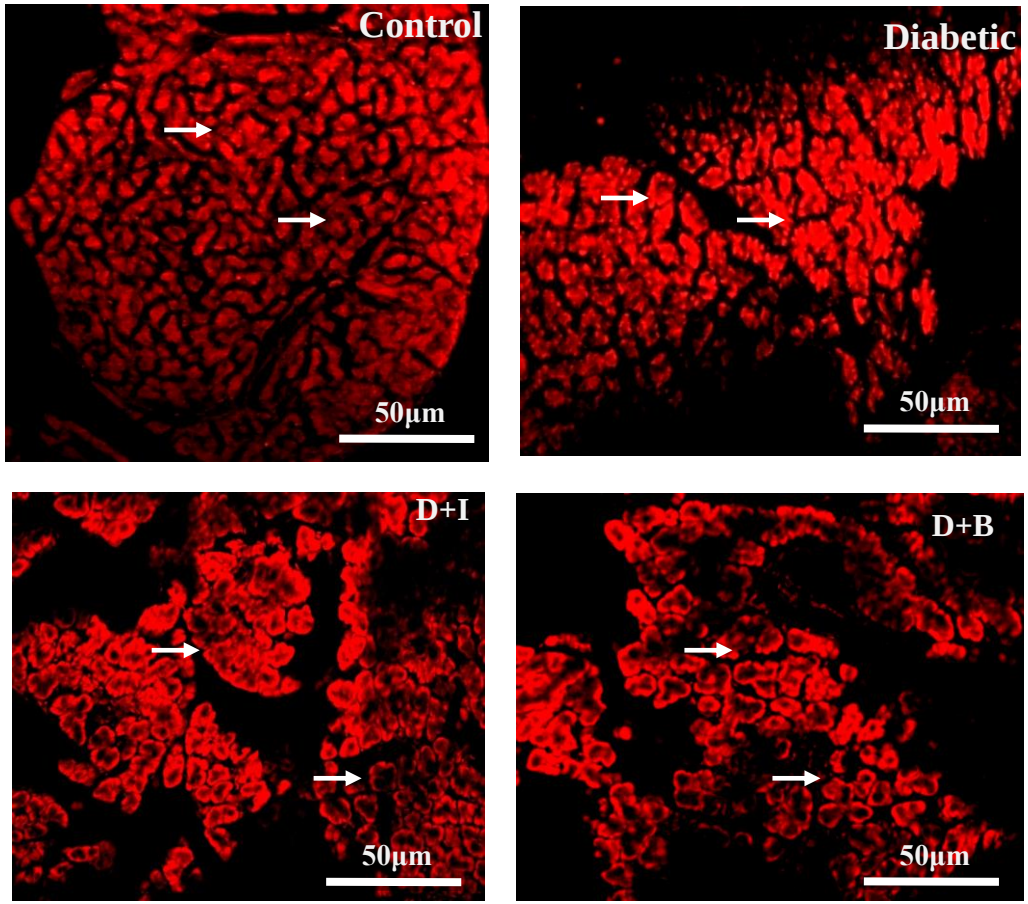
Table-73

Confocal imaging of muscarinic M3 receptor in the brain stem of experimental rat

Experimental groups	Mean Pixel Intensity
Control	31.80 ± 1.30
Diabetic	68.51 ± 3.64[a, g]
D + I	40.75 ± 1.62[a, d]
D + B	46.18 ± 2.08[a, d, h]
D + E	51.35 ± 3.19[a, d, h]
D +G	62.56 ± 3.29[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. Using immunofluorescent muscarinic M3 receptor specific primary antibody and cy5® as secondary antibody. (⟹) in white shows muscarinic M3 receptors. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 75µm.

Figure-99
Histochemistry of TO-PRO®-3 iodide staining in the pancreas experimental rats



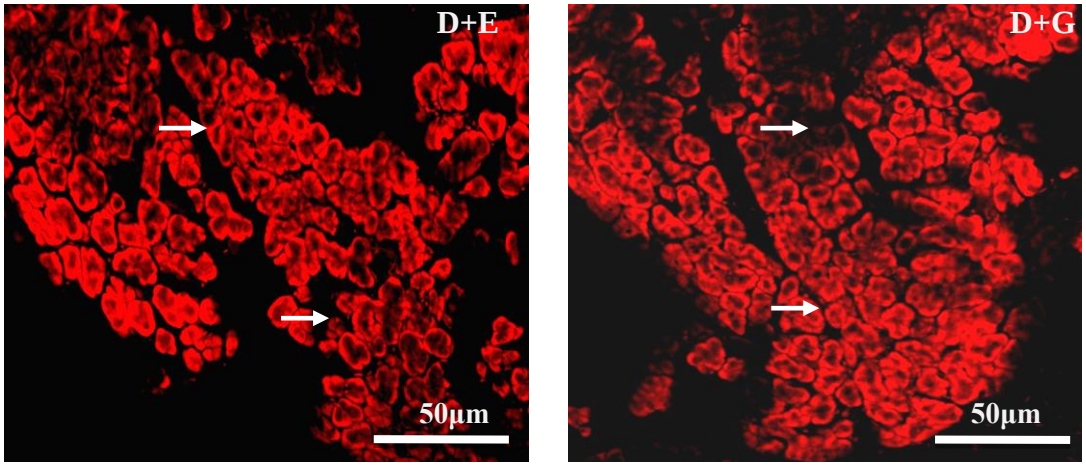


Table-99

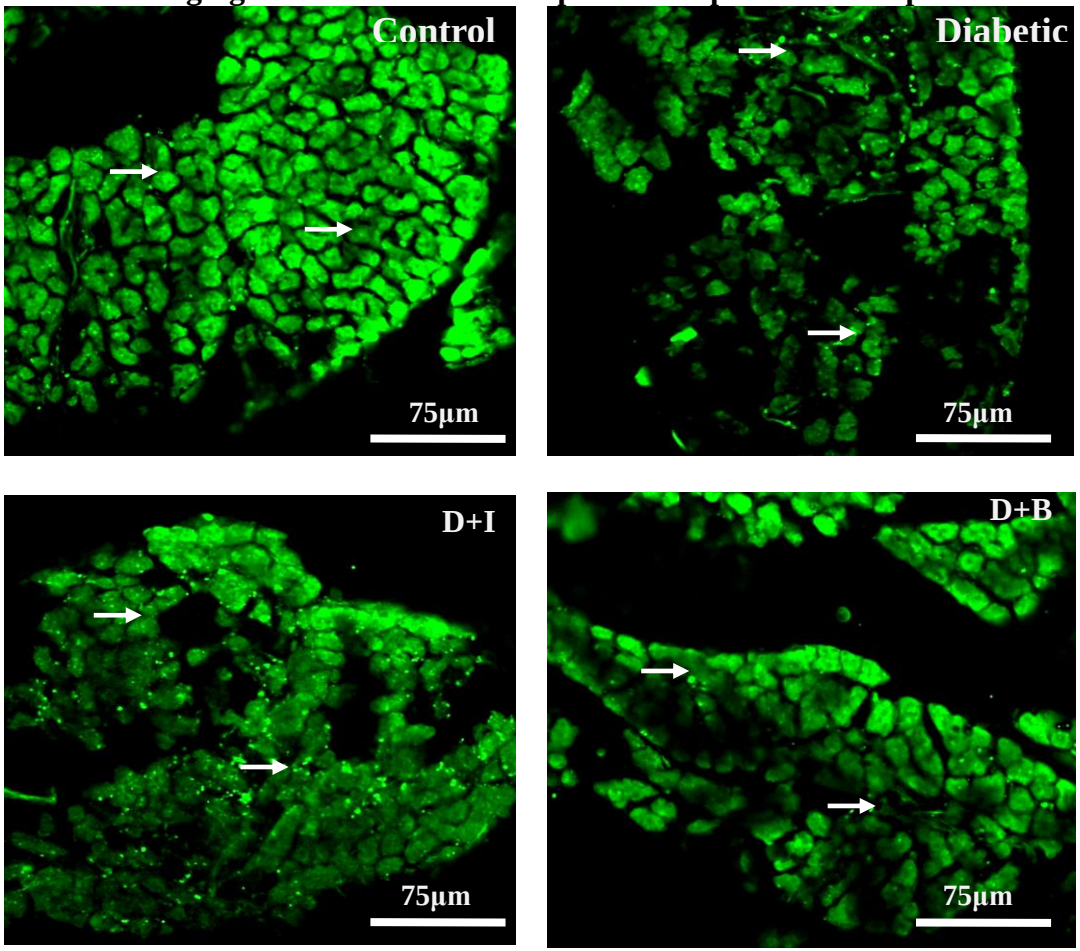
Histochemistry of TO-PRO®-3 iodide staining in the pancreas experimental rats

Experimental groups	Mean Pixel Intensity
Control	74.63 ± 4.89
Diabetic	42.61 ± 2.05[a, g]
D + I	64.58 ± 3.41[a, d]
D + B	58.67 ± 2.98[a, d, h]
D + E	54.94 ± 3.70[a, d, g]
D +G	67.13 ± 4.13[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats. (⟹) in white shows nuclear density. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 50 µm

Figure-100

Confocal imaging of muscarinic M1 receptor in the pancreas of experimental rats



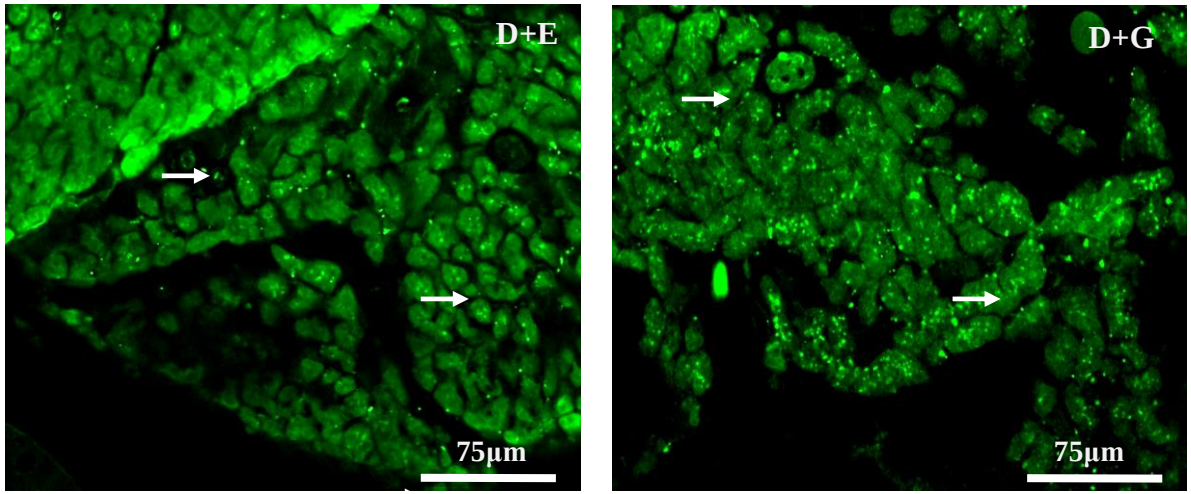


Table-100

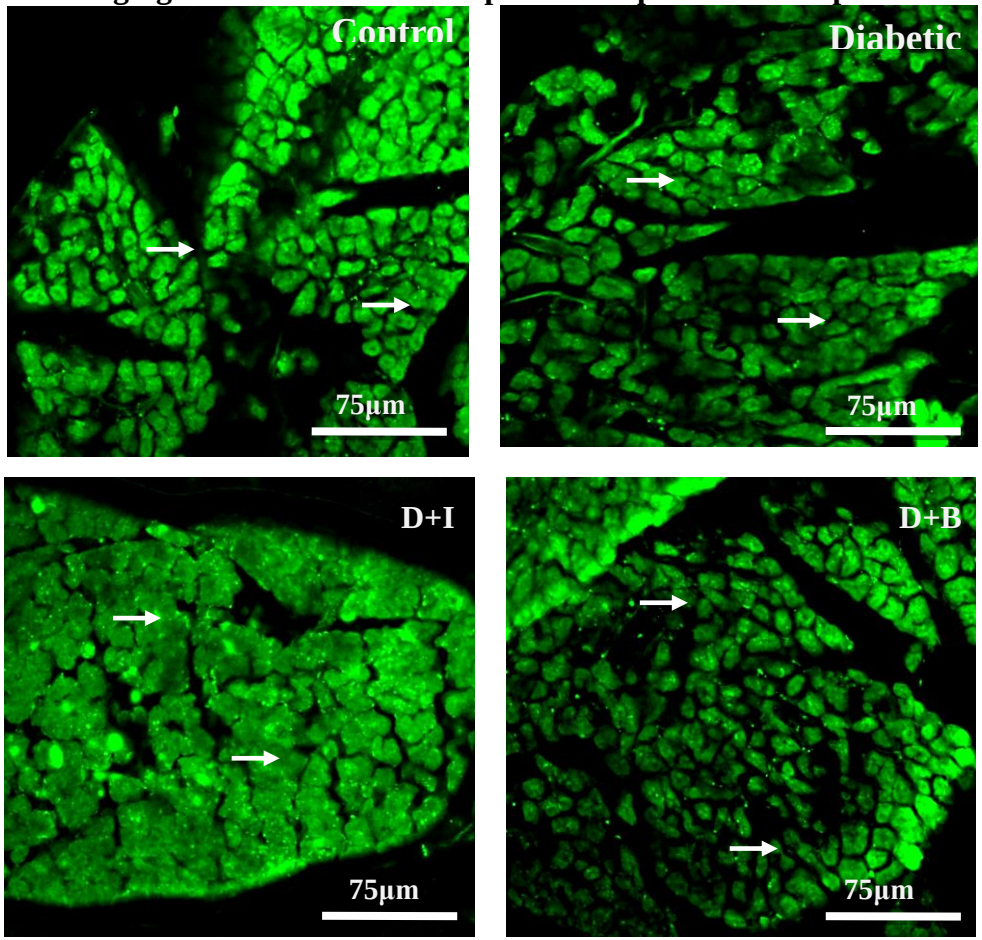
Confocal imaging of muscarinic M1 receptor in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	87.51 ± 5.42
Diabetic	27.12 ± 2.17[a, g]
D + I	51.79 ± 3.18[a, d]
D + B	59.30± 3.59[a, e, h]
D + E	75.11 ± 4.87[a, d, g]
D +G	91.53 ± 5.61[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats.Using immunofluorescent muscarinic M1 receptor specific primary antibody and cy5® as secondary antibody. (⟹) in white shows muscarinic M1 receptors. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar = 75µm.

Figure-101

Confocal imaging of muscarinic M3 receptor in the pancreas of experimental rats



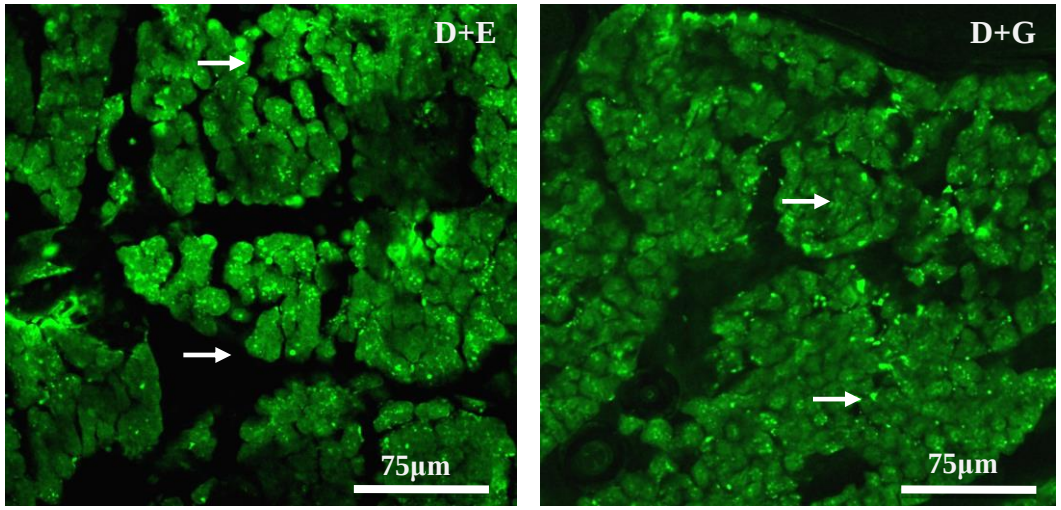


Table-101

Confocal imaging of muscarinic M3 receptor in the pancreas of experimental rats

Experimental groups	Mean Pixel Intensity
Control	76.51 ± 4.71
Diabetic	25.78 ± 2.47[a, g]
D + I	48.57 ± 3.09[a, d]
D + B	57.15± 3.28[a, d, h]
D + E	68.33 ±3.99[b, d, h]
D +G	79.62 ± 4.83[a, d, g]

Values are mean ± S.E.M of 4-6 separate experiments. Each group consist of 6-8 rats. Experimental groups: control, diabetic, D+I (insulin treated), D+B (Baclofen treated), D+E (Vitamin E treated) and D+G (Gymnemic acid treated) diabetic rats.Using immunofluorescent muscarinic M3 receptor specific primary antibody and cy5® as secondary antibody. (⟹)in white shows muscarinic M1 receptors. [a]P<0.001, [b]P<0.05, [c]P<0.01 when compared to Control. [d]p<0.001, [e]P<0.05, [f]P<0.01 when compared to Diabetic group. [g]P<0.001, [h]P<0.05, [i]P<0.01 when compared to D+I. Scale bar =75µm.