MUSCARINIC M1 RECEPTOR GENE EXPRESSION IN STREPTOZOTOCIN INDUCED DIABETIC RATS: REGULATION OF INSULIN SECRETION BY *Aegle marmelose* AND *Costus pictus* LEAF EXTRACTS

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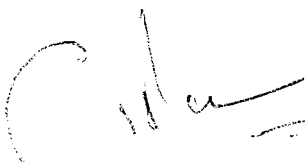Email: cspaulose@cusat.ac.in, paulosecs@yahoo.co.in Fax: 91-0484-2576267, 2577595

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CERTIFICATE

This is to certify that the thesis entitled "**MUSCARINIC M1 RECEPTOR GENE EXPRESSION IN STREPTOZOTOCIN INDUCED DIABETIC RATS: REGULATION OF INSULIN SECRETION BY** *Aegle marmelose* **AND** *Costus pictus* **LEAF EXTRACTS**" is a bonafide record of the research work carried out by Mr. Gireesh. G. under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

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DECLARATION

I do hereby declare that the thesis entitled "**MUSCARINIC M1 RECEPTOR GENE EXPRESSION IN STREPTOZOTOCIN INDUCED DIABETIC RATS: REGULATION OF INSULIN SECRETION BY** *Aegle marmelose* **AND** *Costus pictus* **LEAF EXTRACTS**" 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 Dr. C.S. Paulose, Director, Centre for Neuroscience, Reader & Head, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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ABBREVIATIONS USED IN THE TEXT

5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydroxy tryptamine
8-OH-DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin
ACh	Acetylcholine
AChE	Acetylcholine Esterase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
B_{max}	Maximal binding
BS	Brain stem
cAMP	cyclic Adenosine mono phosphate
CC	Cerebral cortex
CNS	Central nervous system
CSF	Cerbro spinal fluid
DA	Dopamine
DEPC	Di ethyl pyro carbonate
DNA	Deoxy ribonucleic acid
DTT	Dithiothreitol
ECD	Electro chemical detector
EGF	Epidermal growth factor
EPI	Epinephrine
GABA	Gamma aminobutyric acid
GH	Growth Hormone
GK	Glucokinase
GOD	Glucose oxidase
GRP	Gastrin Releasing Peptide
GTP	Guanosine triphosphate

HBSS	Hanks Balanced Salt Solution
HGF	Hepatocyte growth factor
HNF	Hepatocyte nuclear factor
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
HYPO	Hypothalamus
i.p	Intraperitoneally
IAPP	Islet amyloid polypeptide
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
IGF	Insulin like growth factor
IP_3	Inositol triphosphate
K_i	Inhibitory coefficient
K_m	Michaelis Constant
K_d	Dissociation constant
KRB	Krebs Ringer Bicarbonate
MAPK	Mitogen Activated Protein Kinase
MHC	Major histocmpatibilty complex
MIF	Macrophage migration inhibiting factor
MODY	Maturity onset diabetes in the young
mRNA	messenger Ribonucleic acid
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NE	Norepinephrine
NIDDM	Non-insulin dependent diabetis mellitus
NMN	Normetanephrine
p	Level of significance
PACAP	Pituitary adenylate cyclase activating polypeptide
PDGF	Platelet Derived Growth Factor

PDX-1	Pancreas duodenum homeobox gene-1
PEG	Polyethylene glycol
Pi	Inorganic phosphate
PKC	Protein kinase C
PLC	Placental lactogen
PMSF	Phenyl methyl sulfonyl fluoride
POD	Peroxidase
PRL	Prolactin
RIA	Radioimmuno assay
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SMOCC	Second messenger-operated calcium channels
SNc	Substantia nigra pars compacta
STZ	Streptozotocin
TGF	Transforming growth factor
TNF	Tumor necrosis fctor
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
VICC	Voltage – insensitive calcium channels
VIP	Vasoactive intestinal peptide
VMH	Ventro medial hypothalamus
V_{max}	Maximum Velocity
VOCC	Volatge sensitive calcium channels

CONTENTS

Introduction

The term diabetes mellitus describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO,1999). Even though insulin secretion is mainly regulated by changes in circulating concentrations of glucose and other metabolic fuels, stimuli such as neurotransmitters and gastrointestinal hormones make an important contribution to the overall regulation of pancreatic beta cell function. Acetylcholine (ACh), a major neurotransmitter from the autonomic nervous system, regulates the cholinergic stimulation of insulin secretion, through interactions with muscarinic receptors (Satin & Kinard, 1998; Ahren, 2000; Gilon & Henquin, 2001).

The autonomic nervous system plays a prominent role in the regulation of insulin secretion. It has been proposed that neuronal afferent signals delivered to the pancreatic β-cell through the vagus are responsible for the cephalic phase of insulin secretion. These effects are mediated by acetylcholine, which is released from nerve terminals and acts upon muscarinic cholinergic receptors in the β-cell plasma membrane (Sharp *et al.*, 1974; Berthoud *et al.*, 1980; Mathias *et al.*, 1985; Ahren, 2000). Cholinergic agonist carbachol increases insulin secretion from isolated rat islets (Zawalich & Zawalich, 2002). Carbachol stimulated insulin secretion is inhibited by atropine, a general muscarinic antagonist, confirming the role of muscarinic receptors in cholinergic induced insulin secretion. Reverse transcription analysis of rat pancreatic islets indicated that muscarinic M1 and M3 are predominant receptors in the islets (Lismaa *et al.*, 2000). Muscarinic M1 and M3 receptors function differentially regulate glucose induced insulin secretion (Renuka *et al.*, 2006). Increased activity of muscarinic M1 and M3 receptor subtypes stimulate insulin secretion and islet cell proliferation during the

regeneration of pancreas (Renuka *et al.*, 2005) The muscarinic receptor stimulation by acetylcholine leads to activation of phospholipase C, which, in turn, hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to produce Inositol triphosphate (IP3) and diacylglycerol (DAG) (Best & Malaisse, 1983; Zawalich *et al.*, 1989). In pancreatic β-cells, IP3 mobilizes Ca^{2-} from intracellular stores, resulting in an elevation of the intracellular concentration of Ca^{2+} and allowing activation of Ca^{2+}/calmodulin. DAG on the other hand, activates PKC (Nishizuka, 1995; Renstrom *et al.*, 1996). PKC, like Ca^{2+}/calmodulin, accelerates exocytosis of insulin granules (Nakano *et al.*, 2002).

Approaches to the control and prevention of hyperglycemia are central to the management of diabetes mellitus (Herman & Crofford, 1997). The development of new dietary adjuncts and novel antidiabetic agents, which reinstate a normal metabolic environment, thereby reducing the long term complications associated with diabetes is required. Such agents would both ideally stimulate the secretion and improve the action of insulin (Bailey & Flatt, 1995). Throughout the world, many traditional plant treatments for diabetes exist and therein lies a hidden wealth of potentially useful natural products for diabetes control (Bailey & Day, 1989; Gray & Flatt, 1997; Swanston-Flatt *et al.*, 1991). Despite this, few traditional antidiabetic plants have received scientific or medical scrutiny, and the World Health Organization (1980) recommended that this area warrants further attention. The plants provide a potential source of hypoglycemic drugs because many plants and plant derived compounds have been used in the treatment of diabetes. Several medicinal plants have found potential use as hypoglycemic in the Indian system of medicines, including ayurveda. Many Indian plants have been investigated for their beneficial use in different types of diabetes and reports occur in numerous scientific journals (Mukherjee *et al.*, 2006).

Aegle marmelose Corr. (Rutaceae) commonly called as 'Koovalam' in Malayalam and 'Bael' in Hindi is indigenous to India. Preliminary reports indicate *Aegle marmelose* leaf extract exhibits antidiabetic action in glucose-induced hyperglycemic rats (Sachdewa *et al.*, 2001) and in alloxan induced diabetic rats (Ponnachan *et al.*, 1993).

Costus pictus, D Don (Costaceae) is a plant that attained popularity in Kerala recently by its so called anti-diabetic effects. There are no available reports on the pharmacological actions of *Costus pictus* leaf extract.

The present work is to understand the alterations of total Muscarinic and Muscarinic M1 receptors in brain and pancreatic islets of Streptozotocin induced diabetic rats. The work focuses on the evaluation of the antihyperglycemic activity of aqueous extracts of *Aegle marmelose* and *Costus pictus* leaves *in vivo* and the changes in the total Muscarinic and Muscarinic M1 receptors during diabetes and after the treatment with insulin. The insulin secretory activity of *Aegle marmelose* and *Costus pictus* leaf extracts and the effect of cholinergic receptor agonist were investigated *in vitro* using rat primary pancreatic islet culture. Muscarinic M1 receptor kinetics and gene expression during diabetes and regulation of insulin secretion by *Aegle marmelose* and *Costus pictus* leaf extracts will help us to elucidate the role of Muscarinic and Muscarinic M1 receptors in hyperglycemia and the regulatory activity of these plant extracts on insulin secretion through Muscarinic receptors.

OBJECTIVES OF THE PRESENT STUDY ARE:

1. To study the antihyperglycemic activity of aqueous extract *of Aegle marmelose* and *Costus pictus* leaves in streptozotocin (STZ) diabetic animal model.

2. To measure the circulating insulin level in the control, diabetic, insulin treated, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves treated diabetic rats.

3. To study the cholinergic activity using acetylcholine esterase assay in the brain regions - cerebral cortex (CC), brain stem (BS), corpus striatum (CS) and hypothalamus (Hypo) during diabetes, insulin treated, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves treated diabetic rats.

4. To study the total muscarinic, M1 receptor binding parameters in CC, BS, CS and Hypo during diabetes, insulin treated, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves treated diabetic rats.

5. To study the muscarinic M1 receptor binding parameters in the pancreatic islets of experimental rats.

6. To study the expression of muscarinic M1 mRNA in the brain regions during diabetes, insulin treated, aqueous extract of *Aegle marmelose* and Costus *pictus* leaves treated diabetic rats using RT PCR and Real Time PCR.

7. To study the effect of aqueous extract of *Aegle marmelose* and *Costus pictus* leaves on insulin secretion using rat primary islet culture in vitro.

8. To study the effect of cholinergic receptor agonist carbachol, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves on insulin secretion using rat primary islet culture in vitro.

9. To measure the ^{14}C glucose uptake activity of the liver and cerebral cortex of control, diabetic, insulin, *Aegle marmelose* and *Costus pictus* leaves extract treated diabetic rats.

10. To perform neurophysiologic analysis of the electrical activity of the brain using electroencephalogram of experimental rats

Literature Review

Diabetes mellitus is a major global health problem that affects more than 185 million people around the world (Zimmet *et al.*, 2001; Zimmet, 1999; Amos *et al.*, 1997). The disease is an increasingly prevalent metabolic disorder in humans and is characterised by hyperglycemia (Dunne *et al.*, 2004; Kumar *et al.*, 2002). The number of diabetic patients is expected to reach 300 million by the year 2025. The projected increase in the number of diabetic patients will strain the capabilities of healthcare providers the world over (Adeghate *et al.*, 2006). The pancreatic hormones have an important role in the regulation of glucose metabolism. The secretion of insulin by ß-cells of the endocrine pancreas is regulated by glucose and other circulating nutrients. It is also modulated by several hormones and neurotransmitters, among which acetylcholine plays a prominent role.

β-Cell function: physiology and pathophysiology

Islets of Langerhans are microscopic organelles scattered diffusely throughout the pancreas. Each islet contains approximately 2000 cells, which include four types: α, β, δ, and PP cells. The major secretory products of these cells are glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. The α-cell secretes glucagon primarily in response to hypoglycemia, but also to amino acids. The β-cell secretes insulin in response to elevated glucose levels and also responds to other substances such as glucagon and acetylcholine. Insulin responses to intravenous glucose are time-dependent and referred to as first- and second-phase responses. The δ-cell releases somatostatin in response to glucose. The PP cell releases pancreatic polypeptide in response to hypoglycemia and secretin. The functions of these hormones are distinctly different. Glucagon stimulates glycogenolysis in the liver to increase blood glucose

levels. Insulin decreases hepatic glucose production and increases glucose entry into muscle and fat cells. Somatostatin inhibits the secretion of many hormones, including insulin and glucagon, and likely is an intra islet paracrine regulator of α and β cells. The function of pancreatic polypeptide in humans remains unclear (Robertson & Harmon, 2006).

The endocrine pancreas is richly innervated, but the abundance and organization of these innervations are highly variable between species (Kobayashi & Fujita, 1969). Most of the nerve fibers enter the pancreas along the arteries (Miller, 1981; Woods & Porte Jr, 1974). Unmyelinated nerve fibers are found in the neighborhood of all islet cell types at the periphery and within the islet. At some distance from the islets, glial Schwann cells often form a thin sheet around nerve fibers on their travel toward and within the islet. In the vicinity of islet cells, however, it is not rare to see some nerve fibers lacking this glial protection and coming close to or ending blindly 20–30 nm from the endocrine cells (Bock, 1986; Radke & Stach (a), 1986; Radke & Stach (b), 1986; Fujita & Kobayashi, 1979; Shorr & Bloom, 1970; Kobayashi & Fujita, 1969; Watari, 1968; Legg, 1967).

The autonomic innervation of the endocrine pancreas has several origins. Classically, the autonomic nervous system uses two interconnected neurons to control effector functions and is divided into two systems, the sympathetic and the parasympathetic nervous systems, according to the location of the preganglionic cell bodies. However, there are indications suggesting that these two systems are not always independent of each other, but display anatomical interactions (Berthoud & Powley, 1993) or share similar neurotransmitters (Verchere *et al.*, 1996; Liu *et al.*, 1998; Sheikh *et al.*, 1988).

The parasympathetic innervation

The preganglionic fibers of the parasympathetic limb originate from perikarya located in the dorsal motor nucleus of the vagus (Chen *et al.*, 1996; Berthoud & Powley, 1991; Berthoud *et al.*, 1990; Rinaman & Miselis , 1987; Ahrén *et al.*,1986; Louis-Sylvestre, 1987; Luiten *et al.*, 1984; Ionescu *et al.*, 1983) and possibly also in the nucleus ambiguus (Luiten *et al.*,1986; Luiten *et al.*, 1984; Sharkey *et al.*, 1984; Sharkey & Williams, 1983; Weaver, 1980) which are both under the control of the hypothalamus. They are organized in well separated branches traveling within the vagus nerves (cranial nerve X), and through the hepatic, gastric (Berthoud & Powley, 1991; Berthoud *et al.*, 1990) and possibly celiac branches of the vagus (Kinami *et al.*, 1997). They reach intrapancreatic ganglia that are dispersed in the exocrine tissue. These ganglia send unmyelinated postganglionic fibers toward the islets (Berthoud & Powley, 1990; Berthoud *et al.*, 1981; Woods & Porte Jr, 1974;). Preganglionic vagal fibers release ACh that binds to nicotinic receptors on intraganglionic neurons. Postganglionic vagal fibers release several neurotransmitters: ACh, Vasoactive Intestinal Peptide (VIP), gastrin-releasing peptide (GRP), nitric oxide (NO), and pituitary adenylate cyclase-activating polypeptide (PACAP) (Ahrén , 2000; Myojin *et al.*, 2000; Love & Szebeni , 1999; Wang *et al.*, 1999; Ahrén *et al.*, 1999; Havel *et al.*, 1997; Sha *et al.*, 1995; Ekblad *et al.*, 1994; Knuhtsen *et al.*, 1987; Ahrén *et al.*, 1986; Knuhtsen *et al.*, 1985; Bloom *et al.*,1983; Bloom& Edwards, 1981). Cholinergic terminals are found in the neighborhood of all islet cell types at the periphery and within the islet (Love & Szebeni, 1999; Van der Zee *et al.*, 1992; Radke & Stach, 1986; Stach & Radke, 1982; Esterhuizen *et al.*, 1968; Coupland, 1958). The importance of the cholinergic innervation of the endocrine pancreas is attested by the presence of a 10-fold higher activity of choline acetyltransferase and acetylcholinesterase (the enzymes involved in the synthesis and the degradation of

ACh respectively) in the islets than in the surrounding exocrine tissue (Godfrey & Matschinsky, 1975). Cholinergic synapses with endocrine cells have been observed in some species (Golding & Pow, 1990; Voss *et al.*, 1978).

Understanding the organization of the pancreatic innervation permits correct interpretation of some experiments using different cholinergic antagonists. The stimulation of insulin release occurring upon electrical stimulation of vagal nerves in the dog is abolished by both nicotinic and muscarinic antagonists (Ahrén & Taborsky Jr, 1986). In the perfused rat pancreas, nicotine produces an increase of insulin secretion that is blocked by atropine (Miller, 1981). These observations can be explained by the presence of nicotinic receptors on pancreatic ganglia and nerves (Karlsson & Ahrén, 1998; Kirchgessner & Liu, 1998; Stagner & Samols, 1986) and muscarinic receptors on ß-cells.

The overall effect of a parasympathetic stimulation is an increase of insulin secretion because postganglionic fibers contain various neurotransmitters in addition to the classic neurotransmitter ACh. It is important to keep in mind that parasympathetic neurotransmission is the sum of various biological effects. VIP and PACAP stimulate insulin secretion by increasing cAMP levels (Ahrén, 2000). They act on the same family of receptors (Jian *et al.*, 1999) and exert their action by two mechanisms, directly by stimulating ß-cells through the PLC-PKC pathway (Ahrén, 2000), and indirectly by activating intrapancreatic postganglionic nerves that stimulate insulin secretion (Karlsson & Ahrén, 1998).

The sympathetic innervation

The sympathetic innervation of the pancreas originates from preganglionic perikarya located in the thoracic and upper lumbar segments of the spinal cord (Furuzawa *et al.*, 1996). The myelinated axons of these cells traverse the ventral roots

to form the white communicating rami of the thoracic and lumbar nerves that reach the paravertebral sympathetic chain (Chusid, 1979). Preganglionic fibers communicate with a nest of ganglion cells within the paravertebral sympathetic chain or pass through the sympathetic chain, travel through the splanchnic nerves, and reach the celiac (Ahrén, 2000; Furuzawa *et al.*, 1996; Brunicardi *et al.*, 1995; Fox & Powley, 1986; Sharkey & Williams, 1983) and mesenteric ganglia (Furuzawa *et al.*, 1996). Ganglia within the paravertebral sympathetic chain, and the celiac and mesenteric ganglia, give off postganglionic fibers that eventually reach the pancreas. The existence of intrapancreatic sympathetic ganglia has also been reported (Liu *et al.*, 1998; Luiten *et al.*, 1986; Luiten *et al.*, 1984). The preganglionic fibers release ACh that acts on nicotinic receptors on intraganglionic neurons, whereas the postganglionic fibers release several neurotransmitters: norepinephrine, galanin, (Ahrén, 2000; Myojin *et al.*, 2000; Dunning *et al.*, 1988; Ahrén & Taborsky, 1986). A rich supply of adrenergic nerves in close proximity of the islet cells has been observed in several mammalian species (Radke & Stach, 1986; Stach & Radke, 1982; Ahrén *et al.*, 1981; Esterhuizen *et al.*, 1968).

Brain neurotransmitter changes during diabetes

Neurotransmitters have been reported to show significant alterations during hyperglycemia resulting in altered functions causing neuronal degeneration. A significant increase in the catecholamine contents and activity of metabolising enzymes has been reported in experimental diabetes (Gupta *et al.*, 1992). Norepinephrine has been reported to increase in several brain regions during diabetes (Tassava *et al.*, 1992; Chen & Yang, 1991; Wesselmann *et al.*, 1988; Chu *et al.*, 1986; Fushimi *et al.*, 1984; Oreland & Shasken, 1983), but a significant decrease in NE has been reported in hypothalamus (Ohtani *et al.*, 1997), pons and medulla (Ramakrishna

& Namasivayam, 1995). EPI levels were significantly increased in the striatum, hippocampus and hypothalamus of diabetic rats and these changes were reversed to normal by insulin treatment (Ramakrishna & Namasivayam, 1995). Streptozotocin-induced diabetes and acute insulin deficiency were demonstrated to result in increased content of EPI in the supra chiasmatic nucleus. In addition to this, a decreased turnover of dopamine in the ventromedial nucleus in diabetes was found to be reversed by insulin treatment (Oliver *et al.*, 1989). These data indicate that experimental diabetes and acute insulin deficiency result in the rapid onset of detectable alterations in EPI and DA activity in specific hypothalamic nuclei. This can lead to the development of secondary neuroendocrine abnormalities known to occur in the diabetic condition. The DA content was increased in whole brain, (Chen & Yang, 1991; Lackovic *et al.*, 1990) corpus striatum (Chu *et al.*, 1986), cerebral cortex and hypothalamus of diabetic rats (Ohtani *et al.*, 1997; Tassava *et al.*, 1992). The plasma DA content was decreased in diabetic rats (Eswar *et al.*, 2006). Serotonin (5-HT) content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991; Lackovic *et al.*, 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sandrini *et al.*, 1997; Sumiyoshi *et al.*, 1997). Brain tryptophan was also reduced during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993).

Acetylcholine

Cholinergic system plays an important role in physiological and behavioural functions. Acetylcholine acts by binding to specific membrane receptors and can be divided into muscarinic and nicotinic receptors. Cholinergic stimulation of pancreatic β-cells increases insulin secretion (Kaneto *et al.*, 1967). These are mediated by

muscarinic cholinergic, rather than nicotinic receptors (Stubbe & Steffens, 1993; Ahren *et al.*, 1990; Zawalich *et al.*, 1989; Henquin & Nenquin, 1988; Morgan *et al.*, 1985; Grill & Ostenson, 1983) and is dependent on extracellular glucose concentration (Henquin *et al.*, 1988). Acetylcholine stimulated insulin secretion coupling is mediated by complex mechanisms of signal transduction. It has been proposed that ACh activates phospholipid turnover and thereby increases the intracellular calcium level. Normal β-cells' voltage-dependent sodium channels are important for membrane depolarisation. ACh increases sodium influx into the cells (Henquin *et al.*, 1988). ACh hyperpolarises the cell by increasing potassium permeability. Quist (1982) reported that carbachol causes Ca^{2+}-dependent stimulation of phosphate incorporation into phosphatidyl inositol phosphates in the canine heart. Cholinergic stimulation of phosphatidyl inositol phosphates synthesis is blocked by muscarinic antagonist atropine (Brown & Brown, 1983).

Muscarinic receptors

Muscarinic receptors are a family of G protein-coupled receptors that have a primary role in central cholinergic neurotransmission. Specific agonists, which activate postsynaptic muscarinic receptors, stimulate cholinergic signaling (Valentin *et al.*, 2006). The muscarinic acetylcholine receptors are widely distributed throughout the body and subserve numerous vital functions in both the brain and autonomic nervous system (Hassal *et al.*, 1993). Activation of muscarinic receptors in the periphery causes decrease in heart rate, relaxation of blood vessels, constriction in the airways of the lung, increase in the secretions and motility of the various organs of the gastrointestinal tract, increase in the secretions of the lacrimal and sweat glands, and constriction in the iris sphincter and ciliary muscles of the eye (Wess, 1993). In the

brain, muscarinic receptors participate in many important functions such as learning, memory, and the control of posture.

Muscarinic receptors are members of a large family of plasma membrane receptors that transduce the intracellular signals via coupling to guanine nucleotide binding regulatory proteins (G proteins) (Hulme *et al.*, 1990; Bonner, 1989; Nathanson, 1987;). Molecular cloning studies have revealed the existence of five molecularly distinct mammalian muscarinic receptor proteins (Hulme *et al.*, 1990; Bonner, 1989).

All mammalian muscarinic receptor genes share one common feature with several other members of G-protein receptor gene family *i.e.*, their open reading frame contained within a single exon (Bonner *et al.*, 1987). Like all other G protein coupled receptors, the muscarinic receptors are predicted to conform to a generic protein fold consisting of seven hydrophobic transmembrane helices joined by alternating intracellular and extracellular amino-terminal domain, and a cytoplasmic carboxy-terminal domain. The five mammalian muscarinic receptors display a high degree of sequence identity sharing about 145 amino acids. Characteristically all muscarinic receptors contain a very large third cytoplasmic loop, which, except for the proximal portions, displays virtually no sequence identity among the different subtypes (Bonner, 1989). Agonist binding to muscarinic receptors is thought to trigger conformational changes within the helical bundle, which are then transmitted to the cytoplasmic face where the interaction with specific G proteins known to occur. Site directed mutagenesis and receptor-modeling studies suggest that a conserved Asp residue present in TM II of almost all G protein coupled receptors plays a pivotal role in mediating the conformational changes associated with receptor activation (Wess, 1993).

The ligand binding to muscarinic receptors is predicted to occur in a pocket formed by the ring like arrangement of the seven transmembrane domains (Wess *et al.*, 1991; Hulme *et al.*, 1990). Ligand binding appears to be initiated by ion-ion interaction between positively charged amino head present in virtually all muscarinic receptor ligands and a conserved Asp residue located in TM III. In addition a previous mutagenesis study has shown that replacement of the conserved TM III Asp residue in the rat muscarinic M1 receptor with Asn results in a receptor unable to bind to [^{3}H] QNB.

Sequence analysis shows that the hydrophobic core of all muscarinic receptors contains a series of conserved Ser, Thr and Tyr residues, most of which do not occur in other G protein coupled receptors. Pharmacological analysis of mutant M3 muscarinic receptors showed that two Thr residues (Thr231 and Thr234) and four Tyr residues (Tyr148, Tyr506, Tyr529 and Tyr533) are important for high affinity acetylcholine binding (Wess *et al.*, 1991). It has been shown that a Pro 201 to Ala mutant M3 muscarinic receptor exhibits affinities for both muscarinic agonists and antagonists 80-450 times less than those of the wild type (Wess *et al.*, 1993).

In the periphery, among other effects, muscarinic receptors mediate smooth muscle contraction, glandular secretion, and modulation of cardiac rate and force. In the central nervous system there is evidence that muscarinic receptors are involved in motor control, temperature regulation, cardiovascular regulation and memory. Interest in the classification of muscarinic receptors involved in functions at different locations has been heightened by the potential therapeutic application of selective agents in areas such as Alzheimer's disease, Parkinson's disease, asthma, analgesia,

and disorders of intestinal motility and cardiac and urinary bladder function (Caulfield & Birdsall, 1998).

Classification

Muscarinic receptors are widely distributed throughout the central and peripheral nervous system. They have critical functions in learning and memory, attention and motor activity (Levey, 1993; Weiner *et al.*, 1990; Bonner, 1989). The five muscarinic receptor subtypes are designated as M1 - M5. The odd-numbered receptors (M1, M3, and M5) couple to Gq/11, and thus activate phospholipase C, which initiates the phosphatidyl inositol trisphosphate cascade. This leads to the dissociation of phosphatidyl 4, 5- bisphosphates (PIP2) into two components, i.e., IP_3 and DAG. IP_3 mediates Ca^{2+} release from the intracellular pool (endoplasmic reticulum), whereas DAG is responsible for activation of protein kinase C. On the other hand, PIP2 is required for the activation of several membrane protein, such as the "M current" channel and Na^+/Ca^{2+} exchangers, and muscarinic receptor-dependent depletion of PIP2 inhibits the function of these proteins (Suh & Hille, 2005; Winks *et al.*, 2005; Fuster *et al.*, 2004; Meyer *et al.*, 2001; Caulfield & Birdsall, 1998; Bonner *et al.*, 1988; Bonner *et al.*, 1987;). The M1, M2 and M4 subtypes of mAChRs are the predominant receptors in the CNS. These receptors activate a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of ACh release (Volpivelli *et al.*, 2004)

Muscarinic M1 receptor

M1 receptors are predominantly expressed in the forebrain, including the cerebral cortex, hippocampus and corpus striatum, where this sub-type contributes by 50-60% to the total of the muscarinic receptors (Gerber *et al.*, 2001; Miyakawa *et al.*,

2001; Hamilton *et al.*, 1997). The M1 receptor subtype, which is also expressed in peripheral tissues, has been implicated in stress adaptive cardiovascular reflexes and central blood pressure control. Studies have shown that central administration of the M1 specific antagonist pirenzepine lowered the blood pressure (Buccafusco, 1996; Brezenoff & Xiao, 1986). A putative overexpression of the M1 subtype in selected brain areas of spontaneously hypertensive rats has been reported (Scheucher *et al.*, 1991). Muscarinic agonist depolarization of rat isolated superior cervical ganglion is mediated by M1 receptors (Brown *et al.*, 1980). M1 is one of the predominant muscarinic receptor subtypes expressed in pancreatic islets (Gilon & Henquin., 2001). Studies in pancreatic islets revealed that activation of muscarinic receptors is pertusis toxin insensitive and Gq mediated. Muscarinic M1 receptor number decreased in the brainstem at time of pancreatic regeneration without any change in the affinity (Renuka *et al.*, 2006).

Muscarinic M2 receptor

Muscarinic receptor activation in guinea pig heart produces a reduction in force of contraction and a decrease in the rate of beating. These effects are probably the consequence of inhibition of voltage-gated Ca^{2+} channels and activation of inwardly rectifying K^{+} channels, respectively. Extensive studies with many antagonists have defined this response as being mediated by the M2 receptor (Caulfield, 1993). Muscarinic M2 receptors mediate both negative and positive ionotropic responses in the left atrium of the reserpinized rat, latter effect being insensitive to pertusis toxin (Kenakin & Boselli, 1990). Central cholinergic transmission can also be activated by inhibition of the presynaptic M2 acetylcholine autoreceptor using selective antagonists. The presynaptic M2 autoreceptor negatively influences the release of acetylcholine in several brain regions, including the striatum,

hippocampus, and cerebral cortex (Zhank *et al.*, 2002; Kitaichi *et al.*, 1999; Billard *et al.*, 1995;). A direct consequence of brain M2 autoreceptor inhibition is an elevation of acetylcholine release in the synaptic cleft. Methoctramine and other M2 receptor antagonists have been shown to enhance the release of acetylcholine in different brain structures (Stillman *et al.*, 1993; Stillman *et al*, 1996).

Muscarinic M3 receptor

M3 muscarinic receptors are broadly expressed in the brain, although the expression level is not high, compared to those of the M1 and M2 receptors (Levey, 1993). Muscarinic M3 receptor is widely distributed in the peripheral autonomic organs with the highest expression found in the exocrine glands (Candell *et al.*, 1990; Matsui *et al.*, 2000; Kashihara *et al.*, 1992 ; Pedder *et al.*, 1991). Expression of the M3 receptor in the rat pancreatic islets and insulin secreting cell lines has been established (Lismaa, 2000). M3 receptor also triggers direct contractions of smooth muscle, however, it only represents a minor fraction of total muscarinic receptor population in smooth muscle. It is expressed in relatively low density throughout the brain. Studies using knock out mice for M3 receptors gave evidences for the primary importance of these receptors in the peripheral cholinergic system. In urinary bladder, pupillary muscles and intestinal smooth muscles the cholinergic contractions are mediated predominately by M3 receptors (Matsui *et al.*, 2000).

Muscarinic M4 receptor

Muscarinic M4 receptor is known to be abundantly expressed in the striatum (Levey, 1993). Muscarinic M4 receptors act as inhibitory muscarinic autoreceptors in the mouse (Zhang *et al.*, 2002). The neuroblastoma-glioma hybrid cell line NG108-15 expresses M4 mRNA and M4 receptors can be detected readily in radioligand

binding assays (Lazareno *et al.*, 1990). Inhibition of adenylyl cyclase activity by muscarinic agonists in rat corpus striatum is mediated by M4 receptors (Caulfield, 1993; Olianas *et al.*, 1996).

Muscarinic M5 receptor

The M5 receptor was the last muscarinic acetylcholine receptor cloned. Localization studies have revealed that the M5R is abundantly expressed in dopamine-containing neurons of the substantia nigra par compacta, an area of the midbrain providing dopaminergic innervation to the striatum. Concordantly, oxotremorine-mediated dopamine release in the striatum was markedly decreased in M5R-deficient mice . More intriguingly, in M5R-deficient mice, acetylcholine induced dilation of cerebral arteries and arterioles was greatly attenuated (Yamada *et al.*, 2001), suggesting that the M5 receptor might be a suitable target for the treatment of cerebrovascular ischemia. Muscarinic M5 receptor subtype expressed at low levels in the brain (Hulme *et al.*, 1990; Hosey, 1992).

Studies of the M5 receptor have been hampered both by the lack of selective ligands and of tissues or cell lines that endogenously express the native receptor protein. Immunoprecipitation and RT-PCR studies have shown that the M5 receptor is expressed at very low densities in the mammalian brain. However, in situ hybridization studies have demonstrated that M5 transcripts are highly concentrated in the basal ganglia and are the only muscarinic receptor transcripts expressed on dopaminergic neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (Reever *et al.*, 1997). Another potentially useful system is the eosinophilic leukemia cell line (EoL-1) where M5 receptors can be induced on differentiation with interferon-γ (Mita *et al.*, 1996).

Signal transduction by muscarinic activation

Gq-protein-coupled receptors (GqPCRs) are widely distributed in the CNS and play fundamental roles in a variety of neuronal processes. Their activation results in phosphatidyl inositol 4,5-bisphosphate (PIP2) hydrolysis and Ca^{2+} release from intracellular stores via the phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP_3) signaling pathway. Because early GqPCR signaling events occur at the plasma membrane of neurons, they might be influenced by changes in membrane potential (Billups *et al.*, 2006). Muscarinic receptors, which are G protein coupled, stimulate signaling by first binding to G protein complex ($\alpha\beta\gamma$) which provides specificity for coupling to an appropriate effector. The α subunit interacts with an effector protein or ion channel to stimulate or inhibit release of intracellular second messengers. Mutation analysis showed that the G protein is primarily but not exclusively acts through interaction with the third cytoplasmic loop. It is suggested that the short sequences, N terminal 16-21 and C terminal 19 amino acids of the loop play a key role in determining the specificity (Wess *et al.*, 1989).

Cyclic adenosine monophosphate

Adenylate cyclase can be either positively or negatively regulated by G protein coupled receptors resulting in an increase or decrease in the generation of the second messenger, Cyclic adenosine monophosphate (cAMP). The stimulation of muscarinic M2 and M4 receptors endogenously expressed in cell lines, results in the inhibition of adenylate cyclase. G protein reconstitution experiments have shown that M2 receptors inhibit adenylate cyclase through Gi and possibly through the pertusis toxin insensitive Gz. In neuroblastoma SK-N-SH cells which express endogenous muscarinic M3 receptors stimulate adenylate cyclase activity (Baumgold & Fishman, 1988). The muscarinic M1 receptor which ectopically expressed at physiological

levels in A9L cells, was shown to stimulate adenylate cyclase through an IP_3 and Ca^{2+} dependent mechanism (Felder *et al.*, 1989). In contrast, M1 receptors stimulate adenylate cyclase in CHO cells predominantly through an IP_3 and Ca^{2+} independent mechanism that also contained a small Ca dependent component (Gurwitz *et al.*, 1994).

Phospholipase C

The family of phospholipase C (PLC) enzymes has been grouped into three classes, β, γ and δ (Rhee & Choi, 1992). PLC serves as the primary effector for the muscarinic M1 receptor that is coupled through Gq α subunits (Berstein *et al.*, 1992). Muscarinic M1, M3 and M5 receptors can stimulate the production of IP_3, independent of direct PLCβ and G protein interaction (Gusovsky, 1993). This alternate route for the generation of IP_3 involves the tyrosine kinase dependent phosphorylation of PLCγ, a mechanism normally stimulated by growth factors and their receptors (Meisenhelder *et al.*,, 1989). Expression studies revealed that the cloned muscarinic M2 receptor stimulates PLC through a pertusis toxin-sensitive G protein although with lower efficiency than M1 or M3 receptors (Ashkenazi *et al.*, 1987). Inhibition of PLC by an endogenously expressed M2 receptor has been reported in FRTL5 cells suggesting that negative regulation may also occur in some cells (Bizzarri *et al.*, 1990).

Phospholipase A2

Phospholipase A2 catalyze the hydrolysis of membrane phospholipids to generate free arachidonic acid and the corresponding lysophospholipid. Muscarinic receptors have been shown to stimulate the release of arachidonic acid and its eicosanoid metabolites in a variety of tissues including heart, brain and muscle

(Abdel-Latif, 1986). Ectopic transfection experiments indicates that the muscarinic M1, M3 or M5 receptors, but not M2 or M4 receptors are linked to phospholipase A2 activation (Conklin *et al.*, 1988; Felder *et al.*, 1990; Liao *et al.*, 1990). Muscarinic receptor stimulated release of arachidonic acid occurs predominantly through the activation of phospholipase A2 and phosphatidylcholine serves as the primary substrate. Studies suggested that calcium influx, through voltage independent calcium channel activation, and diacylglycerol, through PLC activation were essential for phospholipase A2 activation (Felder *et al.*, 1990; Brooks *et al.*, 1989). In ileal smooth muscle cells, carbachol stimulated phospholipase A2 itself caused calcium influx, implicating an amplification mechanism in phospholipase A2 regulation (Wang *et al.*, 1993).

Phospholipase D

Muscarinic receptor stimulated phospholipase D has been reported in a number of cell types including canine synaptosomes (Qian & Drewes, 1989), rat astrocytoma cells (Martinson, 1990), human neuroblastoma cells (Sandmann & Wurtman, 1991) and rat parotid cells (Guillemain & Rossignol, 1992). Association of muscarinic subtypes with phospholipase D has been shown in human embryonic kidney cells transfected with the M1-M4 receptors. In most cells studied, phospholipase C and D are usually stimulated simultaneously following receptor activation (Liscovitch, 1991).

Calcium influx and release from intracellular stores

Muscarinic receptors typically stimulate biphasic increases in intracellular calcium in most cells. The transient phase represents the release of calcium from IP_3 sensitive intracellular calcium stores. Calcium influx through calcium channels play a

central role in the regulation of multiple signaling pathways activated by muscarinic receptors. In excitable cells such as neurons and muscle cells calcium passes predominantly through voltage sensitive calcium channels (VOCC). In non-excitable cells, such as fibroblasts and epithelial cells, calcium passes through a family of poorly characterised voltage - insensitive calcium channels (VICC) (Fasolato *et al.*, 1994). VICCs open in response to receptor activation and have been classified into (1) receptor operated calcium channels which are second messenger independent, (2) second messenger - operated calcium channels (SMOCCs) and (3) depletion operated calcium channels which open following IP_3 mediated depletion of intracellular stores and provide a source of calcium for refilling the stores.

Insulin secretion regulating factors

Glucose

Glucose is an important regulator of various β-cell processes including insulin biosynthesis and release. Glucose, over short intervals stimulates insulin biosynthesis at the level of translation (Permut *et al.*, 1972). Studies have shown that preproinsulin mRNA levels rise 4-10 folds in response to glucose stimulation. Studies of insulin gene expression in primary cultures of rat islets transfected Insulin I gene 5' flanking sequence suggested that metabolic signal from glucose influx is transmitted through the insulin enhancer (German *et al.*, 1990).

Phosphorylation of glucose to glucose-6-phosphate serves as the rate limiting step in glucose oxidation (Schuit, 1996). Glucokinase acts as sensor during this process. The entry of glucose into β-cells is followed by an acceleration of metabolism that generates one or several signals that close ATP-sensitive K^+ channels in the plasma membrane. The resulting decrease in K^- conductance leads to

depolarisation of the membrane with subsequent opening of voltage dependent Ca^{2+} channels. The rise in the cytoplasmic free Ca^{2+} eventually leads to the exocytosis of insulin containing granules (Dunne, 1991; Gembal *et al.*, 1992). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C within the β-cell (Harris, 1996). It is suggested that PKC may be tonically active and effective in the maintenance of the phosphorylated state of the voltage-gated L-type Ca^{2+} channel, enabling an appropriate function of this channel in the insulin secretory process (Arkhammar, 1994).

Fatty acids

Short chain fatty acids and their derivatives are highly active stimulators of insulin release in sheep (Horino *et al.*, 1968). Exogenous saturated long chain fatty acids markedly potentiated glucose-induced insulin release and elevated long chain acyl-CoA esters in the clonal β-cell line, HIT (Prentki *et al.*, 1992). A novel ester of succinic acid 1, 2, 3-tri-(methyl-succinyl) glycerol ester displayed stimulation of insulin release and biosynthetic activity in pancreatic islets of Goto-Kakizaki rats (Laghmich *et al.*, 1997). A monomethyl ester of succinic acid along with D-glucose is required to maintain the β-cell response to D-glucose (Fernandez *et al.*, 1996).

Amino acids

Amino acids act as potent stimulators of insulin release. L-Tryptophan, which is the precursor of 5-Hydroxytryptamine (5-HT) can act as a stimulator of insulin release (Bird *et al.*, 1980). L-Arginine also stimulates insulin release from pancreatic β-cells. Several *in vitro* studies have suggested the production of nitric oxides from islet nitric oxide system may have a negative regulation of the L-arginine induced secretion of insulin in mice.

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Substrates derived from nutrients

Substrates like pyruvate (Lisa, 1994), citrate, ATP (Tahani *et al.*, 1979), NADH and NADPH may involve in the indirect reflux stimulation triggered by food intake or local islet stimulation through the production of metabolites. The NADH acts as an intracellular regulator of insulin secretion. Heterotrimeric GTP-binding protein Gαi is involved in regulating glucose induced insulin release (Konrad *et al.*, 1995). GTP analogues are also important regulators of insulin secretion (Lucia *et al.*, 1987). Glucose induced insulin secretion is accompanied by an increase in the islet content of cAMP (Rabinovitch *et al.*, 1976).

Glucagon

Glucagon is the hormone secreted by pancreatic α-cells. It has been shown that glucagon has a striking stimulatory effect on insulin release in the absence of glucose (Sevi, 1966). The presence of specific glucagon receptors on isolated rat pancreatic β-cells as well as a subpopulation of α- and δ-cells shows the relevance of glucagon on regulation of insulin secretion. Intra-islet glucagon appears to be a paracrine regulator of cAMP *in vitro* (Schuit, 1996). Glucagon stimulates insulin release by elevating cAMP. cAMP through activation of protein kinase A, increases Ca^{2+} influx through voltage dependent L-type Ca^{2+} channels, thereby elevating Ca^{2+} and accelerating exocytosis (Carina, 1993). Protein phosphorylation by Ca^{2+}/Calmodulin and cAMP dependent protein kinase play a positive role in insulin granule movement which results in potentiation of insulin release from the pancreatic β-cell (Hisatomi, 1996).

Somatostatin

This hormone is secreted by the pancreatic δ-cells of the islets of Langerhans. Somatostatin inhibits insulin release. Its action is dependent on the activation of G-proteins but not associated with the inhibition of the voltage dependent Ca^{2+} currents or adenylate cyclase activity (Renstrom *et al.*, 1996).

Pancreastatin

Pancreastatin is known to be produced in islet β-cells and to inhibit insulin secretion. Pancreastatin is a modulator of the early changes in insulin secretion after increase of glucose concentration within the physiological range (Ahren *et al.*, 1996). It is reported to increase Ca^{2+} in insulin secreting RINm5F cells independent of extracellular calcium (Sanchez *et al.*, 1992).

Amylin

Amylin is a 37-amino acid peptide hormone co-secreted with insulin from pancreatic β-cells. Amylin appears to control plasma glucose via several mechanisms that reduce the rate of glucose appearance in the plasma. Amylin limits nutrient inflow into the gut and nutrient flux from the gut to blood. It is predicted to modulate the flux of glucose from liver to blood by its ability to suppress glucagon secretion. Amylin is absolutely or relatively deficient in type I - diabetes and in insulin requiring type II - diabetes (Young, 1997). It inhibits insulin secretion via an autocrine effect within pancreatic islets. Amylin fibril formation in the pancreas may cause islet cell dysfunction and cell death in type II - diabetes mellitus (Alfredo *et al.*, 1994).

Adrenomedullin

Adrenomedullin is a novel hypotensive adrenal polypeptide isolated from a human pheochromocytoma and is structurally related to calcitonin gene related peptide and amylin. It has been suggested that besides being an adrenal hypotensive peptide, adrenomedullin may be a gut hormone with potential insulinotropic function (Mulder *et al.*, 1996).

Galanin

Galanin is a 29 amino acid neuropeptide localised in the intrinsic nervous system of the entire gastrointestinal tract and the pancreas of man and several animal species (Scheurink *et al.*, 1992). It inhibits insulin secretion in rat, mouse, and also in isolated human islets pig. In isolated rat and mouse islets galanin inhibits insulin secretion by increasing the K^+ permeability and interfering with activation of adenylate cyclase and the activity of protein kinase C and cAMP. Among other functions, galanin inhibits insulin release (Ahren *et al.*, 1991), probably via activation of G proteins (Renstrom, 1996) by the mediation of activated galanin receptors.

Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF), originally identified as cytokines, secreted by T lymphocytes. It was found recently to be both a pituitary hormone and a mediator released by immune cells in response to glucocorticoid stimulation. Recently it has been demonstrated that insulin secreting β-cells of the islets of Langerhans express MIF and its production is regulated by glucose in a time and concentration dependent manner. MIF and insulin were both present within the secretory granules of the pancreatic β-cells and once released, MIF appears to regulate

insulin release in an autocrine fashion. MIF is therefore a glucose dependent islet cell product that regulates insulin secretion in a positive manner and may play an important role in carbohydrate metabolism (Waeber *et al.*, 1997).

Nerve growth factor

Nerve growth factor (NGF) is a neurotropic growth factor that promotes neurite outgrowth during development. This growth factor is capable of modulating β-cell plasticity because it promotes neurite-like outgrowth in fetal and adult pancreatic β-cells from primary cultures (Vidaltamayo *et al.*, 1996) and in RINm5F and insulinoma cells (Polak *et al.*, 1993). In adult rat β-cells, *in vitro* NGF stimulates glucose induced insulin secretion. The presence of the high affinity receptor for NGF has been described in insulinoma cell lines as well as in foetal and adult β-cells. The adult β-cells synthesise and secrete NGF in response to increasing extra cellular glucose concentration (Vidaltamayo *et al.*, 1996). The effect of NGF on insulin secretion is partly mediated by an increase in calcium current through calcium channels (Rosenbaum *et al.*, 2001).

Neuropeptides

Immunocytochemistry has revealed the presence of three neuropeptides in the nerve terminals of pancreatic ganglia and islets of different species: Vasoactive intestinal peptide (VIP), gastrin releasing peptide (GRP) and pituitary adenylate cyclase activating polypeptide (PACAP).

Gastrin releasing peptide

Gastrin releasing peptide (GRP) consists of a 27 amino acid residue. It is localised to pancreatic nerves, including islet nerve terminals of several species. GRP

released from the pancreas after vagal nerve activation and stimulates insulin secretion (Sundler& Bottcher, 1991; Knuhtsen *et al.*, 1987). In islets, activation by GRP receptors is coupled to PLC and phospholipase D (Gregersen & Ahren, 1996, Wahl *et al.*, 1992).

Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) stimulates insulin secretion in a glucose dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP (Klinteberg *et al.*, 1996). VIP increases activity of sympathetic system, including release of catecholamines from the adrenal medulla and lead to the release of the pancreatic glucagon and inhibition of insulin release, by the activation of adrenergic receptors (Jarrhult and Holst, 1978).

Pituitary adenylate cyclase activating polypeptide

Pituitary adenylate cyclase activating polypeptide (PACAP) is localised to the parasympathetic nerves and released by the activation of the vagus nerve (Ahren, 2000). It exists in two forms consisting of 27 and 38 amino acids and show 68% homology (Arimura & Shioda, 1995). PACAP stimulates insulin secretion in a glucose dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP (Klinteberg *et al.*, 1996).

Role of neurotransmitters in insulin regulation & secretion

Acetylcholine

Acetylcholine is one of the principal neurotransmitters of the parasympathetic system. Acetylcholine, through vagal muscarinic and non-vagal muscarinic pathways

(Greenberg & Pokol, 1994) increases insulin secretion (Tassava *et al.*, 1992). They function through muscarinic receptors present on pancreatic islet cells (Ostenson *et al.*, 1993). Acetylcholine agonist, carbachol, at low concentration (10^{-7} M) stimulated insulin secretion at 4 mM and 20 mM concentrations of glucose (Renuka *et al.*, 2006).

Dopamine

Dopamine is reported to inhibit glucose stimulated insulin secretion from pancreatic islets (Tabeuchi *et al.*, 1990). Eswar *et al.*, (2006) reported that dopamine significantly stimulated insulin secretion at a concentration of 10^{-8} M in the presence of high glucose (20mM).Reports show that experimental diabetes and insulin deficiency result in the rapid onset of detectable alterations in dopaminergic activity in specific hypothalamic nuclei. The uptake affinity and velocity of dopamine in synaptosomes decreased significantly during diabetes. The dopamine content was increased in the cerebral cortex and hypothalamus of diabetic rats (Ohtani *et al.*, 1997; Tassava *et al.*, 1992; Shiimzu, 1991). The altered turnover ratio in the limbic forebrain is reported to cause enhanced spontaneous locomotor activity in diabetic rats (Kamei *et al.*, 1994).

High concentrations of dopamine in pancreatic islets can decrease glucose stimulated insulin secretion (Tabeuchi *et al.*, 1990). L-DOPA, the precursor of dopamine had similar effect to that of dopamine (Lindstrom & Sehlin, 1983). Dopamine D3 receptors are implicated in the control of blood glucose levels (Alster & Hillegaart, 1996). Dopamine D1 receptors have also been reported to be present on pancreatic β-cells (Tabeuchi *et al.*, 1990). These clearly indicate the role of dopamine in the regulation of pancreatic function.

Gamma-Aminobutyric acid

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. GABA is reported to present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson *et al.*, 1991). Glutamate decarboxylase, the primary enzyme that is involved in the synthesis of GABA, has been identified as an early target antigen of the T-lymphocyte mediated destruction of pancreatic β-cells causing insulin-dependent diabetes mellitus (Baekkeskov *et al.*, 1990). GABA through its receptors have been demonstrated to attenuate the glucagon and somatostatin secretion from pancreatic α–cells and δ-cells respectively (Gaskins, 1995). It is present in the cytoplasm and in synaptic-like microvesicles (Reetz, 1991) and is co-released with insulin from β-cells in response to glucose. The released GABA inhibits islet α-and δ-cell hormonal secretion in a paracrine manner. During diabetes the destruction of β-cells will lead to decrease in GABA release resulting in the enhancement of glucagon secretion from α-cells leading to hyperglycemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central GABA$_A$ receptors increases plasma glucose concentration (Lang, 1995). GABA$_A$ receptors in brainstem have a regulatory role in pancreatic regeneration (Kaimal *et al.*, 2007) Thus, any impairment in the GABAergic mechanism in the central nervous system and/or in the pancreatic islets is important in the pathogenesis of diabetes.

Serotonin

Serotonin content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991); (Lackovic *et al.*, 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sumiyoshi

et al., 1997; Sandrini *et al.*, 1997). Ohtani *et al.*, (1997) have reported a significant decrease in extracellular concentrations of NE, 5-HT and their metabolites in the ventro medial hypothalamus (VHM). The ratio of 5-HIAA/5-HT was increased. A similar observation was reported by (Ding *et al.*, 1992) with a decrease in 5-HT in cortex (19%) and 5-HT turnover (5-HIAA/5-HT) that increased by 48%. Chu *et al.*, (1986) has reported lower 5-HT levels in both hypothalamus and brainstem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-HIAA and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993). There was a significant increase in 5-HIAA observed at 2-6 hours after insulin administration (Kwok & Juorio, 1987).

Epinephrine and Norepinephrine

These are secreted by the adrenal medulla. Norepinephrine (NE) is a principal neurotransmitter of sympathetic nervous system. These hormones inhibit insulin secretion, both *in vivo* and *in vitro* (Renstrom *et al.*, 1996; Porte, 1967). Epinephrine exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro *et al.*, 1996). NE and EPI - the flight and fright hormones - are released in all stress conditions and are the main regulators of glucose turnover in strenuous exercise (Simartirkis *et al.*, 1990). In severe insulin-induced hypoglycemia, a 15 to 40 -fold increase of epinephrine plays a pivotal role in increasing glucose production independently of glucagon (Gauthier *et al.*, 1980). It is already known that, when used in high doses *in vivo* or *in vitro*, epinephrine reduces

the insulin response to stimulators (Malaisse, 1972). *In vitro* studies with yohimbine showed that the insulin secretion from the pancreatic islets increased significantly suggesting that when the alpha 2-adrenergic receptors are blocked, it enhances islet cell proliferation and insulin secretion (Ani *et al.*, 2006). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte *et al.*, 1966). They also inhibit insulin -stimulated glycogenesis through inactivation of glycogen synthase and activation of phosphorylase with consequent accumulation of glucose-6-phosphate. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phospho-fructokinase. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis. Adrenaline is, however, known to play a secondary role in the physiology of glucose counter-regulation. Indeed, it has been shown to play a critical role in one pathophysiological state, the altered glucose counter-regulation in patients with established insulin-dependent diabetes mellitus (Cryer, 1993). The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore and Randle, (1964), who incubated pancreatic tissue from the rabbit. As judged by Malaisse *et al.*, (1967) the inhibitory effect of EPI on glucose-induced insulin secretion is mediated through the activation of α-adrenoreceptors.

Central muscarinic regulation of glucose homeostasis

The acetylcholine esterase inhibitor soman induced marked and sustained hypertension in rats (Letienne *et al*, 1999). Stimulation of muscarinic receptors in the nucleus tractus solitarius (NTS) of the rat decreases arterial blood pressure and heart rate. Atropine injected into the NTS of rats produced a dose-dependent inhibition of cardiovascular response elicited by injection of acetylcholine into the same site. It is

suggested that cholinergic mechanisms in the NTS are not involved in the tonic regulation of cardiovascular function or the baroreceptor reflex (Tsukamoto *et al.*, 1994).

When carbachol, muscarine, bethanechol, methacholine, or neostigmine was injected into the third cerebral ventricle, it caused a dose-dependent increase in the hepatic venous plasma glucose concentration. However, in the case of 1, 1-dimethylphenyl-4-piperazinium iodide (DMPP) or nicotine, the level of hepatic venous glucose did not differ from that of the saline-treated control rats. The increase in glucose level caused by neostigmine was dose-dependently suppressed by co-administration of atropine. These facts suggest that cholinergic activation of muscarinic receptors in the central nervous system plays a role in increasing hepatic glucose output. Injection of neostigmine, an inhibitor of cholinesterase, into the ventricle resulted in the increase of not only glucose, but also glucagon, epinephrine, and norepinephrine in the hepatic venous plasma. Neostigmine-induced increments in glucose did not occur in adrenalectomized rats. This suggests that the secreted epinephrine acts directly on the liver to increase hepatic glucose output (Iguchi *et al.*, 1986).

The injection of adrenaline and carbachol into the third cerebral ventricle resulted in a marked hyperglycemia associated with increased immunoreactive glucagon. Adrenaline-induced hyperglycemia was not affected by bilateral adrenalectomy, while carbachol-induced hyperglycemia was completely inhibited by adrenalectomy. The injection of somatostatin with adrenaline into the third cerebral ventricle did not influence adrenaline-induced hyperglycemia, while carbachol-induced hyperglycemia was inhibited by co-administration with somatostatin (Iguchi *et al.*, 1985).

Atropine injected into the third cerebral ventricle suppressed epinephrine secretion and dose-dependently inhibited hepatic venous hyperglycemia induced by neostigmine in intact rats. The neostigmine-induced glucagon secretion which occurs in adrenalectomised rats was suppressed by atropine. Atropine also prevented the neostigmine-induced hyperglycemia in adrenalectomised rats receiving constant somatostatin infusion through femoral vein. Phentolamine, propranolol and hexamethonium showed no significant inhibitory effect on neostigmine-induced hyperglycemia, epinephrine and glucagon secretion in intact rats, glucagon secretion in adrenalectomised rats, or hyperglycemia in adrenalectomised-Somato rats. These results suggest that neostigmine-induced epinephrine and glucagon secretion and increased hepatic glucose output stimulated by direct neural innervation to liver is mediated by central muscarinic receptor in fed rats (Iguchi *et al.*, 1990)

Studies by Iguchi *et al.*, (1992) suggest that the glucoregulatory hippocampal activity evoked by the acetylcholine esterase inhibitor, neostigmine transmitted to peripheral organs via the ventromedial hypothalamus. The ventromedial hypothalamus, lateral hypothalamus, paraventricular hypothalamus, and median site of the lateral-preoptic area were involved in increasing the plasma levels of glucose and epinephrine by cholinergic stimulation (Honmura *et al.*, 1992).

Atropine in a dose-dependent manner suppressed the hyperglycemia induced by hippocampal administration of neostigmine, whereas hexamethonium had no significant effect. These observations suggest that the pathway for this experimental hyperglycemia involves, at least in part, the muscarinic cholinergic neurons in the ventromedial hypothalamus (Iguchi *et al.*, 1991). Takahashi *et al.*, (1993) reported that neostigmine induced hyperglycemia affects not only the cholinergic system but also the noradrenergic and dopaminergic systems in the hypothalamus (Takahashi *et al.*, 1993). Muscarinic cholinergic system is reported to participate in the $HgCl_2$-

induced central hyperglycemic effect through the function of the adrenal medulla. Norepinephrine and dopamine content were found to be decreased suggesting that their neurons have hypothalamic glycoregulation (Takahashi *et al.*, 1994).

Microinjections of carbachol or neostigmine into the ventromedial nucleus of the hypothalamus of fed, conscious rats produced marked increases in plasma glucose and lactate, which were suppressed or markedly reduced by previous adrenodemedullation. The reports suggest that cholinergic synapses in the ventromedial hypoythalamus participate in a central glucoregulatory system that increases hepatic glucose production mainly through a stimulation of adrenal medulla epinephrine secretion (Brito *et al.*, 1993).

Neostigmine caused significant increases in serum glucose concentrations, hypothalamic noradrenergic and dopaminergic neuronal activities, and significantly suppressed hypothalamic serotonergic neuronal activity. All these responses to neostigmine were completely inhibited by the co-administration of atropine. These observations emphasize the important role of the interactions between cholinergic (muscarinic) and monoaminergic neurons in the brain (Gotoh & Smythe, 1992). In the ventromedial hypothalamic nucleus, lateral hypothalamus and paraventricular nucleus the cholinergic activity is increased after 2-D glucose administration (Takahashi *et al.*, 1994 & 1996).

Central cholinergic-muscarinic activation with neostigmine stimulates sympathetic nervous activity in the liver, heart, pancreas and interscapular brown adipose tissue (Gotoh & Smythe, 1992). Histamine induction of central nervous system-mediated hyperglycemia involves neuronal transmission not only via H1 receptors but also, at least in part, by muscarinic cholinergic neurons (Nonogaki *et al.*, 1993). The action of acetylcholine within the hypothalamus on the pancreatic hormone secretions is mediated to a large part through sympatho-adrenomedullary

activity. However, a part of the decreased insulin response to glucose may be mediated by direct innervation of the pancreas (Ishikawa *et al.*, 1982).

Intravenous 2-D glucose induced a marked increase in plasma glucose that was not affected by intracerebroventricular administration. However, the hyperglycemia induced by intracerebroventricular 2-D glucose was significantly reduced by previous intracerebroventricular injection of atropine. Central cholinergic neurons participate in the complex neural events responsible for the hyperglycemic response to neurocytoglucopenia and to stressful situations (Brito *et al.*, 2001). Intravenous administration of 2-D glucose caused neuroglycopenia and marked hyperglycemia. The cholinergic activity was increased after 2-D glucose administration (Takahashi *et al.*, 1996).

Peripheral muscarinic receptor alterations in diabetes

Autonomic neuropathy is a major complication of chronic diabetes and is responsible for disturbances in the cardiovascular system and other organs. Early cardiac disturbances have been attributed to defective vagal control of the heart (Carrier *et al.*, 1984). Streptozotocin (STZ) induced diabetes caused a variety of abnormalities including alterations in the muscarinic receptors (Latifpour *et al.*, 1991). Muscarinic acetylcholine receptors are reported to be decreased in the atrium of STZ induced diabetic rats (Mardon *et al.*, 1999).

Tonq *et al.*, (2006) reported that STZ-induced diabetes increases mRNA and protein expression of the M2-mAChR in the urothelium as well as the muscle layer. The myocardium of STZ induced diabetic rats exhibited an increase in Gi function by the increased inhibition of guanyliminodiphosphate-mediated adenylyl cyclase and the superhigh affinity for carbachol of the muscarinic receptors. This functional alteration of Gi is suggested to be related to the cardiac dysfunction that is associated with

diabetes. The cerebral blood flow response to muscarinic receptor agonist decreased in the brain regions of diabetic rats (Pelligrino *et al.*, 1992).

Bladder dysfunction is a common complication of diabetes mellitus and is attributed in part to peripheral neuropathy. [^{3}H]quinuclidinyl benzylate (QNB) binding studies revealed that the receptor number is higher in the diabetic animals showing a direct correlation between the diabetes-induced biochemical and functional alterations in muscarinic receptor properties of rat bladder (Latifpour *et al.*, 1989). In STZ induced diabetes, inositol phosphate production in the bladder is found to be enhanced after muscarinic agonist stimulation (Mimata *et al.*, 1995). The bladder contractile response to muscarinic agonist, arecaidine propargyl ester (APE), was significantly increased in the diabetic rats. The M2 receptor is the dominant muscarinic subtype in animal bladders. There was an over-expression of M2 receptor resulting in hyper-contractility in the bladder of diabetic rats (Tong *et al.*, 1999; 2002). The M3 and M2-recptor protein and mRNA in the bladder tissue were significantly increased in diabetic rats (Tong, & Cheng, 2002; Tong *et al.*, 2002). STZ-induced diabetes caused a variety of abnormalities including a down regulation in the density of M3 muscarinic receptors in the rat prostrate and insulin, but myo-inositol could not prevent the development of these abnormalities (Latifpour *et al.*, 1991; Fukumoto *et al.*, 1993).

The inhibitory M2 receptors on parasympathetic nerves in the trachea and ileum are hyperfunctional in diabetic rats. In the trachea the function of post-junctional M3 muscarinic receptors is also increased in diabetes (Coulson *et al.*, 2002). In [^{3}H]QNB binding studies for muscarinic receptor of the STZ rats, in the parotid gland the receptor number was decreased and the affinity of receptors decreased in the submandibular gland. The decrease in salivary secretion of STZ rats is not only induced by a water loss, but also closely associated with the lowered

susceptibility of the muscarinic receptors (Watanabe *et al.*, 2001). Studies of Latifpour & McNeill (1984) on long-term STZ-induced diabetes revealed that ventricular β adrenergic and muscarinic receptors demonstrated a large reduction in their densities as compared with their age-matched controls.

Insulin-induced net hepatic glucose uptake depends on the sensing by muscarinic, intrahepatic nerves of a glucose concentration gradient between portal vein and hepatic artery. The function of these intrahepatic nerves is impaired in diabetic animals (Stumpel *et al.*, 1998). Muscarinic receptor number increased in the pancreatic islets of diabetic rats. Cholinergic-induced insulin release was also higher in STZ induced diabetes than in normal islets (Ostenson & Grill, 1987).

Insulin partly reversed the changes observed in the STZ-treated rats. There was a decrease in the muscarinic receptor number and axonal transport of receptor-bound opiate in STZ induced hyperglycemia suggesting that impaired axonal transport of receptors partly involved in the neurological disturbance which is seen in diabetic patients (Laduron & Janssen, 1986).

Plants as antidiabetic agents

Plants still remain a major source for drug discovery inspite of the great development of synthetic molecules. Consequently, the uses of traditional plant extract in the treatment of various diseases have been flourished (Fabricant & Farnsworth, 2001). According to the World Health Organisation (WHO), over than 150 plants are known to be used for the treatment of diabetes mellitus and the study of hypoglycemic plants is then encouraged (Marles & Farnsworth, 1995). The ethnobotanical information reports about 800 plants that possess anti-diabetic potential (Alarcon-Aguilara *et al.*, 1998). Several such herbs have shown anti-diabetic activity when assessed using presently available experimental techniques

A wide array of plant derived active principles representing numerous chemical compounds have demonstrated activity consistent with their possible use in the treatment of NIDDM (Bailey & Day, 1989; Ivorra *et al.*, 1988; Marles & Farnsworth, 1995). Among these are alkaloids, glycosides, galactomannan gun, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions. Even the discovery of widely used hypoglycemic drug, metformin came from the traditional approach of using Galega officinalis . Thus, plants are a potential source of anti-diabetic drugs but this fact has not gained enough momentum in the scientific community. The reasons may be many including lack of belief among the practitioners of conventional medicine over alternative medicine, alternative forms of medicine are not very well-defined, possibility of quacks practising such medicine providing alluring and magical cures and natural drugs vary tremendously in content, quality and safety (Grover *et al.*, 2002).

In modern medicine, no satisfactory effective therapy is till available to cure the diabetes mellitus. Though insulin therapy is also used for the management of diabetes mellitus but there are several drawbacks like insulin resistance (Piedrola *et al.*, 2001), anorexia nervosa, brain atrophy and fatty liver (Yaryura-Tobias *et al.*, 2001) after chronic treatment. In recent years, there has been renewed interest in plant medicine (Dubey *et al.*, 1994; Prince *et al.*, 1998; Ladeji *et al.*, 2003) for the treatment against different diseases as herbal drugs are generally out of toxic effect (Geetha *et al.*, 1994; Rao *et al.*, 2003) reported from research work conducted on experimental model animal.

Traditional antidiabetic plants of India

Historical accounts reveal that as early as 700 -200 BC, Diabetes mellitus was a well recognized disease in India. In India, indigenous remedies have been used in the treatment of Diabetes mellitus since the time of Charaka & Sushruta (6th century BC) (Grover & Vats, 2001). Since ancient times, plants have been an exemplary source of medicine. Ayurveda and other Indian literature mention the use of plants in treatment of various human ailments. India has about 45,000 plant species and among them, several thousands have been claimed to possess medicinal properties. Research conducted in last few decades on plants mentioned in ancient literature or used traditionally for diabetes have shown anti-diabetic property (Grover *et al.*, 2002).

Trigonella foenum graecum: Methi or Mutti (Hindi) and Fenugreek (English) - It is found as a wild plant and also cultivated in Northern India. The hypoglycemic effect of fenugreek seeds has been demonstrated in experimentally induced diabetic rats, dogs, mice and healthy volunteers (both IDDM and NIDDM) (Ribes *et al.*, 1984; Riyad *et al.*, 1988; Alarcon-Aguilara *et al.*, 1998).

Swertia chirayita: Chirata (Hindi) - It is mainly found in temperate Himalayas between the height of 1200 and 1300 m. Various crude extracts and its isolated fractions have shown hypoglycemic activity in various animal models. Oral administration of ethanolic extracts (95%) and hexane fraction of *Swertia chirayita* (10, 50 and 100 mg/kg) to normal, glucose fed and STZ induced diabetic rats significantly lowered blood glucose in all groups of animals (Sekar *et al.*, 1987).

Momordica charantia: Karela (Hindi) and Bitter Gourd (English) - It is a very common folklore remedy for diabetes. Extract of fruit pulp, seed, leaves and whole plant of *Momordica charantia* has shown hypoglycemic effect in various animal models (Sharma *et al.*, 1960; Gupta and Seth, 1962; Jose *et al.*, 1976; Vimla Devi *et al.*, 1977; Kedar and Chakrabarti, 1982).

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Phyllanthus niruri: Jangli Amla (Hindi) - It is used traditionally in management of dropsy and other ailments and has been mentioned in Ayurveda as a potential diuretic, hypotensive and hypoglycemic drug. In a clinical observation, oral administration of a preparation of the whole plant of *P. amarus* (syn. *Phyllanthus niruri*) (5 gm/day in divided doses) for 10 days to 9 mild hypertensives (4 with DM) reduces blood glucose (5_/50 mg) in diabetic as well as non-diabetic subjects along with significant reduction in systolic blood pressure. No harmful side effects were noted in this study (Srividya & Periwal, 1995).

Tinospora cordifolia: Amarta or Guduci (Hindi) - It is found in forests throughout India and is widely used in Ayurveda as tonic, vitalizer and as a remedy for DM and metabolic disorders (Nandkarni, 1954; Chopra *et al.*, 1958).

Allium cepa: Pyaj (Hindi) and Onion (English) - It is cultivated throughout India and is an important dietary constituent. Various ether soluble fractions of onion as a single oral dose (0.25 mg/kg) showed significant hypoglycemic effect in normal fasted rabbits. Ethyl ether extract showed most potent hypoglycemic action (Augusti, 1973).

Allium sativum: Lahasun (Hindi) and Garlic (English) - It is a perennial herb cultivated throughout India and is commonly used as a food ingredient. Oral administration of 0.25 gm/kg of ethanol, petroleum ether, ethyl ether extract of *Allium sativum* causes 18.9, 17.9, 26.2% reduction in blood sugar in alloxan-diabetic rabbits (150 mg/kg IV) (Jain and Vyas, 1975).

Aloe vera or *Aloe barbadensis*: Ghee Kunwar and Kumar panthu (Hindi) - It is cultivated or grows wildly as hedgerows in the drier part of India. It is used in Ayurveda for managing painful conditions and is also mentioned in folk medicine of Arabian Peninsula for management of diabetes. Extracts of aloe gum effectively

increased glucose tolerance in both normal and diabetic rats (Al- Awadi & Gumaa, 1987).

Azadirachta indica: Nim or Neem (Hindi) - It is a medium to large size tree found throughout India in deciduous forests and is also widely cultivated. Hydroalcoholic extract of *Azadirachta indica* showed hypoglycemic and anti-hyperglycemic effect in normal, glucose fed and STZ diabetic rats (Chattopadhyay *et al.*, 1987).

Gymnema sylvestre: Gudmar or Merasingi (Hindi) and Periploca of the woods (English) Anti-hyperglycemic effect of dried leaf powder of *Gymnema sylvestre* was seen in alloxanized rabbits along with decrease in the activity of gluconeogenic enzymes and reversal of pathological changes in the liver initiated during the hyperglycemic phase (Shanmugasundaram *et al.*, 1983).

Aegle marmelose

Medicinal plants have formed the basis for Indian traditional medicine systems. *Aegle marmelose* Corr. (Rutaceae) commonly called as 'Koovalam' in Malayalam and 'Bael' in Hindi is indigenous to India. It is a medium sized, armed deciduous tree found wild, especially in dry forests and is also cultivated throughout Indian subcontinent for its fruit. The fruit are globose with smooth, hard and aromatic rind. The ripe fruit is used for digestive and stomachic complications. Leaves, fruits, stem and roots of *Aegle marmelose* have been used in ethno medicine for several medicinal properties: astringent, antidiarrheal, antidysenteric, demulcent, antipyretic, antiscourbutic, haemostatic, aphrodisiac and as an antidote to snake venom (Nandkarni, 1976; Kirtikar & Basu, 1935). *Aegle marmelose* is also known as herbal medicine for the treatment of diabetes mellitus (Alam *et al.*, 1990; Prakash, 1992). Preliminary report indicates blood glucose lowering activity in green leaves of *Aegle*

marmelose (Chakrabarti *et al.*, 1960). Oral administration of aqueous decoction of *Aegle marmelose* root bark (1 ml/100 g) showed hypoglycemic effect, which was maximum (44%) at 3 h in normal fasted rats. In addition, the same extract completely prevented peak rise of blood sugar at 1 h in OGTT (Karunanyake *et al.*, 1984). Ponnachan *et al.* (1993) have observed that the crude aqueous leaf extract (1 g/kg for 30 days) exhibit hypoglycemic effect in alloxan induced diabetic rats. Aqueous leaf extract reversed the increase in Km values of liver malate dehydrogenase enzyme (Seema *et al.*, 1996) and improved histopathological alterations in the pancreatic and kidney tissues of streptozotocin (STZ) induced diabetic rats (Das *et al.*, 1996).

The aqueous extracts of fruits have also been reported to possess hypoglycemic activity (Kamalakkannan & Prince, 2003, 2004). Aqueous seed extract of *Aegle marmelose* possess antidiabetic and hypolipidemic effects in diabetic rats. (Kesari *et al.*, 2006) *Aegle marmelose* extract effectively reduced the oxidative stress induced by alloxan and produced a reduction in blood sugar(Sabu *et al.*, 2004). Anandharajan *et al.*,(2006) reported that methanolic extracts of *Aegle marmelose* activate glucose transport in a PI3 kinase-dependent fashion. *Aegle marmelose* root extract treated animals showed significant inhibitory activity against castor oil-induced diarrhea(Mazumder *et al.*, 2006). *Aegle marmelose* fruit extract exhibits protective effects on the pancreas of streptozotocin induced diabetic rats (Kamalakkannan & Prince, 2005)

Scopoletin (7-hydroxy-6-methoxy coumarin) was isolated from the leaves of *Aegle marmelose* and evaluated for its potential to regulate hyperthyroidism, lipid peroxidation and hyperglycemia in levo-thyroxine-induced hyperthyroid rats. Scopoletin (1.00 mg/kg, p.o.) administered daily for 7 days to levo-thyroxine-treated animals decreased the levels of serum thyroid hormones and glucose as well as

hepatic glucose-6-phosphatase activity, demonstrating its potential to regulate hyperthyroidism and hyperglycemia (Panda & Kar, 2006).

The leaves of *Aegle marmelose* Correa were reported as a source of aegeline (Chatterjee *et al.*, 1959). An examination of the fruits by various workers has revealed the occurrence of a coumarin termed 'marmelosin' (Asima & Sudhangsu, 1949). There are no available reports on the pharmacological action of *Aegle marmelose* seeds till date, therefore, the effect of aqueous extract of *Aegle marmelose* seeds on blood glucose and lipids in normal and streptozotocin induced diabetic rats has been investigated.

Costus pictus D. Don

The genus Costus Linn. belongs to family Costaceae, which has been separated from family Zingiberaceae on the basis of the presence of spirally arranged leaves and rhizomes being free from aromatic essential oils. More than 100 species of the genus are distributed in the tropics all over the world.

Costus pictus D. Don commonly known as Spiral ginger, Stepladder or Insulin plant is a plant originated in Mexico. In India it is grown in gardens as ornamental plant especially in Kerala in every home.. Red painted stem enhances the beauty of the glossy linear leaves and strongly spiralling canes. The flowers are in a terminal cone, yellow in colour with an orange red tip and this lasts for 3 – 4 days. Usually the plant grows up to 2 –3 m and spread 1.5 – 2 m. The flowers are displayed in a dramatic form high above the leaves. While the flowers do not produce an aroma, they do make a beautiful effect sitting atop of the tall spiraling stems. Propagation is carried out through stem cuttings and also from rhizomes.

Costus pictus, D Don is a plant attained popularity in Kerala recently by its so called anti-diabetic effects. There are no available reports on the pharmacological actions of *Costus pictus* leaf extract.

Alterations of glucose transport during diabetes

In diabetes mellitus apart from raised blood glucose levels, disturbances in the metabolism of a number of other biomolecules such as glycogen, lipids, proteins and glycoproteins have also been reported (Randle *et al.*, 1963; Williamson *et al.*, 1968). Treatment with insulin generally rectifies these disturbances in diabetic state as it increases the peripheral utilisation of glucose by influencing key enzymes of glucose metabolic pathways (Exton *et al.*, 1966; Lenzen *et al.*, 1990). The liver plays a major role in insulin-regulated glucose homoeostasis through the balance between glucose utilization and glucose production, both processes being tightly coordinated (Nevado *et al.*, 2006). More recently, it has been shown that glucose uptake and release required a family of membrane facilitated-diffusion glucose transporters which are expressed in a tissue-specific manner . In muscle and fat, GLUT-4 is the main isoform of glucose transporters (Burant *et al.*, 1991). In adipose tissue the concentrations of GLUT-4 protein and mRNA are markedly decreased after 2-3 weeks of diabetes, and they are restored by insulin therapy (Berger *et al.*, 1989; Garvey *et al.*, 1989), whereas in skeletal muscle the concentrations of GLUT-4 protein and mRNA are marginally altered (Garvey *et al.*, 1989; Bourey *et al.*, 1990). In liver, GLUT-2 is the main isoform of glucose transporters (Thorens *et al.*, 1988). Much less information is available concerning the expression of GLUT-2 in liver of diabetic rats, and the results are somewhat contradictory.

Electrophysiological changes during diabetes

Neuroelectrophysiological recordings represent a non-invasive and reproducible method of detecting central and peripheral nervous system alterations in diabetes mellitus (Morano *et al.*, 1996). Diabetes mellitus is associated with chronic complications such as nephropathy, angiopathy, retinopathy and peripheral neuropathy. In diabetic patients, hyperglycemia may precipitate seizures, and in experimental diabetes, indications for an increased neuronal excitability have been found (Anderson *et al.*, 2006). Neurophysiological alterations have also been described in animal models of diabetes, in particular in rats. In the peripheral nervous system (PNS) of diabetic rats the time course of neurophysiological changes is well established. Deficits in both motor and sensory nerve conduction velocity (MNCV and SNCV, respectively) can be detected within weeks after the onset of diabetes and increase up to 2–3 months after diabetes onset, remaining relatively stable thereafter (Moore *et al.*, 1980; Cameron *et al.*, 1986; Brismar *et al.*, 1987; Kappelle *et al.*, 1993). Studies of MNCV and SNCV in diabetic rats have made important contributions to the elucidation of the pathogenesis of the effects of diabetes on the PNS, as well as in the development of putative pharmacotherapy. Neurophysiological alterations have also been reported in the CNS of diabetic rats. Less is known about the underlying mechanisms of alterations in the CNS in diabetic rats. Cerebral metabolic (Knudsen *et al.*, 1989; Kumar and Menon, 1993) and vascular (Duckrow *et al.*, 1987; Jakobsen *et al.*, 1990) disturbances have been demonstrated within weeks after diabetes induction. However, the severity of these disturbances appears to be limited compared with the PNS (Biessels *et al.*, 1994), possibly leading to a less hostile neuronal microenvironment.

Recent pharmacological and gene-targeting studies have unraveled a wealth of knowledge about the diverse functions of the muscarinic acetylcholine receptor

subtypes. Based on these findings, many receptor subtype-selective ligands have been generated, some of which are clinically effective without any significant adverse effects. These approaches are very likely to lead to the future development of therapeutics for several disorders involving muscarinic acetylcholine receptor signals (Ishii & Kurachi, 2006). The present work is to understand the alterations of Muscarinic and Muscarinic M1 receptors in brain and pancreatic islets during diabetes and the regulation of insulin secretion by *Aegle marmelose* and *Costus pictus* leaf extracts. Studies on the alterations of Muscarinic and Muscarinic M1 receptors in hyperglycemia and the regulatory activity of these plant extracts on insulin secretion through Muscarinic receptors can be used as molecular data for therapeutic management of diabetes.

Materials and Methods

BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India.

CHEMICALS USED IN THE STUDY

Biochemicals: (Sigma Chemical Co., St. Louis, USA.)

Acetylthiocholine iodide, ethylene diamine tetra acetic acid-EDTA, HEPES - [n' (2-hydroxy ethyl)] piperazine-n'-[2-ethanesulfonic acid], citric acid, Tris HCl, foetal calf serum (heat inactivated), D-glucose, calcium chloride, collagenase type XI and bovine serum albumin fraction V, pirenzepine, atropine, RPMI-1640 medium.

Radiochemicals

Quinuclidinylbenzilate, L-[Benzilic-4,4'-^{3}H]-[4-^{3}H] (Sp. Activity 42 Ci/mmol) was purchased from NEN life sciences products Inc., Boston, U.S.A.

Radioimmunoassay kits for insulin and ^{14}C Glucose were purchased from Bhabha Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals

Random hexamers, Taq DNA polymerase, human placental RNAse inhibitor, dNTPS and DNA molecular weight markers were purchased from Bangalore Genei, India. Reverse transcriptase enzyme MuMLV, was obtained from Amersham Life Science, UK. Tri-reagent kit was purchased from MRC,

USA. PCR primers used in this study were synthesised by Sigma Chemical Co., St. Louis, USA. Real Time PCR Taqman probe assays on demand were purchased from Applied Biosystems, Foster City, CA, USA.

ANIMALS

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

PLANT MATERIAL

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

PREPARATION OF PLANT EXTRACTS

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

Aegle marmelose (L.) Correa.

Costus pictus, (D.) Don.

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 anesthesia (Junod *et al.*, 1969). Streptozotocin was given at a dose of 65mg/Kg body weight (Hohenegger & Rudas, 1971; Arison *et al.*, 1967).

DETERMINATION OF BLOOD GLUCOSE

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

DETERMINATION OF ANTI-DIABETIC POTENTIAL OF *Aegle marmelose* AND *Costus pictus* LEAF EXTRACTS

Animals used in this study were randomly divided into the following groups. Each group consisted of 4-6 animals.

a) Group 1: Control (given citrate buffer injection)
b) Group 2: Diabetic
c) Group 3: Diabetic rats treated with insulin
d) Group 4: Diabetic rats treated with aqueous extract of *Aegle marmelose* leaves
e) Group 5: Diabetic rats treated with aqueous extract of *Costus pictus* leaves

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 (Sasaki & Bunag, 1983). The last injection was given 24 hr before sacrificing the diabetic rats.

Aqueous extract of *Aegle marmelose* was given orally to the 4th group of diabetic rats in the dosage of 1g/Kg body weight (Ponnachan et al.,1993) at 24 hour intervals. Aqueous extract of *Costus pictus* leaves was given orally to the 5th group of diabetic rats in the dosage of 250mg/ Kg body weight. Blood samples were collected from the tail vein at 0 hours (Before the start of the experiment), 3rd day, 6th day, 10th day and 14th 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 1st Day (before the start of the experiment), 7th day and 15th day.

GLUCOSE TOLERANCE TEST

Normal Wistar rats were fasted overnight. They were divided into four groups containing four to five animals each. Initial serum glucose was estimated by collecting the blood from tail vain. Control rats (Group 1) were given glucose (2g/ Kg, body weight) orally. Three different concentrations (100, 250 and 500 mg/ Kg, body weight) of aqueous extract of *Costus pictus* leaves was administered orally 30 minutes before oral administration of 2g/ Kg glucose solution to second, third and fourth groups respectively. Blood samples were collected from the tail vein at 30, 60 and 120 min. after the glucose loading and serum glucose levels were measured.

SACRIFICE AND TISSUE PREPARATION

The animals were then sacrificed on 15th day by decapitation. The cerebral cortex, corpus striatum, brain stem, cerebellum and hypothalamus were dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966). The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -70° C until assay.

ESTIMATION OF BLOOD GLUCOSE

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

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

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

$$H_2O_2 + \text{Phenol} \quad \text{4-aminoantipyrene} \xrightarrow{\text{(Peroxidase)}} \text{Coloured complex} + H_2O$$
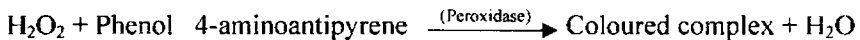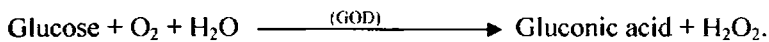
The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm in a spectrophotometer (Milton Roy Genesys 5 Spectronic).

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [^{125}I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the

antibody bound and free insulin were separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples.

Assay Protocol

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

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

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

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

ACETYLCHOLINE ESTERASE ASSAY

Acetylcholine esterase asssay was done using the spectrophotometric method of Ellman *et al.*, (1961). The homogenate (10%) was prepared in sodium phosphate buffer (30mM, pH-7). One ml of 1% Triton x 100 was added to the homogenate to release the membrane bound enzyme and centrifuged at 10,000

rpm for 30 minutes at 4°C. Different concentrations of acetylthiocholine iodide were used as substrate. The mercaptan formed as a result of the hydrolysis of the ester reacts with an oxidising agent 5,5' -dithiobis (2-Nitrobenzoate) absorbs at 412 nm.

MUSCARINIC RECEPTOR BINDING STUDIES USING [3H] RADIOLIGANDS

Binding studies in the Brain regions
[3H]QNB binding

[3H]QNB binding assay in cerebral cortex (CC), brain stem (BS), corpus striatum (CS) and hypothalamus (HYPO) was done according to the modified procedure of Yamamura & Snyder (1981). Brain tissues were homogenised in a polytron homogeniser 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 receptor binding parameter assays were done using different concentrations i.e., 0.1-2.5nM of [3H] QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non-specific binding was determined using 100µM Atropine. 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. Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

Muscarinic M1 receptor binding assays were done using different concentrations i.e., 0.1-2.5nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non-specific binding was determined using 100µM pirenzepine. Tubes were incubated at 220C 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. Protein was measured by the method of Lowry et al., (1951) using bovine serum albumin as standard.

Muscarinic M1 receptor binding studies in the Pancreatic islets

[^{3}H] QNB binding
Isolation of pancreatic islets

Pancreatic islets were isolated from male weanling Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell, 1968). The pancreas was aseptically dissected out into a sterile petridish containing ice cold HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers, 1985) with the following composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM CaCl$_2$, 0.8mM MgSO$_4$, 1mM KH$_2$PO$_4$, 14.3mM KHCO$_3$ and 10mM HEPES. Autoclaved triple distilled water was used in the preparation of the buffer. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2ml 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. Islets visible as yellowish white

spheres were were then homogenised for 30seconds in a polytron homogeniser with 10 ml medium consisting of 50mM Na_2HPO_4/NaH_2PO_4 and 2mM $MgCl_2$ with the addition of BSA (1mg/ml), bacitracin (0.2mg/ml), aprotinin (500 kallikrein inhibitor units/ml), pH 7.4. The homogenate was then centrifuged at 30,000xg for 30 minutes, the pellets were resuspended in appropriate volume of the same buffer. Binding assays were done using different concentrations i.e., 0.1-5nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250μl containing 250-300μg protein. Non-specific binding was determined using 100μM pirenzepine. Competition studies were carried out with 3.5nM [^{3}H]QNB in each tube with pirenzepine concentrations varying from 10^{-9} - 10^{-4}M of pirenzepine. Tubes were incubated at 22°C for 2 hours and after incubation filtered rapidly through GF/C filters (Whatman). The filters were washed with ice cold phosphate assay buffer. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

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 radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

Nonlinear regression analysis for displacement curve

The displacement data were analysed by nonlinear regression using GraphPad Prism software, GraphPad Inc., USA. The concentration of the competing drug that competes for half the specific binding was defined as EC_{50} which is same as IC_{50} (Unnerstall, 1990). The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors (Cheng, 1973).

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Isolation of RNA

RNA was isolated from the brain regions of control and experimental rats using the Tri reagent (MRC., USA). 25-50 mg tissue homogenates were made in 0.5 ml Tri Reagent. The homogenate was kept in the room temperature for 5 minutes. 50µl of bromochloropropane (BCP) was added to the homogenate, 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 8 minutes at 4°C. RNA precipitate forms 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 as one absorbance $_{260}$ = 42µg.

RT PCR Primers

The following primers were used for muscarinic M1, receptor and β-actin RT-PCR studies.

5'- GCA.CAG.GCA.CCC.ACC.AAG.CAG -3'

5'- AGA GCA GCA GCA GGC GGA ACG -3'   M1

PRODUCT SIZE: 373 bp

5'- CAA CTT TAC CTT GGC CAC TAC C -3'

5'- TAC GAC TGC AAA CAC TCT ACA CC -3'   β-ACTIN

PRODUCT SIZE: 150bp

RT-PCR of M1 receptor and β-actin

RT-PCR was carried out in a total reaction volume of 20µl reaction mixture in 0.2ml tubes. RT-PCR was performed in an Eppendorf Personal thermocycler. cDNA synthesis of 2µg RNA was performed in a reaction mixture containing 40 Units of MuMLV reverse transcriptase, 2mM dithiothreitol, 4 Units of human placental RNAse inhibitor, 0.5µg of random hexamer and 0.25mM dNTPS (dATP, dCTP, dGTP and dTTP). The tubes were then incubated at 37°C for one hour. After incubation the reverse transcriptase, MuMLV, was inactivated by heating at a temperature of 95°C.

Polymerase Chain Reaction of M1 receptor and β–actin

Polymerase Chain Reaction (PCR) was carried out in a 20μl volume reaction mixture containing 4μl of cDNA, 0.25mM dNTPS - dATP, dCTP, dGTP and dTTP, 0.5units of Taq DNA polymerase and 10 picomoles of specific primers.

Thermocycling profile used for PCR of M1 receptor

94°C -- 5 min   --- Initial Denaturation

94°C -- 1 min   --- Denaturation

62°C -- 1 min   --- Annealing   30 cycles

72°C -- 1 min   --- Extension

72°C -- 7 min   --- Final Extension

Thermocycling profile used for PCR of β- actin

94°C -- 5 min   --- Initial Denaturation

94°C -- 30 sec   --- Denaturation

58°C -- 30 sec   --- Annealing   30 cycles

72°C -- 30 sec   --- Extension

72°C -- 5 min   --- Final Extension

Analysis of RT-PCR products

The Polymerase Chain Reaction product was loaded on a 2.0% agarose gel with ethidium bromide. Bromophenol blue was used as the indicator dye. 48V current was used for all the run. The image was captured using an Imagemaster gel documentation system (Pharmacia Biotech) and the bands were densitometrically analysed using Gel Quant software. Muscarinic M1 receptor mRNA expression in the brain regions-CS, CC, HYPO and BS of control and experimental rats were analysed.

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.

Quantitative real-time PCR assays

Real-time PCR assays were performed in 96-well plates in a ABI 7300 real-time PCR instrument (Applied Biosystems). 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 TaqMan probe for Muscarinic M1 gene and endogenous control (β-actin) and 12.5 µl of Taqman 2X Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNAse free water. The thermal cycle conditions were as follows:

50 °C	---	2 min	
95 °C	---	10 min	
95 °C	---	15 sec	40 cycles
60 °C	---	01 min	

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).

INSULIN SECRETION STUDIES WITH *Aegle marmelose*, *Costus pictus* LEAF EXTRACTS AND CHOLINERGIC AGONIST CARBACHOL *IN VITRO*

Pancreatic islets were isolated by collagenase digestion and islets were incubated in RPMI-1640 medium for 16 hours in $5\%CO_2$ at 37°C for fibroblast attachment. Islets were harvested after 16 hours and used for secretion studies.

Insulin secretion study - 1 hour

Islets were harvested after removing the fibroblasts and resuspended in Krebs Ringer Bicarbonater buffer, pH 7.3 (KRB). To study the effect of aqueous extract of *Aegle marmelose* leaves , aqueous extract of *Costus pictus* leaves and cholinergic agonist carbachol on insulin secretion, the isolated islets were incubated for 1hour at 37°C with 0.25, 0.5, 1, 2 and 5mg/ ml concentrations of *Aegle marmelose* , 0.25, 0.5, 1, 2 and 5mg/ ml concentrations of *Costus pictus* leaf extract , 10^{-7} M concentrations of carbachol and two different concentrations of glucose i.e., (i) 4mM glucose and (ii) 20mM glucose. After incubation, cells were centrifuged at 1,500xg for 10 minutes at 4°C and the supernatant were transferred to fresh tubes for insulin assay by radioimmunoassay.

Insulin secretion study - 24 hours

The islets were harvested after removing the fibroblasts and cultured for 24hours in RPMI-1640 medium. Insulin secretion study was carried out by preincubating the cells in 4mM and 20mM glucose concentrations with 0.25, 0.5,

1, 2 and 5mg/ ml concentrations of *Aegle marmelose*, 0.25, 0.5, 1, 2 and 5mg/ ml concentrations of *Costus pictus* leaf extract, 10^{-7} M concentrations of carbachol. The cells were then harvested and washed with fresh KRB and then incubated for another 1 hour in the presence of same concentrations of glucose, carbachol, *Aegle marmelose* leaf extract and Costus *pictus* leaf extract. At the end of incubation period the medium was collected and insulin content was measured by RIA method using kit from BARC, Mumbai.

MEASUREMENT OF GLUCOSE UPTAKE ACTIVITY

Glucose uptake activity was measured using D-[^{14}C] glucose by the modified procedure of Crane & Mandelstan, (1960). After dissection slices of liver and cerebral cortex of control and experimental rats were transferred to 3 ml of Kreb's Ringer Buffer (KRB), pH 7.4, containing 8mM glucose and 32mM mannitol, 0.1 % bovine serum albumin and 2mM sodium pyruvate. These tissues are then incubated for 30 minutes and one hour at 37°C with the addition of 20,000dpm/tube radio labelled glucose. The gas phase in the flasks during incubation period was 95% oxygen and 5% CO_2. After incubation, tissues were removed, washed in incubation medium to remove unbound D-[^{14}C] glucose and hydrolysed in 0.5 ml 1M NaOH(85°C, 1 h) and counted for ^{14}C radioactivity with cocktail-T in a Wallac 1409 liquid scintillation counter . ^{14}C glucose uptake was expressed as dpm/mg protein.

EEG ANALYSIS IN CONTROL AND EXPERIMENTAL RATS

Spontaneous electrical activity of brain regions of the control and experimental rats were carried with Neurocare TM Wingraph Digital EEG system. EEG analysis was done by placing electrodes in right and left frontal, parietal, occipital and temporal areas of the scalp of experimental rats and electrode placed on the ear was considered as reference. Each electrode was placed 10-20 percent

away from the neighbouring electrode. The EEG recording data were analysed for the brain activity in different brain areas of control and experimental rats.

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).

Results

BODY WEIGHT

The body weight was significantly decreased ($p<0.001$) in the diabetic rats when compared to control group. After insulin treatment, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves supplementation for 14 days, the body weight was reversed to near the initial level (Table-1).

BLOOD GLUCOSE LEVEL

Blood glucose level of all rats before streptozotocin administration was within the normal range. Streptozotocin administration led to a significant increase ($p<0.001$) in blood glucose level of diabetic group when compared to control group. Insulin treatment, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves treatment was able to significantly reduce ($p<0.001$) the increased blood glucose level to near the control value when compared to diabetic group (Table-2).

CIRCULATING INSULIN LEVEL

There was a significant decrease in the plasma insulin level of the diabetic group when compared to control group ($p<0.001$). Insulin treatment, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves supplementation for 14 days significantly increased ($p<0.001$) the plasma insulin level to near control level when compared to diabetic group (Figure-1).

GLUCOSE TOLERANCE BY *Costus pictus* LEAF EXTRACT

Administration of 2.0 g glucose/ Kg body wt. to normal rats increased serum glucose levels from 81.73 to 133.6 mg/dl after 60 minutes but returned to near control value after 120 minutes. Administration of 250 mg/Kg of *Costus pictus* leaf extract decreased the elevation of serum glucose level significantly (p<0.001) at 60 min, whereas 100mg/ Kg body wt. and 500mg/ Kg body wt. reduced the elevated glucose level to near control value after 120 minutes (Figure-2).

ACETYLCHOLINE ESTERASE ACTIVITY IN THE BRAIN REGIONS OF EXPERIMENTAL RATS

Cerebral cortex

Acetylcholine esterase kinetics studies showed that V_{max} significantly increased (p<0.05) in the cerebral cortex of diabetic group with significant decrease (p<0.05) in the K_m when compared to control. Insulin and aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the V_{max} (p<0.05) to near control value when compared to diabetic group. There was no significant reversal in the K_m of treated diabetic rats (Table-3, Figure-3). Aqueous extract of *Costus pictus* leaves treatment was able to significantly reversed the V_{max} (p<0.05) to near control value when compared to diabetic group. There was no significant reduction in the K_m of treated diabetic rats (Table-4, Figure-4).

Brainstem

The V_{max} of AChE significantly increased (p<0.05) in the brainstem of diabetic rats with no significant change in K_m when compared to control group. Insulin treatment and aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the V_{max} (p<0.05) to near control value when compared to diabetic group

(Table-5, Figure-5). Aqueous extract of *Costus pictus* leaves treatment significantly reverse the V_{max} (p<0.05) to near control value when compared to diabetic group (Table-6, Figure-6).

Corpus striatum

Acetylcholine esterase kinetics studies showed that V_{max} was significantly decreased (p<0.001) in the corpus striatum of diabetic group with with no significant change in K_m. Insulin treatment and aqueous extract of *Aegle marmelose* leaves treatment was significantly reverse the V_{max} (p<0.001) to near control value when compared to diabetic group (Table-7, Figure-7). Aqueous extract of *Costus pictus* leaves treatment was significantly reverse the V_{max} (p<0.001) to near control value when compared to diabetic group (Table-8, Figure-8).

Hypothalamus

Acetylcholine esterase kinetics studies showed that V_{max} was significantly increased (p<0.001) in the hypothalamus of diabetic group with a significant decrease in K_m (p<0.05) when compared to control group. Insulin treatment was able to significantly reverse the V_{max} (p<0.001) to near control value when compared to diabetic group. There was no significant reversal in the K_m of insulin treated diabetic rats. The aqueous extract of *Aegle marmelose* leaves treatment was able to significantly reverse the V_{max} (p<0.001) with no significant change in K_m to near control value when compared to diabetic group (Table-9, Fig.-9). Aqueous extract of *Costus pictus* leaves treatment significantly reverse the V_{max} (p<0.001) to near control value with no significant change in K_m when compared to diabetic group (Table-10, Fig.-10).

CENTRAL MUSCARINIC RECEPTOR ALTERATIONS DURING DIABETES AND AFTER THE TREATMENT WITH INSULIN, *Aegle marmelose* AND *Costus pictus* LEAF EXTRACTS

Cerebral cortex

I) Total Muscarinic receptor analysis

a) Scatchard analysis of [^{3}H] QNB binding against atropine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated, Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic 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} was decreased significantly ($p<0.001$) in diabetic rats with a significant decrease ($p<0.01$) in the K_d when compared to control group. In insulin treated diabetic rats B_{max} significantly ($p<0.001$) reversed to near control value when compared to diabetic group. K_d also significantly ($p<0.01$) reversed to near control value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the B_{max} ($p<0.001$) and K_d ($p<0.01$) to near control value when compared to diabetic group (Table-11 & Fig-11). Aqueous extract of *Costus pictus* leaves treatment also significantly reverse the B_{max} ($p<0.01$) and K_d ($p<0.01$) to near control value when compared to diabetic group (Table-13 & Fig-13).

b) Displacement analysis of [^{3}H]QNB using atropine

The competition curve for atropine against [^{3}H]QNB fitted for one sited model in all groups. The log (EC_{50}) did not change in all the experimental groups. The K_i decreased in diabetic condition and reversed back to near control value in

insulin, *Aegle marmelose* and *Costus pictus* leaf extract treated diabetic rats (Table-12 & Fig-12; Table-14 & Fig-14).

II) Muscarinic M1 receptor analysis

a) Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated , Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic rats

Binding analysis of Muscarinic M1 receptors was done using [^{3}H]QNB and M1 subtype specific antagonist pirenzepine. The B_{max} was decreased significantly ($p<0.001$) in diabetic group when compared to control group. The K_d also decreased significantly when compared to control group ($p<0.01$). In insulin treated diabetic rats B_{max} was significantly ($p<0.001$) reversed back to near control value when compared to diabetic group. K_d also significantly ($p<0.01$) reversed back to near control value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the B_{max} ($p<0.001$) and K_d ($p<0.01$) to near control value when compared to diabetic group (Table-15 & Fig-15). Aqueous extract of *Costus pictus* leaves treatment significantly reverse the B_{max} ($p<0.001$) and K_d ($p<0.05$) to near control value when compared to diabetic group (Table-19 & Fig-19).

b) Displacement analysis of [^{3}H]QNB using pirenzepine

The competition curve for pirenzepine against [^{3}H]QNB fitted for one sited model in all groups. The log (EC_{50}) did not change in all the experimental groups. The K_i decreased in diabetic condition and reversed back to near control value in

insulin, *Aegle marmelose* and *Costus pictus* treated diabetic rats (Table-16 & Fig-16 & Table-20 & Fig-20).

III) RT- PCR and Real Time-PCR analysis

RT-PCR and Real Time -PCR analysis showed that the muscarinic M1 receptor mRNA decreased in diabetic condition and it reversed to near control value in insulin treated, *Aegle marmelose* and *Costus pictus* treated diabetic rats (Table-17 & Fig-17, Table-18 & Fig-18; Table-21 & Fig-21, Table-22 & Fig-22).

Brainstem

I) Total Muscarinic receptor analysis

a) *Scatchard analysis of [³H] QNB binding against atropine in the brainstem of Control, Diabetic, Diabetic+Insulin treated , Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic rats*

The Scatchard analysis showed that the B_{max} was increased significantly ($p<0.001$) in the brainstem of diabetic rats with a significant decrease ($p<0.001$) in the K_d when compared to control rats. In insulin treated diabetic rats, B_{max} was significantly ($p<0.01$) reversed back to near control value when compared to diabetic group but there is no reversal of K_d value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment was able to significantly reverse the B_{max} ($p<0.01$) to near control value when compared to diabetic group with no significant change in the K_d (Table-23 & Fig-23). Aqueous extract of *Costus pictus* leaves treatment significantly reverse the B_{max} ($p<0.01$) to near control value when compared to diabetic group without any change in K_d value (Table-25 & Fig.25).

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b) Displacement analysis of [³H]QNB using Atropine

In the displacement analysis, the competitive curve fitted to a one-sited model in all groups with Hill slope values near to unity. The log (EC_{50}) did not alter in all the experimental groups. The K_i showed an increase in diabetic condition (Table24- & Fig-24, Table- 26 & Fig- 26).

II) Muscarinic M1 receptor analysis

a) Scatchard analysis of [³H] QNB binding against pirenzepine in the brainstem of Control, Diabetic, Diabetic+Insulin treated, Diabetic+Aegle marmelose and Diabetic+Costus pictus treated rats

The Scatchard analysis showed that the B_{max} was decreased significantly $(p<0.01)$ in diabetic condition when compared to control group. The K_d also decreased significantly when compared to control group $(p<0.05)$. In insulin treated diabetic condition B_{max} and K_d was significantly $(p<0.05)$ reversed back to near control value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment to significantly reverse the B_{max} $(p<0.01)$ to near control value when compared to diabetic group. The K_d value also reversed back to near control value when compared to diabetic group $(p<0.05)$ (Table27- & Fig-27). Aqueous extract of *Costus pictus* leaves treatment also significantly reversed the B_{max} $(p<0.001)$ and K_d to near control value when compared to diabetic group (Table-31 & Fig-31).

b) Displacement analysis of [^{3}H]QNB using pirenzepine

The competition curve for pirenzepine against [^{3}H]QNB fitted for one sited model in all groups. The log (EC_{50}) did not change in all the experimental groups. The K_i decreased in diabetic condition and reversed back to near control value in insulin, *Aegle marmelose* and *Costus pictus* treated diabetic rats (Table-28 & Fig-28; Table-32 & Fig- 32).

III) RT-PCR and Real Time-PCR analysis

RT-PCR and Real Time -PCR analysis showed that the muscarinic M1 receptor mRNA decreased in diabetic condition and it reversed to control in insulin treated, *Aegle marmelose* and Costus pictus treated diabetic rats (Table-29 & Fig-29, Table-30 & Fig-30: Table-33 & Fig-33, Table-34 & Fig-34).

Hypothalamus

I) Total Muscarinic receptor analysis

a) Scatchard analysis of [^{3}H] QNB binding against atropine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated , Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic rats

The Scatchard analysis for hypothalamic total muscarinic receptor status showed that the B_{max} was increased significantly (p<0.001) in diabetic condition with a significant decrease (p<0.01) in the K_d when compared to control group. In insulin treated diabetic condition B_{max} was significantly (p<0.001) reversed back to near control value when compared to diabetic group but there is no reversal of K_d value

when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the B_{max} (p<0.001) to near control value when compared to diabetic group without any change in K_d (Table-35 & Fig-35). Aqueous extract of *Costus pictus* leaves treatment significantly reverse the B_{max} (p<0.001) to near control value when compared to diabetic group with out a change in K_d value (Table-37 & Fig-37).

b) Displacement analysis of [^3H]QNB using Atropine

In the displacement analysis, the competitive curve fitted to a one-sited model in all groups with Hill slope values near to unity. The log (EC_{50}) did not alter in all the experimental groups. The K_i decreased in diabetic condition and reversed back to near control value in insulin, *Aegle marmelose* and *Costus pictus* treated diabetic rats (Table-36 & Fig-36; Table-38& Fig-38).

II) Muscarinic M1 receptor analysis

a) Scatchard analysis of [^3H] QNB binding against pirenzepine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated, Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic rats

Scatchard analysis for hypothalamus muscarinic M1 receptors showed that there was a significant decrease in B_{max} (p<0.001) and K_d (p<0.001) in diabetic rats when compared to control group. In insulin treated diabetic condition B_{max} and K_d was significantly (p<0.001) reversed back to near control value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the B_{max} (p<0.001) and K_d (p<0.05) to near control value when compared to diabetic group (Table-39 & Fig-39). Aqueous extract of *Costus pictus* leaves treatment

significantly reverse the B_{max} (p<0.001) and K_d (p<0.05) to near control value when compared to diabetic group (Table-43 & Fig-43).

b) Displacement analysis of [^{3}H] QNB using pirenzepine

In the displacement analysis, the competitive curve fitted to a one-sited model in all groups with Hill slope values near to unity. The log (EC_{50}) did not alter in all the experimental groups. The K_i showed a decrease in diabetic condition (Table 40- & Fig-40; Table 44- & Fig-44).

III) RT- PCR and Real Time-PCR analysis

RT-PCR and Real Time -PCR analysis showed that the muscarinic M1 receptor mRNA decreased in diabetic condition and it reversed to control level in insulin treated, *Aegle marmelose* and *Costus pictus* treated diabetic rats (Table-41 & Fig-41 & Table-42 & Fig-42; Table-45 & Fig-45, Table-46 & Fig-46).

Corpus striatum

I) Total Muscarinic receptor analysis

a) Scatchard analysis of [^{3}H] QNB binding against atropine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated , Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic rats

The Scatchard analysis showed that the B_{max} and K_d of the [^{3}H]QNB receptor binding decreased significantly (p<0.001) in the corpus striatum of diabetic rats when compared to control group. In insulin treated diabetic group B_{max} and K_d was significantly (p<0.001) reversed back to near control value when compared to diabetic

group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reverse the B_{max} and K_d (p<0.001) to near control value when compared to diabetic group (Table-47 & Fig-47). Aqueous extract of *Costus pictus* leaves treatment significantly reverse the B_{max} and K_d (p<0.001) to near control value when compared to diabetic group (Table-49 & Fig-49).

b) Displacement analysis of [³H]QNB using Atropine

In the displacement analysis, the competitive curve fitted to a one-sited model in all groups with Hill slope values near to unity. The log (EC_{50}) did not alter in all the experimental groups. The K_i decreased in diabetic condition (Table-48 & Fig-48; Table-50 & Fig-50).

II) Muscarinic M1 receptor analysis

a) Scatchard analysis of [³H] QNB binding against pirenzepine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated, Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic rats

The Scatchard analysis showed that the B_{max} of muscarinic M1 receptors of corpus striatum was increased significantly (p<0.001) in diabetic condition when compared to control group while the K_d was decreased significantly when compared to control group (p<0.001). In insulin treated diabetic rats B_{max} was significantly (p<0.001) reversed back to near control value when compared to diabetic group but K_d was not reversed back to near control value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment was able to significantly reversed the B_{max} (p<0.001) and K_d (p<0.001) to near control value when compared to diabetic group (Table-51 & Fig-51). Aqueous extract of *Costus pictus* leaves

treatment significantly reverse the B_{max} (p<0.001) and K_d (p<0.001) to near control value when compared to diabetic group (Table-55 & Fig-55).

b) Displacement analysis of [^{3}H]QNB using pirenzepine

In the displacement analysis, the competitive curve fitted to a one-site model in all the experimental conditions. Hill slopes were near unity confirming the one-site model. There were no changes in the log (EC_{50}) values. The K_i value decreased in diabetic condition (Table-52 & Fig-52; Table-56 & Fig-56).

III) RT- PCR and Real Time -PCR analysis

RT-PCR and Real Time -PCR analysis showed that the muscarinic M1 receptor mRNA increased in diabetic condition and it reversed to control value in insulin treated, *Aegle marmelose* and *Costus pictus* treated diabetic rats (Table-53 & Fig-53, Table-54 & Fig-54; Table-57 & Fig-57, Table-58 & Fig-58).

PANCREATIC MUSCARINIC RECEPTOR ALTERATIONS DURING DIABETES AND AFTER THE TREATMENT WITH INSULIN, *Aegle marmelose* AND *Costus pictus* LEAF EXTRACTS

I) Muscarinic M1 receptor analysis

a) Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the pancreatic islets of Control, Diabetic, Diabetic+Insulin treated, Diabetic+Aegle marmelose and Diabetic+Costus pictus treated diabetic rats

Scatchard analysis for pancreatic islets muscarinic M1 receptors showed that the B_{max} was decreased significantly (p<0.01) in diabetic rats when compared to

control group while the K_d was increased significantly ($p<0.05$) when compared to control group. In insulin treated diabetic condition B_{max} and K_d was significantly ($p<0.05$) reversed to near control value when compared to diabetic group. Aqueous extract of *Aegle marmelose* leaves treatment significantly reversed the B_{max} and K_d ($p<0.05$) to near control value when compared to diabetic group (Table-59 & Fig-59). Aqueous extract of *Costus pictus* leaves treatment also significantly reverse the B_{max} and K_d ($p<0.05$) to near control value when compared to diabetic group (Table-62 & Fig-62).

b) Displacement analysis of [³H] QNB using pirenzepine

In the displacement analysis, the competitive curve fitted to a one-site model in all the experimental conditions. Hill slopes were near unity confirming the one-site model. There were no changes in the log (EC_{50}) values. The Ki value decreased in diabetic condition (Table-60 & Fig-60; Table-63 & Fig-63).

II) RT- PCR and Real Time -PCR analysis

RT-PCR and Real Time -PCR analysis showed that the muscarinic M1 receptor mRNA increased in diabetic condition and it reversed to near control value in insulin treated, *Aegle marmelose* and *Costus pictus* treated diabetic rats (Table-61 & Fig-61, Table-64 & Fig-64).

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS

Effect of *Aegle marmelose* leaf extract on 1 hour Glucose Induced Insulin Secretion *in vitro*

The isolated islets incubated for 1 hour with 0.25, 0.5, 1, 2 and 5mg/ml concentrations of *Aegle marmelose* leaf extract and two different concentrations of

glucose, 4mM and 20mM. In 4mM glucose concentration, *Aegle marmelose* leaf extract significantly increased (p<0.001) insulin secretion in all the concentrations in a dose dependent manner when compared to control. In 20mM glucose concentration *Aegle marmelose* leaf extract significantly increased (p<0.001) insulin secretion when compared to control. Maximum insulin secretion was obtained at a concentration of 2mg/ml concentration in both the glucose concentrations (Fig -65).

Effect of *Aegle marmelose* leaf extract on 24 hours Glucose Induced Insulin Secretion *in vitro*

Islets were incubated with 0.25, 0.5, 1, 2 and 5mg/ml concentrations of *Aegle marmelose* leaf extract and two different concentrations of glucose, 4mM and 20mM in 24 hours *in vitro* culture. In the long term incubation studies also *Aegle marmelose* leaf extract significantly increased glucose induced insulin secretion by all the concentrations except higher concentration (5mg/ml) where there was an inhibition of insulin secretion. In 4mM glucose concentration the maximum insulin secretion was obtained at a concentration of 1mg/ ml of *Aegle marmelose* leaf extract (p<0.001) . In 20mM glucose concentration 0.5 & 1mg/ ml concentration of *Aegle marmelose* leaf extract significantly increased (p<0.001) insulin secretion when compared to control with maximum insulin secretion obtained at a concentration of 1mg/ml in both the glucose concentrations (Fig -66).

Effect of *Costus pictus* leaf extract on 1 hour Glucose Induced Insulin Secretion *in vitro*

The isolated islets incubated for 1 hour with 0.25, 0.5, 1, 2 and 5mg/ml concentrations of *Costus pictus* leaf extract and two different concentrations of glucose, 4mM and 20mM. The result showed that *Costus pictus* leaf extract

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significantly increased (p<0.001) glucose induced insulin secretion in all the concentrations at both 4mM and 20mM glucose with maximum secretion obtained at a concentration of 1mg/ ml (Fig -67).

Effect of *Costus pictus* leaf extract on 24 hours Glucose Induced Insulin Secretion *in vitro*

Islets were incubated with 0.25, 0.5, 1, 2 and 5mg/ml concentrations of *Costus pictus* leaf extract and two different concentrations of glucose, 4mM and 20mM in 24 hours *in vitro* culture. In the long term incubation studies also *Costus pictus* leaf extract significantly increased (p<0.001) glucose induced insulin secretion in all the concentrations at both 4mM and 20mM glucose with maximum secretion obtained at a concentration of 0.5mg/ ml (Fig -68).

Effect of Cholinergic Agonist Carbachol and *Aegle marmelose* leaf extract on 1 hour Glucose induced Insulin Secretion *in vitro*

The isolated islets incubated for 1 hour with 0.5, 1, 2 and 5mg/ml concentrations of *Aegle marmelose* leaf extract in the presence of 10^{-7} M concentrations of carbachol at two different concentrations of glucose, 4mM and 20mM. It showed that of all the concentrations of *Aegle marmelose* leaf extract in the presence of 10^{-7} M carbachol significantly enhanced (p<0.001) glucose induced insulin secretion at both 4mM and 20mM glucose concentration. The maximum insulin secretion obtained at a concentration of 2 mg/ml and 10^{-7} M Carbachol (Fig-69).

Effect of Cholinergic Agonist Carbachol and *Aegle marmelose* leaf extract on 24 hours Glucose induced Insulin Secretion *in vitro*

Islets were incubated with 0.25, 0.5, 1, 2 and 5mg/ml concentrations of *Aegle marmelose* leaf extract in the presence of 10^{-7} M concentrations of carbachol and two different concentrations of glucose, 4mM and 20mM in 24 hours *in vitro* culture. It showed that the maximum insulin secretion obtained at a concentrations of 2 mg/ml concentrations of *Aegle marmelose* leaf extract and 10^{-7} M Carbachol combinations at both 4mM and 20mM glucose concentration (p<0.001) (Fig-70).

Effect of Cholinergic Agonist Carbachol and *Costus pictus* leaf extract on 1 hour Glucose induced Insulin Secretion *in vitro*

The isolated islets incubated for 1 hour with 0.5, 1, 2 and 5mg/ml concentrations of *Costus pictus* leaf extract in the presence of 10^{-7} M concentrations of carbachol at two different concentrations of glucose, 4mM and 20mM. It showed that all the concentrations of *Costus pictus* leaf extract in the presence of 10^{-7} M carbachol significantly enhanced (p<0.001) glucose induced insulin secretion at both 4mM and 20mM glucose concentration. In 4mM glucose concentration the maximum insulin secretion obtained at concentrations of 2 mg/ml and 10^{-7} M Carbachol. In 20mM glucose concentration the maximum insulin secretion obtained at a concentration of 1mg/ml and 10^{-7} M Carbachol (Fig-71).

Effect of Cholinergic Agonist Carbachol and *Costus pictus* leaf extract on 24 hours Glucose induced Insulin Secretion *in vitro*

Islets were incubated with 0.5, 1, 2 and 5mg/ml concentrations of *Costus pictus* leaf extract in the presence of 10^{-7} M concentrations of carbachol and two different concentrations of glucose, 4mM and 20mM in 24 hours *in vitro* culture. It

showed that all the concentrations of *Costus pictus* leaf extract and Carbachol combinations enhanced glucose induced insulin secretion at both 4mM and 20mM glucose concentration. The maximum insulin secretion was obtained at a concentrations of 2 mg/ml and 10^{-7} M Carbachol (p<0.001) (Fig-72).

Effect of plant extracts on glucose uptake by liver and cerebral cortex of experimental rats

Fourteen days after various drug treatment (insulin, aqueous extract of *Aegle marmelose* and *Costus pictus* leaves) liver and cerebral cortex were incubated with ^{14}C glucose (20,000dpm/ tube for 30 minutes and one hour). The liver of diabetic rats showed a significant decrease in ^{14}C glucose uptake when compared with control liver after 30 minutes (p<0.001) and 1 hour incubation (p<0.05). Insulin treatment Treatment with *Aegle marmelose* and Costus *pictus* leaf extract significantly increased (p<0.05) ^{14}C glucose uptake when compared with diabetic group after 1 hour incubation (Table-65). The cerebral cortex of diabetic rats showed a significant decrease (p<0.05) in ^{14}C glucose uptake when compared with control cerebral cortex after 30 minute and 1 hour incubation (p<0.01). Insulin treatment Treatment with *Aegle marmelose* (p<0.05) *Costus pictus* significantly increased (p<0.05) ^{14}C glucose uptake when compared with diabetic group after 1 hour incubation (Table-66).

Electroencephalogram analysis in experimental rats

Electroencephalogram analysis showed that there is a change in the brain activity of frontal, parietal, occipital and temporal areas of diabetic rats when compared to control rats. Treatment with insulin, *Aegle marmelose* and *Costus pictus* leaf extract reversed the brain activity to near control range (Fig. 73 - Fig. 77).

Discussion

BLOOD GLUCOSE & BODY WEIGHT

Increased blood glucose and decreased body weight during diabetes is similar with previous reports as a result of the marked destruction of insulin secreting pancreatic islet β-cells by streptozotocin (Junod *et al.*, 1969). Hyperglycemia occurs as a result of increased glycogenolysis, decreased glycogenesis, increased gluconeogenesis, impaired glucose transport across membranes and almost complete suppression of the conversion of glucose into fatty acids through acetyl-CoA. Our results showed that administration of *Aegle marmelose* and *Costus pictus* leaf extracts to STZ diabetic rats normalizes blood glucose levels. The glucose lowering activity of *Aegle marmelose* leaf extract confirmed the previous reports (Ponnachan *et al.*, 1993).

Glucose tolerance test was carried out to find out the effective dose (the quantity of leaf extract that can bring down the glucose level in the blood to the control level of *Costus pictus* leaf extract. *Costus pictus* leaf extract was administered to diabetic rats at a dose of 250mg/ Kg body weight and showed a significant glucose lowering activity. From this data it is clear that *Costus pictus* leaf extract has anti hyperglycemic activity. As far as the molecular action as well as pharmacological activities of *Costus pictus* leaf extract is concerned, no previous reports have been demonstrated. This is the first scientific study to demonstrate the anti hyperglycemic activity of *Costus pictus* leaf extract. The results suggest that the mode of action of the plant extract is probably mediated by an enhanced secretion of insulin and enhanced tissue glucose utilization. The decreased body weight in the diabetic rats is due to excessive breakdown of tissue proteins. Treatment of diabetic rats with insulin,

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Aegle marmelose and *Costus pictus* leaf extracts improved body weight significantly which indicate prevention of muscle tissue damage due to hyperglycemic condition.

CIRCULATING INSULIN LEVEL

There was a significant decrease in the circulating insulin level of diabetic rats when compared to control group. The increase in insulin levels in *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats attribute to the stimulation of the surviving beta cells by the extracts, which in turn exerts an antihyperglycemic action. Reports are available to show that antidiabetic plants are known to increase circulating insulin levels (Lamela *et al.*, 1985). Thus, it can be suggested that the *Aegle marmelose* and *Costus pictus* leaf extracts induce the release of insulin thereby potentiating its effect. A possible mechanism of action is that the extracts stimulate the residual pancreatic β-cell function or produced the antihyperglycemia through an extra-pancreatic mechanism, probably increasing peripheral utilization of glucose. This data confirmed the antihyperglycemic activity of *Aegle marmelose* and *Costus pictus* leaf extracts.

CENTRAL ACETYLCHOLINE ESTERASE ACTIVITY

Acetylcholine is the primary neurotransmitter of the cholinergic system, and its activity is regulated by acetylcholine esterase (AChE). The termination of nerve impulse transmission is accomplished through the degradation of acetylcholine into choline and acetyl CoA by AChE (Weihua Xie *et al.*, 2000). Acetylcholine esterase activity has been used as a marker for cholinergic activity (Goodman & Soliman, 1991; Ellman *et al.*, 1961)). It is well recognized that diabetes mellitus results in

altered membrane functions in several tissues (Alberti *et al.*, 1982; Osterby., 1988 and Striker *et al.*, 1993). Membrane alterations have been recognized as the underlying primary biochemical defect (Alberti *et al.*, 1982). It has been well established that there is a marked change in the acetylcholine esterase in diabetic condition. Akmayev *et al.*, (1978) showed that there is difference in distribution of the enzyme in the neurons of the central vagal nuclei and medulla oblongata in normal and diabetic adult male rats. It is suggested that the changes in the plasma glucose or insulin levels is influenced by the activity of cholinergic neurons. Thus central cholinergic activity will be implicated in the insulin secretion.

Central cholinergic activity was studied in experimental rats after using AChE as marker. Our results showed an increase in V_{max} and decrease in K_m in cerebral cortex and hypothalamus of diabetic rats when compared to control group. In brainstem there was an increase in V_{max} of diabetic group without a change in K_m when compared to control rats. This study support the reported delayed nerve transmission and impaired brain functions (Bartus *et al.*, 1982; Davis *et al.*, 1983; Clements, 1979; Carrington *et al.*, 1991). In corpus striatum of diabetic rats there was decrease in activity of enzyme when compared to control group. Decreased V_{max} without alteration in K_m for erythrocyte AChE from diabetic patients has been reported (Suhail & Rizvi, 1989). The decrease was observed to have a negative correlation with the blood glucose level.

The activation of central cholinergic system by administration of cholinergic agonist into the third cerebral ventricle reported to produce hyperglycemia in rats (Iguchi *et al.*, 1985). When carbachol, muscarine, bethanechol, methacholine, or neostigmine was injected into the third cerebral ventricle, it caused a dose-dependent increase in the hepatic venous plasma glucose concentration (Iguchi *et al.*, 1986). In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats

AChE activity was reversed back to near control value. Our results showed that diabetic state clearly influenced the kinetic properties of AChE enzyme and the reversal of AChE activity to near control value found in the insulin, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats brain regions is a compensatory mechanism to maintain the normoglycemic level.

CENTRAL MUSCARINIC RECEPTOR ALTERATIONS

Over the past decade, the role of muscarinic receptors in health was given much scientific study. The potential therapeutic value of various cholinergic agonists and antagonists have received increasing attention (Zwieten & Doods, 1995; Zwieten *et al.*, 1995). Muscarinic receptors are a family of G protein-coupled receptors that have a primary role in central cholinergic neurotransmission. Specific agonists, which activate postsynaptic muscarinic receptors, stimulate cholinergic signaling (Valentin *et al.*, 2006). It is known that different parts of the brain, particularly the hypothalamus and the brainstem, are important centers involved in the monitoring of glucose status The effect of the cholinergic agonist blocked by the muscarinic antagonist atropine shows the involvement of muscarinic receptors in the central cholinergic glucose homeostasis. The M1 muscarinic receptor is one of five known muscarinic subtypes in the cholinergic nervous system (Bonner *et al.*, 1987; Hulme *et al.*, 1990; van Zwieten & Doods, 1995). The M1, M2 and M4 subtypes of mAChRs are the predominant receptors in the CNS. These receptors activate a multitude of signaling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of Ach release (Volpivelli *et al.*, 2004).

Cerebral cortex

The RT-PCR and HPLC studies revealed that the M1 receptor was present in a relatively high density in the cerebral cortex (Jian *et al.*, 1994; Oki *et al.*, 2005). Cholinergic agonist carbachol normalized glucose-stimulated insulin secretion and glucose tolerance in mice subjected to a high-fat diet. Carbachol also potentiated glucose-stimulated insulin secretion from isolated islets with higher efficiency in high fat-fed mice (Ahren *et al.*, 1999).

Binding studies using [^{3}H]QNB and muscarinic general antagonist atropine revealed that total muscarinic receptors are decreased in the cerebral cortex during diabetic condition. In insulin, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats, binding parameters were reversed to near control values. In these groups, the animals maintained the near control glucose and circulating insulin levels.

Central cholinergic neurons participate in the complex neural events responsible for the hyperglycemic response to neurocytoglucopenia and to stressful situations. The hyperglycemia induced by intracerebroventricular 2-deoxyglucose (2-DG) was significantly reduced by previous intracerebroventricular injection of atropine (Brito *et al.*, 2001). Atropine injected into the third cerebral ventricle suppressed epinephrine secretion and dose-dependently inhibited hepatic venous hyperglycemia induced by neostigmine in intact rats (Iguchi *et al.*, 1999) The down regulation of muscarinic receptors during diabetes is a compensatory mechanism to facilitate insulin secretion and maintenance of normoglycemia in diabetic rats.

Muscarinic M1 receptor changes during diabetes were studied using subtype specific antagonist, pirenzepine and [^{3}H]QNB . Muscarinic M1 receptors were decreased in diabetic rats, with a decrease in K_d indicating an increase in the affinity of receptors during diabetic state. In insulin, *Aegle marmelose*, *Costus pictus* leaf extracts treated diabetic rats binding parameters are reversed to near control values.

Muscarinic receptors are reported to be involved in the release of NE in the central nervous system (Appasundaram *et al.*, 1998). In the PC cell lines addition of cholinergic stimulation results in the release of NE and muscarinic M1 receptors are involved in the NE release. Down regulation of the muscarinic M1 receptor in the central nervous system helps to regulate the NE and EPI secretion which are inhibitory to insulin secretion. Real Time-PCR analysis also revealed a down regulation of the muscarinic M1 receptor mRNA level during diabetic condition. This is in concordant with our receptor binding studies.

Corpus striatum

Densities of M1 receptor subtype were highest in the corpus striatum (Oki *et al.*, 2005). The corpus striatum is the largest component of the basal ganglia. Corpus striatum regulates endocrine functions indirectly through the secretion of other hormones like thyroxin. Binding studies using [^{3}H]QNB revealed that total muscarinic receptors decreased in corpus striatum during diabetic condition. In insulin treated and *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats binding parameters were reversed to near control values. Muscarinic M1 receptor changes were studied in experimental rats using subtype specific antagonist pirenzepine. Muscarinic M1 receptors are increased during diabetic state. In insulin, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats binding parameters are reversed back to near control values. RT-PCR analysis also revealed an up regulation of the muscarinic M1 receptor mRNA level during diabetic condition. This is in concordant with our receptor binding studies.

Hypothalamus

Specialized subgroups of hypothalamic neurons exhibit specific excitatory or inhibitory electrical responses to changes in extracellular levels of glucose (Burdakov *et al.*, 2005). Hypothalamic centers involved in the regulation of energy balance and endogenous glucose production constantly sense fuel availability by receiving and integrating inputs from circulating nutrients and hormones such as insulin and leptin. In response to these peripheral signals, the hypothalamus sends out efferent impulses that restrain food intake and endogenous glucose production. This promotes energy homeostasis and keeps blood glucose levels in the normal range. Disruption of this intricate neural control is likely to occur in type 2 diabetes and obesity which contribute to defects of glucose homeostasis and insulin resistance common to both diseases (Demuro & Obici, 2006). Hypothalamus is the centre involved in the neuroendocrine regulation. It is the region of the central nervous system where the autonomic and endocrine systems are integrated. Hypothalamic paraventricular nucleus (PVN) serves as the major neuroendocrine and autonomic output centre. In the PVN information from all over the brain is integrated and there are several other hypothalamic nuclei that also feed their information into this nucleus. Microinjection of $HgCl_2$ and neostigmine into the third ventricle under anesthesia caused marked hyperglycemia. In medulloadrenalectomized and atropine-coadministered rats, no marked hyperglycemia was induced by $HgCl_2$ or neostigmine. These results show that the muscarinic cholinergic system participates in the $HgCl_2$-induced central hyperglycemic effect through the function of the adrenal medulla (Takahashi *et al.*, 1994).

The cholinergic glucoregulatory hippocampal activity transmitted to peripheral organs via the ventromedial hypothalamus (Iguchi *et al.*, 1992). The ventromedial hypothalamus (VMH), lateral hypothalamus, paraventricular

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hypothalamus and median site of the lateral preoptic area were involved in increasing the plasma glucose and epinephrine levels (Honmura *et al.*, 1992). The muscarinic antagonist atropine suppressed the hyperglycemia induced by hippocampus administration of neostigmine in a dose-dependent manner, suggesting the involvement of muscarinic receptors of the VMH in the glucoregulation (Iguchi *et al.*, 1991).

General muscarinic antagonist, $[^{3}H]QNB$ binding showed that total muscarinic receptors are increased in the hypothalamus during diabetes with a significant decrease in the K_d when compared to control group. The ventromedial hypothalamus, lateral hypothalamus, paraventricular hypothalamus, and median site of the lateral-preoptic are involved in increasing the plasma levels of glucose and epinephrine by cholinergic stimulation (Honmura *et al.*, 1992). In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats, B_{max} reversed to near control values. Receptor binding studies using muscarinic M1 subtype specific antagonist pirenzepine showed that M1 receptors decreased during diabetes with an increase in affinity of the receptors when compared to control group. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats, binding parameters were reversed back to near control values. The increased activity of the total muscarinic receptors and down regulation of Muscarinic M1 receptors could help the maintenance of normoglycemia.. RT-PCR studies showed that the receptor mRNA decreased during diabetic condition. Previous studies demonstrated that the distribution of mRNA of muscarinic receptor generally parallels with the distribution of their protein.

Brain stem

Brain stem along with hypothalamus serves as the key centre of the central nervous system regulating the body homeostasis. Stimulation of the peripheral vagus nerve leads to an increase in circulating insulin levels. Anatomical studies suggest that the origin of these vagal efferent fibres is nucleus ambiguus and dorsal motor nucleus directly innervating pancreas (Bereiter *et al.*, 1981).

The total muscarinic receptors of the brain stem are found to be increased during diabetic condition. Muscarinic M1 receptor changes were studied in experimental rats using subtype specific antagonist pirenzepine. Muscarinic M1 receptors are decreased during diabetic state. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats, binding parameters were reversed back to near control values.

The dorsal motor nucleus of the vagus nerve is located in the brain stem. It is connected to the endocrine pancreas exclusively *via* vagal fibres and has a role in neurally mediated insulin release. Nucleus ambiguus stimulation reported to increase plasma insulin levels in rats (Bereiter *et al.*, 1981). . The insulin was reported to be mitogenic and stimulated pancreatic β-cell proliferation *in vitro*. RT-PCR analysis also revealed a down regulation of the muscarinic M1 receptor mRNA level during diabetic condition. This is in concordant with our receptor binding studies.

MUSCARINIC M1 RECEPTORS ALTERATIONS IN THE PANCREAS

Expression of muscarinic receptors in rat islets, RINm5F cells, and INS-1 cells was established by reverse transcriptase-polymerase chain reaction (RT-PCR) and quantified by RNase protection. Both methods indicated that M1 and M3 receptors

were expressed approximately equally in the various cellular preparations (Lismaa *et al.*, 2000).

The autonomic nervous system plays an important role in the insulin release. Physiological insulin secretion is initiated by glucose and augmented by nervous and humoral systems (Ahren *et al.*, 1986). The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several different neurotransmitters are stored within the terminals of these nerves, both acetylcholine and noradrenalin, and several neuropeptides. Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion. Insulin secretion is stimulated by parasympathetic nerves and inhibited by sympathetic nerves (Ahren, 2000). Acetylcholine mediates insulin release through vagal stimulation. Acetylcholine acts through the activation of Gq-phospholipase C. It stimulates Ca^{2-} influx through the voltage dependent L-type Ca^{2+} channel that is primarily activated by glucose. Studies showed that M1 and M3 are the major muscarinic receptors present in the pancreas (Lismaa *et al.*, 2000). During diabetic condition M1 receptors are decreased. The muscarinic M1 receptors are decreased in number during diabetes with an increase in affinity.

Muscarinic M1 receptor changes were studied in experimental rats using subtype specific antagonist pirenzepine. Muscarinic M1 receptors are decreased in diabetic rats while K_d was increased when compared to control group. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats, binding parameters were reversed back to near control values.

Administration of choline to rats elevates serum insulin. Pretreatment with a peripheral muscarinic acetylcholine receptor antagonist atropine methylnitrate, blocked the choline-induced increase in blood insulin. The increase in serum insulin elicited by choline was also prevented by pretreatment with the M1 antagonist,

pirenzepine, or the M1 + M3 antagonist, 4-DAMP. Pretreatment with, an antagonist of ganglionic nicotinic acetylcholine receptors, hexamethonium prevented the choline-induced increase in serum insulin. Choline increased the acetylcholine content of the pancreas, and enhanced acetylcholine release from minced pancreas, which suggests that choline stimulates insulin secretion indirectly by enhancing acetylcholine synthesis and release (Ilcol *et al.*, 2003).

Muscarinic M1 receptors are involved in the glucose induced insulin secretion. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats Muscarinic M1 receptor status reversed to near control level. It helps to increase the insulin secretion from remaining β-cells to maintain the normal glucose level. RT-PCR analysis also revealed a down regulation of the muscarinic M1 receptor mRNA level during diabetic condition. This is concordant with our receptor binding studies.

STIMULATION OF INSULIN SYNTHESIS AND SECRETION FROM PANCREATIC β-CELL *IN VITRO* BY *Aegle marmelose* & *Costus pictus* LEAF EXTRACTS

To understand the mechanisms by which *Aegle marmelose* and *Costus pictus* ameliorates hyperglycemia, *in vitro* insulin secretion study using rat primary islet culture was carried out. Signal-transduction in the pancreatic β-cell and thereby the insulin secretory process is regulated by a sophisticated interplay between glucose and a plethora of additional factors including other nutrients, neurotransmitters, islet generated factors and systemic growth factors. The coupling of glucose metabolism to electrical activity remains central in all models of β-cell stimulus-secretion coupling. The resting membrane potential of the β-cell is set by the ATP-sensitive potassium

(KATP) channel (Ashcroft & Rorsman, 1990). Incubation of the pancreatic β-cells with stimulatory glucose concentrations leads to the activation of a cascade of reactions, which ends in the exocytosis of stored insulin. This complex of processes starts with the uptake of glucose by the β-cell high-K_m/low affinity glucose transporter GLUT2 and proceeds with the conversion of glucose into glucose-6-phosphate by the β-cell isoform of glucokinase (Randel, 1993; Matschinsky, 1996). Metabolism of glucose in glycolysis and the Krebs cycle results in the generation of ATP. Elevation in the ATP/ADP ratio leads to closure of the KATP, which in turn results in depolarization of the plasma membrane. The subsequent opening of voltage-gated L-type Ca^{2+} channels leads to an increase in the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, which promotes insulin secretion (Berggren & Larsson, 1994).

Isolated pancreatic islets were incubated for one hour with five different concentrations (0.25, 0.5, 1, 2 & 5 mg/ml) of *Aegle marmelose* and *Costus pictus* leaf extracts separately in the presence of 4mM and 20mM glucose concentration, which would represent normal and diabetic conditions respectively. In one hour pancreatic islet cell culture, *Aegle marmelose* and *Costus pictus* leaf extracts enhanced glucose stimulated insulin secretion significantly at both the concentrations (4mM and 20mM) of glucose when compared to control.

Twenty four hours islet cell culture was done to study the long-term effect of *Aegle marmelose* on insulin synthesis and release from the isolated islets. Long-term insulin secretion studies showed that all the concentrations of *Aegle marmelose* except in higher concentration (5mg/ml) enhanced glucose stimulated insulin secretion significantly at both the concentrations (4mM and 20mM) of glucose when compared to control. In 24 hour culture *Costus pictus* leaf extract enhanced glucose stimulated insulin secretion significantly at both the concentrations (4mM and 20mM) of glucose when compared to control.

These experiments revealed that aqueous extract of *Aegle marmelose* and *Costus pictus* enhanced insulin secretion. The enhancement of insulin secretion by the *Aegle marmelose* and *Costus pictus* extracts correlates with the blood glucose and circulating insulin level data and can be attributed to the stimulation of the surviving beta cells by the extracts, which in turn exerts an antihyperglycemic action. Similar reports have been observed from some previous studies (Gray & Flatt, 1997; 1999).

MUSCARINIC STIMULATION OF INSULIN SYNTHESIS AND SECRETION FROM PANCREATIC β-CELL *IN VITRO*

Activation of the parasympathetic branch of the autonomic nervous system has long been known to increase insulin secretion and peripheral glucose uptake (Porte & Woods, 1990). Cholinergic stimulation of pancreatic β-cells increases insulin secretion. This effect is mediated by muscarinic receptors.

Isolated pancreatic islets were incubated for one hour with four different concentrations (0.5, 1, 2 & 5 mg/ml) of *Aegle marmelose* leaf extract separately in the presence 10^{-7}M carbachol and four different concentrations (0.5, 1, 2 & 5 mg/ml) of *Costus pictus* leaf extract separately in the presence 10^{-7}M carbachol in 4mM and 20mM glucose concentration. Carbachol at low concentration (10^{-8}M) stimulated insulin secretion. In one hour pancreatic islet cell culture all the four concentrations (0.5, 1, 2 & 5 mg/ml) of *Aegle marmelose* and *Costus pictus* leaf extracts in the presence of 10^{-7}M carbachol enhanced glucose induced insulin secretion when compared to 10^{-7}M carbachol alone.

Twenty four hours islet cell culture was done to study the long-term effect of cholinergic agonist carbachol and *Aegle marmelose*, *Costus pictus* leaf extracts on insulin synthesis and release from the isolated islets. The presence of insulin

synthesis/secretion stimulators in the 24 hours islet cell cultures showed that they capacitate the ability of the viable cells to synthesise and secrete the insulin. Cholinergic agonist showed stimulatory effect in the long-term studies also (Renuka *et al.*, 2006). Twenty four hours islet cell culture also showed similar results as in one hour *in vitro* culture.

Acetylcholine stimulation-insulin secretion coupling is mediated by complex mechanisms of signal transduction and several factors are involved. ACh is released from cholinergic synapses on β-cells during the cephalic phase of digestion causing a transient increase in insulin secretion. It has been proposed that ACh activates phospholipid turnover and thereby increases the intracellular calcium levels. IP_3 mediates Ca^{2+} mobilization from intracellular Ca^{2+} stores and plays an important role in insulin secretion from pancreatic β-cells (Laychock, 1990). IP_3 exerts its action through receptors that are ligand-activated, Ca^{2+} selective channels. IP_3 receptors have been localized to the endoplasmic reticulum, nucleus and insulin granules (Yoo *et al.*, 1990).

PKC plays an important role in mediating insulin secretion in response to cholinergic stimulation (Persaud *et al.*, 1989; Wollheim & Regazzi, 1990). PKC also mediates densensitisation in many cell types. Activation of PKC by carbamylcholine leads to densensitisation and TPA (phorbol 12-myristate 13-acetate) treatment inactivates PKC leading to the inhibition of the densitisation process in islets (Verspohl & Wienecke, 1998). It is also reported that the desensitisation of PLC – coupled muscarinic receptors is mediated by PKC (Haga *et al.*, 1990). The inhibition of insulin secretion by the addition of high concentration of carbamylcholine is the result of the receptor desensitisation by PKC.

In the present *in vitro* study we observed an increase in insulin release when islets were incubated with various concentrations of the *Aegle marmelose* and *Costus*

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pictus leaf extracts and the combinations of these plants extracts with cholinergic agonist carbachol. This finding agrees with *in vivo* results thus strengthening the evidence that the extract acts as a stimulator of insulin secretion. Similarly insulin secretoagogue effect has been reported in plants such as *Agaricus campestris* (Gray and Flatt, 1998); *Viscum album* (Gray and Flatt, 1999) and *Urtica dioica* (Farzami *et al.*, 2003) in isolated islets. Our study confirmed that the regulatory activity of these plant extracts on insulin secretion is through Muscarinic receptors.

GLUCOSE UPTAKE STUDY

The plasma glucose level is tightly controlled throughout life in the normal individual. The stability of the plasma glucose level is a reflection of the balance between the rates of whole body glucose production and glucose utilisation. Each of these processes is tightly regulated by the levels of hormones and substrates in blood (Alan, 1999). The liver plays a major role in insulin-regulated glucose homoeostasis through the balance between glucose utilization and glucose production, both processes being tightly coordinated (Nevado *et al.*, 2006; Carmen *et al.*, 2005). The glucose dependence of liver glucose uptake is influenced by the route of glucose delivery and the prevailing insulin levels (Chen *et al.*, 2004).

Fourteen days after the STZ injection, liver and cerebral cortex slices of control, diabetic, insulin treated, *Aegle marmelose* and *Costus pictus* treated diabetic rats were incubated with ^{14}C glucose for 30 minutes and one hour. In the liver and cerebral cortex of diabetic rats there was a significant reduction in the glucose uptake activity when compared to control group. Treatment with insulin, *Aegle marmelose* and *Costus pictus* extracts enhanced glucose uptake in the liver and cerebral cortex.

One of the underlying mechanisms of glucose lowering activity is suggested to be due to stimulation of peripheral glucose utilization. Since STZ induced diabetes was accompanied by insulin resistance, *Aegle marmelose* and *Costus pictus* leaf extracts treatment can act by improving sensitivity. In diabetes, the decrease in body weight is associated with decreased rate of glucose utilization and impaired carbohydrate metabolism. *Aegle marmelose* and *Costus pictus* leaf extracts treatment seems to have regulated these disturbances at the cellular level.

The incorporation of ^{14}C - glucose was found to be significantly decreased in the liver slices of diabetic rats. The present result of *in vitro* ^{14}C – glucose uptake in diabetic rat liver are in accordance with the reports that glucose utilisation is inhibited in diabetic conditions due to lack of insulin that in turn decreases transport of glucose across the cell wall of hepatocytes (Chaikoff, 1951; Hemandex & Sols, 1963). *Aegle marmelose* and *Costus pictus* leaf extracts treatment of diabetic rats significantly enhanced the *in vitro* ^{14}C – glucose uptake in liver slices which could possibly be due to the regulation caused by these plant extracts at the level of glucose transport system. The incorporation of ^{14}C - glucose was found to be significantly decreased in the cerebral cortex slices of diabetic rats. Tuonq *et al.*, (1984) reported that slices from rat cerebral cortex incubated in the presence of 2-deoxy[^{3}H]glucose accumulate the sugar mainly in the form of its phosphorylated derivative.

ELECTROPHYSIOLOGICAL CHANGES DURING DIABETES

Neuroelectrophysiological recordings represent a non-invasive and reproducible method of detecting central and peripheral nervous system alterations in diabetes mellitus (Morano *et al.*, 1996). Neurophysiological alterations have been described in animal models of diabetes, in particular in rats. Deficits in both motor

and sensory nerve conduction velocity (MNCV and SNCV, respectively) can be detected within weeks after the onset of diabetes and increase up to 2–3 months after diabetes onset, remaining relatively stable thereafter (Moore *et al.*, 1980; Cameron *et al.*, 1986; Brismar *et al.*, 1987; Kappelle *et al.*, 1993). Studies of MNCV and SNCV in diabetic rats have made important contributions to the elucidation of the pathogenesis of the effects of diabetes on the PNS, as well as in the development of putative pharmacotherapy.

The control, diabetic, insulin treated, *Aegle marmelose* and *Costus pictus* treated diabetic rats underwent EEG analysis. The diabetic rats showed a change in the EEG pattern compared to the control rats. Treatment with insulin, *Aegle marmelose* and *Costus pictus* brought the wave patterns to the near control levels.

Diabetes mellitus is associated with chronic complications such as nephropathy, angiopathy, retinopathy and peripheral neuropathy. In diabetic patients, hyperglycemia may precipitate seizures, and in experimental diabetes, indications for an increased neuronal excitability have been found (Anderson *et al.*, 2006). These changes in the diabetic cause the altered wave patterns. In our study it was found that the neurological disturbances can be reduced by the administration of *Aegle marmelose* and *Costus pictus* leaf extracts.

Thus our results suggest that the acetylcholine acting through muscarinic and specifically muscarinic M1 receptor subtype of receptors regulate the glucose homeostasis. *Aegle marmelose* and *Costus pictus* leaf extracts have a potential role in the insulin synthesis and secretion from the pancreatic β- cell, mediating its function through muscarinic receptors of acetylcholine. This has immense clinical significance in the management of diabetes.

Summary

1. Streptozotocin induced diabetic rats were used as model to study the alterations of total Muscarinic, Muscarinic M1 receptors and their regulation by *Aegle marmelose* and *Costus pictus* leaf extracts in insulin secretion.

2. Antihyperglycemic activity of *Aegle marmelose* and *Costus pictus* leaf extracts were evaluated by the blood glucose and circulating insulin level measurement of experimental rats.

3. Acetylcholine esterase activity has been used as a marker for cholinergic activity. Acetylcholine esterase activity was measured in the brain regions. During diabetic stage it increased in the cerebral cortex, brainstem and hypothalamus while in corpus striatum it decreased. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats the activity of the enzyme reversed to near control value.

4. Muscarinic receptor functional status was analysed by Scatchard and displacement analysis using specific ligands, [³H]QNB- general muscarinic antagonist, atropine- non radioactive general muscarinic antagonist and pirenzepine- non radioactive muscarinic M1 antagonist.. Receptor binding parameters was confirmed by studying the mRNA status of the corresponding receptor using RT-PCR and Real-Time PCR. During diabetes, total muscarinic receptors were down regulated in cerebral cortex and corpus striatum while in brainstem and hypothalamus it was up regulated. Muscarinic M1 receptors were down regulated in the cerebral cortex, brainstem and

hypothalamus while it was up regulated in corpus striatum. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats the activity of the receptor reversed to near control value. Muscarinic M1 receptors were down regulated in the pancreas during diabetes. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats the activity of the receptor reversed to near control value.

5. *In vitro* studies showed that *Aegle marmelose* and *Costus pictus* leaf extracts induced glucose stimulated insulin secretion in pancreatic islets. *Aegle marmelose* and *Costus pictus* leaf extracts further increased glucose induced insulin secretion in the presence of muscarinic agonist carbachol.

6. *In vitro* ^{14}C glucose uptake studies showed glucose uptake by liver and cerebral cortex was decreased in diabetic group. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats liver and cerebral cortex showed an increase in ^{14}C glucose uptake.

7. A prominent brain activity difference was observed in diabetic rats when compared to control rats by EEG analysis. In insulin treated, *Aegle marmelose* and *Costus pictus* leaf extracts treated diabetic rats brain activity was revered to near control range.

It is evident from our results that brain and pancreatic muscarinic and muscarinic M1 receptor functional balance plays a major role in regulating the insulin secretion. The present study showed the regulatory role of *Aegle marmelose* and *Costus pictus* leaf extracts on glucose homeostasis and insulin

secretion. Thus, our results suggest that total muscarinic and muscarinic M1 receptor binding parameters are involved in the insulin synthesis and secretion. Gene expression studies confirm these results. *Aegle marmelose* and *Costus pictus* leaf extracts regulate insulin synthesis and secretion mediated through Muscarinic receptors.

Conclusion

We conclude from our studies that cholinergic system through muscarinic receptors play an important role in the regulation of glucose homeostasis and insulin secretion. Cholinergic activity as indicated by acetylcholine esterase, a marker for cholinergic system, increased in the brain regions - cerebral cortex, brain stem, and hypothalamus and decreased in corpus striatum during diabetes. Treatment of diabetic rats with insulin, *Aegle marmelose* and *Costus pictus* leaf extracts reversed the enzyme status to near normal value. The functional changes in the muscarinic receptors studied in the brain regions and showed that the total muscarinic receptors down regulated in cerebral cortex and corpus striatum. There was an up regulation of total muscarinic receptors in brainstem and hypothalamus. Central Muscarinic M1 receptors were decreased in cerebral cortex, brainstem and hypothalamus during diabetes. There was an up regulation of Muscarinic M1 receptors during diabetes. Pancreatic muscarinic M1 receptor activity was decreased during diabetes. Treatment of diabetic rats with insulin, *Aegle marmelose* and *Costus pictus* leaf extracts reversed these receptor alterations to near control range. These alterations in the muscarinic receptors regulate sympathetic activity and maintain glucose level. Gene expression studies by RT-PCR and Real-Time PCR also showed a similar change in the mRNA level of Muscarinic M1 receptors. *In vitro* studies showed that *Aegle marmelose* and *Costus pictus* leaf extracts induced glucose stimulated insulin secretion in pancreatic islets. *Aegle marmelose* and *Costus pictus* leaf extracts further increased glucose induced insulin secretion in the presence of muscarinic agonist carbachol suggesting the importance of muscarinic receptors in insulin secretion.

Thus we conclude that the Central muscarinic and muscarinic M1 receptor subtypes functional difference regulates insulin synthesis and secretion, which in turn

control the glucose homeostasis. *Aegle marmelose* and *Costus pictus* leaf extracts have a regulatory role in the insulin secretion and glucose homeostasis through muscarinic receptors.

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Publications

1. Balarama Kaimal S , **Gireesh G**, Paulose C. S., Decreased $GABA_A$ receptor function in the brain stem during pancreatic regeneration in rats (2007) (*Neurochemical Research*-DOI.10.1007/S 11064.007-9283-3.)

Abstracts/ presentations

♦ **G.Gireesh** and C.S.Paulose (2006) Muscarinic M1 receptor gene expression in the cerebral cortex of streptozotocin induced diabetic rats: antihyperglycemic role of *costus pictus* leaf extract ; Proceedings of the International Conference on Ethnopharmacology and Alternative Medicine & Fifth Annual Conference of National Society of Ethnpharmacology at Amala Cancer Centre, Thrissur, Kerala , India .

♦ **G.Gireesh** and C.S.Paulose (2006) Antihyperglycemic activity of *Aegle marmelose* leaf extract: Muscarinic M1 receptor gene expression in the cerebral cortex of streptozotocin induced diabetic rats. Proceedings of the National Conference on Biotechnology and Economic Development- A Kerala Scenario at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India.

♦ Jerry Ignatius, CS Paulose, Santhosh K Thomas, Reas Khan S, and **G Gireesh** (2006) Upregulation of neurotransmitter receptors- a possible mechanism for accelerated fracture healing. The Journal of Kerala Orthopedic Association. Vol No.20, No.1 31-38.

- Reas Khan S, **G.Gireesh** and C.S.Paulose (2005) Decreased Glutamate decarboxylase activity in the cerebellum of pilocarpine induced epileptic rats. International Symposium on Advances in research on neurodegenerative diseases at University of Madras , Chennai.

- **G.Gireesh,** Benjamin George, P.S. John and C.S.Paulose (2004) Enhancement of ultrasound induced cell proliferation by neurotransmitters; Proceedings of the International Conference on Biotechnology and Neuroscience at Centre for Neuroscience, Dept. of Biotechnology, Cochin University of Science and Technology, Cochin-22, Kerala, India.

- **G.Gireesh,** S. Rakesh, Jackson James, K. Prakash Kumar and C.S.Paulose (2004) Effect of *Aegle marmelose* on acetylcholine esterase activity in the cerebral cortex of streptozotocin induced diabetic rats; Proceedings of the International Conference on Biotechnology and Neuroscience at Centre for Neuroscience, Dept. of Biotechnology, Cochin University of Science and Technology, Cochin-22, Kerala, India

- Santhosh k Thomas, **G.Gireesh,** S. Rakesh and C.S.Paulose (2004) Effect of neurotransmitters on bone marrow cell proliferation; Proceedings of the International Conference on Biotechnology and Neuroscience at Centre for Neuroscience, Dept. of Biotechnology, Cochin University of Science and Technology, Cochin-22, Kerala, India

◆ **G.Gireesh,** S. Reas Khan, Chathu Finla, and C.S.Paulose (2004) Decreased glutamate decarboxylase and increased glutamate dehydrogenase activity in the cerebellum of alcoholic rats; Proceedings of the International Neuroscience Conference at School of Life Sciences, University of Hyderabad, Hyderabad, India

◆ Santhosh k Thomas, **G.Gireesh** and C.S.Paulose (2003) Enhanced Catalase activity in the corpus striatum of alcoholic rats at International Medical Science Academy (IMSA) Annual Conference at Cochin

Table-1

Body weight (g) of Experimental rats

Animal status	Initial	7th day	14th day
Control	190.0 ± 10.0	202.5 ± 14.3	213.3 ± 14.5
Diabetic	210.0 ± 5.7	176.6 ± 3.3	153.7 ± 2.3 *** ΦΦΦ
Diabetic + Insulin treated (D+I)	226.6 ± 13.3	196.6 ± 3.3	190.2 ± 2.0 ψψψ
Diabetic + *Aegle marmelose* treated (D+A)	193.3 ± 6.6	183.3 ± 8.8	180.0 ± 3.5 ψψψ
Diabetic + *Costus pictus* treated (D+C)	203.3 ± 8.8	176.6 ± 3.3	173.7 ± 2.3 ψψψ

Values are mean ± S.E.M of 4-6 rats in each group
*** $p<0.001$ when compared with control
ΦΦΦ $p<0.001$ when compared with initial weight
ψψψ $p<0.001$ when compared with diabetic group

Table-2

Blood glucose (mg/dl) level in Experimental rats

Animal status	0 day (Before STZ injection)	3rd day (Initial)	6th day	10th day	14th day (Final)
Control	87.1 ± 1.1	92.5 ± 1.1	88.4 ± 0.8	103.2 ± 2.1	99.3 ± 1.21
Diabetic	76.7 ± 1.7	257.1 ± 0.4	305.1 ± 0.9	307.7 ± 0.4	307.9±1.2 ***
D + I	86.2 ± 0.7	258.8 ± 0.6	301.4 ± 0.9	192.8 ± 1.3	140.0 ± 1.1 ᵛᵛᵛ ���
D + A	95.6 ± 2.9	254.3 ± 2.3	300.8 ± 1.3	195.8 ± 2.9	120.0 ± 15.9 ᵛᵛᵛ ���
D + C	102.5 ± 1.2	259.8 ± 2.5	301.5 ± 2.1	205.0 ± 2.8	124.0±12.9 ᵛᵛᵛ ���

Values are mean ± S.E.M of 4-6 rats in each group
*** P<0.001 when compared to control
ᵛᵛᵛ P<0.001 when compared to diabetic group
φφφ p<0.001 when compared with initial reading

Figure – 1

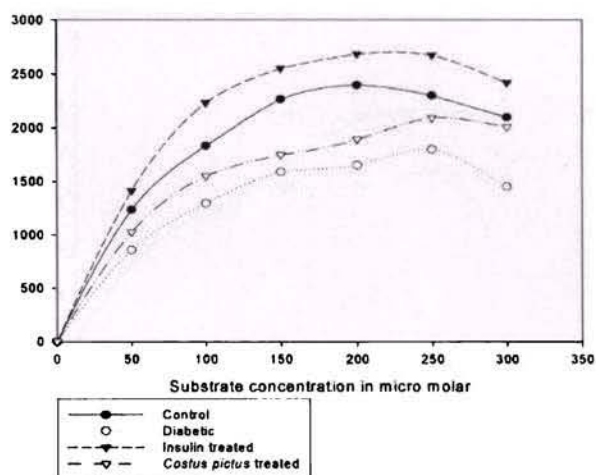**Circulating insulin level in the plasma of experimental rats**
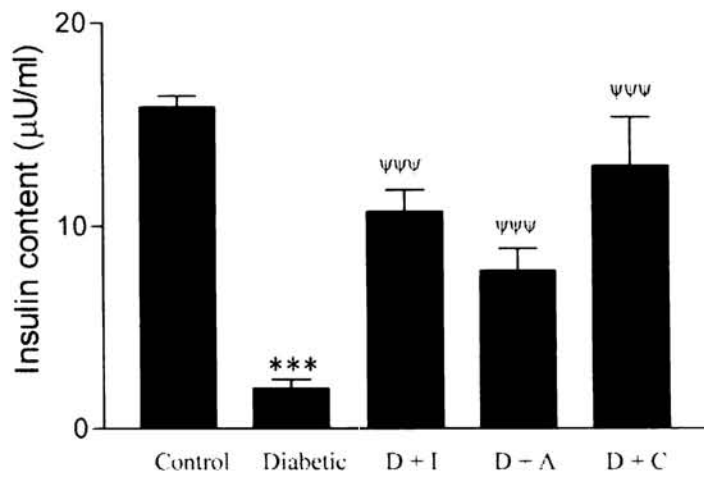
Values are mean ± S.E.M of 4-6 separate experiments
*** P<0.001 when compared to control,
ΨΨΨ P<0.001 when compared to diabetic group

Figure – 2

Effect of *Costus pictus* on glucose tolerance in glucose loaded rats
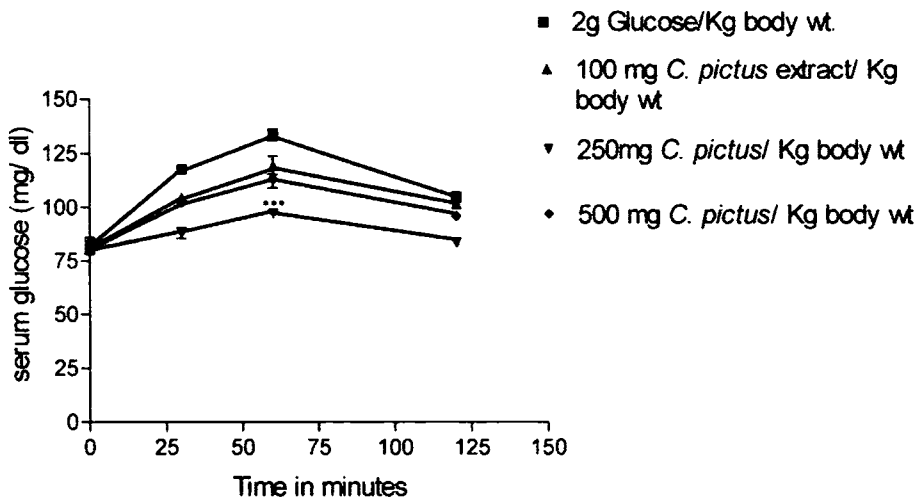
***p<0.001 when compared to 2g Glucose/ Kg body wt. group

Figure – 3

Acetylcholine esterase activity in the cerebral cortex of Control, Diabetic, D+I and D+A group rats

Table-3

Acetylcholine esterase activity in the cerebral cortex of Control, Diabetic, D+I and D+A group rats

Animal status	V_{max} (µmoles/min/mg protein)	K_m (µM)
Control	589.4 ± 48.2	81.4 ± 10.1
Diabetic	842.5 ± 69.9 *	55.4 ± 7.5 *
Diabetic + Insulin treated (D+I)	503.6 ± 29.3 ᵚ	59.2 ± 8.9
Diabetic + *Aegle marmelose* treated (D+A)	625.6 ± 3.2 ᵚ	59.2 ± 8.9

Values are mean ± S.E.M of 4-6 separate experiments
*$P<0.05$ when compared to control, ᵚ $P<0.05$ when compared to diabetic group

Figure – 4

Acetylcholine esterase activity in the cerebral cortex of Control, Diabetic, D+I and D+C group rats

Table-4

Acetylcholine esterase activity in the cerebral cortex of Control, Diabetic, D+I and D+C group rats

Animal status	V_{max} (μmoles/min/mg protein)	K_m (μM)
Control	589.4 ± 48.2	81.4 ± 10.1
Diabetic	842.5 ± 69.9 *	55.4 ± 7.5 *
Diabetic + Insulin treated (D+I)	503.6 ± 29.3 ᵛ	59.2 ± 8.9
Diabetic + *Costus pictus* (D+C)	546.2 ± 88.5 ᵛ	54.2 ± 7.2

Values are mean ± S.E.M of 4-6 separate experiments
*P<0.05 when compared to control
ᵛ P<0.05 when compared to diabetic group

Figure – 5

Acetylcholine esterase activity in the brainstem of Control, Diabetic, D+I and D+A group rats

Table-5

Acetylcholine esterase activity in the brainstem of Control, Diabetic, D+I and D+A group rats

Animal status	V_{max} (µmoles/min/mg protein)	K_m (µM)
Control	626.1 ± 12.0	55.8 ± 15.4
Diabetic	881.2 ± 43.7*	59.1 ± 18.3
Diabetic + Insulin treated (D+I)	644.2 ± 66.8 ᵛ	51.6 ± 7.9
Diabetic + *Aegle marmelose* treated (D+A)	652.9 ± 29.1 ᵛ	54.1 ± 13.3

Values are mean ± S.E.M of 4-6 separate experiments
*P<0.05 when compared to control,
ᵛ P<0.05 when compared to diabetic group

Figure – 6

Acetylcholine esterase activity in the brainstem of Control, Diabetic, D+I and D+C group rats

Substrate concentration in micro molar

Control
Diabetic
Diabetic + Insulin treated
Diabetic + Costuspictus treated

Table-6

Acetylcholine esterase activity in the brainstem of Control, Diabetic, D+I and D+C group rats

Animal status	V_{max} (µmoles/min/mg protein)	K_m (µM)
Control	626.1 ± 12.0	55.8 ± 15.4
Diabetic	881.2 ± 43.7 *	59.1 ± 18.3
Diabetic + Insulin treated (D+I)	644.2 ± 66.8 ᵛ	51.6 ± 7.9
Diabetic + *Costus pictus* treated (D+C)	708.1 ± 43.5 ᵛ	50.0 ± 10.1

Values are mean ± S.E.M of 4-6 separate experiments
*P<0.05 when compared to control
ᵛP<0.05 when compared to diabetic group

Figure – 7

Acetylcholine esterase activity in the corpus striatum of Control, Diabetic, D+I and D+A group rats

Control
Diabetic
Insulin treated
Aegle marmelose treated

Table-7

Acetylcholine esterase activity in the corpus striatum of Control, Diabetic, D+I and D+A group rats

Animal status	V_{max} (µmoles/min/mg protein)	K_m (µM)
Control	2420.0 ± 11.5	51.0 ± 0.5
Diabetic	1631.3 ± 16.1***	51.3 ± 0.8
Diabetic + Insulin treated (D+I)	2647.0 ± 26.5 ▼▼▼	51.0 ± 0.5
Diabetic + *Aegle marmelose* treated (D+A)	2471.0 ± 12.4 ▼▼▼	52.6 ± 0.3

Values are mean ± S.E.M of 4-6 separate experiments
*** $P<0.001$ when compared to control,
▼▼▼ $P<0.001$ when compared to diabetic group

Figure – 8

Acetylcholine esterase activity in the corpus striatum of Control, Diabetic, D+I and D+C group rats

Substrate concentration in micro molar

Control
Diabetic
Insulin treated
Costus pictus treated

Table-8

Acetylcholine esterase activity in the corpus striatum of Control, Diabetic, D+I and D+C group rats

Animal status	V_{max} (µmoles/min/mg protein)	K_m (µM)
Control	2420.0 ± 11.5	51.0 ± 0.5
Diabetic	1631.3 ± 16.1***	51.3 ± 0.8
Diabetic + Insulin treated (D+I)	2647.0 ± 26.5 ▼▼▼	51.0 ± 0.5
Diabetic + *Costus pictus* treated (D+C)	2022.0 ± 13.3 ▼▼▼	53.0 ± 1.0

Values are mean ± S.E.M of 4-6 separate experiments
*** P<0.001 when compared to control,
▼▼▼ P<0.001 when compared to diabetic group

Figure – 9

Acetylcholine esterase activity in the hypothalamus of Control, Diabetic, D+I and D+A group rats

Substrate concentration in micro molar

- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treated

Table-9

Acetylcholine esterase activity in the hypothalamus of Control, Diabetic, D+I and D+A group rats

Animal status	V_{max} (μmoles/min/mg protein)	K_m (μM)
Control	973.3 ± 12.0	71.6 ± 4.4
Diabetic	1191.3 ± 13.9***	58.3 ± 2.0*
Diabetic + Insulin treated (D+I)	766.6 ± 24.0 ▼▼▼	53.3 ± 2.4
Diabetic + *Aegle marmelose* treated (D+A)	973.3 ± 14.5 ▼▼▼	60.6± 1.7

Values are mean ± S.E.M of 4-6 separate experiments
*$P<0.05$ when compared to control, *** $P<0.001$ when compared to control
▼▼▼ $P<0.001$ when compared to diabetic group

Figure – 10

Acetylcholine esterase activity in the hypothalamus of Control, Diabetic, D+I and D+C group rats

Substrate concentration in micro molar

- ● Control
- ○ Diabetic
- ▼ Insulin treated
- ▽ Costus pictus treated

Table-10

Acetylcholine esterase activity in the hypothalamus of Control, Diabetic, D+I and D+C group rats

Animal status	V_{max} (µmoles/min/mg protein)	K_m (µM)
Control	973.3 ± 12.0	71.6 ± 4.4
Diabetic	1191.3 ± 13.9***	58.3 ± 2.0*
Diabetic + Insulin treated (D+I)	766.6 ± 24.0 ▼▼▼	53.3 ± 2.4
Diabetic + Costus pictus treated (D+C)	830.6 ± 20.6 ▼▼▼	67.6 ± 1.4

Values are mean ± S.E.M of 4-6 separate experiments
*$P<0.05$ when compared to control, *** $P<0.001$ when compared to control
▼▼▼ $P<0.001$ when compared to diabetic group

Figure – 11

Scatchard analysis of [³H] QNB binding against atropine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Table-11
Scatchard analysis of [³H] QNB binding against atropine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	710.00± 5.77	0.90 ± 0.10
Diabetic	290.00 ± 5.77***	0.27 ± 0.02**
Diabetic + Insulin treated	656.66 ± 29.06 ᵚ ᵚ ᵚ	0.93 ± 0.06ᵚ ᵚ
Diabetic + *Aegle marmelose* treated	680.00± 62.45 ᵚ ᵚ ᵚ	1.15 ± 0.18 ᵚ ᵚ

Values are mean ± S.E.M of 4-6 separate experiments
** P<0.01 when compared to diabetic group, *** P<0.001 when compared to control
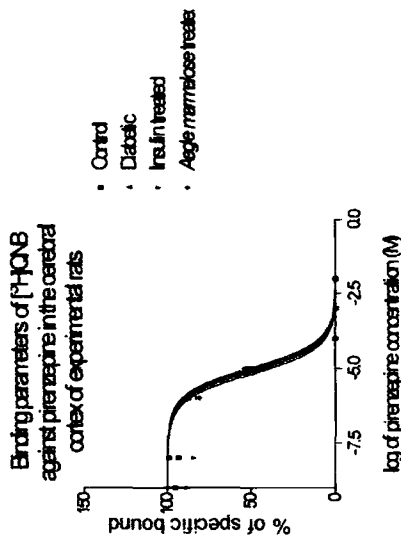ᵚᵚ P<0.01 when compared to diabetic group, ᵚ ᵚ ᵚ P<0.001 when compared to diabetic group

Table-12

Binding parameters of [³H]QNB against atropine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

| Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-6.403	1.31×10^{-7}	-0.9807
Diabetic	One-site	-6.552	9.35×10^{-8}	-0.9731
Insulin treated diabetic	One-site	-6.485	1.09×10^{-7}	-0.9918
Aegle marmelose treated diabetic	One-site	-6.136	2.43×10^{-7}	-0.9981

Values are mean of 4-6 separate experiments

Figure – 12
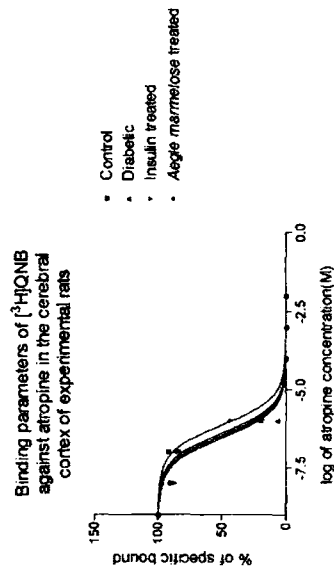
Data were fitted with iterative nonlinear regression software (Prism, GraphPad. San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Binding parameters of [³H]QNB against atropine in the cerebral cortex of experimental rats

- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treated

Figure – 13

Scatchard analysis of [³H] QNB binding against atropine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Table 13

Scatchard analysis of [³H] QNB binding against atropine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	710.00± 5.77	0.90 ± 0.10
Diabetic	290.00 ± 5.77 ***	0.27 ± 0.02 **
Diabetic + Insulin treated	656.66 ± 29.06 ᵛᵛᵛ	0.93 ± 0.06 ᵛᵛ
Diabetic + *Costus pictus* treated	450.00± 5.77 ᵛᵛ	0.56 ± 0.06 ᵛᵛ

Values are mean ± S.E.M of 4-6 separate experiments
** P<0.01 when compared to diabetic group, *** P<0.001 when compared to control
ᵛᵛ P<0.01 when compared to diabetic group, ᵛᵛᵛ P<0.001 when compared to diabetic group

Table-14

Binding parameters of [³H]QNB against atropine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-6.403	1.31×10^{-7}	0.9807
Diabetic	One-site	-6.552	9.35×10^{-8}	0.9731
Insulin treated diabetic	One-site	-6.481	1.10×10^{-7}	0.9917
Costus pictus treated diabetic	One-site	-6.123	2.51×10^{-7}	-0.9914

Values are mean of 4-6 separate experiments

Figure 14

Binding parameters of [³H]QNB against atropine in the cerebral cortex of experimental rats

- Control
- Diabetic
- Insulin treated
- Costus pictus treated
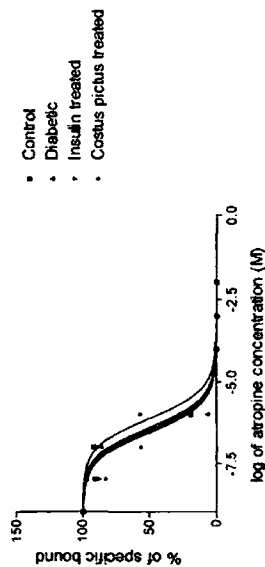
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure 15

Scatchard analysis of [³H] QNB binding against pirenzepine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Table-15

Scatchard analysis of [³H] QNB binding against pirenzepine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	2126.60± 14.50	2.62 ± 0.06
Diabetic	1433.30 ± 28.40***	1.35 ± 0.14**
Diabetic + Insulin treated	2023.30 ± 14.50 ᵛᵛᵛ	2.30 ± 0.18 ᵛᵛ
Diabetic + *Aegle marmelose* treated	2110.0± 30.50 ᵛᵛᵛ	2.39 ± 0.25 ᵛᵛ

Values are mean ± S.E.M of 4-6 separate experiments
** P<0.01 when compared to diabetic group, *** P<0.001 when compared to control
ᵛᵛ P<0.01 when compared to diabetic group, ᵛᵛᵛ P<0.001 when compared to diabetic group

Table-16

Binding parameters of [^{3}H]QNB against pirenzepine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-5.053	3.93 x 10^{-6}	-0.9904
Diabetic	One-site	-4.989	4.55 x 10^{-6}	-0.9928
Insulin treated diabetic	One-site	-5.205	2.77 x 10^{-6}	-0.9664
Aegle marmelose treated diabetic	One-site	-5.122	3.35 x 10^{-6}	-0.9914

Values are mean of 4-6 separate experiments

Figure – 16

Binding parameters of [^{3}H]QNB against pirenzepine in the cerebral cortex of experimental rats

- ■ Control
- ◆ Diabetic
- ▲ Insulin treated
- ● *Aegle marmelose* treated

Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 17

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the cerebral cortex of Control, Diabetic, Diabetic+Insulin and Diabetic+*Aegle marmelose* treated rats

373 bp
M1 amplicon

150 bp
β-actin
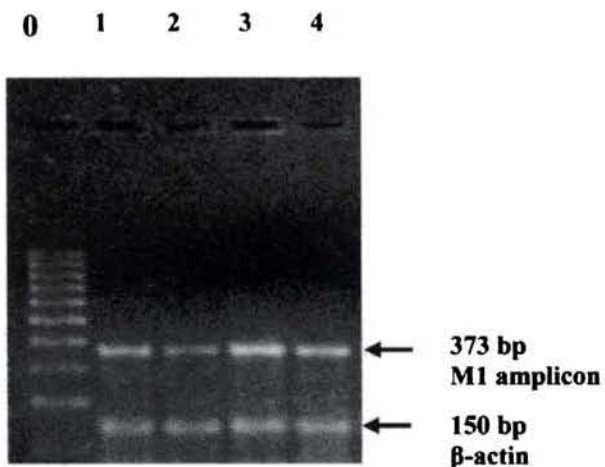
Table-17

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	15782	1
2	15009	0.95
3	17861	1.13
4	16650	1.05

0-1000 bp ladder, 1-Control, 2-Diabetic, 3-Diabetes + Insulin treated, 4- Diabetes + *Aegle marmelose* treated

Figure -18

Real Time amplification of mRNA from the cerebral cortex of Control, Diabetic, Diabetic+Insulin and Diabetic+*Aegle marmelose* treated rats
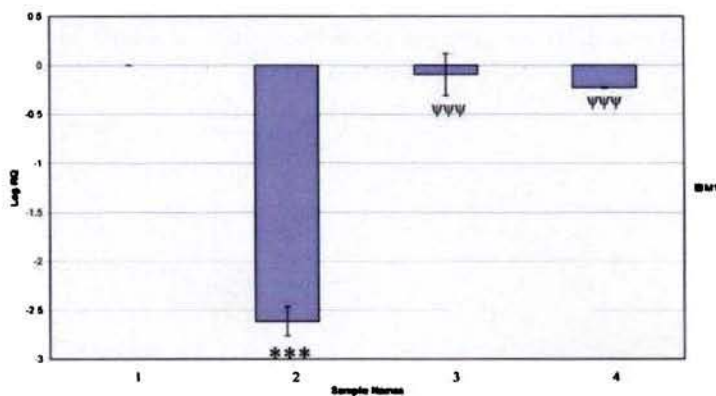
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Aegle marmelose* treated

Table -18

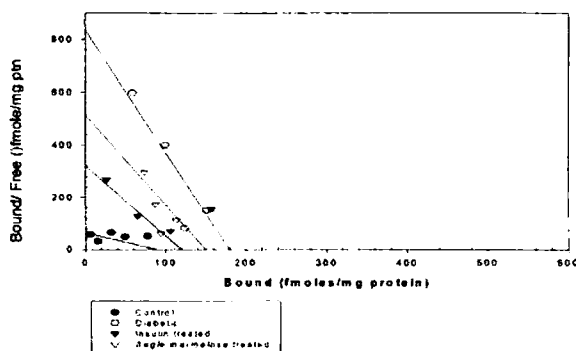| Experimental group	RQ Value
Control	0
Diabetic	-2.61 ± -0.15***
Diabetic + Insulin treated	-0.09 ± -0.21 ᵛᵛᵛ
Diabetic + *Aegle marmelose* treated	-0.23 ± 0.01 ᵛᵛᵛ

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

*** $p < 0.001$ when compared with control

ᵛᵛᵛ $p < 0.001$ when compared with diabetic group

Figure – 19

Scatchard analysis of [³H] QNB binding against pirenzepine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Table-19

Scatchard analysis of [³H] QNB binding against pirenzepine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	2126.60± 14.50	2.62 ± 0.06
Diabetic	1433.30 ± 28.40***	1.35 ± 0.14**
Diabetic + Insulin treated	2023.30 ± 14.50 ᵜᵜᵜ	2.30 ± 0.18 ᵜᵜ
Diabetic + *Costus pictus* treated	2083.30± 33.30 ᵜᵜᵜ	2.35 ± 0.27 ᵜ

Values are mean ± S.E.M of 4-6 separate experiments
** P<0.01 when compared to diabetic group, *** P<0.001 when compared to control,
ᵜ P<0.05 when compared to diabetic group, ᵜᵜ P<0.01 when compared to diabetic group, ᵜᵜᵜ P<0.001 when compared to diabetic group

Table-20

Binding parameters of [³H]QNB against pirenzepine in the cerebral cortex of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.053	3.93×10^{-6}	-0.9904
Diabetic	One-site	-4.989	4.5×10^{-6}	-0.9928
Insulin treated diabetic	One-site	-5.205	2.7×10^{-6}	-0.9664
Costus pictus treated diabetic	One-site	-4.955	4.9×10^{-6}	-0.9914

Values are mean of 4-6 separate experiments

Figure – 20

Binding parameters of [³H]QNB against pirenzepine in the cerebral cortex of experimental rats

- Control
- Diabetic
- Insulin treated
- Costus pictus treated
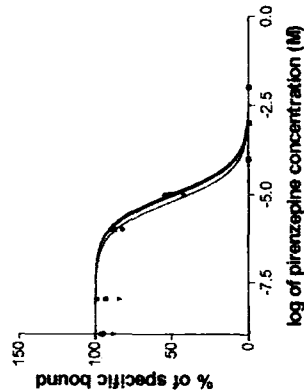
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure – 21

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the cerebral cortex of Control, Diabetic, Diabetic+Insulin and Diabetic+*Costus pictus* treated group rats

373 bp
M1 amplicon

150 bp
β-actin
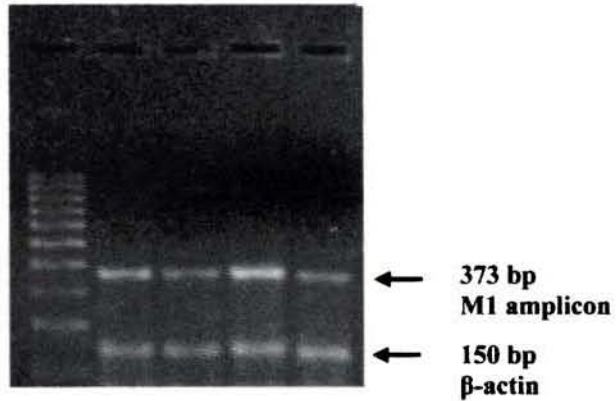
Table-21

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	15782	1
2	15009	0.95
3	17861	1.13
4	15315	0.97

0-1000 bp ladder, 1-Control, 2-Diabetic, 3- Diabetes +Insulin treated, 4- Diabetes +*Costus pictus* treated

Figure -22

Real Time amplification of Muscarinic M1 receptor mRNA from the cerebral cortex of Control, Diabetic, Diabetic+Insulin and Diabetic+*Costus pictus* treated group rats
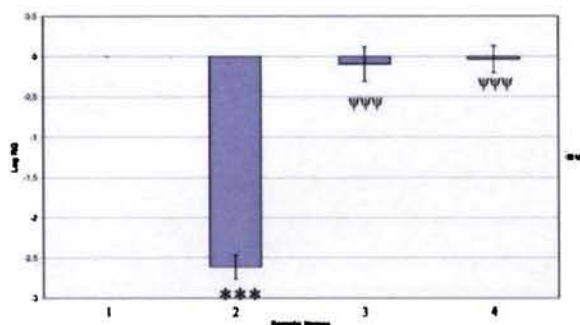
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Costus pictus* treated

Table -22

Experimental group	RQ Value
Control	0
Diabetic	-2.61 ± -0.15***
Diabetic + Insulin treated	-0.09 ± -0.21 ▼▼▼
Diabetic + *Costus pictus* treated	-0.03 ± 0.16 ▼▼▼

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

*** $p<0.001$ when compared with control
▼▼▼ $p<0.001$ when compared with diabetic group

Figure – 23

Scatchard analysis of [³H] QNB binding against atropine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats

Table-23

Scatchard analysis of [³H] QNB binding against atropine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	82.50± 7.50	1.30 ± 0.10
Diabetic	182.50 ± 2.50***	0.22 ± 0.06***
Diabetic + Insulin treated	127.50 ± 2.50 ᵛᵛ	0.24 ± 0.05
Diabetic + *Aegle marmelose* treated	137.50± 2.80 ᵛᵛ	0.22 ± 0.02

Values are mean ± S.E.M of 4-6 separate experiments
*** P<0.001 when compared to control
ᵛᵛ P<0.01 when compared to diabetic group

Table-24

Binding parameters of [³H]QNB against atropine in the brainstem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.601	3.57×10^{-9}	0.9269
Diabetic	One-site	-8.026	1.34×10^{-9}	0.9323
Insulin treated diabetic	One-site	-7.868	1.93×10^{-9}	0.9819
Aegle marmelose treated diabetic	One-site	-7.559	3.94×10^{-9}	0.8019

Values are mean of 4-6 separate experiments

Figure – 24

Binding parameters of [³H]QNB against atropine in the brainstem of experimental rats
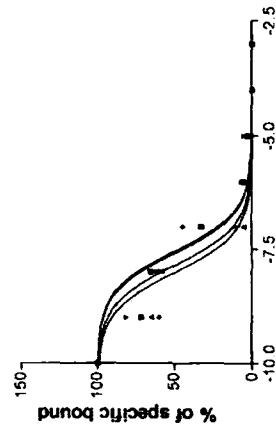
- Control
- Diabetic
- Insulin treated
- *Aegle marmelos*

Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 25

Scatchard analysis of [³H] QNB binding against atropine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats
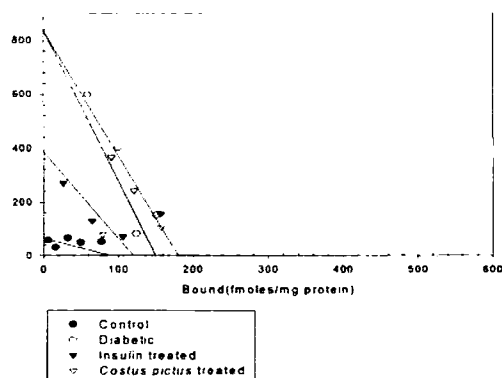
Table-25

Scatchard analysis of [³H] QNB binding against atropine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	Bmax (fmoles/mg protein)	Kd (nM)
Control	82.50± 7.50	1.30 ± 0.10
Diabetic	182.50 ± 2.50***	0.22 ± 0.06***
Diabetic + Insulin treated	127.50 ± 2.50 ΨΨ	0.24 ± 0.05
Diabetic + *Costus pictus* treated	145.00± 5.00 ΨΨ	0.21 ± 0.03

Values are mean ± S.E.M of 4-6 separate experiments
*** P<0.001 when compared to control, ΨΨ P<0.01 when compared to diabetic group

Table – 26

Binding parameters of [³H] QNB against atropine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.601	3.57×10^{-9}	0.9269
Diabetic	One-site	-8.026	1.34×10^{-9}	0.9323
Insulin treated diabetic	One-site	-7.868	1.93×10^{-9}	0.9819
Costus pictus treated diabetic	One-site	-7.703	2.83×10^{-9}	0.9037

Values are mean of 4-6 separate experiments

Figure-26

Binding parameters of [³H]QNB against atropine in the brainstem of experimental rats
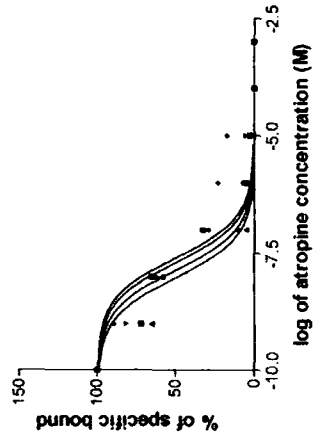
- Control
- Diabetic
- Insulin treated
- *Costus pictus* treated

Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure 27

Scatchard analysis of [³H] QNB binding against pirenzepine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Table – 27

Scatchard analysis of [³H] QNB binding against pirenzepine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Animal status	Bmax (fmoles/mg protein)	Kd (nM)
Control	815.00± 45.00	0.89 ± 0.03
Diabetic	620.00 ± 30.00**	0.58 ± 0.02*
Diabetic + Insulin treated	733.33 ± 16.66 ᵚ	0.73 ± 0.06 ᵚ
Diabetic + *Aegle marmelose* treated	833.33± 33.33 ᵚᵚ	0.78 ± 0.06ᵚ

Values are mean ± S.E.M of 4-6 separate experiments

* P<0.05 when compared to control group, ** P<0.01 when compared to control group

ᵚ P<0.05 when compared to diabetic group, ᵚ ᵚ P<0.01 when compared to diabetic group

Table – 28

Binding parameters of [³H]QNB against pirenzepine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-5.359	1.09×10^{-6}	0.7588
Diabetic	One-site	-5.902	3.13×10^{-7}	0.9644
Insulin treated diabetic	One-site	-5.215	1.52×10^{-6}	0.9782
Aegle marmelose treated diabetic	One-site	-6.193	1.60×10^{-7}	0.7294

Values are mean of 4-6 separate experiments

Figure – 28

Binding parameters of [3H] QNB against pirenzepine in the brain stem of experimental rats
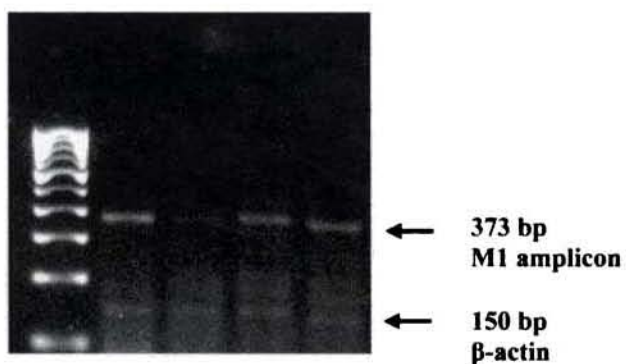
- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treated
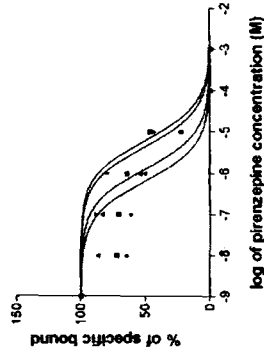
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 29

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the brainstem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

← 373 bp M1 amplicon

← 150 bp β-actin
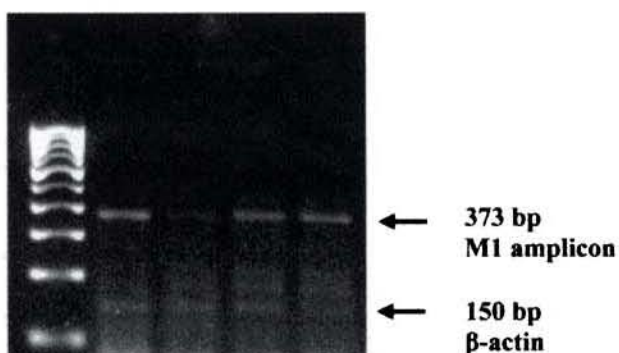
Table 29

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	6634	1
2	4603	0.69
3	6154	0.93
4	5815	0.88

0-1000 bp ladder, 1-Control, 2-Diabetic, 3- Diabetes +Insulin treated, 4- Diabetes +*Aegle marmelose* treated

Figure -30

Real Time amplification of Muscarinic M1 receptor mRNA from the Brainstem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats
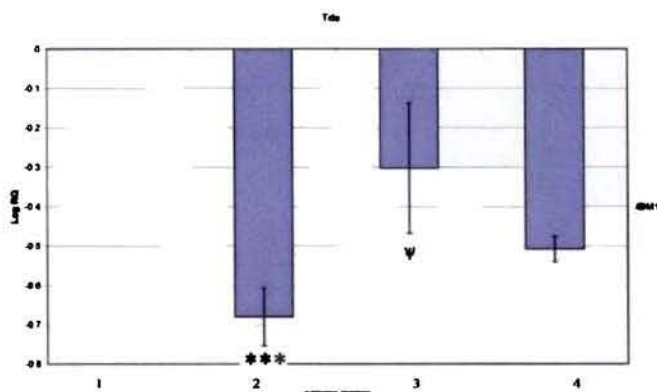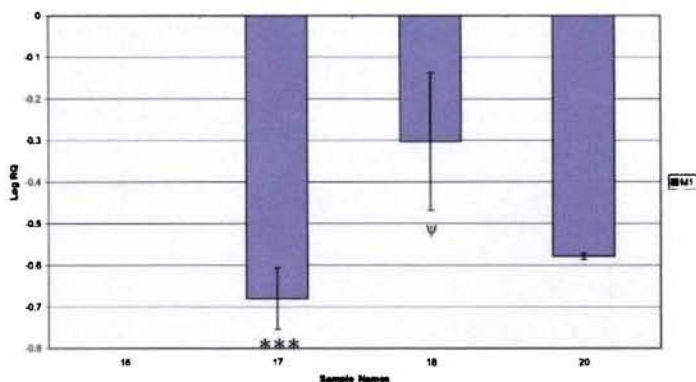

1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Aegle marmelose* treated

Table -30

| Experimental group	RQ Value
Control	0
Diabetic	-0.68 ± -0.07***
Diabetic + Insulin treated	-0.30 ± -0.16 ᵛ
Diabetic + *Aegle marmelose* treated	-0.50 ± 0.03

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

*** $p<0.001$ when compared with control

ᵛ $p<0.05$ when compared with diabetic group

Figure 31

Scatchard analysis of [³H] QNB binding against pirenzepine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Table 31

Scatchard analysis of [³H] QNB binding against pirenzepine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	815.00± 45.00	0.89 ± 0.03
Diabetic	620.00 ± 30.00**	0.58 ± 0.02*
Diabetic + Insulin treated	733.33 ± 16.66 ᵛ	0.73 ± 0.06 ᵛ
Diabetic + *Costus pictus* treated	786.66± 6.66 ᵛᵛᵛ	0.79 ± 0.02 ᵛ

Values are mean ± S.E.M of 4-6 separate experiments.

* $P<0.05$ when compared to control group,** $P<0.01$ when compared to control group

ᵛ $P<0.05$ when compared to diabetic group, ᵛᵛᵛ $P<0.001$ when compared to diabetic group

Table 32

Binding parameters of [³H]QNB against pirenzepine in the brain stem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+Costus pictus treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-5.359	1.09×10^{-6}	0.7588
Diabetic	One-site	-5.902	3.13×10^{-7}	0.9644
Insulin treated diabetic	One-site	-5.215	1.52×10^{-6}	0.9782
Costus pictus treated diabetic	One-site	-6.695	5.04×10^{-6}	0.8313

Values are mean of 4-6 separate experiments

Figure 32
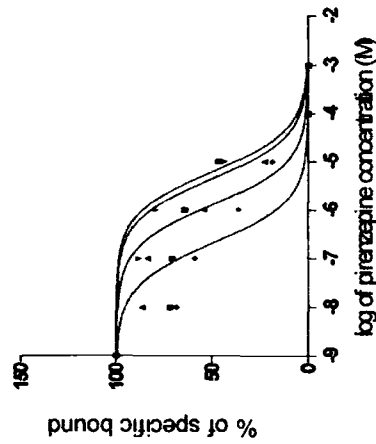
• Control
• Diabetic
• Insulin treated
• Costus pictus treated

Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure 33

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the brainstem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Table 33

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	6634	1
2	4603	0.69
3	6154	0.93
4	5750	0.86

0-1000 bp ladder, 1-Control, 2-Diabetic, 3- Diabetes +Insulin treated, 4- Diabetes +*Costus pictus* treated

Figure -34

Real Time amplification of Muscarinic M1 receptor mRNA from the Brainstem of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Costus pictus* treated

Table -34

Experimental group	RQ Value
Control	0
Diabetic	-0.68 ± -0.07 ***
Diabetic + Insulin treated	-0.30 ± -0.16 ᵛ
Diabetic + *Costus pictus* treated	-0.57 ± 0.01

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

*** p<0.001 when compared with control

ᵛ p<0.05 when compared with diabetic group

Figure 35

Scatchard analysis of [^{3}H] QNB binding against atropine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats
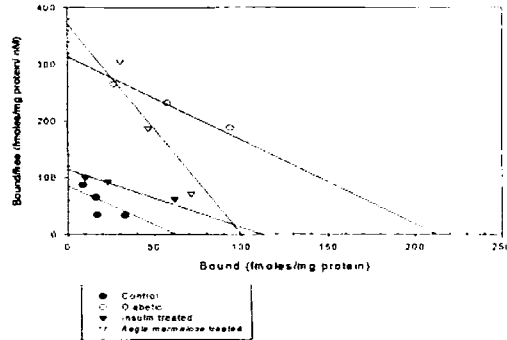
Table – 35

Scatchard analysis of [^{3}H] QNB binding against atropine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats

Animal status	Bmax (fmoles/mg protein)	Kd (nM)
Control	86.50± 6.50	1.40 ± 0.20
Diabetic	207.50 ± 12.50***	0.71 ± 0.03**
Diabetic + Insulin treated	120.00 ± 10.00 ♥♥♥	0.23 ± 0.03
Diabetic + *Aegle marmelose* treated	90.00± 10.00 ♥♥♥	0.21 ± 0.07

Values are mean ± S.E.M of 4-6 separate experiments
** P<0.01 when compared to control, *** P<0.001 when compared to control,
♥♥♥ P<0.001 when compared to diabetic group.

Table – 36

Binding parameters of [³H]QNB against atropine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.272	5.34×10^{-8}	0.9618
Diabetic	One-site	-7.765	1.71×10^{-9}	0.8883
Insulin treated diabetic	One-site	-7.521	3.01×10^{-8}	0.9606
Aegle marmelose treated diabetic	One-site	-7.301	5.00×10^{-8}	0.9526

Values are mean of 4-6 separate experiments

Figure – 36

Binding parameters of [³H]QNB against atropine in the hypothalamus of experimental rats

- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treated
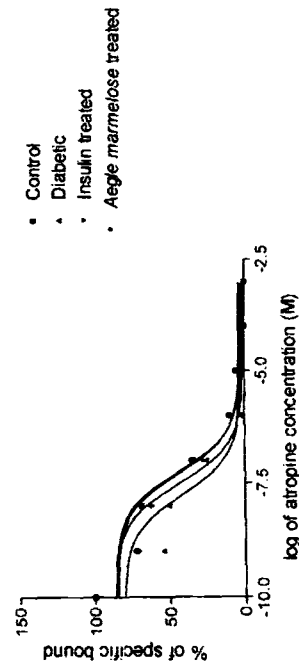
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure 37

Scatchard analysis of [³H] QNB binding against atropine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats
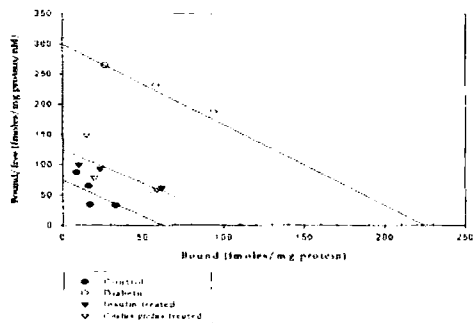
Table 37

Scatchard analysis of [³H] QNB binding against atropine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	86.50± 6.50	1.40 ± 0.20
Diabetic	207.50 ± 12.50***	0.71 ± 0.03**
Diabetic + Insulin treated	120.00 ± 10.00 ᵛᵛᵛ	0.23 ± 0.03
Diabetic + *Costus pictus* treated	76.50± 1.50 ᵛᵛᵛ	0.25 ± 0.15

Values are mean ± S.E.M of 4-6 separate experiments
*** $P<0.001$ when compared to control ** $P<0.01$ when compared to control,
ᵛᵛᵛ $P<0.001$ when compared to diabetic group

Table 38

Binding parameters of [³H]QNB against atropine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.272	5.34×10^{-8}	0.9618
Diabetic	One-site	-7.762	1.73×10^{-9}	0.8883
Insulin treated diabetic	One-site	-7.520	3.01×10^{-8}	0.9606
Costus pictus treated diabetic	One-site	-7.657	2.20×10^{-8}	0.9893

Values are mean of 4-6 separate experiments

Fig 38
Binding parameters of [³H]QNB against atropine in the hypothalamus of experimental rats

- Control
- Diabetic
- Insulin treated
- *Costus pictus* treated

% of specific bound

log of atropine concentration (M)
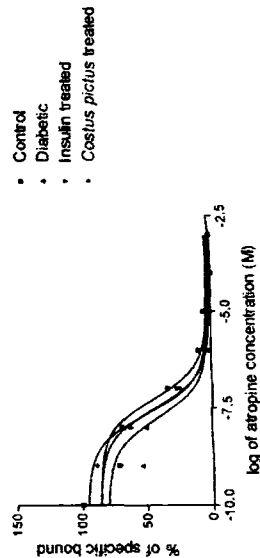
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure 39

Scatchard analysis of [³H] QNB binding against pirenzepine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelos* treated group rats
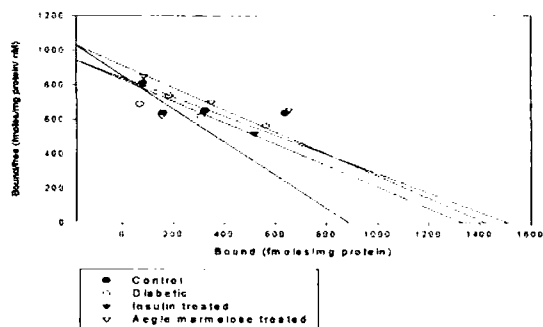

Table 39

Scatchard analysis of [³H] QNB binding against pirenzepine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	1533.33± 33.33	1.47 ± 0.19
Diabetic	923.33 ± 23.33***	0.86 ± 0.02***
Diabetic + Insulin treated	1363.33 ± 31.79ᵛ ᵛ ᵛ	1.30 ± 0.30 ᵛ
Diabetic + *Aegle marmelose* treated	1480.00± 46.18 ᵛ ᵛ ᵛ	1.36 ± 0.12 ᵛ

Values are mean ± S.E.M of 4-6 separate experiments
*** P<0.001 when compared to control, ᵛ P<0.05 when compared to diabetic group,
ᵛ ᵛ ᵛ P<0.001 when compared to diabetic group

Table – 40

### Binding parameters of [³H]QNB against pirenzepine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-5.295	1.69×10^{-6}	0.6446
Diabetic	One-site	-5.190	2.15×10^{-6}	0.8872
Insulin treated diabetic	One-site	-5.003	3.30×10^{-6}	0.9774
Aegle marmelose treated diabetic	One-site	-5.214	2.03×10^{-6}	0.9014

Values are mean of 4-6 separate experiments

Figure – 40

Binding parameters of [³H]QNB against pirenzepine in the hypothalamus of experimental rats

- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treated
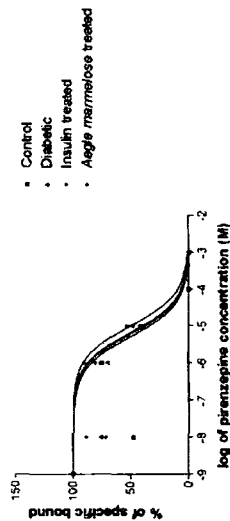
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure - 41

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

373 bp M1 amplicon

150 bp β-actin
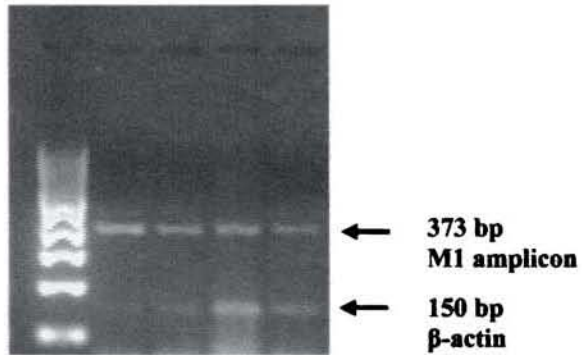
Table – 41

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	12529	1
2	10726	0.85
3	7519	0.6
4	10095	0.80

0-1000 bp ladder, 1-Control, 2-Diabetic, 3- Diabetes +Insulin treated, 4- Diabetes +*Aegle marmelose* treated

Figure -42

Real Time amplification of Muscarinic M1 receptor mRNA from the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats
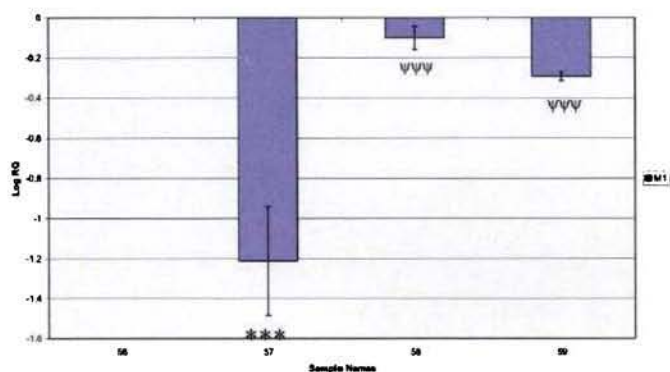
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Aegle marmelose* treated

Table -42

Experimental group	RQ Value
Control	0
Diabetic	1.21± 0.27***
Diabetic + Insulin treated	0.10 ± -0.06ᵛᵛᵛ
Diabetic + *Aegle marmelose* treated	0.29 ± 0.02ᵛᵛᵛ

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

*** $p<0.001$ when compared with control

ᵛᵛᵛ $p<0.001$ when compared with diabetic group

Figure – 43

Scatchard analysis of [³H] QNB binding against pirenzepine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats
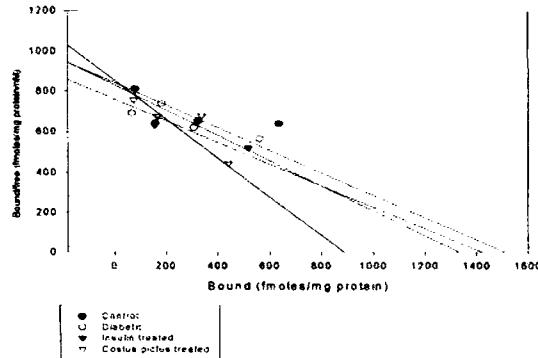
Table 43

**Scatchard analysis of [³H] QNB binding against pirenzepine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats**

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	1533.33± 33.33	2.51 ± 0.20
Diabetic	923.33 ± 23.33***	1.17 ± 0.09***
Diabetic + Insulin treated	1363.33 ± 31.79ᵛᵛᵛ	1.89 ± 0.16 ᵛ
Diabetic + *Costus pictus* treated	1348.33± 24.21 ᵛᵛᵛ	1.36 ± 0.13 ᵛ

Values are mean ± S.E.M of 4-6 separate experiments
*** P<0.001 when compared to control
ᵛ P<0.05 when compared to diabetic group, ᵛᵛᵛ P<0.001 when compared to diabetic group

Table – 44

Binding parameters of [³H]QNB against pirenzepine in the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-5.295	1.26×10^{-6}	0.6446
Diabetic	One-site	-5.190	1.61×10^{-6}	0.8872
Insulin treated diabetic	One-site	-5.003	2.48×10^{-6}	0.9774
Costus pictus treated diabetic	One-site	-5.023	2.37×10^{-6}	0.9325

Values are mean of 4-6 separate experiments

Figure – 44

Binding parameters of [³H]QNB against pirenzepine in the hypothalamus of experimental rats

- Control
- Diabetic
- Insulin treated
- *Costus pictus* treated
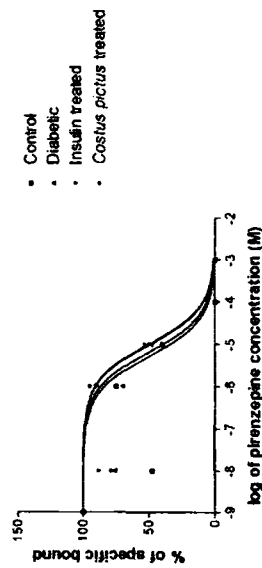
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 45

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

373 bp M1 amplicon

150 bp β-actin
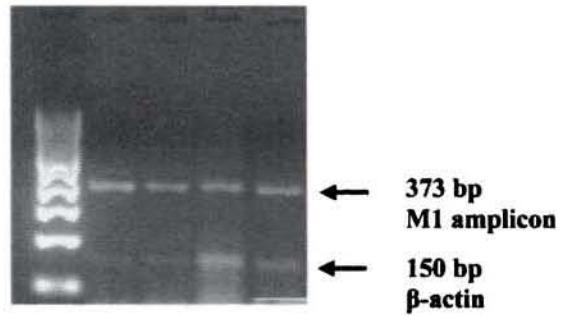
Table – 45

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	12529	1
2	10726	0.85
3	7519	0.6
4	16857	1.97

0-1000 bp ladder, 1-Control, 2-Diabetic, 3-Diabetes +Insulin treated, 4-Diabetes +*Costus pictus* treated

Figure -46

Real Time amplification of Muscarinic M1 receptor mRNA from the hypothalamus of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats
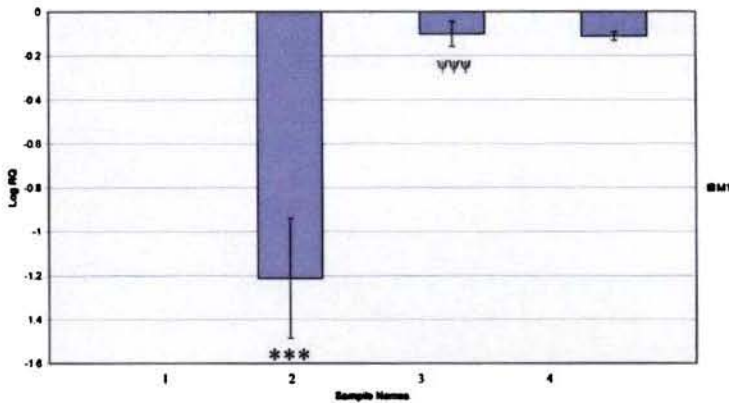
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Costus pictus* treated

Table -46

Experimental group	RQ Value
Control	0
Diabetic	1.21± 0.27***
Diabetic + Insulin treated	0.10 ± -0.06ᵛᵛᵛ
Diabetic + *Costus pictus* treated	0.11 ± 0.01ᵛᵛᵛ

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

*** p<0.001 when compared with control
ᵛᵛᵛ p<0.001when compared with diabetic group

Figure – 47

Scatchard analysis of [³H] QNB binding against atropine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Table – 47

Scatchard analysis of [³H] QNB binding against atropine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	214.00 ± 3.05	0.19 ± 0.01
Diabetic	150.00 ± 5.77***	0.27 ± 0.05***
Diabetic + Insulin treated	184.00 ± 3.05 ᵛᵛᵛ	0.17± 0.05 ᵛᵛᵛ
Diabetic + *Aegle marmelose* treated	220.00 ± 4.41 ᵛᵛᵛ	0.19 ± 0.03 ᵛᵛᵛ

Values are mean ± S.E.M of 4-6 separate experiments
*** P<0.001 when compared to control
ᵛᵛᵛ P<0.001 when compared to diabetic group

Table – 48
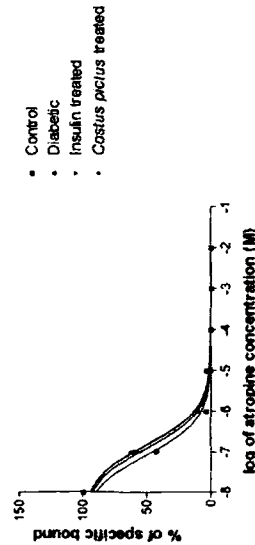
Binding parameters of [³H] QNB against atropine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.076	2.10×10^{-8}	0.9832
Diabetic	One-site	-6.818	3.80×10^{-8}	0.9883
Insulin treated diabetic	One-site	-6.913	3.05×10^{-8}	0.9889
Aegle marmelose treated diabetic	One-site	-6.695	5.04×10^{-8}	0.9803

Values are mean of 4-6 separate experiments

Figure – 48

Binding parameters of [³H] QNB against atropine in the corpus striatum of experimental rats

- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treated
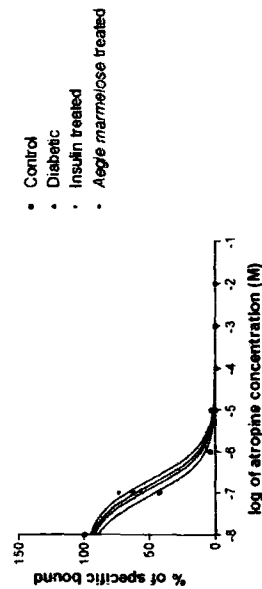
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 49

Scatchard analysis of [3H] QNB binding against atropine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+Costus pictus treated group rats

Table – 49

Scatchard analysis of [^{3}H] QNB binding against atropine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	214.00 ± 3.05	0.19 ± 0.01
Diabetic	150.00 ± 5.77***	0.27 ± 0.05***
Diabetic + Insulin treated	184.00 ± 3.05 ▼▼▼	0.17 ± 0.05 ▼▼▼
Diabetic + *Costus pictus* treated	188.00 ± 4.16 ▼▼▼	0.13 ± 0.06 ▼▼▼

Values are mean ± S.E.M of 4-6 separate experiments
*** $P < 0.001$ when compared to control
▼▼▼ $P < 0.001$ when compared to diabetic group

Table – 50

Binding parameters of [³H]QNB against atropine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.076	2.10×10^{-8}	0.9832
Diabetic	One-site	-6.818	3.80×10^{-8}	0.9883
Insulin treated diabetic	One-site	-6.913	3.05×10^{-8}	0.9889
Costus pictus treated diabetic	One-site	-6.827	3.72×10^{-8}	0.9950

Values are mean of 4-6 separate experiments

Figure – 50

Binding parameters of [³H]QNB against atropine in the corpus striatum of experimental rats

- Control
- Diabetic
- Insulin treated
- *Costus pictus* treated

Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 51

Scatchard analysis of [3H] QNB binding against pirenzepine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats
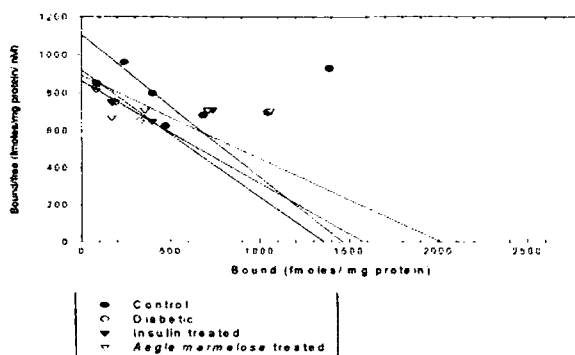
Table – 51

**Scatchard analysis of [3H] QNB binding against pirenzepine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats**

Animal status	Bmax (fmoles/mg protein)	Kd (nM)
Control	1460.00± 30.55	1.34 ± 0.02
Diabetic	2060.00 ± 30.55***	0.45 ± 0.02***
Diabetic + Insulin treated	1550.00 ± 28.86 ΨΨΨ	0.56 ± 0.01
Diabetic + *Aegle marmelose* treated	1400.00± 11.54 ΨΨΨ	1.53 ± 0.13ΨΨΨ

Values are mean ± S.E.M of 4-6 separate experiments
 *** P<0.001 when compared to control
ΨΨΨ P<0.001 when compared to diabetic group

Table – 52

Binding parameters of [³H]QNB against pirenzepine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.107	2.23 x 10^{-8}	0.7224
Diabetic	One-site	-6.459	9.92 x 10^{-8}	0.7444
Insulin treated diabetic	One-site	-7.322	4.76 x 10^{-8}	0.6826
Aegle marmelose treated diabetic	One-site	-6.908	3.52 x 10^{-8}	0.6836

Values are mean of 4-6 separate experiments

Figure – 52

Binding parameters of [³H]QNB against pirenzepine in the corpus striatum of experimental rats

- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treated
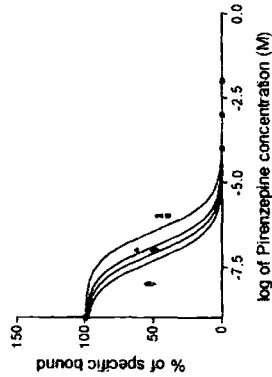
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 53

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats

← 373 bp M1 amplicon

← 150 bp β-actin
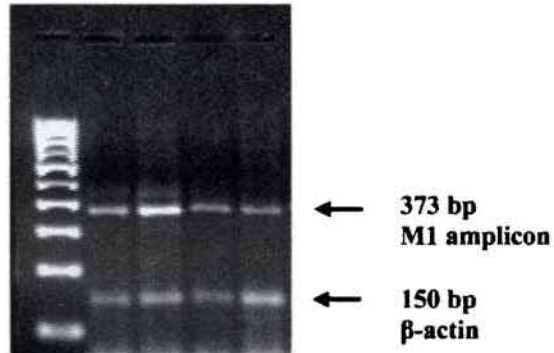
Table – 53

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	16073	1
2	29941	1.863
3	17018	1.059
4	13093	0.815

0-1000 bp ladder, 1-Control, 2-Diabetic, 3-Diabetes +Insulin treated, 4-Diabetes +*Aegle marmelose* treated

Figure -54

Real Time amplification of Muscarinic M1 receptor mRNA from the Corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats
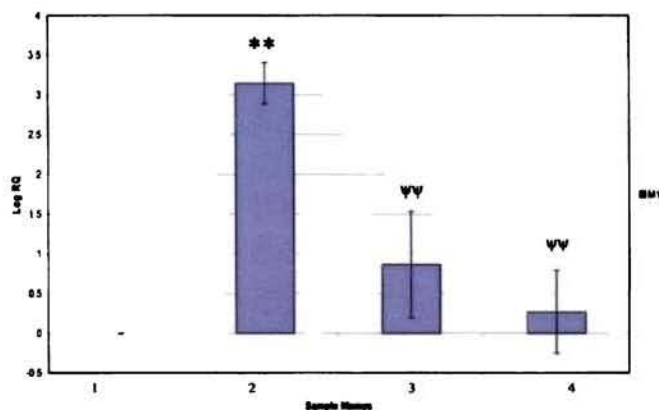
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Aegle marmelose* treated

Table -54

Experimental group	RQ Value
Control	0
Diabetic	3.14± 0.25**
Diabetic + Insulin treated	0.86 ± -0.66ᵛᵛ
Diabetic + *Costus pictus* treated	0.26 ± 0.52ᵛᵛ

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta$CT method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

** $p < 0.01$ when compared with control

ᵛᵛ$p < 0.01$ when compared with diabetic group

Figure – 55

Scatchard analysis of [3H] QNB binding against pirenzepine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Table-55

## Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	1460.00± 30.55	1.34 ± 0.02
Diabetic	2060.00 ± 30.55***	0.45 ± 0.02***
Diabetic + Insulin treated	1550.00 ± 28.86 ᵛᵛᵛ	0.56 ± 0.01
Diabetic + *Costus pictus* treated	1633.33± 72.64 ᵛᵛᵛ	1.53 ± 0.13 ᵛᵛᵛ

Values are mean ± S.E.M of 4-6 separate experiments
*** $P<0.001$ when compared to control,
ᵛᵛᵛ $P<0.001$ when compared to diabetic group

Table-56

Binding parameters of [³H]QNB against pirenzepine in the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-7.107	2.23×10^{-8}	0.7224
Diabetic	One-site	-6.459	9.92×10^{-8}	0.7444
Insulin treated diabetic	One-site	-7.322	4.76×10^{-8}	0.6826
Costus pictus treated diabetic	One-site	-6.726	5.36×10^{-8}	0.7603

Values are mean of 4-6 separate experiments

Figure – 56

Binding parameters of [³H] QNB against pirenzepine in the corpus striatum of experimental rats

- Control
- Diabetic
- Insulin treated
- *Costus pictus* treated

Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure – 57

RT-PCR amplification product of Muscarinic M1 receptor mRNA from the corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

← 373 bp M1 amplicon

← 150 bp β-actin
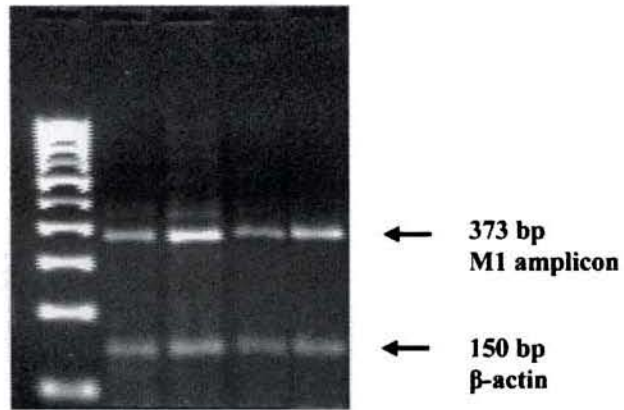
Table-57

Band properties of M1 receptor mRNA RT-PCR amplification

Lane No	Area	Relation
1	16073	1
2	29941	1.863
3	17018	1.059
4	20446	1.272

0-1000 bp ladder, 1-Control, 2-Diabetic, 3-Diabetes +Insulin treated, 4-Diabetes +*Aegle marmelose* treated

Figure -58

Real Time amplification of Muscarinic M1 receptor mRNA from the Corpus striatum of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats
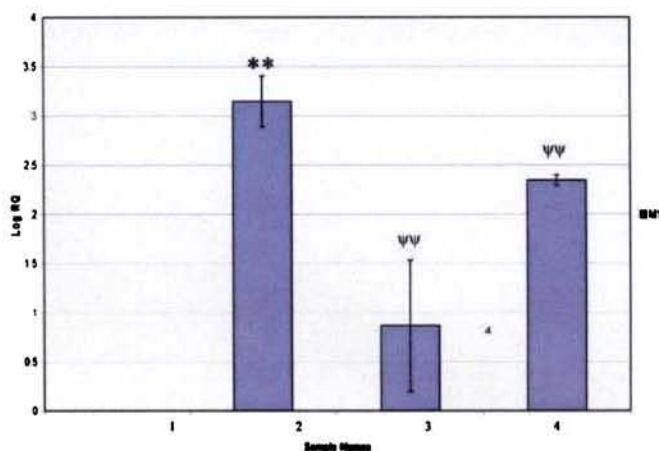
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Costus pictus* treated

Table -58

Experimental group	RQ Value
Control	0
Diabetic	1.21 ± 0.27**
Diabetic + Insulin treated	0.10 ± -0.06ᵛᵛ
Diabetic + *Costus pictus* treated	0.29 ± 0.02ᵛᵛ

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

** $p<0.01$ when compared with control

ᵛᵛ $p<0.01$ when compared with diabetic group

Figure – 59

Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the pancreatic Islets of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats

Table-59

Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the pancreatic Islets of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats

Animal status	B$_{max}$ (fmoles/mg protein)	K$_d$ (nM)
Control	132.83± 18.61	2.30 ± 0.15
Diabetic	47.33 ± 5.45**	2.96 ± 0.09*
Diabetic + Insulin treated	107.33 ± 9.40$^{\Psi}$	2.09 ± 0.05 $^{\Psi}$
Diabetic + *Aegle marmelose* treated	88.66± 6.96 $^{\Psi}$	1.90 ± 0.17 $^{\Psi}$

Values are mean ± S.E.M of 4-6 separate experiments
* P<0.05 when compared to control, ** P<0.01 when compared to control,
$^{\Psi}$ P<0.05 when compared to diabetic group

Table-60

Binding parameters of [³H]QNB against pirenzepine in the pancreas of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Aegle marmelose* treated group rats
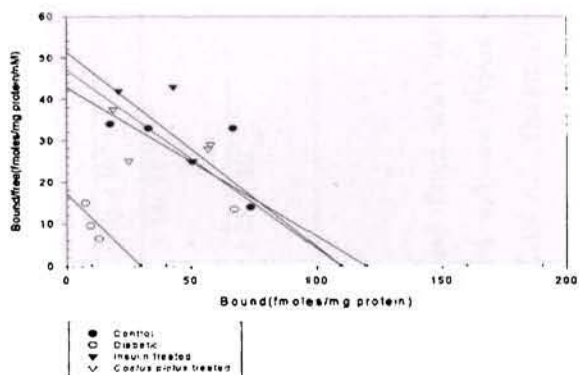
Experimental Group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-6.701	1.24x 10^{-7}	0.7249
Diabetic	One-site	-7.030	5.83 x 10^{-8}	0.8698
Insulin treated diabetic	One-site	-6.803	9.84x 10^{-8}	0.8730
Aegle marmelose treated diabetic	One-site	-8.100	4.96 x 10^{-8}	0.7166

Values are mean of 4-6 separate experiments

Figure 60

Binding parameters of [³H]QNB against atropine in the hypothalamus of experimental rats

- Control
- Diabetic
- Insulin treated
- *Aegle marmelose* treat
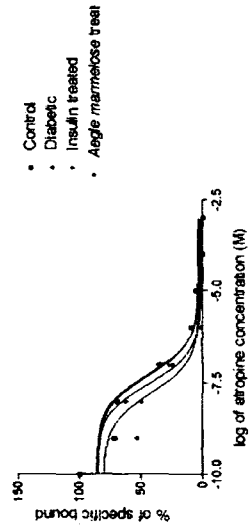
Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure -61

Real Time amplification of Muscarinic M1 receptor mRNA from the pancreas of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Aegle marmelose* treated group rats
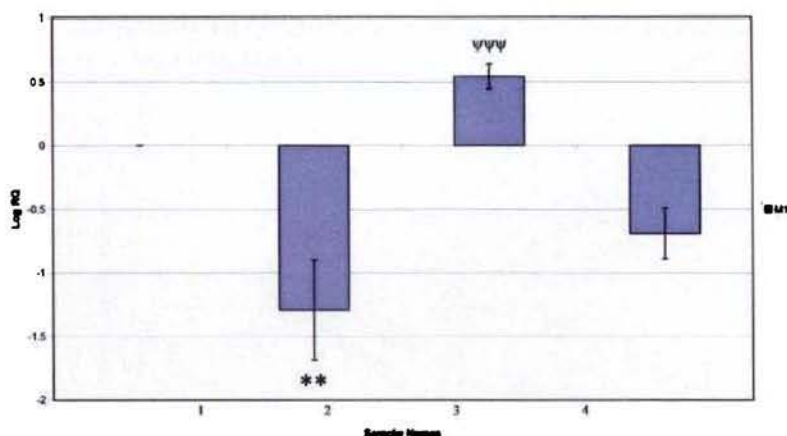
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Aegle marmelose* treated

Figure -61

Experimental group	RQ Value
Control	0
Diabetic	-1.29±0.39 **
Diabetic + Insulin treated	0.54±0.10 ▼▼▼
Diabetic + *Aegle marmelose* treated	-0.69±0.19

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

** $p<0.01$ when compared with control

▼▼▼ $p<0.001$ when compared with diabetic group

Figure – 62

Scatchard analysis of [³H] QNB binding against pirenzepine in the pancreatic Islets of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats


Table – 62

Scatchard analysis of [3H] QNB binding against pirenzepine in the pancreatic islets of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	132.83± 18.61	2.30 ± 0.15
Diabetic	47.33 ± 5.45**	2.96 ± 0.09*
Diabetic + Insulin treated	107.33 ± 9.40ᵛ	2.09 ± 0.05 ᵛ
Diabetic + *Costus pictus* treated	106.00± 18.14 ᵛ	2.09 ± 0.23 ᵛ

Values are mean ± S.E.M of 4-6 separate experiments
* $P<0.05$ when compared to control, ** $P<0.01$ when compared to control
ᵛ $P<0.05$ when compared to diabetic group

Table – 63

Binding parameters of [³H]QNB against pirenzepine in the pancreas of of Control, Diabetic, Diabetic+Insulin treated and Diabetic+ *Costus pictus* treated group rats

Experimental group	Best-fit model	Log (EC$_{50}$)	Ki	Hill slope
Control	One-site	-6.701	1.24x 10^{-7}	0.7249
Diabetic	One-site	-7.030	5.83 x 10^{-8}	0.8698
Insulin treated diabetic	One-site	-6.803	9.84x 10^{-8}	0.8730
Costus pictus treated diabetic	One-site	-6.698	1.25 x 10^{-7}	0.9049

Values are mean of 4-6 separate experiments

Figure 63

Binding parameters of [³H]QNB against pirenzepine in the pancreas of experimental rats
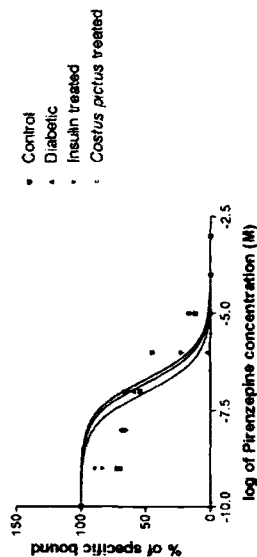
- Control
- Diabetic
- Insulin treated
- *Costus pictus* treated

Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. EC$_{50}$ is the concentration of the competitor that competes for half the specific binding.

Figure -64

Real Time amplification of Muscarinic M1 receptor mRNA from the pancreas of Control, Diabetic, Diabetic+Insulin treated and Diabetic+*Costus pictus* treated group rats
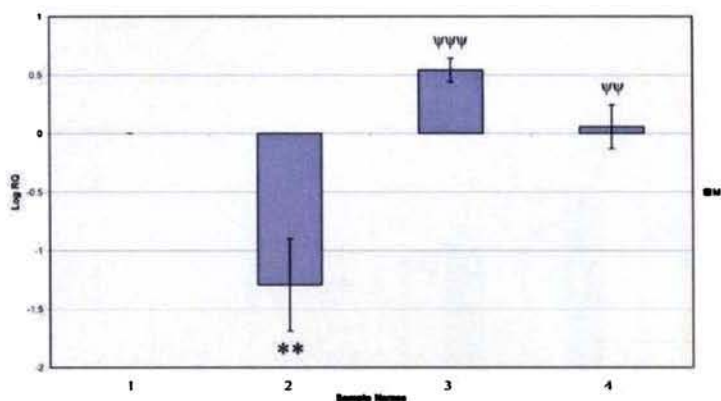
1 – Control
2 - Diabetic
3 – Diabetic + Insulin treated
4 – Diabetic + *Costus pictus* treated

Table -64

Experimental group	RQ Value
Control	0
Diabetic	-1.29±0.39**
Diabetic + Insulin treated	0.54±0.10ᵛᵛᵛ
Diabetic + *Costus pictus* treated	0.05±0.18ᵛᵛ

Values are mean ± S.D of 4-6 separate experiments. Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $\Delta\Delta CT$ method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

** $p<0.01$ when compared with control
ᵛᵛ$p<0.01$ when compared with diabetic group
ᵛᵛᵛ$p<0.001$ when compared with diabetic group

Figure - 65

Effect of *Aegle marmeolse* leaf extract on glucose induced insulin secretion from Pancreatic islets in one hour *in vitro* culture

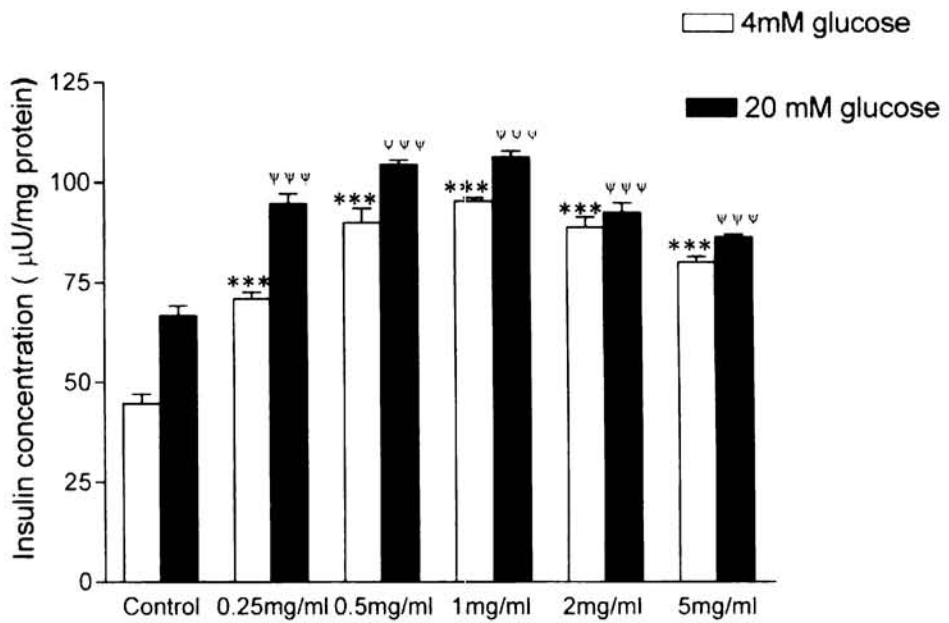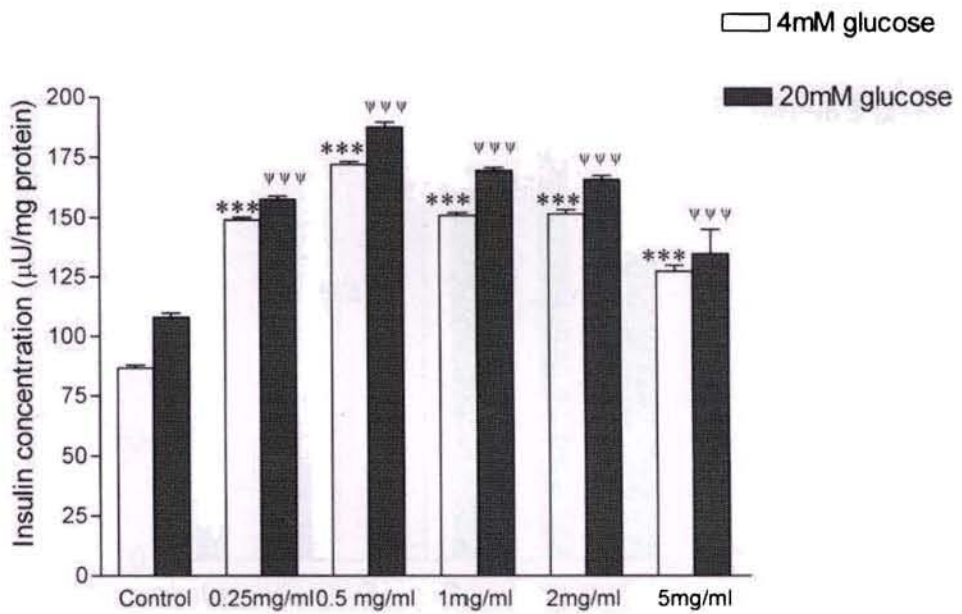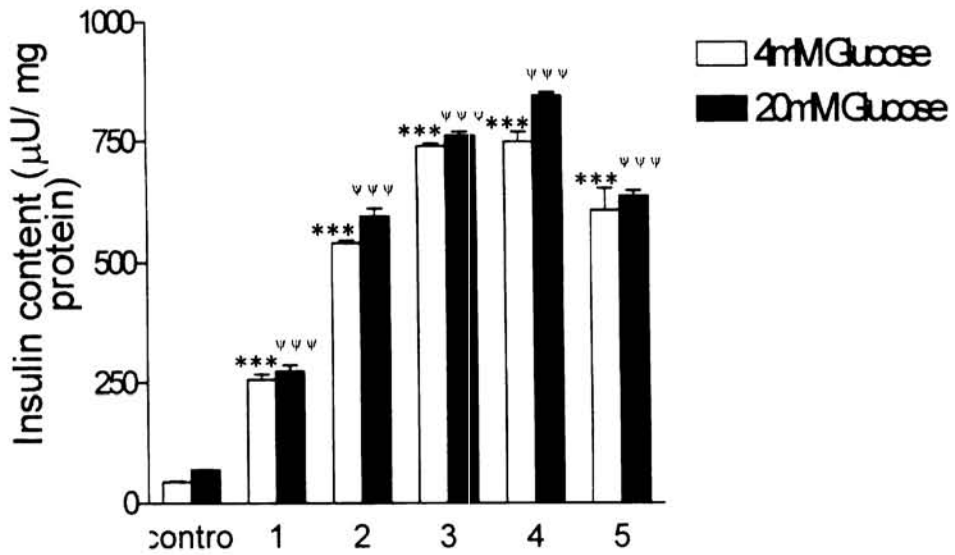***P<0.001   when compared to respective control (medium only)
ᵛ ᵛ ᵛ P<0.001   when compared to respective control (medium only)

Figure -66

Effect of *Aegle marmeolse* leaf extract on glucose induced insulin secretion from Pancreatic islets in 24 hours *in vitro* culture
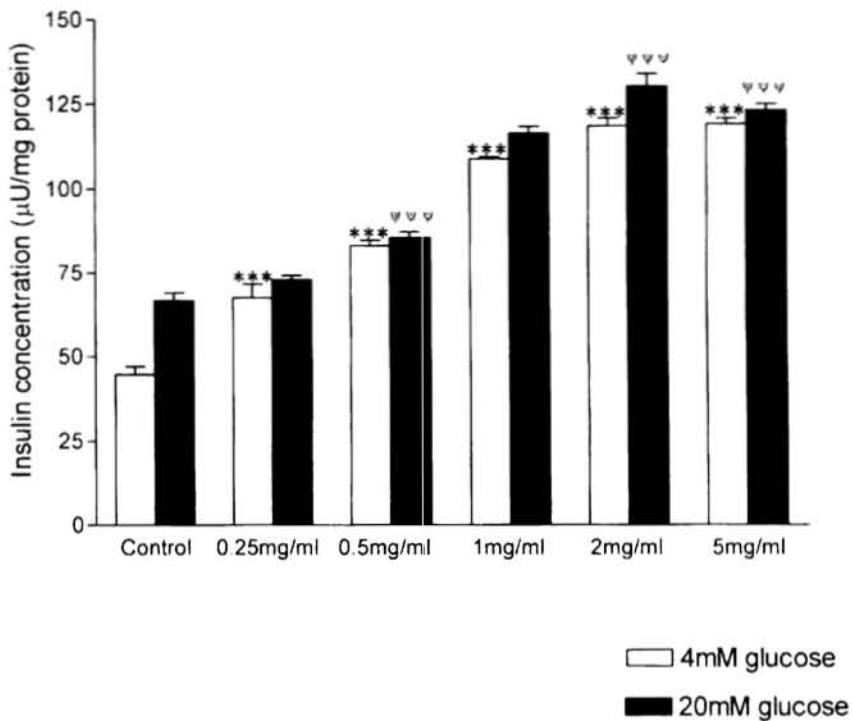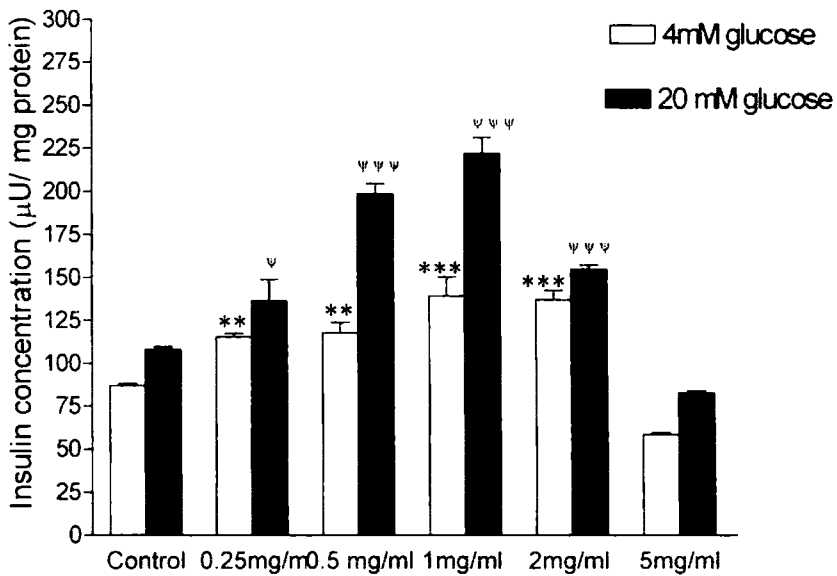
**P<0.01   when compared to respective control (medium only)
***P<0.001   when compared to respective control (medium only)
Ψ P<0.05 when compared to respective control (medium only)
ΨΨΨ P<0.001 when compared to respective control (medium only)

Figure 67

Effect of *Costus pictus* leaf extract on glucose induced insulin secretion from Pancreatic islets in one hour *in vitro* culture

***P<0.001   when compared to respective control (medium only)
ᵛᵛᵛ P<0.001   when compared to respective control (medium only)

Figure 68

Effect of *Costus pictus* leaf extract on glucose induced insulin secretion from Pancreatic islets in 24 hours *in vitro* culture

***P<0.001 when compared to respective control (medium only)
ᵛᵛᵛ P<0.001 when compared to respective control (medium only)

Figure – 69

Effect of cholinergic receptor agonist carbachol and leaf extract of *Aegle marmelose* on insulin secretion from pancreatic islets in one hour *in vitro* culture

Control – Medium only
1- 10^{-7} M Carbachol
1- 10^{-7} M Carbachol + 0.5 mg/ml *Aegle marmelose*
3- 10^{-7} M Carbachol + 1 mg/ml *Aegle marmelose*
4- 10^{-7} M Carbachol + 2 mg/ml *Aegle marmelose*
5- 10^{-7} M Carbachol + 5 mg/ml *Aegle marmelose*

***$P<0.001$ when compared to respective control 10^{-7} M Carbachol at 4mM glucose concentration
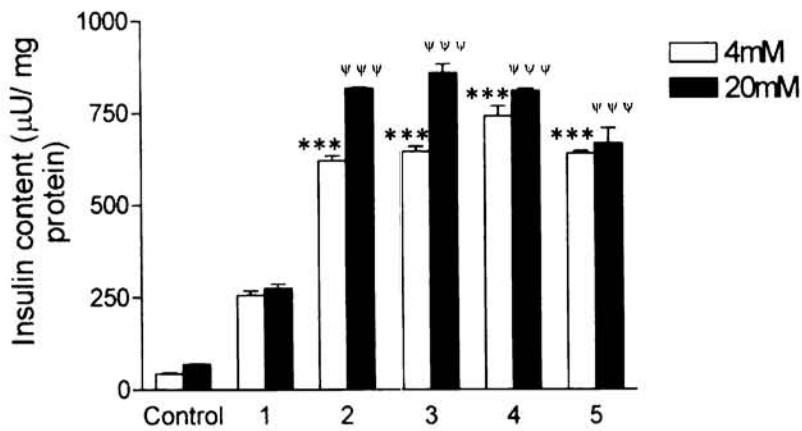ѰѰѰ $P<0.001$ when compared to respective control 10^{-7} M Carbachol at 20mM glucose concentration

Figure – 70

Effect of cholinergic receptor agonist carbachol and leaf extract of *Aegle marmelose* on insulin secretion from pancreatic islets in 24 hour *in vitro* culture
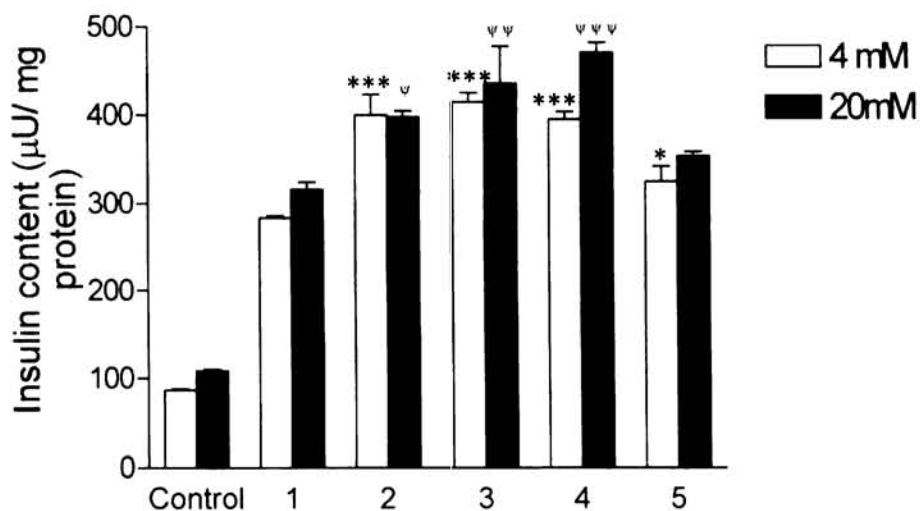
Control - Medium only
1- 10^{-7} M Carbachol
1- 10^{-7} M Carbachol + 0.5 mg/ml *Aegle marmelose*
3- 10^{-7} M Carbachol + 1 mg/ml *Aegle marmelose*
4- 10^{-7} M Carbachol + 2 mg/ml *Aegle marmelose*
5- 10^{-7} M Carbachol + 5 mg/ml *Aegle marmelose*

*$P<0.05$ when compared to respective control 10^{-7} M Carbachol at 4mM glucose concentration
***$P<0.001$ when compared to respective control 10^{-7} M Carbachol at 4mM glucose concentration
ᵛ $P<0.05$ when compared to respective control 10^{-7} M Carbachol at 20mM glucose concentration
ᵛ ᵛ $P<0.01$ when compared to respective control 10^{-7} M Carbachol at 20mM glucose concentration
ᵛ ᵛ ᵛ $P<0.001$ when compared to respective control 10^{-7} M Carbachol at 20mM glucose concentration

Figure – 71

Effect of cholinergic receptor agonist carbachol and leaf extract of *Costus pictus* on insulin secretion from pancreatic islets in one hour *in vitro* culture

Control – Medium only
1- 10^{-7} M Carbachol
2- 10^{-7} M Carbachol + 0.5 mg *Costus pictus*
3- 10^{-7} M Carbachol + 1 mg *Costus pictus*
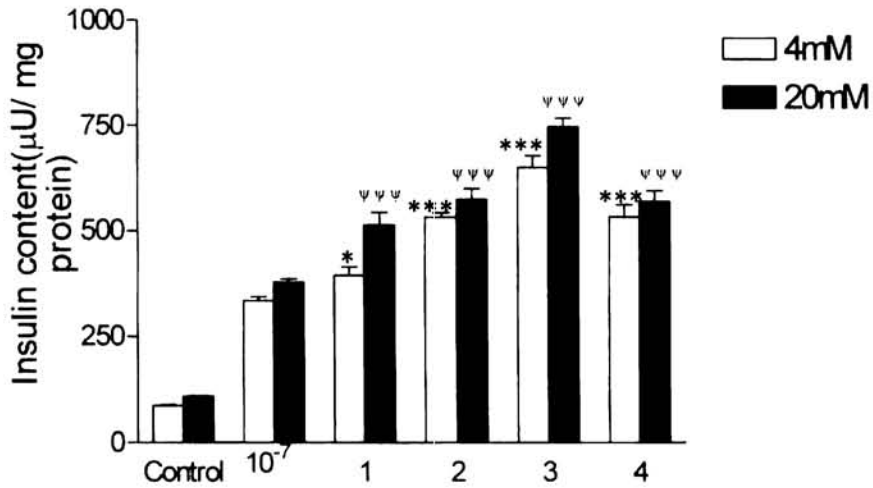4- 10^{-7} M Carbachol + 2 mg *Costus pictus*
5- 10^{-7} M Carbachol + 5 mg *Costus pictus*

***$P<0.001$ when compared to respective control 10^{-7} M Carbachol at 4mM glucose concentration
ΨΨΨ $P<0.001$ when compared to respective control 10^{-7} M Carbachol at 20mM glucose concentration

Figure – 72

Effect of cholinergic receptor agonist carbachol and leaf extract of *Costus pictus* on insulin secretion from pancreatic islets in 24 hours *in vitro* culture

Control – Medium only
1- 10^{-7} M Carbachol
2- 10^{-7} M Carbachol + 0.5 mg/ml *Costus pictus*
3- 10^{-7}M Carbachol + 1 mg /ml *Costus pictus*
4- 10^{-7}M Carbachol + 2 mg/ml *Costus pictus*
5- 10^{-7}M Carbachol + 5 mg/ml *Costus pictus*

***P<0.001 when compared to respective control 10^{-7} M Carbachol at 4mM glucose concentration
ΨΨΨ P<0.001 when compared to respective control 10^{-7} M Carbachol at 20mM glucose concentration

Table 65

Effect of *Aegle marmelose* and *Costus pictus* leaf extract on ^{14}C glucose uptake in the liver of experimental rats

Animal status	^{14}C glucose counts(dpm/mg protein)	
	30 minutes	60 minutes
Control	252.30 ± 5.80	351.66 ± 16.41
Diabetic	184.33 ± 3.48***	208.10 ± 10.99*
Diabetic + Insulin treated	206.30 ± 1.87	329.66 ± 19.83$^{\Psi}$
Diabetic + *Aegle marmelose* treated	231.96 ± 6.09	291.00 ± 9.53$^{\Psi\Psi}$
Diabetic + *Costus pictus* treated	219.33 ± 10.86	315.50 ± 46.94$^{\Psi\Psi}$

Values are mean ± S.E.M of 4-6 separate experiments
*P<0.05 when compared to control group, *** P<0.001 when compared to control
$^{\Psi}$ P<0.05 when compared to diabetic group, $^{\Psi\Psi}$ P<0.01 when compared to diabetic group

Table 66

Effect of *Aegle marmelose* and *Costus pictus* leaf extract on ^{14}C glucose uptake in the cerebral cortex of experimental rats

Animal status	^{14}C glucose counts(dpm/mg protein)	
	30 minutes	60 minutes
Control	307.00 ± 7.93	347.66 ± 9.59
Diabetic	217.66 ± 33.84*	264.66 ± 5.17**
Diabetic + Insulin treated	252.33 ± 13.34	305.66 ± 8.09 $^{\Psi}$
Diabetic + *Aegle marmelose* treated	244.33 ± 6.98	330.00 ± 7.63 $^{\Psi}$
Diabetic + *Costus pictus* treated	178.00 ± 15.56	316.33 ± 20.46 $^{\Psi}$

Values are mean ± S.E.M of 4-6 separate experiments
*P<0.05 when compared to control group, ** P<0.01 when compared to control group,
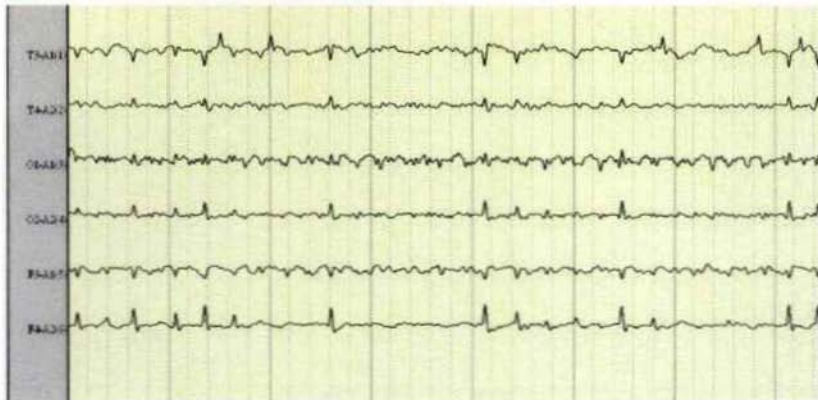$^{\Psi}$ P<0.05 when compared to diabetic group

Figure -73

EEG of control rats
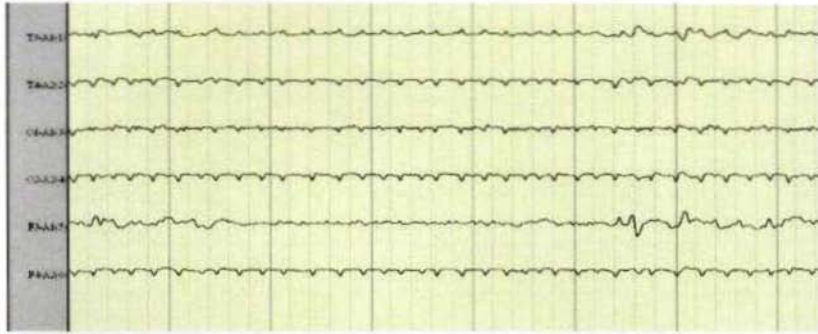
Figure -74

EEG of diabetic rats

F3	Frontal lobe	left		F4	Frontal lobe	right
T3	Temporal lobe	left		T4	Temporal lobe	right
O1	Occipital lobe	left		O2	Occipital lobe	right
A1	Reference	left		A2	Reference	right

Figure -75

EEG of insulin treated rats
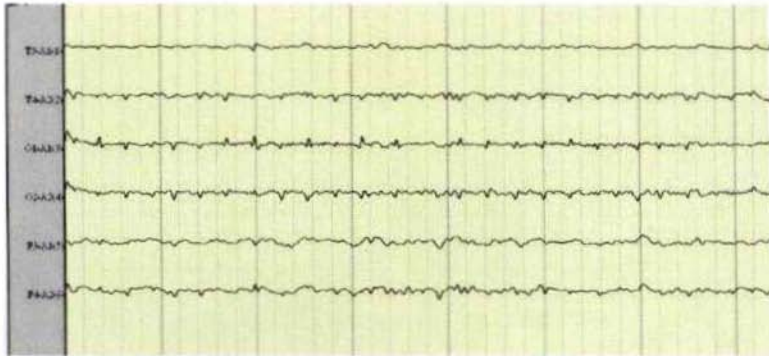
Figure -76

EEG of *Aegle marmelose* treated rats
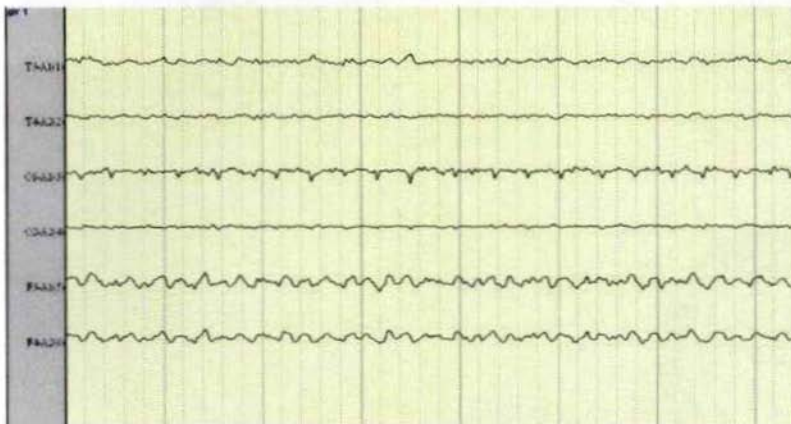
F3	Frontal lobe	left		F4	Frontal lobe	right
T3	Temporal lobe	left		T4	Temporal lobe	right
O1	Occipital lobe	left		O2	Occipital lobe	right
A1	Reference	left		A2	Reference	right

Figure -77

EEG of *Costus pictus* treated rats

F3	Frontal lobe	left		F4	Frontal lobe	right
T3	Temporal lobe	left		T4	Temporal lobe	right
O1	Occipital lobe	left		O2	Occipital lobe	right
A1	Reference	left		A2	Reference	right