DIABETES

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and altered carbohydrate, lipid and protein metabolisms, which further lead to increased risk of many secondary complications including cardiovascular diseases (Keen et al., 1982; Pickup and Williams, 2003).

HISTORY

In 1679, it was detected that the urine of a diabetic person had a sweet taste that gave the disorder the name diabetes. Diabetes mellitus is derived from the Greek word “diabetes” - to siphon / pass through and the Latin word “mellitus” - sweet as honey.

Diabetes is known since long and the description about this disorder comes from the ancient manuscripts, where ancient Indian physicians Charak and Sushruta (600–400 BC) described it as Madhumeha (passing a large volume of sweet urine) and they have also mentioned about the health status of the patients wherein they have mentioned that, some patients were lean and had severe polyuria, thirst and dehydration, while others were stout, ate excessive amounts of food, and were sedentary (Ambady and Snehalata, 2009). The circulating glucose level in diabetics varies from patient to patient depending on the severity of the disease; there are further associated signs and symptoms based on which, diabetes can be classified as either insulin dependent diabetes mellitus (IDDM) or non-insulin dependent diabetes mellitus (NIDDM) (Keen et al., 1982; WHO, 1985). This system of classification of diabetes was widely accepted after it was...
published in WHO report in 1985 and, going along its lines, diabetes type I is a condition wherein there is severe to absolute lack of insulin, which may be due to reduction in the number of beta cells as, the beta cells are in acute auto immune and environmental insult (Keen et al., 1982; Pickup and William, 2003). IDDM is also known for its onset in childhood and it is also termed as juvenile onset diabetes or childhood onset diabetes. NIDDM is known for its onset in adults and mostly related to obesity, which results from a defect in insulin secretion caused mainly due to prior insulin resistance wherein, the body is unable to respond properly to insulin. This defective response mainly involves problems related to insulin receptor on cell membranes but still, the specific defects are not very clear. Gestational diabetes, genetic defects of β cell function, genetic defects in insulin action, endocrinopathies and uncommon forms of immune mediated diabetes etc are few other forms of diabetes.

PRESENT STATUS

In 2000, according to the World Health Organization, there are at least 171 million diabetics world over constituting about 2.8% of the population. According to the American Diabetes Association, there are about 6.2 million people who are undiagnosed and about 41 million people who can be considered pre-diabetic (American Diabetes Association, 2005). There is also an increase in the rate of incidence of diabetes in developing countries following the trend of urbanization and changes in lifestyle. As far as the Indian status is concerned, according to a multi center epidemiological study in early 1970s by Indian Council of Medical Research (ICMR), the prevalence of diabetes in the urban and rural populations

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above 14 years of age was around 2.3 and 1.5 respectively (Ahuja, 1979). Since then, if we carefully examine the studies carried out from different parts of India from 1971-2000, one can conclude that, there has been a 10 fold increase in the reported incidence of diabetes in the urban areas compared to 1.2 percent in 1971 (Ahuja, 1979; Tripathy et al., 1971; Gupta et al., 1978; Patel, 1986; WHO, 1980; Ramachandran et al., 1988; Verma et al., 1986; Rao et al., 1989; Ahuja, 1991; Ramaiya et al., 1990; Ramachandran et al., 1992, 1997; Shah et al., 1998; Asha Bai et al., 1999; Zargar et al., 2000; Kutty et al., 2000; Misra et al., 2001; Iyer et al., 2001; Mohan et al., 2001). Talking about the rural areas, (as presently large part of India can be considered rural), WHO assumes that and urbanization is expected to affect nearly around 46% by 2030 (WHO, 2002), and so, the rural areas undergoing rapid urbanization might be expected to make a considerable contribution to the overall diabetic population. The change in lifestyle of rural areas of India has led to an explosion of metabolic disorders such as diabetes, CVD, and hypertension (Ramachandran et al., 2008; Mohan et al., 2008). Studies across Asian countries have shown similar trend, and which includes countries like Thailand, Malaysia, Bangladesh, and Pakistan is an indication of a large pool of pre-diabetic subjects in India, Asia and ultimately across the world (Sicree., et al., 2006).

With the data available from the rural and urban areas the average expenditure of an individual for diabetic treatment is approximately Rs.10,000/- per month in urban areas and approximately Rs. 6,260/- pm in the rural areas of India (Ramachandran et al., 2007). The expenditure incurred for management of
diabetes is considerable for the individual as well as the health care system; apart from that, individuals are also affected indirectly as many face premature retirement and mortality due to absence from work or inability to work and poor health status. Ultimately, pain, pressure and stress are the indirect cost paid by the diabetic individuals (Ramachandran et al., 2009).

WHY PLANTS?

India has been using plants for the cure many diseases since ages and, there are about 45,000 plant species of which, several of them have been proven to be having medicinal significance (Grover et al., 2002). Many plants used by the diverge communities in India for anti diabetic purpose and, a number of plants are recognized that show varying range or degree of hypoglycemic and anti hyperglycemic activity (Grover et al., 2002).

Plants have been involved in the development of modern medicine as, the biologically active compounds derived from plants have played an important role in combating pain and several diseases. As for example, over 70% of Monographs in the British Pharmacopoeia (1932) were on plant-derived products. Thus plants can be considered as an extensive source of drugs and, many of the currently available drugs have been directly or indirectly derived form these extensive sources only. The ethanobotanical reports provide evidence in support to of plants as, there are about 800 plants reported to be having anti diabetic potential (Alarcon-Aguilara et al., 1998). Moreover, great interest has been shown by people in favour of medicinal plants in recent times as, most
often there are chances of side effects if individuals depend on synthetic drugs (Day, 1998).

THE AYURVEDIC APPROACH TO DIABETES FROM PAST TO PRESENT:

Ayurveda, the ancient healing system from India, has steadily increased in popularity in the western world in recent years. This 5,000 year old system of medicine recommends a combination of lifestyle management (which includes diet, exercise and meditation), and treatment with specific herbs and minerals to cure various diseases. Indian has not only been using this system of medicinal therapy but also recognizes alternative systems of heath care as, varied interdependent fields like Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy have developed in a very well planned and organized manner and are still developing side by side with the field of allopathy (Subramanian et al., 2003). Presently, there are over a million people using herbal medicine on a regular basis either as, over the counter drugs prescribed by non-allopathic remedies or, of same spices as home remedies (Gautam et al., 2003). Nobel-laureate Ernst Boris Chain in his famous article "The quest for new biodynamic substances" wrote "In China and India there has been an extensive drive aimed at the systematic study of medicinal plants, traditionally used in these countries in folklore medicine; this has failed, so far, to bring to light new classes of compounds with interesting pharmacologic activities. As far as drug research is concerned, therefore, we cannot expect many major surprises to come from the study of plant constituents" (Chain, 1967). India, by adopting the approach of reverse pharmacology and by undertaking research in a triangular manner,
including fields like modern medicine, Indian system of medicine and life and pharmaceutical science at national level, has almost disproved the Nobel laureate in a very short period of time by noted novel activities in the field and with collaborative teamwork in research and development (Mashelkar et al., 2003; Patwardhan et al., 2004). At present there are about 400 registered medical colleges in India involved in training more than 500,000 non-allopathic practitioners in the field of Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy, which includes logical and systematic approach towards pathogenesis and diagnostics deriving successful and affordable solutions. Hence this system of alternate therapy is no more a folklore or traditional practice (Vaidya, 1992).

**CADMIUM**

Cd is a white, lustrous, tarnishable metal. It belongs to group IIb of the periodic table. The valence shell of cadmium atom has two s electrons outside a filled 4d orbital. With this arrangement of electrons in the last two shells of the atom and relatively low first and second ionization energies, Cd forms only a stable +2 state. Most of its compounds are ionic, but it forms a few covalently-bound compounds. It readily reacts with non oxidizing acids to liberate hydrogen and form Cd\(^{2+}\). Cadmium was discovered in 1817, but it was not until the 20th century that it was used for industrial purposes. Its main use is in electroplating and galvanizing because of its resistance to corrosion. It is also used to make cathode material in nickel-Cd batteries, pigment, and plastics. Cadmium is highly toxic and is ranked seventh among the top 20 hazardous substances on the
ATSDR/EPA priority list (ATSDR, 2001). A very large amount of cadmium (approximately 4000 to 13,000 t) is released into the atmosphere by the nickel-cadmium battery industry alone; along with some other industries and ores, the amount of cadmium released into the atmosphere reaches around 30,000 t every year (ATSDR, 2005). Cadmium keeps on accumulating in animals and humans as it has a very long half life and is not degraded in the environment and thereby a facilitating gradual entry into the food chain (ATSDR, 2005). The metal has no known biological functions, but is found as a trace element in biological systems. In humans, the concentration of Cd at birth is close to zero, but because the metal has such long biological half-life (approximately 30 yr in humans), the concentration gradually increases with age (Goyer, 1975). The concentration of Cd found in an individual, however, is dependent on the level and route of exposure. Cadmium is found almost everywhere in the environment at least in a relatively low concentration, for example, the average concentration of cadmium in soil and water is 0.33 μg/g, (Sanchez-Martin et al, 2000) and 1μg/L (ATSDR, 1998) respectively. The constant mining process and the industrial waste have led to a high risk of cadmium exposure in humans and animals; humans are primarily exposed to this toxicant through food and, through inhalation, predominantly via cigarette smoking (ATSDR, 1998). The average concentration of cadmium ranges between 2 to 40 ppb in food and 1ppb in drinking water and between 1,000 to 3,000 ppb in cigarettes in the United States (Goyer, 1996). Vegetables have major contribution in cadmium accumulation in humans than any other food of animal origin. This may be due to the common usage of sludge
as fertilizer (McKenna and Chaney, 1995). Another factor responsible for increased levels of cadmium in fruits, vegetables and cereals is their cultivation in industrial areas where the water is polluted leading to accumulation of Cd in these cultivated food materials (Cai et al., 1995).

All toxicants cause some detrimental or harmful effect on the normal physiological functions occurring in organisms and the degree of lesion being variable. In laboratory animals, cadmium poisoning leads to hepatic and testicular injury primarily while, chronic exposure leads to renal damage and osteotoxicities (Dudley et al., 1985; Goyer et al., 1995; Shaikh et al., 1999). The tissue accumulation of cadmium is through the formation of Cd–metallothionein (MT) complex which provides a relative tolerance form the harmful effects of this metal in the organism (Masters et al., 1994). However, when the amount of accumulation of cadmium increases further than the pool of MT, the non-MT-bound Cd ions now leads to hepato- and nephrotoxicity (Goyer et al., 1989).

Coming to the mechanistic aspect of Cadmium induced toxicity, one of the possible mechanisms by which these metals produce injury to the cellular compartment is by generation of free radicals and lipid peroxidation. Co treatment with potent antioxidants can reduce the level of cadmium hepatotoxicity as well as nephrotoxicity (Nomiyama et al., 1998; Shaikh et al., 1999).

Hyperglycemia from acute Cd administration is associated with nephrotoxicity. Moreover, Cd aggravates hyperglycemia and nephrotoxicity in experimentally induced diabetic animals (Jin et al., 1999). Parenteral administration of Cd has
been reported to elevate the concentration of blood glucose (hyperglycemia) and/or to increase the urinary excretion of glucose (glucosuria) (Voinar, 1952; Havu, 1969; Sporn, Dinu et al., 1970; Ghafghazi and Mennear, 1973; 1975; Ithakissios, Ghafghazi). It is of interest that, Ishizaki & Fukushima (1968) found glucosuria in well over 90% of Itai-Itai patients (population endemically exposed to Cd). Furthermore, glucosuria has also been documented in industrially exposed workers (Bonnell, et al., 1959). Elevation of blood glucose or maintenance of normal blood glucose in presence of glucosuria could result not only from increased production and/or release of glucose into the circulation, but also from reduced tissue uptake and utilization of circulating glucose. Havu (1969) showed that, intramuscular injection of Cd in the fish, Corpus scorpious leads to accumulation of this heavy metal in the islet tissue, which was associated with necrotic lesions of beta cells, hyperglycemia and glucosuria. Parenteral administration of Cd has also been found to impair glucose tolerance in the mouse and rat (Ghafghazi and Mennear, 1973, 1975; Ithakissios et al., 1975).

**EXERCISE**

Exercise can be classified into two types: a) anaerobic and b) aerobic based essentially on the metabolic energy source utilized while performing any activity (Michael et al., 2006). Anaerobic type of exercise leads to higher intensities of muscular contraction and these vigorous contractions ultimately lead to the generation of lactic acid and energy in the form of adenosine triphosphate (Michael et al., 2006). In other words, anaerobic exercise is the ability of an
individual to work at a very high intensity in a very short period of time, may be within 5 to 30 seconds, while, aerobic exercise is the one which requires lower rates of muscular contraction but, these regular contractions are for relatively longer duration of time, which leads to the usage of carbohydrates, fats and some protein by mitochondrial oxidation within the muscle (Michael et al., 2006). Organisms primarily use aerobic method for their metabolism and energy production (Michael et al., 2006). There is a very significant difference between the two types of exercise, as far as their effect on the circulating blood glucose is concerned; the aerobic type of exercise tends to decrease the blood sugar level during and after while, the anaerobic type of exercise that lasts for few seconds to minutes leads to a tremendous increase in the circulating blood sugar level (Michael et al., 2006).

As described earlier, the incidence of diabetes is on the increase and is largely related to the prevalence of obesity and sedentary lifestyle. It is well known since past long time, even before 19th century, that, the blood glucose concentrations typically increase with diabetes (Riddell et al, 2002). In 1950s American physician E.P. Joslin described about the significance of exercise to effectively manage his patient's symptoms and, his idea of managing blood glucose with effective exercise to keep away all the other secondary complications, was very successful. According to American Diabetes Association, exercise is an important tool for the treatment of diabetes (American Diabetes Association, 1998). Insulin action on entire body in an individual improves if undergoing proper exercise (King et al., 1988; Mikines et al., 1989), especially in skeletal
muscle, which is the major site for insulin stimulated glucose uptake. This may be mediated via the increase in GLUT-4 level and/or translocation, improving insulin-stimulated glucose transport in skeletal muscle (Ploug et al., 1990; Rodnick et al., 1992). There are several other reports from animal experimentation (Riggs et al., 1992) and clinical trials involving humans (Gul et al., 2001) about reported beneficial effect of exercise in diabetic subjects in terms of insulin sensitivity and decrement in the circulating blood glucose levels. Increased oxidative stress has been proposed to play a major role in the pathogenesis of micro and macro vascular complications of diabetes (Giugliano et al., 1996). Regular exercise has been shown to strengthen antioxidant defenses in non diabetic animals and may decrease resting and, acute exercise induced oxidative stress (Alessio and Goldfarb, 1988; Sen et al., 1992; Sen et al., 1994; Sen, 1995; Powers et al., 1999). Low load eccentric exercise is used to prevent muscle injury and, clinical studies have found that such load eccentric training indeed can protect skeletal muscles from lengthening related injury induced by intense exercise. Induction of nitric oxide formation may play a role in destruction of beta cells during the development of type 1 diabetes (Corbbet et al., 1993). Antioxidant defense mechanisms are important for protection of cells and tissues from oxidative damage and consist of non enzymatic antioxidants and antioxidant enzymes, which include SOD, Cat, GPx, and GSH (Nadler and Winter, 1996). Reduced antioxidant levels as a result of increased free radical production in experimental diabetes have been reported by many authors (Grankvist et al., 1981; Kanteret al., 2003). Regular physical exercise has been
reported to be effective in prevention and delay of onset of NIDDM, and to increase insulin sensitivity and ameliorate glucose metabolism (Derouich and Boutayeb, 2002).

**ALLOXAN**

Alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5, 6-dioxyuracil) was first described by Brugnatelli in 1818. Wholer and Liebig used the name "alloxan" and described its synthesis by uric acid oxidation. The diabetogenic properties of this drug were reported many years later by Dunn et al. (1943), who studied the effect of its administration in rabbits and reported specific necrosis of pancreatic islets. Alloxan exerts its diabetogenic action when it is administered parenterally intravenously, intraperitoneally or subcutaneously. The dose of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status. The most frequently used intravenous dose of this drug to induce diabetes in rats is 65 mg/kg body weight (Gruppuso et al., 1990; Boylan et al., 1992). When alloxan is given intraperitoneally or subcutaneously its effective dose must be 2–3 times higher. The intraperitoneal dose below 150 mg/kg body weight may be insufficient for inducing diabetes in the rat (Katsumata et al., 1992). Alloxan is a hydrophilic and unstable substance: its half-life at neutral pH and 37° C is about 1.5 minutes and is longer at lower temperatures (Lenzn and Munday, 1991). On the other hand, when a diabetogenic dose is used, the time of alloxan decomposition is sufficient to allow it to reach the pancreas in amounts that are deleterious (Malaisiee, 1982). The action of alloxan in the pancreas is preceded by its rapid uptake by insulin–secreting cells and, has been proposed
to be one of the important features determining alloxan dibetogenicity. Another aspect concerns the formation of reactive oxygen species (Zang et al., 1992). One of the targets of the reactive oxygen species is DNA of pancreatic islets. Its fragmentation takes place in B-cells exposed to alloxan (Takasu et al., 1991; Sakurai and Ogiso, 1995). DNA damage stimulates poly ADP-ribose, a process participating in DNA repair. Some inhibitors of poly ADP-ribosylation can partially restrict alloxan toxicity. This effect is, however, suggested to be due to their ability to scavenge free radicals rather than the restriction of poly ADP-ribosylation initiated by alloxan (Grankvist et al., 1979) and non-enzymatic scavengers of hydroxyl radicals like melatonin (Ebelt et al., 2000) were also found to protect against alloxan toxicity.

**MELATONIN**

Melatonin (N-Acetyl 5-methoxytryptamine), is a ubiquitous molecule which plays important role in many physiological functions. Earlier, it was believed to be involved in the neuroendocrine system and mainly in reproductive physiology (Reiter, 1973; Reiter, 1980) and later its role in controlling circadian rhythm disorder was elucidated (Arendt, 1998; Reiter, 1991, Coto-Montes and Hardeland 2000). The antioxidant properties of melatonin are a recently discovered fact (Tomas et al., 2007). Pineal is the main source of melatonin secretion and it regulates the circadian rhythm, with low serum levels during the day and higher levels at night. The time of secretion of melatonin is observed to vary depending on the length of light-dark cycles. In few of the photoperiodic species, it is this signaling property of melatonin which helps them in the synchronization of
number of physiological activities and neuroendocrine signals associated with seasonal changes (Von et al., 2002). Melatonin, as well as its metabolites (Tan et al., 2001), possess redox properties because of the presence of an electron rich system which allows these molecules to act as electron donors (Allegra et al., 2003). It has been reported that melatonin can effectively normalize the impaired antioxidative status in rats with streptozotocin-induced diabetes (Anwar and Meki, 2003). Similarly, long-term melatonin administration reduced hyperlipidemia and hyperinsulinemia, and restored altered ratios of polyunsaturated fatty acids in serum and tissues of diabetic rats (Nishida, 2005). Treatment with insulin and melatonin suppress hyperglycemia, prevent oxidative damage and restore completely endothelial function in the aorta and corpus cavernosum of diabetic rats (Paskaloglu et al., 2004).

In view of these above mentioned reports and review on the role of alternative nutraceuticals on alleviation of symptoms of diabetes, the present work was designed with the following objectives in mind:

- To evaluate the efficacy of an extract of common plants when given in combination as an antidiabetic, antilipidemic and antioxidant agent.
- To evaluate the therapeutic benefit of the plants chosen together with melatonin in alloxan induced diabetic rats.
- To assess the beneficial role of exercise together with dietary administration of herbal extract and melatonin in alloxan induced diabetic rats.
• To study the potentiating effect on diabetic manifestations and free radical induced tissue oxidative by prior cadmium intoxication and the efficacy of herbal combination and melatonin is combating the same.
MATERIALS AND METHODS

-pocket diagram

AMIMALS AND MAINTAINANCE

Female albino rats of wistar strain (200-250 g) of 180 days old were obtained from Sun Pharmaceuticals Ltd., Baroda and maintained in the animal house at 20 ± 2° C with light and dark cycles of 12 :12 h respectively. Animals were provided with standard rodent pellet diet purchased from M/S Pranav Agro Industries limited. Food and water were provided ad libitum. Animal experiments were conducted according to the guidelines of CPCSEA (827/ac/04/CPCSEA). Following the treatment schedule, animals were sacrificed and selected tissues were separated and stored at -80 C till biochemical assay. Blood was collected prior to sacrifice by keeping the animals under light ether anesthesia and the separated serum obtained was used for further analysis. During the entire treatment schedule body weight, food and water were monitored on a daily basis.

INDUCTION OF TYPE I DIABETES

To induce diabetes, Alloxan monohydrate obtained from Sigma Chemicals, USA was used. Animals were fasted overnight prior to alloxanization and alloxan was administered intraperitoneally at a dosage of 120mg/kg body weight. The animals were monitored for food and water intake, body weight and mortality for the next six to seven days before analyzing their blood glucose level. Blood was withdrawn from the orbital sinus of alloxan treated animals after a period of seven days and animals showing a glucose level above 300mg/dl were only considered to be diabetic and were used for treatment further.
PREPARATION OF PLANT EXTRACT

Pterocarpus marsupium (VIJAYSAR)
The heart wood powder of *Pterocarpus marsupium* bark was purchased from Sri. Gayatri Pharmaceuticals Private Limited, Rajpipla and authenticated by Prof. M. Daniel (Head, Department of Botany, M.S.University of Baroda). The powder was extracted with HPLC grade methanol using soxhelt (Borosil Glass Works, Mumbai, India) at boiling temperature (60 °C) up to 10 h; a dark brown colour extract was obtained. This dark brown extract was cooled and filtered to remove the residue. The extract was concentrated on rotavapour under reduced pressure and then dried to get a powder. The dried powder was diluted in CMC (500mg/kg body weight) (Narendhirakannan *et al.*, 2005).

Ocimum sanctum (TULSI)
Fresh leaves of Tulsi were purchased from local market in Baroda and authenticated by Prof. M. Daniel (Head, Department of Botany, M.S.University of Baroda). The leaves were shade dried, and ground in a mixer to get a fine powder. The powder was extracted with HPLC grade methanol using soxhelt (Borosil Glass Works, Mumbai, India) at boiling temperature (60 °C) up to 10 h; a dark brown colour extract was obtained. This dark brown extract was cooled and filtered to remove the residue. The extract was concentrated on rotavapour under reduced pressure and then dried to get a powder. The dried powder was diluted with saline in required proportion for the study (Narendhirakannan *et al.*, 2005).
The extract obtained from both the plants separately was dissolved together in desired concentration in (0.33%) Carboxy methyl cellulose (CMC) and was administered to the animals orally using a gavage.

Dose: Vijaysar: 500mg/Kg bodyweight
Tulsi: 500mg/Kg bodyweight

(Plant extract preparation used in Chapters 1, 3, 5 and 7)

**Cadmium Chloride (CdCl₂)**

Animals were exposed to cadmium toxicity prior to induction of diabetes. A realistic dose of cadmium (5.12mg/Kg bw) was given to the animals by oral gavage for 45 days for inducing Cd toxicity. After exposure of the animals to Cd, same animals were made diabetic by intraperitoneal injection of Alloxan (120mg/Kg BW). The treatment schedule with extract/melatonin in confirmed diabetes animals and the control non diabetic animals was carried out in prior Cd exposed animals.

Dose of Cadmium Chloride (CdCl₂): CdCl₂ was dissolved in distilled water and a dose of 5.12mg/Kg bodyweight was given to the rats for a period of 45 days at 17 hours. (Cadmium treatment used in Chapters 4, 5, 6 and 7)

**POLYHERBAL EXTRACT PREPARATION**

**Details of plants selected for the study**

Seeds of *Cassia fistula*(Fabaceae), and leaves of *Langerstromia flos-regineae*(Lythraceae), *Murraya koenigii*(Rutaceae), *Annona squamosa*(Annonaceae), *Ocimum sanctum*(Lamiaceae), *Coccinia indica*(Cucurbitaceae) and *Mangifera indica*(Anacardiaceae) were used for the
preparation of a polyherbal extract. The plant material after collection was identified by Prof. M. Daniel (Head, Department of Botany, M.S. University of Baroda, Vadodara).

**PREPARATION OF POLYHERBAL EXTRACT (PE):**

Equal amount (250 grams) of fresh leaves/seeds was plucked and separated from the twigs. Leaves were chopped into small pieces and shade dried and then ground in a mixer along with the seeds of *Cassia fistula* which were dried separately to get a powder mixture. The powder was extracted with distilled water using soxhelt at boiling temperature (100 °C) up to 10 h; a dark brown coloured extract was obtained. This dark brown extract was cooled and filtered to remove the residue. The extract was concentrated on rotavapour under reduced pressure and then dried to get a powder (Kesari et al., 2005). The dried powder was diluted with saline in required proportion for the study. The PE was administered to animals by orally gavage. (Kesari et al., 2005).

(Polyherbal Extract preparation used in Chapters 10 and 11)

**Rationale behind dosage:**

The allopathic drugs prescribed for routine treatment of diabetes do not act at all the possible sites of lesion associated with diabetes, and moreover some also have side-effects. Natural herbs are have no side effects and hence could possibly be used as an effective medicine against diabetes.

**DOSAGE:**

Extract: 500mg/kg body weight per day given at 8000 hr.
SWIMMING PROTOCOL FOR EXERCISE

Animals were subjected to swimming exercises and were made to swim in a tank with a dimension (150X90X70) lengthXbreathXheight, filled with water to a depth of 30–45 cm, once per day between 0830 and 9000. Animals were acclimatized by making them to swim for 5 days prior to the commencement of the experimental schedule. The acclimatized animals were divided into different experimental groups where and were subjected to swimming exercise for 15 days for 30min, as described previously (Kiraly et al., 2007).

(Swimming exercise performed in Chapters 8, 9, 10 and 11)

**ORAL GLUCOSE TOLERANCE TEST:**

At the end of treatment schedule animals were fasted overnight and glucose tolerance test was done by feeding them orally with a glucose solution at a dose of 2g/kg body weight. Blood was collected from the retro orbital sinus at 0min, 30min, 60min, 90min, and 120min after glucose load. Serum was separated was done and glucose was estimated in all the collected samples to get a tolerance curve for all the experimental groups.

**INSULIN RESPONSE TEST:**

Response to insulin was checked by injecting Insulin to the rats at a dose of 1U/kg body weight intraperitoneally (i.p) in the fed state a day following the completion of treatment and blood was collected at 0, 30, 60, 90, and 120 min from the retro orbital sinus under mild ether anesthesia. Serum was separated
and used to estimate the glucose level and an insulin response curve was drawn to evaluate the results.

**EXPERIMENTAL GROUPS:**

**NON-DIABETIC CONTROL (NC)**

Animals in this group received saline as vehicle.

**CMC CONTROL (CMC)**

Control rats treated with Carboxymethyl cellulose as vehicle through oral gavage.

**EXTRACT (NC+E)**

Control rats treated with plant extract at a dose of 500mg/kg body weight at 8000 hrs for a period of 15 days.

**MELATONIN LOW DOSE (NC+ML)**

Control rats treated with 1mg/kg bodyweight of melatonin, at 1800hrs for a period of 15 days.

**MELATONIN HIGH DOSE (NC+MH)**

Control rats treated with 10mg/kg bodyweight of melatonin, at 1800hrs for a period of 15 days.

**POLYHERBAL EXTRACT (NC+PE)**
Control rats treated with plant extract mixture at a dose of 500mg/kg body weight at 8000 hrs for a period of 15days.

**SWIMMING (NC+S)**

Control rats exposed to swimming exercise daily for 30 minutes for 15 days.

**SWIMMING + MELATONIN LOW DOSE (NC+S+Mₐ)**

Control rats exposed to swimming exercise daily were treated with plant extract mixture at a dose of 500mg/kg body weight at 8000 hrs for a period of 15days.

**SWIMMING + POLYHERBAL EXTRACT (NC+S+PE)**

Control rats exposed to swimming exercise daily were treated with plant extract mixture at a dose of 500mg/kg body weight at 8000 hrs for a period of 15days.

**POLYHERBAL EXTRACT + SWIMMING + MELATONIN LOW DOSE (NC+PE+S+Mₐ)**

Control rats exposed to swimming exercise were treated with plant extract mixture at a dose of 500mg/kg body weight at 8000 hrs for a period of 15days.

**CONTROL + EXTRACT + MELATONIN HIGH DOSE (NC + E + Mₘ)**

Control rats treated with plant extract and melatonin at a dose of 500mg/kg body weight and 10mg/kg bodyweight respectively at 8000 hrs and 18 hrs for a period of 15days.

**CONTROL + CADMIUM (NC + Cd)**
Control rats treated with 5.12 mg/kg bodyweight of cadmium chloride salt dissolved in (0.9 N) saline, at 8000hrs for a period of 45 days.

CONTROL + CADMIUM + EXTRACT (NC+Cd+E)

Control rats treated with cadmium (5.12mg/kg bodyweight) for 45 days followed by administration of plant extract at a dose of 500mg/kg body weight at 8000 hrs for a period of 15 days.

CONTROL + CADMIUM+MELATONIN HIGH DOSE (NC + Cd+ M_H)

Control rats treated with cadmium (5.12mg/kg bodyweight) for 45 days followed by administration of 10mg/kg bodyweight melatonin, at 1800hrs for a period of 15 days.

CONTROL + CADMIUM + MELATONIN HIGH DOSE + EXTRACT (NC+Cd+M_H+E)

Control rats treated with cadmium (5.12mg/kg bodyweight) for 45 days were followed by administration of 10mg/kg bodyweight melatonin as well as extract, at 1800hrs and 8000 hrs respectively for a period of 15 days.

DIABETIC (DC)

Diabetic rats treated with saline as vehicle.

DIABETIC RATS TREATED WITH EXTRACT (DC+E)

Diabetic rats treated with plant extract at a dose of 500mg/kg body weight at 8:00hrs for a period of 15 days.
Control diabetic rats treated with 1mg/kg bodyweight of melatonin, at 1800hrs for a period of 15 days.

Control diabetic rats treated with 10mg/kg bodyweight of melatonin, at 1800hrs for a period of 15 days.

Diabetic rats treated with plant extract and melatonin at a dose of 500mg/kg body weight and 10mg/kg bodyweight respectively at 8000 hrs and 18 hrs for a period of 15 days.

Control rats treated with 5.12 mg/kg bodyweight of cadmium chloride salt dissolved in (0.1 N) saline, at 8000hrs for a period of 45 days, were made diabetic by alloxan administration.

Control rats treated with cadmium (5.12mg/kg bodyweight) for 45 days were made diabetic followed by administration of plant extract at a dose of 500mg/kg body weight at 8000 hrs for a period of 15 days.
Control rats treated with cadmium (5.12mg/kg bodyweight) for 45 days were made diabetic followed by administration of 10mg/kg bodyweight melatonin, at 1800hrs for a period of 15 days.

\[ \text{DIABETIC + CADMIUM + MELATONIN HIGH DOSE + EXTRACT (DC+Cd+Mn+E)} \]

Control rats treated with cadmium (5.12mg/kg bodyweight) for 45 days were made diabetic followed by administration of 10mg/kg bodyweight melatonin as well as extract, at 1800hrs and 8000 hrs respectively for a period of 15 days.

\[ \text{DIABETIC + SWIMMING (CC+S)} \]

Diabetic rats exposed to swimming exercise daily for 30 minutes for 15 days.

\[ \text{DIABETIC + SWIMMING + MELATONIN LOW DOSE (DC+S+Ml)} \]

Diabetic rats exposed to swimming exercise daily were treated with plant extract mixture at a dose of 500mg/kg body weight at 8000 hrs for a period of 15days.

\[ \text{DIABETIC + SWIMMING + POLYHERBAL EXTRACT (DC+S+PE)} \]

Diabetic rats exposed to swimming exercise daily were treated with plant extract mixture at a dose of 500mg/kg body weight at 8000 hrs for a period of 15days.

\[ \text{DIABETIC + POLYHERBAL EXTRACT + SWIMMING + MELATONIN LOW DOSE (DC+PE+S+Ml)} \]
Diabetic rats exposed to swimming exercise were treated with plant extract mixture at a dose of 500mg/kg body weight at 8000 hrs for a period of 15 days.

METHODS (SERUM AND TISSUE ANALYSIS)

GLUCOSE

Method: Kit based assay from Agappe Diagnostics

► PRINCIPLE

Glucose is oxidized by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of the enzyme peroxidase (POD) oxidizes phenol which combines with 4-aminoantipyrine to produce a red colored quinoneimine dye. The intensity of the color developed is proportional to glucose concentration in the sample.

► PROCEDURE

In the sample tube, 10 μl of serum was added, in the blank tube 10 μl of redistilled water was added and in the standard tube 10 μl of glucose standard (100 mg/dl) was added. 1 ml of working enzyme reagent was added in all the tubes. The contents were thoroughly mixed and incubated at 37°C for 15 minutes. The optical density of the sample and the standard were measured against blank in a spectrophotometer at 505 nm. The amount of glucose is expressed as mg/dl of serum.
INSULIN

METHOD: Rat Insulin ELISA (MERCODIA, Sweden)

► PRINCIPLE:

Mercodia Rat Insulin ELISA is a solid phase two site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti insulin antibodies and anti insulin antibodies bound to microtitration well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3, 3', 5, 5'-tetramethylbenzidine. The reaction is stopped by adding acid to give colorimetric endpoint that is read spectrophotometrically.

► PROCEDURE:

To 25 microlit calibrator or sample add 50microlit of enzyme conjugate and incubate it for 2hours at room temperature. Wash about six times and then add 200microlit of TMB substrate and again incubate for 15min before adding the stop solution 50microlit. Read the absorbance at 450nm and evaluate.

CORTICOSTERONE

METHOD: ELISA based kit from Immuno-Technology & Steroid Laboratory Department of Reproductive Biomedicine, National Institute of Health and Family Welfare.Munirka, New Delhi-

► PRINCIPLE:
The Elisa of corticosterone is a competitive solid phase assay. Serum samples or standards are incubated in antibody coated wells with horse radish peroxidase conjugate. After incubation, the liquid contents of the wells are decanted and the wells are washed in running tap water for removing the unbound enzyme conjugate. The bound enzyme activity is measured by developing colored product from colorless substrate after incubation. Quantity of color developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

► REAGENT PROVIDED

Reagents are stable until the expiry date of the kit, if stored at 2-8°C.

1. Enzyme conjugate: HRP-corticosterone conjugate (8 ml), ready to use
2. Corticosterone-Ab coated–96 wells: (8 well/strip) ready to use
3. Six standards: (each vial with 1.0 ml of standards), ready to use. The standard range from 0 to 60ng/ml in stripped serum with 0.1% thimerosal. The exact concentration is marked on each vial.
4. TMB/H$_2$O$_2$ solution (11 ml)
5. Stop solution (12.0ml 0.5 M H$_2$SO$_4$)

► PROCEDURE:

50microlit of standard or sample is dispensed in the wells and then 25microlit of enzyme conjugate is added and incubated at RT for 60min. After thorough washing step 100microlit of substrate is added and incubated for 15min before adding the stop solution and reading is taken at 450nm.
Results are obtained from the standard curve by interpolation. The curve serves for the determination of corticosterone concentration in sample measured at the same time as the standards.

i) Standards curve (sigmoid in nature)
Plot on semi-logarithmic paper optical density or % $A/A_0$ on the vertical axis and the corticosterone concentrations ($\mu$g/dl) of the standard on the horizontal axis.

1. Samples
For each sample, locate the OD or $%A/A_0$ on the vertical axis and read the corresponding corticosterone concentration on the horizontal axis. It will show directly the concentration in terms of $\mu$g/dl in serum

$$ao = \text{optical density at zero dose}$$

$$a = \text{optical density at varying concentrations of standard dose or unknown sample}$$

PROGESTERONE

METHOD: ELISA based kit method by shrivastav et al., 2009 from NIHFW, New Delhi.

PRINCIPLE:
The ELISA of progesterone is a competitive solid phase assay. Serum samples or standards are incubated in antibody coated wells with HRP conjugate. After incubation the liquid contents are washed to remove the unbound enzyme conjugate. The bound enzyme activity is measured by developing colored product from colorless substrate after incubation. Quantity of color developed is
directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

► Chemicals and Reagents

All solvents, chemicals, and salts used in the present study were of analytical grade and were used without prior purification. All steroids used for the synthesis and cross-reactivity were obtained from Sterloids, Inc., Newport, USA. Bovine serum albumin, N-hydroxy succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), complete Freund's adjuvant, gelatin, and thimerosal were purchased from Sigma Chemical Company, St. Louis, MO, USA. Horseradish peroxidase and tetramethylbenzidine=H2O2 solution were purchased from Bangalore Genei, Bangalore, India, and Arista Biochemical, USA, respectively. Microtitre plates were procured from Greiner, Germany.

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Buffers

A. The most frequently used buffer was 10mM phosphate (10mM PB) pH 7.0, (Na2HPO4·2H2O: 0.895 gm=L and NaH2PO4·2H2O: 0.39 gm=L) containing 0.9 % NaCl (10mM PBS) and 0.1% NaN3.

B. HRP conjugate dilution buffer was 10mM acetate buffer (10mM AB) pH 5.6, (CH3COONa: 0.84 gm=L and 1N CH3COOH 1.5mL=L), containing 0.1% thimerosal and dextran T-70, 0.3% BSA.

C. Microtitre well blocking and stabilizing buffer was 10mM PB containing 0.9 % NaCl, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70,
ethylene diamine tetra acetic acid: di-potassium salt (EDTA: K salt), and 0.01% gentamicin sulfate.

► PROCEDURE:
50 microlit of standard or sample is dispensed in the wells and then 100 microlit of enzyme conjugate is added and incubated at RT for 60 min. After thorough washing step 100 microlit of substrate is added and incubated for 15 min before adding the stop solution and reading is taken at 450 nm.

► PRINCIPLE:
The Biocheck E2 EIA is based on the principle of competitive binding between E2 in the test specimen and E2 HRP conjugate for a constant amount of rabbit anti-estradiol. In incubation, goat anti IgG coated wells are incubated with 25 microlit E2 standards and samples. 100 microlit Estradiol HRP conjugate and 50 microlit rabbit anti Estradiol reagent at RT for 90 min. During incubation, a fixed amount of HRP labeled E2 competes with the endogenous E2 in the standards or sample for a fixed number of binding sites of the specific E2 antibody. Thus, the amount of E2 peroxidase bound in the well progressively decreases as the concentration of E2 in the specimen increases. Unbound E2 peroxidase conjugate is then removed and the wells washed. Next a solution of TMB reagent is added and incubated at RT for 20 min resulting in the development of blue color. The color development is stopped with addition of in
HCL and the absorbance is measured spectrophotometrically at 450nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled E2 in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The E2 concentration of the specimen run concurrently with the standard can be calculated from the curve.

PROCEDURE:
Secure the desired number of wells in the holder and dispense 25 microlit of standard or sample. Next add 100 microlit Estradiol HRP conjugate reagent into each well. Then add 50 microlit of anti rabbit estradiol reagent to each well, mix well for 30 seconds and incubate at room temperature for 90 minutes. After incubation rinse and flick the wells with distilled water and then dispense 100microlit of TMB reagent. Incubate again for 20 minutes at room temperature. Then add 100microlit stop solution and read the absorbance at 450nm within 15minutes.

SERUM CHOLESTEROL

METHOD: kit based assay from Accurex biomedical Pvt Ltd.

PRINCIPLE
Cholesterol esterase hydrolysis cholesterol esters into free cholesterol and fatty acids. In the second reaction cholesterol oxidase converts cholesterol to cholest 4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol to produce red
quinoneimine dye which has absorbance maximum at 510 nm (500-530). The intensity of the red colour is proportional to the amount of total cholesterol in the specimen.

► PROCEDURE:
Take 0.01ml of serum or standard in the tubes and add 1ml reagent to each. Incubate the assay mixture for 10 minutes at room temperature and then read at 510nm against blank.

► TRIGLYCERIDES

Method: Kit based assay from Accurex Biomedical Pvt Ltd.

► PRINCIPLE:
Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol-3-phosphate which is oxidized by glycerol phosphate oxidase to dihydroacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red colored compound.

\[
\text{Triglycerides} \xrightarrow{\text{LPL}^*} \text{Fatty acids + Glycerol}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\\text{GK}^*} \text{Glycerol-3-phosphate + ADP}
\]

\[
\text{Glycerol-3-phosphate + O}_2 \xrightarrow{\\text{GPO}^*} \text{Dihydroxyacetone phosphate + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{Phenolic chromogen} \xrightarrow{\\text{POD}^*} \text{Red color compound}
\]

► PROCEDURE:
Take 0.01ml of serum or standard in the tubes and add 1ml reagent to each. Incubate the assay mixture for 20 minutes at room temperature and then read at 510nm against blank.

**HDL CHOLESTEROL**

Method: Kit based assay from Nicholas Piramal India Limited.

► PRINCIPLE

Chylomirons, VLDL (Very low density lipoproteins), and LDL (Low density lipoproteins) are precipitated by adding phosphotungstic acid and magnesium ions to the sample. Centrifugation leaves only the HDL (High density lipoproteins) in the supernatant. The cholesterol content in it is determined enzymatically.

► PROCEDURE

Take 100 microlit serum or standard in tubes and add 1ml of reagent to it. Mix it well and incubate for 10 minutes at 20 – 30 C and then read against blank at 546nm.

**SGPT**

Method: kit based assay from Agappe Diagnostics Ltd.

► PRINCIPLE

The rate of NADH consumption is measured photometrically and is directly proportional to the ALAT (Alanine Amino Transferase) concentration in the sample.

ALAT
L - Alanine + 2 - Oxoglutarate $\leftrightarrow$ L - Glutamate + Pyruvate

LDH

Pyruvate + NADH + H\(^+\) $\leftrightarrow$ L - Lactate + NAD\(^+\)

► PROCEDURE
Take 100 µl of sample of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 5 minutes. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 340 nm.

SGOT

Method: kit based assay from Crest Biosystems, a division of coral clinical system.

► PRINCIPLE
The rate of NADH consumption is measured photometrically and is directly proportional to the ASAT (Aspartate Amino Transferase) concentration in the sample.

ALAT

L - Alanine + 2 - Oxoglutarate $\leftrightarrow$ L - Glutamate + Oxaloacetate

MDH

Oxaloacetate + NADH + H\(^+\) $\leftrightarrow$ L - Lactate + NAD\(^+\)

► PROCEDURE
Take 100 µl of sample of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 5 minutes. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 340 nm.

**ALKALINE PHOSPHATASE**

**Method:** kit (p-NPP method) from Reckon diagnostics Pvt Ltd.

► **PRINCIPLE**

The increase in absorbance due to formation of 4 - nitrophenolate is rate of is measured photo metrically and is directly proportional to the ALP (Alkaline Phosphatase) activity in the sample.

\[
P - \text{Nitrophenylphosphate} + H_2O \rightarrow \text{Phosphate} + p - \text{Nitrophenol}
\]

► **PROCEDURE**

Take 20 µl of sample of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 1 minute. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 405 nm.

**ACID PHOSPHATASE**

**Method:** kit (Kinetic method) from Aspen Laboratories

► **PRINCIPLE**

The enzymatic reaction sequence employed in the assay of Acid Phosphatase is as follows:

\[
\alpha - \text{Naphthylphosphate} + H_2O \rightarrow \alpha - \text{Naphthol} + \text{Inorganic Phosphate}
\]

36
α - Naphthol + Fast Red TR → Diazo Dye (CHromophore)

The α - Naphthol released from the substrate α - Naphthylphosphate by acid phosphatase is coupled with Fast Red TR to produce a coloured complex which absorbs light at 405nm. The reaction can be quantitated photometrically because the coupling reaction is instantaneous.

► PROCEDURE

Take 20 µl of sample of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 1 minute. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 405 nm.

► PROTEIN

METHOD: The protein content of the tissue extracts was estimated by the method of Lowry et al., (1951).

► PRINCIPLE: In alkaline solution, copper ions and protein molecules in the sample form a complex with the amino acids containing phenolic hydroxyl group, viz., (tyrosine and tryptophan) and reacts with Folin Ciocalteau reagent to give a blue colour due to the reaction of phosphomolybdate. The intensity of colour is propotional to the concentration of proteins.

► PROCEDURE:

A set of tubes containing BSA in the concentration range of (0-100µg) was used. The volume in each tube was made to 1 ml with d/w. 5 ml of freshly prepared alkaline copper sulphate solution was added in each of these tubes, mixed thoroughly and were incubated at room temperature for 10 min. In each of these
tubes 0.5 ml of folin-ciocalteau reagent was added and the contents were mixed immediately. It was allowed to stand for 30 min at room temperature for the color to develop. The absorbance of each tube at 660 nm was recorded. A standard curve of absorbance at 660 nm versus µg of BSA was plotted to determine the amount of protein in the sample.

**GLYCOGEN**

**METHOD:** Glycogen by Seifter et al, (1950)

**PRINCIPLE**

Glycogen present in the tissue is first hydrolysed to glucose and then estimated as per known weight of tissue by using a conversion factor of 1.11. (1gm glycogen yields 1.11 gm of glucose on complete hydrolysis.) Fresh tissue is digested in hot KOH solution and glycogen is precipitated to glucose using ethyl alcohol. This type of precipitation is critical, glycogen should be entirely precipitated and the ppt is then suspended in water. The suspension is then treated with anthrone reagent prepared in sulphuric acid. H₂SO₄ reduces anthrone in presence of glucose to develop a green colour. The colour intensity is directly proportional to the amount of glucose.

\[
\text{Glycogen + H}_2\text{SO}_4 + \text{anthrone (yellow)} \\
\downarrow\text{oxidised} \\
\text{hydroxyl methyl furfural + anthrone (green)}
\]
PROCEDURE:

Pre-weighed pieces of tissue were digested with 2 ml of 30% potassium hydroxide for 20 minutes in a boiling water bath. The contents were cooled in an ice bath and 2.5 ml of 95% ethanol was added, thoroughly mixed and glycogen was precipitated by bringing the contents to boiling in a water bath. The supernatant was decanted and the tubes were allowed to drain on a filter paper for few minutes.

GLYCOGEN PHOSPHORYLASE:


PRINCIPLE: Glycogen phosphorylase cleaves the phosphoric bond of α-1,4 linkages between glucose molecules, to yield glucose -1-phosphate. The property of synthesizing glycogen from glucose-1-phosphate by liberating inorganic phosphorus is made use of in this procedure.

PROCEDURE: In the sample tube were added 0.2 ml of sodium citrate buffer (0.1M pH5.9), 0.3 ml of potassium fluoride (0.154M), 0.05 ml of glucose-1-phosphate (0.2M) and homogenate (20 mg/ml). The incubation was carried out at 37°C for 30 minutes. The reaction was terminated by adding 1 ml of trichloroacetic acid (10%). In the control tubes all the contents were added along with trichloroacetic acid prior to incubation. The tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant fluid was analyzed for phosphorus content according to the method of Fiske and Subbarow (1925) as described below.
To the supernatant fluid, 0.4 ml of sulphuric acid (10N) and 0.8 ml of ammonium molybdate (2.5%) were added and the tubes were allowed to stand for 10 minutes. After 10 minutes 0.4 ml of ANSA was added. After 10 minutes, the intensity of the blue color developed was read at 660 nm against the reagent blank. Appropriate standards were run along with each assay. The enzyme activity is expressed as µ moles of Pi released/mg protein/15 minutes.

**GLUCOSE-6-PHOSPHATASE:**

Method: Harper (1963)

**PRINCIPLE:** Glucose-6-phosphatase catalyses the reaction Glucose-6-phosphate + H₂O $\rightarrow$ glucose + phosphate The rate of the reaction is measured by the increase of inorganic phosphate with time.

**PROCEDURE:** In the sample tube were added homogenate (25 mg/ml in citrate buffer pH 6.5), and 0.1 ml of glucose-6-phosphate (0.08M). The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by adding 2 ml of trichloroacetic acid. In the control tubes, all the reagents were added as above except for glucose-6-phosphate. The tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant fluid was analyzed for phosphorus content according to the method of Fiske and Subbarow (1925) as described below.

To the supernatant fluid, 5 ml of ammonium molybdate (2.5%) and 1 ml of ANSA were added. After 10 minutes, the intensity of the blue color developed was read at 660 nm against the reagent blank. Appropriate standards were run along with
each assay. The enzyme activity is expressed as μ moles of Pi released/ mg protein/15 minutes.

CHOLESTEROL

METHOD: M. Crawford (1958).

► PRINCIPLE:
The method depends upon interaction of FeCl₃ and H₂SO₄ with cholesterol in presence of glacial acetic acid solution. The resulting red – purple colour is measured spectrophotometrically. The content of total cholesterol in dry lipid sample determines the intensity of colour. The exact chemical nature of the reaction is however not known

► PROCEDURE:
The remaining 2 ml solution from lipid extraction (see total lipids) was added to a test tube and dried similarly in oven at 60°C. After the tubes were dried completely, 3 ml of working ferric chloride reagent was added to the tubes and the tubes were heated in a water bath to boiling for 5 minutes. The tubes were cooled and 2 ml of concentrated sulphuric acid was added to each tube in ice bath and were allowed to cool for half an hour. In the blank tube, 3 ml of ferric chloride reagent and 2 ml of concentrated sulphuric acid were added in a similar manner. In the standard tube, 3 ml of cholesterol standard (75μg) and 2 ml of concentrated sulphuric acid were added. The color developed was read at 540 nm against the blank reagent in a spectrophotometer. The amount of cholesterol is expressed as mg/100 mg of tissue.
**TOTAL LIPID**

METHOD: Folch *et al.*, (1957)

► PRINCIPLE:

Lipids are soluble in some organic solvents. This property of specific solubility in non-polar solvents is utilized for extracting lipids from tissues. In biological materials the lipids are generally bound to proteins and they are therefore extracted with a mixture of methanol and chloroform. Inclusion of methanol in the extraction medium helps in breaking the bonds between the lipids and proteins.

► PROCEDURE:

Pre-weighed tissue was crushed along with fine and clean sand particles in a test tube with a clean glass rod. 5 ml of chloroform-methanol mixture (2:1 v/v) and 2 ml of calcium chloride (0.2%) were added to the lysate and kept over night. The upper layer was removed with a syringe and the remaining solution was filtered through wathman filter paper in a graduated tube and the volume was made up to 4 ml with chloroform-methanol mixture. 2 ml of this content was added to pre-weighed lipid tubes, which were kept in an oven at 60°C for drying. After the tubes were dried completely, they were weighed again to get the difference in weight, which was taken as the amount of total lipid. The amount of total lipid is expressed as mg/100 mg of tissue.
GLUTATHIONE PEROXIDASE (GPx)

METHOD: Rotruck et al. (1973)

► PRINCIPLE:

Glutathione peroxidase catalyzes the reduction of hydrogen peroxide by reduced glutathione resulting in H₂O and oxidized glutathione which is then instantly and continuously converted into GSH by and excess of GR used with NADPH providing for a constant level of GSH. Reduced glutathione acts as a reductant. The estimation is based on the oxidation of GSH by 5,5′-dithio bis 2, nitro benzoic acid (DTNB) to measure the total glutathione content of biological samples.

GPx

ROOH + 2GSH → ROH + H₂O + GSSG

H₂O₂ + 2GSH → 2H₂O

► PROCEDURE:

The assay mixture containing 0.4 ml of phosphate buffer (0.4 M, pH 7.0), 0.1 ml sodium azide (10 mM), 0.2 ml of reduced glutathione (4 mM) and 0.2 ml of tissue homogenate was mixed and 0.1 ml of H₂O₂ (30 mM) was added and made up to 2.0 ml with water. The tubes were incubated at 37 °C for 10 min along with control tubes containing all reagents except the enzyme. The reaction was terminated by addition of 0.5 ml of 10% TCA which was then centrifuged at 4000 rpm for 10 min at 4 degree C. 1 ml of this supernatant was taken and added to 3.0 ml of disodium hydrogen phosphate and 1.0 ml DTNB solution (40 mg/100 ml of 1% sodium citrate). The color formed was measured at 412
nm. The blank contained disodium hydrogen phosphate and 1.0 ml of DTNB solution.

GLUTATHIONE REDUCED

METHOD: Beutler et al. (1963).

► PRINCIPLE:
Glutathione is a major non protein thiol present in the same tissue. The sulphhydryl groups in glutathione reduce the 5,5'-dithio bis-2-nitro benzoic acid to form one mole of 5-thio-2-nitro benzoate per mole of SH, this has an intense yellow color with an absorbance at 412nm and can be used to measure –SH group.

► PROCEDURE:
The test system contained tissue extract of 100 microlit, 1ml precipitating reagent solution, 3ml phosphate buffer and 0.5ml DTNB. Absorbance was recorded against a blank containing precipitating reagent, phosphate buffer and DTNB solution. GSH was taken as standard, mixed well and the absorbance recorded at 412nm within 1 min of adding DTNB.

LIPID PEROXIDATION

METHOD: Beuge and Aust, (1978)

► PRINCIPLE:
Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives
thiobarbituric acid reactive substance (TBARS). TBARS gives a characteristic pink color that can be measured calorimetrically at 532 nm.

PROCEDURE: The test system contained tissue extract of 1ml, 1ml TBA reagent solution, was kept in water bath (90-100°C) for 20 minutes, allowed to cool and centrifuged at 3000 rpm for 15 minutes. Absorbance was recorded against a blank recorded at 532nm.

SUPEROXIDE DISMUTASE.


► Principle:

Pyrogallol auto-oxidizes at 420nm at pH 8.0. SOD inhibits this auto oxidation of pyrogallol in a rate limiting fashion. 50% inhibition of pyrogallol auto oxidation is equivalent to 1 I.U. of enzyme.

► PROCEDURE:

The final assay mixture contained 1 ml of potassium phosphate buffer (0.2 M, pH 8.2), 10μl of homogenate and 50 μl of pyrogallol. For control tubes required volume of distilled water was taken in place of tissue homogenate. This was used to determine the uninhibited auto oxidation of pyrogallol. The reaction was started by the addition of pyrogallol and the change in optical density was recorded for 180 seconds at 30 second intervals. Change in absorbance / minute was calculated from the reading. The SOD activity was expressed as IU per mg
protein. One unit of SOD activity being defined as the amount of enzyme required to cause 50 % inhibition of pyragailol auto-oxidation.

**CATALASE**

METHOD: - Sinha et al. (1972)

► PRINCIPLE

Briefly, the assay mixture containing 0.5 ml of 0.2M H2O2, 1 ml of sodium phosphate buffer and 0.4 ml distilled water was mixed with 0.1 ml of cell extract was added to initiate the reaction. Then, 2ml dichromate-acetic acid reagent was added after 15, 34, 45, and 60s, to arrest the reaction. To the control tube, the enzyme was added after the addition of the dichromate acetic acid reagent. The tubes were then heated for 10 min, allowed to cool, and the green color developed was read at 590 nm against blank containing all components except the enzyme on a spectrophotometer. The activity of Catalase was expressed as units/mg protein (1 unit is the amount of enzyme that utilizes 1μmol of H2O2 consumed/ min).

**ASCORBIC ACID**

METHOD: Roe et al. (1954).

► PRINCIPLE:

Ascorbic acid is oxidized to dehydroascorbic acid in presence of norit (activated nimal charcoal). It is then coupled with 2,4 Dinitro phenyl hydrazine (mild reducing agent). H2SO4 converts DNPH into a red coloured compound which is assayed colorimetrically.
PROCEDURE:
Homogenize the weighed amount of tissue with 6% TCA under cold condition and make upto desired dilution. To the sample of clear extract add animal charcoal (0.5 gms/25ml), shake well and let it stand for 15 mins. Filter through wathman filter paper # 42. Take aliquot of extract containing not more than 4ml. Take three tubes and label them as blank, standard and sample. Pipette out reagent or homogenate extract.
Take homogenate 4ml in sample tube, 6% TCA in blank tube and standard ascorbic acid in standard tube. Add about 1ml of 2,4 DNPH in each tube, then add thiourea (about 3 drops) in each tube and incubate at 37°C. for 3 hrs or in boiling water bath for 15 mins. Transfer to icebath then add 5ml of 85% Sulphuric acid in each tube, mix carefully and allow it to stand for 30 mins. Take reading at 540nm in photoelectric colorimeter with green filter.

WESTERN BLOT ANALYSIS

GLUT 4 EXPRESSION IN MUSCLE TISSUE

Sample Preparation: Plasma membrane and cytosolic fractions from muscle tissue of control and experimental animals were prepared as described by Dombrosokki et al. (1996) and Kristiansen et al. (2001).

Reagents
1. Protease inhibitor: Commercially available protease inhibitor cocktail (Sigma Chemical Company, USA.) was used.
2. **Phosphatase inhibitor**: 47 mg Trisodium phosphate (Na$_3$PO$_4$), 42 mg Sodium fluoride (NaF), 4 mg Sodium orthovanadate (Na$_3$VO$_4$), and 635 mg β-glycerophosphate were dissolved in 10 ml with homogenization buffer.

3. **Buffer - A (pH 7.0)**: 84 mg of sodium bicarbonate (NaHCO$_3$) was dissolved in 75 ml of distilled water and pH adjusted to 7. To this, 8.5575 g sucrose, 1.742 mg phenyl methyl sulfonyl fluoride (PMSF) and 32.5 mg sodium azide (Na$_3$N), protease (10 μl/ml) and phosphatase inhibitors (100 μl/ml) were added and made up to 100 ml with distilled water.

4. **Buffer - B (pH 7.0)**: 84 mg of sodium bicarbonate (NaHCO$_3$) was dissolved in 75 ml of distilled water and pH adjusted to 7. To this, 1.742 mg PMSF, 32.5 mg sodium azide and protease inhibitor were added and made up to 100 ml with distilled water.

**Subcellular fractionations**

Muscle tissue from control and experimental animals were simultaneously processed for preparation of different fractions. All steps were carried out on ice or at 4°C. Tissue (~1 g) was first cleaned of all visible fat, nerve, and blood vessels and minced in buffer – A. The minced tissue was homogenized (1 g/ 1.5 ml of buffer-A) using a polytron equipped homogenizer at a precise low setting. The resulting homogenate was centrifuged at 1,300 xg for 10 min. The supernatant was centrifuged at 1, 90,000 xg for 1 hour. The resultant supernatant was saved, and sampled as a cytosolic fraction for GLUT4 protein analysis.
IRS-1 protein expression

► Reagents

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2. **Phosphatase inhibitor**: 47 mg Trisodium phosphate (Na$_3$PO$_4$), 42 mg Sodium fluoride (NaF), 4mg Sodium orthovanadate (Na$_3$VO$_4$), and 635mg β-glycerophosphate were dissolved in 10ml with homogenization buffer.

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ml of buffer-A) using a polytron equipped homogenizer at a precise low setting. The resulting homogenate was centrifuged at 1,300xg for 10 min. The supernatant was centrifuged at 1, 90,000xg for 1 hour. Protein concentration in the sample was determined prior to the western blot analysis.

**BLOT ANALYSIS**

**Separation of proteins**

Proteins were separated by SDS - Polyacrylamide gel electrophoresis as described by Laemmli (1970).

**Principle**

SDS - PAGE involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecule, acquiring a high net negative charge that is proportional to the length of the polypeptide chain. When loaded on to a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. Molecular weight protein marker that produces bands of known size is used to help identify proteins of interest.

**Reagents**

1) **Acrylamide/Bis (30% T, 2.67% C)**

29.2 g acrylamide and 800 g N'N'-bis-methylene-acrylamide were dissolved in 100 ml of deionized water. Filtered and stored at 4°C in the dark (30 days maximum).

2) **10% (w/v) SDS**
10 g SDS was dissolved in 90 ml water with gentle stirring and brought to 100 ml with deionized water.

3) 1.5 M Tris – HCl (pH 8.8)
18.15 g Tris base was dissolved in 80 ml deionized water. Adjusted to pH 8.8 with 6 N HCl and brought total volume to 100 ml with deionized water and stored at 4°C.

4) 0.5 M Tris – HCl (pH 6.8)
6 g Tris base was dissolved in 60 ml deionized water. Adjusted to pH 6.8 with 6N HCl and brought the total volume to 100 ml with deionized water and stored at 4°C.

5) 10% APS (Fresh daily)
100 mg ammonium persulfate was dissolved in 1 ml of deionized water.

6) N' N'- Tetramethyl ethylene diamine (TEMED)
Commercially available.

7) Sample buffer (SDS Reducing buffer) (pH 6.8)
1.25 ml 0.5 M Tris-HCl (pH 6.8), 2.5 ml glycerol, 2 ml 10% (w/v) SDS were added to 0.2 ml of 0.5% (w/v) bromophenol blue and brought total volume to 9.5ml with 3.55 ml deionized water. Stored at room temperature. 50μl β-mercaptoethanol was added to 950 μl of sample buffer prior to use.

8) 10X electrophoresis buffer (pH 8.3)
30.3 g Tris base, 144.0 g Glycine and 10 g SDS were dissolved in 800 ml deionized water and brought total volume to 1 litre with deionized water and stored at 4°C.

**Procedure**

**Preparation of Gel (10ml)**

10 % running gel was prepared by mixing the reagents as shown below.

- Deionized H$_2$O: $-4.1$ ml
- 30% degased acrylamide/bis: $-3.3$ ml
- 1.5 M Tris – HCl (pH 8.8): $-2.5$ ml
- 10 % (w/v) SDS: $-0.1$ ml

7 % running gel was prepared by mixing the reagents as shown below.

- Deionized H$_2$O: $-5.1$ ml
- 30% degased acrylamide/bis: $-2.3$ ml
- 1.5 M Tris – HCl (pH 8.8): $-2.5$ ml
- 10 % (w/v) SDS: $-0.1$ ml

Gently mixed and degassed the mixture for 15 minutes. 50 μl of 10% APS and 5 μl TEMED were added prior to pouring the gel and swirled gently to initiate polymerization. This mixture was poured into 1 mm thickness gel casting plate setup and allowed to 20–30 minutes for polymerization.

5 % stacking gel was prepared by mixing the reagents as given below.

- Deionized H$_2$O: $-5.7$ ml
- 30% degased acrylamide/bis: $-1.7$ ml
- 0.5 M Tris – HCl (pH 6.8): $-2.5$ ml
10 \% (w/v) SDS -- 0.1 ml

Gently mixed and degassed the mixture for 15 minutes. 50\micro litre of 10% APS and 10\micro litre TEMED were added prior to pouring the gel and swirled gently to initiate polymerization. This mixture was added on top of the 10\% running gel. Then, the comb (1mm thickness) was inserted and allowed to form the well.

**Separation of proteins**

Equal volume (25\micro g) of samples from adipose tissue of control and experimental animals were diluted with sample buffer (1:2), heated at 95\degree C for 4 minutes and then cooled on ice for 5 minutes. Samples were loaded to 10\% and 7\% SDS–PAGE in a Bio–Rad miniature slab gel apparatus. The prestained broad range protein molecular weight markers used were myosin (198kDa), \(\beta\)-galactosidase (116kDa), bovine serum albumin (85kDa), ovalbumin (54kDa), carbonic anhydrase (37kDa), soybean trypsin inhibitor (29kDa), lysozyme (19kDa) and aprotinin (6kDa) (Bio–Rad). Electrophoresis of protein was performed at 100V (constant) until the dye front reaches the bottom of the running gel.

**Transfer of proteins to the membrane and immunoblotting**

**Reagents**

i. **Transfer buffer**

3 g Tris and 25 g glycine were dissolved in 800 ml of double distilled water and made upto 1000 ml with 200 ml of methanol.

ii. **Polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences Ltd., UK)**
iii. TBS

4 g of NaCl and 10 ml of 1M Tris HCl (pH 7.6) were dissolved in 450 ml of distilled water, adjusted the pH to 7.6 and made up to 500 ml with distilled water.

iv. TBS-T

500µl of Tween-20 was dissolved in 500 ml of TBS and the pH was adjusted to 7.6.

v. 5% Blocking solution

500 mg of blocking reagent was dissolved in 10 ml of TBS-T solution.

vi. Primary antibody

Procedure

After the separation of proteins by SDS-PAGE, the stacking gel was cut and discarded; the separating gel was briefly rinsed in distilled water 2-3 min and then equilibrated in cold transfer buffer under gentle agitation for 5-10 min. In the mean time, fibre pad and Whatman paper and PVDF membrane were soaked in cold transfer buffer. Transfer sandwich was assembled in the following order from anode (+) to cathode (-).

a) + ve end
b) Fibre pad
c) Filter paper soaked in transfer buffer
d) PVDF Membrane
e) Gel
f) Filter paper soaked in transfer buffer

g) Fiber pad

h) --ve end

The setup was placed in the transfer apparatus filled with cold transfer buffer and subjected to an electric current at 100V for 1h under cold condition. After the transfer, the PVDF blot was removed from the transfer system blocked the unreacted sites on the membrane to reduce the amount of non-specific binding during subsequent steps in the assay using 5% blocking solution for 1 h. After the blocking is over, decanted the blocking solution and rinsed the membrane in TBS-T. After the blocking is over, decanted the blocking solution and rinsed the membrane in TBS-T and incubated for 1 h in GLUT4/ Akt/ Phospho Akt primary antibody at room temperature, which was diluted 1:1000 with TBS-T. Following incubation, the blot was washed for three times (5 minutes each) with TBS-T. After washing, the blot was incubated for 1 h with horseradish peroxidase conjugated rabbit secondary antibody, which was diluted 1:5000 with TBS-T. Following incubation, the blot was washed for three times (5 minutes each) with TBS-T. Drained the excess wash buffer from the washed blot and placed them, protein side up on a sheet of Saran Wrap™. The detection reagent mixture [an equal volume of detection solution 1 with detection solution 2 (Enhanced Chemiluminescence, Amersham Biosciences, UK.)] was pipetted on to the blot and incubated for 30-60 seconds and drained off excess reagent. The blotted protein was quantified using Quantity one software system (Bio-Rad).
Stripping and reprobing the membrane

Membrane was stored wet wrapped in Saran Wrap™ in a refrigerator (2-8°C) after immunodetection. The membrane was submerged in stripping buffer [62.5 mM Tris-HCl (pH 6.7); 2% SDS; 100 mM 2-mercaptoethanol] and incubated at 50°C for 30 minutes with occasional agitation. Then the membrane was washed for 2 times (each 10 minutes) in TBS-T at room temperature using large volume of wash buffer. The membrane was blocked by immersing in 5% blocking solution for 1 hour at room temperature. After the blocking is over, decanted the blocking solution and rinsed the membrane in TBS-T and incubated for 1 h in β-actin antibody at room temperature, which was diluted 1:5000 with TBS-T. The immunodetection protocol was repeated as detailed previously.

HISTOLOGY

Pancreas from the splenic region is removed at the time of sacrifice and is fixed in formaline for histological studies. Paraffin sections of 5 micron thickness were cut and staining was done using Haematoxylin Eosin (HE).

STATISTICAL ANALYSIS:

One way ANOVA with Bonferroni index post test was performed using GraphPad Prism version 3.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com. Confidence limit was taken as 95%. For multiple factor analysis TWO WAY ANOVA followed by one way ANOVA was used.
Chapter 1.

COMBINATION THERAPY WITH *PTEROCARPUS MARSUPIUM* AND *OCIMUM SANCTUM* EFFECTIVELY AMELIORATES METABOLIC AND OXIDATIVE STRESS ALTERATIONS ASSOCIATED WITH TYPE I DIABETES: STUDIES ON ALLOXAN INDUCED DIABETIC FEMALE WISTAR RATS.

Diabetes mellitus (DM) is a metabolic disorder with attendant altered metabolic profile affecting carbohydrates, lipids and proteins with a predilection/predisposition towards vascular disorders (Keen and Tang Fui, 1982; Pickup and Williams, 2003). The multiple etiologies associated with diabetes are primarily due to defective insulin secretion and/or insulin action (Baquer et al., 1998). According to WHO, DM is one of the commonest endocrine disorders affecting about 100 million individuals worldwide which is approximately six percent of the world population and expected to intensify five times than the present scenario in another 10 years (WHO/Acadia, 1992; ADA, 1997). Prevalence of DM in Indian population is about 35 million as per a survey conducted by Ramachandran et al (2008), with about 13 million of these cases assumed to go undiagnosed, of which around 50% cases are from rural and about 30% cases from urban areas of India.

Recent findings suggest that, a disturbance in the pro-oxidant to anti-oxidant balance or, their homeostasis can add to the complications of almost all diseases. The increase in pro-oxidants is due to essentially an over production of free radicals and the consequent oxidative stress proving to be detrimental and, a number of theories have been suggested for the increase in pro-oxidants. Depletion in dietary and/or body antioxidants such as Vitamin C (Ascorbic acid -
AA) and melatonin may contribute to poor and ineffective free radical scavenging activity and consequent oxidative stress (Dringen, 2000; Schulz et al., 2000).

It is well accepted that, the high oxidative stress in diabetics considerably contributes to the complication of this disease (Baines, 1991; Baynes and Thorpe, 1999; Ceriello, 2000) and excessive production of free radicals is an observed phenomenon in association with diabetes (Baines, 1991; Chang et al., 1993; Young et al., 1995; Baines and Thorpe, 1999). Glucose oxidation is believed to be a major factor adding to the level of oxidative stress, as glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Wolff and Dean, 1987; Jiang et al., 1990). Non-enzymatic protein glycosylation associated with diabetes also has a role in diabetic complications.

Plants have played a major role in providing diverse products or ingredients to cure many diseases and pain since ages and, have helped in the development of modern medicine (British pharmacopoeia, 1932). Ayurveda, one of the most ancient healthcare systems, is believed to be in practice much before 2500 B.C; this Indian system of treatment therapy involves complex medicinal preparations, which are mostly based on plant products. More than 1250 plants are used for various ayurvedic preparations to cure many diseases. Most of
these preparations, which are based on naturally available plants and their ingredients hardly, have any side effect (Sukh Dev, 1999).

Vijayasar (*Pterocarpus marsupium*) is known for the treatment of diabetes since very long and it has some unique and unidentified features in protecting the pancreatic beta cells and their regeneration (Chakaravarthy *et al.*, 1981, Subramanian, 1981). The bioactive compounds of vijayasar like (-) epicatechin (a flavonoid), marsupin (benzofuranone), and pterosupin (a dihydrochalcone) have been shown to decrease blood glucose level in diabetics comparable to the effect of metformin (Marles and Farnsworth, 1995; Manickam *et al.*, 1997).

Tulsi leaves are similarly studied for their hypoglycaemic and antioxidative properties; it is shown to decrease blood glucose level in diabetic rats by 26% but most significant is the ability of tulsi leaf extract to reduce lipid peroxidation and glutathione levels (Jyoti *et al.*, 2004). Leaves of tulsi are very rich in oils and, presence of eugenol helps in reducing the oxidative stress significantly (Uma Devi and Ganasoundri, 1999). A long study carried out by Eshrat *et al.* (2001), for eight weeks showed that, the aqueous extract of tulsi leaves was a very effective antioxidant as, it could decrease the oxidative stress in circulating plasma and erythrocytes.

Since diabetic manifestations involve hyperglycaemia and free radical associated damage, a combination of the two identified plants, *Pterocarpus marsupium* (Vijayasar) and *Ocimum sanctum* (Tulsi), has been used in the present study to target both, metabolic dysregulation and oxidative stress, associated with diabetic manifestations.
Methanolic extracts of the two plants were prepared separately and then administered to the animals as a mixture of both at a dosage of 500mg/kg body weight, and its effect was checked by assessing glycaemic status and serum lipid and hormone profile together with glucose tolerance and insulin response tests. Further, tissue load of metabolites (glycogen, cholesterol and protein), enzymes of carbohydrate metabolism (glucose-6-phosphatase, glycogen phosphorylase), enzymatic and non-enzymatic antioxidant status together with lipid peroxidation levels and serum markers of hepatic and renal damage were also assessed.
Results

Since treatment of both non-diabetic and diabetic rats with CMC, vehicle for the extract, has not shown any significant difference compared to non-diabetic and diabetic control rats, the data for CMC treatment is not shown.

Glycaemic changes (Table 1.3)

Non-diabetic control rats supplemented with extract has shown significant decrease in fasting serum glucose level with no apparent change in the fed state. Diabetic rats have shown significant hyperglycaemia in both fasted and fed states. However, supplementation with extract showed significant anti-hyperglycaemic effect with the decrement in fasted state being greater than the fed state on a percentage basis.

Oral Glucose Tolerance Test (OGTT) (Figs 1.1, 1.2, Table 1.5) Treatment with extract has shown no significant difference in terms of per minute glucose elevation or clearance rate compared to non-diabetic rats. Diabetic rats subsequent to glucose challenge have shown significantly greater increment in both elevation and clearance rates. Diabetic rats treated with extract have shown significant decrement in both the rates compared to diabetic rats. These differences in elevation and clearance rates have been justified by the observed greater E: C ratio in diabetic animals and reduced ratio in diabetic animals treated with extract.

Insulin Response Test (IRT) (Figs 1.3, 1.4, Table 1.6)

The percentage decrement in serum glucose levels subsequent to insulin administration is nearly the same in non-diabetic, extract treated non-diabetic as
well as diabetic rats while, the same was significantly higher in extract treated diabetic rats.

**Carbohydrate Metabolism (Figs 1.5, 1.6; Table 1.7)**
The tissue glycogen load of both liver and muscle was significantly lower in diabetic rats in conjunction with significantly increased phosphorylase activity and hepatic glucose 6 phosphatase activity with no significant difference in extract treated non-diabetic rats. Diabetic rats treated with extract have shown significant increment in tissue glycogen content and decrement in phosphorylase and glucose-6-phosphatase activities.

**Tissue lipid and Cholesterol (Table 1.9)**
Though there is a mild lipid and cholesterol lowering effect of extract in non-diabetic rats, the levels of both the metabolites were significantly decreased in diabetic rats. Diabetic rats treated with extract have however shown a significant retrieval in the levels of both the metabolites in all the tissues.

**Tissue Protein content (Table 1.7)**
Extract had no significant effect in the protein content of tissues in non-diabetic rats. Tissue protein contents were however decreased in diabetic rats and supplementation with extract has shown significant recovery in tissue protein content.

**Serum Lipids (Table 1.8)**
Except for an increase in serum triglycerides and VLDL levels of extract treated non-diabetic rats, there was no effect on any of the serum cholesterol fractions. Diabetic rats have shown significant increment in serum triglycerides as well as
cholesterol fractions, the levels of which were significantly decreased on treatment with extract.

**Serum Hormone Profile (Table 1.4)**

**Corticosterone (Cort), insulin, Oestrogen (E$_2$) and Progesterone (P4)**

Of the three hormones assayed, while oestrogen and progesterone did not show any significant change, corticosterone was significantly reduced in extract treated non-diabetic animals. Diabetic animals showed significant decrement in corticosterone and progesterone levels while, oestrogen titre was significantly increased. Though extract treated diabetic animals depicted a reversal of corticosterone and oestrogen titres towards non-diabetic levels, progesterone showed a further decrease.

**Oxidative stress parameters.**

**Lipid Peroxidation (LPO) (Fig 1.7)**

Diabetic animals showed a significant increment in LPO in liver, muscle and kidney. Though extract treatment to non-diabetic animals had no significant effect, extract treated diabetic animals showed significant reduction towards non-diabetic levels.

**Reduced Glutathione (GSH) (Table 1.11)**

Diabetic animals showed significant decrement in hepatic, muscle and renal GSH contents. While, extract treatment to non-diabetic animals was marked by significant increment to above normal levels, extract treatment of diabetic animals showed an increment in GSH content in all the three tissues. Though the
hepatic GSH content showed a significant increment, muscle and renal GSH contents were reverted to normal levels.

**Ascorbic Acid (Table 1.11)**

Diabetic animals showed significant decrement in hepatic, muscle and renal ascorbic acid contents. While, extract treatment to non-diabetic animals had no significant effect, diabetic animals treated with extract showed significant increment though not attaining the non-diabetic levels.

**Catalase (Table 1.10)**

Diabetic animals showed significant decrement in hepatic, muscle and renal catalase activity. While extract treatment to non-diabetics did not show any remarkable effect, diabetic animals treated with extract showed significant increment towards non-diabetic levels, though still with a deficit.

**Glutathion Peroxidase (GPx) (Table 1.10)**

Diabetic animals showed significant decrement in hepatic, muscle and renal GPx activity. Extract treatment increased the levels of enzyme activity, though still significantly lesser than non-diabetic levels

**Superoxide Dismutase (SOD) (Table 1.10)**

Diabetic animals showed a significant decrement in hepatic, muscle and renal SOD activity. Extract treatment to non-diabetic animals showed non-significant increment while, treatment of diabetic animals depicted significant increment.

**Serum Markers of Hepatic and Renal Function**

**SGPT and SGOT (Table 1.12)**
The serum level of SGOT in non-diabetic animals was nearly double that of SGPT. Diabetic animals showed significant increment in serum levels activity of both the enzymes and, extract treatment decreased the levels of both the enzymes, more so of SGOT. However, the levels of both the enzymes were significantly higher than non-diabetic animals.

**ALP and ACP (Table 1.12)**

The serum level of ALP activity was significantly higher than that of ACP in non-diabetic animals. Diabetes tended to increase the levels of both the enzymes significantly, more so of ALP. Extract treatment to non-diabetic animals was of no significant effect while, treatment of diabetic animals showed significant amelioration, more so of ALP.

**Urea and Creatinine (Fig. 1.8, 1.9)**

Diabetes was marked by significant increment in serum levels of urea and creatinine. Treatment of non-diabetic animals with extract was of no significant consequence but, treatment of diabetic animals tended to significantly minimize the levels of urea and creatinine though, still significantly higher than those of the diabetic levels.

**Immunoblot Analysis (Fig 1.10)**

Immunoblot for GLUT-4 showed a significant decrement in its expression in the diabetic group as compared to the non-diabetic group. The administration of PM+OS extract had shown an increment in GLUT-4 expression in extract treated diabetic rats.
Histology (Plate 1)

There was an improvement in the β cell architecture in PM+OS extract treated diabetic rats. The β cell integrity is well maintained in the extract treated diabetic rats similar to that in control rats.
FIGURES AND TABLES

Table 1.1: Body weight (g), food intake (g/animal/day) and water intake (ml/animal/day) in all the experimental groups

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>INITIAL BW</th>
<th>FINAL BW</th>
<th>FOOD INTAKE</th>
<th>WATER INTAKE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>253.3±5.9</td>
<td>263.3±7.27</td>
<td>16.15±0.01</td>
<td>35.56±1.15</td>
</tr>
<tr>
<td>NC+E</td>
<td>228.3±2.13</td>
<td>234.2±1.76</td>
<td>13.07±1.21</td>
<td>19.67±0.12</td>
</tr>
<tr>
<td>DC</td>
<td>186.6±17.09</td>
<td>189.8±6.01</td>
<td>25.13±0.33</td>
<td>73.5±0.12</td>
</tr>
<tr>
<td>DC+E</td>
<td>233.3±4.14</td>
<td>245.6±3.15</td>
<td>19.64±2.11</td>
<td>62.67±1.21</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC

Table 1.2: Relative weights (g/100g of body weight) of liver, muscle, kidney, spleen and adrenal of all the experimental groups

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>LIVER</th>
<th>MUSCLE</th>
<th>KIDNEY</th>
<th>SPLEEN</th>
<th>ADRENAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>2.22±0.14</td>
<td>0.49±0.01</td>
<td>1.68±0.033</td>
<td>0.31±0.07</td>
<td>0.018±0.0014</td>
</tr>
<tr>
<td>NC+E</td>
<td>2.55±0.14</td>
<td>0.56±0.056a</td>
<td>1.57±0.12</td>
<td>0.23±0.021</td>
<td>0.020±0.0012</td>
</tr>
<tr>
<td>DC</td>
<td>3.36±0.001c</td>
<td>1.015±0.02b</td>
<td>0.89±0.01c</td>
<td>0.27±0.006</td>
<td>0.031±0.0010c</td>
</tr>
<tr>
<td>DC+E</td>
<td>2.92±0.041†</td>
<td>1.17±0.36†</td>
<td>0.76±0.08</td>
<td>0.21±0.004@</td>
<td>0.022±0.004#</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC
Table 1.3: Levels of Fasting and Fed Serum Glucose (mg/dl) in Extract Treated Non-diabetic and Diabetic Rat

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>FASTING</th>
<th>FED</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>94.33±5.10</td>
<td>114.33±3.18</td>
</tr>
<tr>
<td>NC+E</td>
<td>87.83±6.27°</td>
<td>111.63±5.90</td>
</tr>
<tr>
<td>DC</td>
<td>444.0 ±22.21°</td>
<td>652.0±7.69°</td>
</tr>
<tr>
<td>DC+E</td>
<td>196.0 ±7.71*</td>
<td>400.0±13.58*</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE. NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC = Diabetic Control and DC+E = Diabetic Control+extract.

a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC.

Table 1.4: Serum hormone profile of extract treated non-diabetic and diabetic rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>INSULIN (pg/ml)</th>
<th>CORTICOSTERONE (ng/ml)</th>
<th>ESTRADIOL (pg/ml)</th>
<th>PROGESTERONE (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0.36±0.01</td>
<td>8.95±0.59</td>
<td>0.19±0.002</td>
<td>66.05±3.48</td>
</tr>
<tr>
<td>NC+E</td>
<td>0.46±0.02b</td>
<td>6.30±0.11°</td>
<td>0.16±0.001</td>
<td>61.90±2.17</td>
</tr>
<tr>
<td>DC</td>
<td>0.16±0.01*</td>
<td>25.0±1.45°</td>
<td>1.98±0.0012*</td>
<td>54.68±1.74°</td>
</tr>
<tr>
<td>DC+E</td>
<td>0.31±0.01*</td>
<td>12.12±1.21*</td>
<td>0.97±0.003&quot;</td>
<td>29.17±2.60*</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE. NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC = Diabetic Control and DC+E = Diabetic Control+extract.

a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC.
Figure 1.1: Serum glucose levels in response to oral glucose tolerance test (OGTT) within a time range of 0 to 120 minutes in all the experimental groups.

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, •) p< 0.005 •) p< 0.0005 compared to DC

Figure 1.2: Area under curve for OGTT in all experimental groups
Figure 1.3: Serum glucose levels in response to insulin administration (IRT) within a time range of 0 to 120 minutes of all the experimental groups

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, (c) p< 0.005 •) p< 0.0005 compared to DC

Figure 1.4: Area under curve for IRT in all experimental groups

NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control
Table 1.5: Elevation and clearance rates of glucose during OGTT in extract treated diabetic and non-diabetic rats.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>RATE OF ELEVATION</th>
<th>RATE OF CLEARENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.21</td>
<td>0.50</td>
</tr>
<tr>
<td>NC+E</td>
<td>1.11</td>
<td>0.40</td>
</tr>
<tr>
<td>DC</td>
<td>9.13</td>
<td>0.97</td>
</tr>
<tr>
<td>DC+E</td>
<td>5.84</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Table 1.6: Clearance and elevation rates of glucose during IRT in extract treated diabetic and non-diabetic rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>RATE OF CLEARENCE</th>
<th>RATE OF ELEVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>2.1</td>
<td>0.56</td>
</tr>
<tr>
<td>NC+E</td>
<td>2.18</td>
<td>0.62</td>
</tr>
<tr>
<td>DC</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>DC+E</td>
<td>2.36</td>
<td>1.55</td>
</tr>
</tbody>
</table>
Table 1.7: Tissue Protein and Glycogen content (mg/100 mg tissue) in Control and Treated Rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>PROTEIN</th>
<th>GLYCOGEN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Muscle</td>
</tr>
<tr>
<td>NC</td>
<td>16.38±1.24</td>
<td>9.18±1.41</td>
</tr>
<tr>
<td>NC+E</td>
<td>17.06±1.82</td>
<td>9.75±0.80</td>
</tr>
<tr>
<td>DC</td>
<td>13.62±0.87a</td>
<td>5.37±0.73e</td>
</tr>
<tr>
<td>DC+E</td>
<td>15.95±0.90*</td>
<td>7.03±0.70*</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 *) p< 0.0005 compared to DC
Figure 1.5: Hepatic and muscle glycogen phosphorylase activity in extract treated non-diabetic and diabetic rats

![Glycogen Phosphorylase Graph](image1)

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, •) p< 0.005 •) p< 0.0005 compared to DC

Figure 1.6: Hepatic Glucose-6-phosphatase activity in extract treated non-diabetic and diabetic rats

![Glucose-6-Phosphatase Graph](image2)

Data are expressed as Means±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, •) p< 0.005 •) p< 0.0005 compared to DC
Table 1.8: Serum lipid profile (mg/dl) of extract treated non-diabetic and diabetic rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>CHO</th>
<th>TG</th>
<th>LDL</th>
<th>VLDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>80.00±2.31</td>
<td>68.67±3.44</td>
<td>15.00±1.73</td>
<td>13.33±1.73</td>
<td>50±1.76</td>
</tr>
<tr>
<td>NC+E</td>
<td>71.33±3.20 b</td>
<td>98.21±6.07 e</td>
<td>10.33±1.44 e</td>
<td>11.33±2.67</td>
<td>50.00±4.63 e</td>
</tr>
<tr>
<td>DC</td>
<td>97.00±4.33 d</td>
<td>140.00±2.33 e</td>
<td>30.66±0.86 e</td>
<td>22.22±2.89 e</td>
<td>45.33±2.60 e</td>
</tr>
<tr>
<td>DC+E</td>
<td>69.66±3.44 e</td>
<td>78.33±3.38 e</td>
<td>6.66±0.29 e</td>
<td>15.51±1.16 e</td>
<td>47±4.93 e</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC

Table 1.9a: Tissue Cholesterol content (mg/100mg tissue) in Control and Treated Rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>CHOLESTEROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>NC</td>
<td>0.28±0.005</td>
</tr>
<tr>
<td>NC+E</td>
<td>0.14±0.02*</td>
</tr>
<tr>
<td>DC</td>
<td>0.60±0.004</td>
</tr>
<tr>
<td>DC+E</td>
<td>0.28±0.01*</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC
Table 1.9b: Tissue lipid content (mg/100mg tissue) in Control and Treated Rats

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Muscle</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.21±0.71</td>
<td>1.56±0.43</td>
<td>0.73±0.06</td>
</tr>
<tr>
<td></td>
<td>3.56±0.65*</td>
<td>1.01±0.3</td>
<td>0.66±0.06</td>
</tr>
<tr>
<td></td>
<td>6.32±0.81*</td>
<td>2.08±0.355</td>
<td>0.93±0.08*</td>
</tr>
<tr>
<td></td>
<td>5.09±0.59*</td>
<td>1.71±0.37#</td>
<td>0.80±0.06*</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE.
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC = Diabetic Control and DC+E = Diabetic Control+extract.
a) p<0.05, b) p<0.025 c) p<0.01, d) p<0.005, e) p<0.0005 compared to NC and *) p<0.05 #) p<0.025 @) p< 0.01, o) p< 0.005 +) p< 0.0005 compared to DC.
Figure 1.7: Levels of LPO in extract treated diabetic and non-diabetic rats

LIPID PEROXIDATION

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC
<table>
<thead>
<tr>
<th>GROUPS</th>
<th>SOD</th>
<th>CATALASE</th>
<th>GPX</th>
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<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney</td>
<td>Muscle</td>
</tr>
<tr>
<td>NC</td>
<td>8.1(±0.89)</td>
<td>10.9±6.00</td>
<td>5.4±20.39</td>
</tr>
<tr>
<td>NC+E-C</td>
<td>9.6±50.57</td>
<td>11.6±20.98</td>
<td>4.9±90.04</td>
</tr>
<tr>
<td>DC</td>
<td>4.9±40.44</td>
<td>6.5±60.47</td>
<td>2.7±90.14</td>
</tr>
<tr>
<td>DC+E</td>
<td>7.3±90.26</td>
<td>9.9±90.84</td>
<td>3.5±90.46</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE. NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC = Diabetic Control and DC+E = Diabetic Control+Extract.

* p<0.05, ** p<0.025, *** p<0.01, **** p<0.005 compared to NC and **** p<0.0005 compared to DC.
Table 1.11: Tissue non-enzymatic anti-oxidant status (mg/100 mg tissue) in extract treated non-diabetic and diabetic rats

<table>
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<tr>
<th>GROUPS</th>
<th>GSH</th>
<th>ASCORBIC ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Muscle</td>
</tr>
<tr>
<td>NC</td>
<td>30.23±2.58</td>
<td>14.36±1.51</td>
</tr>
<tr>
<td>NC+E</td>
<td>36.47±1.14b</td>
<td>19.37±3.45b</td>
</tr>
<tr>
<td>DC</td>
<td>10.61±1.28a</td>
<td>12.44±1.37</td>
</tr>
<tr>
<td>DC+E</td>
<td>17.67±1.23a</td>
<td>13.42±1.86</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC

Table 1.12: Serum Markers of Hepatic Dysfunction in Control and Extract Treated Rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>SGPT U/L</th>
<th>SGOT U/L</th>
<th>ALP U/L</th>
<th>ACP U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>40.00±4.04</td>
<td>70.00±2.96</td>
<td>205.0±2.648</td>
<td>8.5±0.86</td>
</tr>
<tr>
<td>NC+E</td>
<td>37.00±3.79</td>
<td>74.00±1.76</td>
<td>198.0±3.18</td>
<td>8.6±0.18</td>
</tr>
<tr>
<td>DC</td>
<td>123.00± 5.86a</td>
<td>290.0±5.78a</td>
<td>471.0±2.33a</td>
<td>12.6±0.61d</td>
</tr>
<tr>
<td>DC+E</td>
<td>115.00±5.93</td>
<td>192.0±6.39*</td>
<td>287.0±3.18*</td>
<td>10.5±0.86e</td>
</tr>
</tbody>
</table>

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, o) p< 0.005 •) p< 0.0005 compared to DC
Figure 1.8: Serum Urea level in extract treated non-diabetic and diabetic rats

![UREA Graph]

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, (c) p< 0.005 •) p< 0.0005 compared to DC

Figure 1.9: Serum Creatinine level in extract treated non-diabetic and diabetic rats

![CREATININE Graph]

Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p< 0.0005 compared to NC and *) p< 0.05 #) p< 0.025 @) p< 0.01, (c) p< 0.005 •) p< 0.0005 compared to DC
Data are expressed as Mean±SE
NC = Non-diabetic Control, NC+E = Non-diabetic Control+Extract, DC= Diabetic Control and DC+E = Diabetic Control+extract
a) p<0.05, b) p< 0.025 c) p<0.01, d) p< 0.005, e) p<0.0005 compared to NC and *) p< 0.05 #) p<0.025 @) p< 0.01, o) p<0.005 •) p<0.0005 compared to DC

Figure 1.10. (A)

<table>
<thead>
<tr>
<th>LANES</th>
<th>GROUPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NC</td>
</tr>
<tr>
<td>2</td>
<td>CMC</td>
</tr>
<tr>
<td>3</td>
<td>NC+M(H)</td>
</tr>
<tr>
<td>4</td>
<td>DC</td>
</tr>
<tr>
<td>5</td>
<td>DC+E+M(H)</td>
</tr>
</tbody>
</table>

Glut 4 protein expression normalised to B-actin

Figure 1.10. (B) Immunoblot analysis of Glut-4 protein expression.

(B) Semi quantification analysis of Glut-4 protein using scanning densitometry. Signals of Glut-4 in immunoblot were quantified arbitrarily. Bars represent means of ± S.E. of independent experiments and a representative immunoblot is shown here.
Figure A: Transverse section of pancreas of non diabetic rat showing an islet. Note the intact islet histoarchitecture (450X)

Figure B: Transverse section of pancreas of non diabetic rat treated with extract. Note the normal appearing islet with well formed islet cells. (450X)

Figure C: Transverse section of pancreas of diabetic rat. Note the islet cell destruction and the wider intercellular spaces. (450X)

Figure D: Transverse section of pancreas of diabetic rat treated with extract. Note the recovery of islet with marked islet integrity compared to diabetic rats. (450X)
DISCUSSION

No doubt, *Pterocarpus marsupium* (PM) and *Ocimum sanctum* (OS) have been evaluated individually for their ability to ameliorate diabetic hyperglycaemia and associated metabolic alterations and oxidative stress in recent times (Vats *et al.*, 2004; Chandra *et al.*, 2008; Gayathri and Kannabiran, 2008). These studies have shown anti-hyperglycaemic or hypoglycaemic effects of both in animals rendered mildly (blood glucose 150mg/dl – 200mg/dl) or moderately diabetic (blood glucose 200mg/dl – 250mg/dl) with PM being more effective in glycaemic regulation and, OS more effective in minimizing oxidative stress (Vats *et al.*, 2002; Dhanabal *et al.*, 2006; Narendhirakannan *et al.*, 2006). In this behest, the present study has tried to evaluate the combined efficacy of PM and OS in the control of diabetic manifestations. Apart from this uniqueness of attempting a combinational therapy, other novel aspects of the study are 1) a holistic evaluation of the many attendant manifestations like hyperglycaemia, dyslipidemia, tissue metabolite load, metabolic alterations, tissue oxidative stress, hepatic and renal toxicity markers, serum insulin level, glucose tolerance, insulin sensitivity and, insulin sensitive glucose uptake as marked by muscle GLUT-4 protein profile, 2) Short term efficacy with a treatment duration of 15 days and 3) that too in severely diabetic animals with blood glucose levels exceeding 600 mg/dl in fed state and 400 mg/dl in overnight fasted state. Since diabetes is a serious endocrine disorder with multiple etiologies and a spectrum of primary and secondary complications/manifestations, the need of the hour is a systematic evaluation of an ideal combination of herbal principles to combat holistically the plethora of diabetic complications (cause or effect). This is in keeping with the recommendation of World Health Organization to initiate
programmes designed in the use of medicinal plants as a part of traditional health care system, especially in developing countries (WHO, 1978). Subsequently, the 31st assembly of WHO suggested the need for a thorough inventory and systematic evaluation of the efficacy, safety and standardization of medicinal plants for the treatment of diabetes (Farnsworth, 1980). Though, the current available treatment schedules in the form of hypoglycaemicals, do help manage glycaemic level, are nevertheless unsuccessful in preventing secondary complications and, moreover, are also fallible in terms of side effects on long term basis (Rang et al., 1991). It is therefore necessary to target an effective combination of herbal principles providing a holistic management/cure of diabetes with no attendant side effects. The present study and the discussion that follows are in this context a meaningful exploration, much against the recent dictum of phytotherapy scientific community and, journal compelled studies on single plants and/or isolated active principles.

The combination of PM and OS (PM + OS) used in the present study for a short duration of 15 days, has a potent hypoglycaemic effect in severely diabetic animals as, the glycaemic levels showed a decrease from 470% high in diabetic animals to 250% in PM + OS treated diabetic animals. More significant is the change in serum insulin titre which showed a recovery to just about 11% of control animals in extract treated diabetic animals compared to 54% deficit in diabetic animals. Of the many recent studies on hypoglycaemic effect of PM and OS (Halim et al., 2001; Grover et al., 2002; Sethi et al., 2004; Dhanabal et al., 2006), there are only two studies which measured serum insulin level, and both have revealed a lower efficacy in recovery from the hypoinsulinemia recorded in diabetic animals. In one of these studies, Narendhirakannan et al. (2006)
recorded recovery in insulin level to 38% of control animals in mildly diabetic rats (blood glucose 250mg/dl) treated with 200mg/kg of OS extract for 30 days. In the other study, Gayathri and Kannabiran (2008) showed recovery in insulin level to 13% of control rats in mildly diabetic animals treated with 500mg/kg PM extract for as long as 12 weeks. Considering severe diabetic state and short duration (15 days) treatment period, the 11% deficit in insulin level recorded in diabetic animals herein is significantly more effective than the above reports. Apparently, a mixture of PM and OS is more potent in raising serum insulin (the primary cause of all the diabetic manifestations) level even in animals with severely compromised beta cells and insulin secretion. The *raison d'etre* for the increased serum insulin level could be an insulinogenic and/or insulin secretomimetic action of the extract as, many available reports on PM and OS extracts suggest so (Aggarwal et al., 1996; Vats et al., 2004; Dhanabal et al., 2006; Grover et al., 2006; Hannan et al., 2006; Narendhirakannan et al., 2006; Gayathri and Kannabiran, 2008). The histological observation of more robust islets with greater number of β cells recorded in the present study in PM+OS extract treated diabetic rats suggest the possible therapeutic potential of the extract to promote proliferation of surviving β cells (consequent to alloxan destruction of β cells) and/or regeneration of islet β cells. This inference stands validated by the findings of β cell proliferation and/or regeneration on usage of PM extract (Chakravarthy et al., 1980; Ahmed et al., 1991; Manickam et al., 1997).

The favourable influence of the extract mixture in combating diabetic hyperglycaemia is well confirmed by the greater glucose tolerance and insulin sensitivity in extract administered diabetic rats. Glucose elevation rate (E) which essentially provides an index of glucose tolerance is much higher in diabetic
animals (9.1mg/min) and significantly lesser in PM+OS treated diabetic animals (5.8mg/min) as against identical lower E· values of 1.21 and 1.11 mg/min in control and extract treated control rats respectively. Type I diabetes characterized by hypoinsulinemia is insulin sensitive (hence referred to as IDDM) and insulin sensitivity would be greater to administered insulin compared to non-diabetic animals. This is clearly manifest in the observed greater decrease in blood glucose by 120 mins on insulin administration in diabetic rats (45%) as against a meagre response (12%) in control rats. Clearly, the extract treated diabetic rats also show greater insulin sensitivity as seen by the 53% decrease in blood glucose as against the mere 7.6% decrease in extract treated control rats. Further, maximal hypoglycaemia attained on insulin administration was nearly identical in all groups (56% in control, 55% in extract treated control, 45% in diabetic and 53% in extract treated diabetic) with the difference that, whereas this decrease occurred within 30 minutes of insulin challenge in both the control rats, the same was achieved in both the diabetic groups only by 120 mins. This slow lethargic response to insulin in experimental rats suggests dysregulation of insulin mediated glucose disposal mechanisms due to diabetic induction. There are no comparable evaluations of GTT or IRT in severely diabetic animals for either of the two plants used in the present study.

Pertinent insulin mediated glucose disposal mechanisms involve hepatic glucose transport and glycogenesis with reduced gluconeogenesis and peripheral glucose uptake by muscle and adipose tissue. Some previous studies using PM or OS extract individually in animals with mild or moderate diabetes have documented varying degrees of increased tissue metabolite load (glycogen and protein) and decreased activity of gluconeogenic enzymes (Grover et al., 84)
The present study reveals substantial recovery in hepatic and muscle glycogen contents and hepatic, muscle and renal protein contents on PM+OS extract treatment from the significantly decreased levels characteristic of diabetic state. The extract induced recovery of tissue glycogen contents in diabetic rats is well corroborated by decreased glycogenolysis as marked by significant decrement in glycogen phosphorylase activity in extract treated animals. Ability of PM+OS extract to combat diabetes induced gluconeogenesis is indicated by the significant decrement in G-6-Pase activity from the elevated activity level in diabetic rats. Tissue glycogen stores can be taken to reflect insulin status as, insulin promotes glycogenesis by way of stimulation of glycogen synthase and inhibition of glycogen phosphorylase. The observed depletion of tissue glycogen stores in alloxanized diabetic rats is attributable to diminished activity of glycogen synthetase concomitant to the observed increased activity of glycogen phosphorylase. These changes can be related with the hyperglycaemic manifestation of diabetes. Administration of PM+OS extract tends to restore tissue glycogen levels by increasing glycogen synthase activity and concurrently decreasing glycogen phosphorylase activity as seen herein. Glucose-6-phosphatase is an important regulatory enzyme of gluconeogenic pathway (Minnassian and Mitheux, 1994). The activity of this enzyme in liver is known to increase during diabetes (Horecker et al., 1975) contributing to decrease in glycolytic flux. Under normal physiological conditions, insulin is a suppressor of gluconeogenic enzymes (Baquer et al., 1998). The increased activity of glucose-6-phosphatase in the liver of diabetic rats is effectively countermanded by treatment with PM+OS extract and, the recorded
increase in serum insulin level in extract treated diabetic rats is self explanatory. The observed decrease in tissue protein contents can be related with increased protein catabolism and the entry of amino acids into liver feeding the pathway of gluconeogenesis in diabetic rats (Rannels et al., 1997). In this context, Dighe et al. (1984) have opined that, elevated rate of proteolysis under uncontrolled diabetic conditions, occurs due to a derangement in glucagon mediated cyclic-AMP formation under hypoinsulinemia. These reports account for the herein recorded tissue protein contents in diabetic rats. Apparently, the increase in tissue protein content in diabetic rats is suggestive of a check on proteolysis brought about by a recovery in insulin level.

Another important mechanism of insulin induced glucose disposal involves increased peripheral glucose uptake by muscle and adipose tissue through activation of GLUT-4 (Insulin sensitive glucose transporter) and downstream signaling entities like IRS, PRPPKinase, PPARgamma, PI3 Kinase etc. Impaired GLUT-4 translocation and attenuated expression of PI3Kinase and PPARgamma under diabetic conditions and upregulation of GLUT-4 translocation and enhanced expression of all the above at the transcriptional and translational levels in presence of insulin have all been studied (Okada et al., 1994; Ciaraldi et al., 1995; Tsakiridis et al., 1995; Laville et al., 1996; Desvergne and Wahli, 1999; Ntambi and Yonum-cheul, 2000). In the present study, cytosolic GLUT-4 expression in the muscle of diabetic animals is significantly decreased and, administration of PM+OS extract mixture has been found to be very effective in reversing the decline in GLUT-4 expression. This encouraging result on GLUT-4 expression, provides ample evidence for the efficacy of the PM+OS extract on glucose uptake at the molecular level. Pertinently, both Insulin
and Pterocarpus marsupium extract individually has been shown to upregulate the expressions of GLUT-4, PPARgamma and PI3 kinase in L6 muscle cell lines (Anandharajan et al., 2005). Based on this report and the current observations, it can be surmised that, the PM+OS combination extract somehow functions like an insulinomimetic agent. In a nutshell, the observations on tissue glycogen content, glycogen phosphorylase, G-6-Pase and total GLUT-4 expression in muscles tend to suggest the competence of the extract mixture to enhance peripheral glucose uptake and disposal through appropriate metabolic pathways and maintain glycaemic regulation in diabetic animals.

Dyslipidemia is an essential accompaniment of diabetes. Whereas hypertriglyceridemia and hypercholesterolemia could be considered more of a consequence of Type I diabetes, the same could be a cause or consequence of Type 2 diabetes. Irrespective of cause or consequence, dyslipidemia is a diabetic manifestation and even in the present study significant elevations are recorded for serum TG (103%), TC (21%), LDL (146%) and VLDL (115%) along with tissue (hepatic – 114%, muscle – 137%, kidney – 56%) cholesterol and lipid contents, in diabetic rats. Some of the previous studies have shown a better lipid lowering effect of PM extract or its constituent fraction (Jahroni and Ray, 1993; Grover et al., 2005; Dhanabal et al., 2006; Gayathri and Kannabiran, 2008.) while, OS extract had been shown to exert minimal effect (Eshrat et al., 2001; Eshrat and Mukhopadhyay, 2006). Since the higher tissue and serum lipids in diabetes contribute to many secondary complications affecting the cardiovascular system, it is essential for an antidiabetic preparation to have significant hypolipidemic and/or hypocholesterolemic effect. The present study on PM+OS combination extract in this context shows very potent effect in
maintaining hepatic cholesterol content and serum triglycerides, VLDL and HDL levels to near normal non-diabetic state while, lowering serum cholesterol and LDL to even below the non-diabetic control levels. Muscle and kidney cholesterol contents showed a slow response though, the reduction from the diabetic high was to the tune of 50%. Clearly, a combination of PM and OS has the greatest lipid lowering potential and exerts corrective measures on the metabolic machinery responsible for diabetic dyslipidemia.

Similar to dyslipidemia, oxidative stress is another feature which can be both cause and consequence of diabetes and, persistent oxidative stress results in many secondary complications of serious nature. Enough evidence is available to show that, diabetic patients are under oxidative stress and that, increased oxidative stress contributes to the development and progression of diabetes and associated complications (Bonnefont – Rousselot et al., 2000; Maritum et al., 2003). Hyperglycaemia can, not only engender free radicals but, also impair endogenous antioxidant defence system (Saxena et al., 1993; Maritum et al., 2003). Compromised antioxidant system, denoted by increased lipid peroxidation and decreased levels of both non-enzymatic and enzymatic antioxidants, is a feature of diabetes and, such changes have been noted in alloxan or streptozotocin induced diabetes (Bonnefont- Rousselot et al., 2000; Maritim et al., 2003). In the present study, evaluation of oxidative stress in the form of levels of LPO, non-enzymatic (GSH) and enzymatic (SOD, Cat, and GPx) antioxidants in liver, muscle and kidney has depicted an increase in LPO and decrease in antioxidants ranging from 30%-50% at an average, indicating co-existence of metabolic disturbances and oxidative stress in diabetic animals. Therefore, any treatment for diabetes should address to contain oxidative stress
and restoring the antioxidant defense system apart from exerting glycaemic control. The desired effect of any anti-diabetic drug is no doubt normoglycaemia but apart from insulin, only a limited number of drugs such as melatonin, probucol, vitamin C and E plus β Carotene and α-lipoic acid are capable of reversing the severe diabetic hyperglycaemia (Maritum et al., 2003). However, most of the antioxidants fail to achieve glucoregulation and hence the suggestion that they may be given as adjuvants to insulin therapy or hyperglycaemic anti diabetic drugs in vogue (Maritum et al., 2003). On the other hand, continuous treatment with insulin or hypoglycaemics though may help achieve a stable glycaemic state with some favourable effect on anti oxidant/oxidant balance, in reality, total normalization of antioxidant status seems very remote and not achieved (Sharma et al., 1998; Bonnefont- Rousselot et al., 2000). It is here, that, traditional herbal preparations could be of invaluable help. Of the two plants, PM and OS used in this study, though the former is more potent in glycaemic and lipid regulation with only milder recovery effect on endogenous antioxidant levels (Joshi et al., 2004), the latter is more potent in reversing oxidative stress by normalizing antioxidant status (Eshrat et al., 2001; Sethi et al., 2004; Eshrat and Mukhopadhyay, 2006; Chandra et al., 2008). It is in this context, the results of the present study strongly justify the usage of a combination therapy with PM+OS mixture and meet the objective of achieving a holistic amelioration and cure of diabetes as, the PM+OS extract mixture has not only succeeded in effecting glycaemic regulation with rectification of dyslipidemia but also in restoring the antioxidant levels to the pre-diabetic status. Interestingly, oxidative stress in diabetic animals is paralleled with an increase in serum

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corticosterone level. Treatment with PM+OS mixture is found to be remarkably successful in restoring the serum corticosterone level as well.

Disturbances in carbohydrate, lipid and protein metabolisms together with oxidative stress are likely to affect hepatic and renal functions in severe diabetic condition. Accordingly, the present study has recorded significant increment in serum markers of hepatic (SGPT- 207%, SGOT- 286%, ALP- 130%, ACP- 48%) and renal (Urea - 309%, Creatinine- 50%) damage in diabetic rats. Increased levels of SGOT and SGPT under insulin deficiency (Fleig et al., 1970) have been related with increased gluconeogenesis and ketogenesis during diabetes. Moreover, increased levels of these enzymes together with ALP and ACP are reported to be associated with liver dysfunction and leakage into blood stream in diabetes (Ohaeri, 2001). Negative nitrogen balance with enhanced tissue proteolysis and decreased protein synthesis can contribute to increased serum urea and creatinine levels, indicating impaired renal functions in diabetic animals (Jensen et al., 1981; Garvery, 1992). The renal and hepatoprotective effects of PM+OS mixture are clearly inferable from the significant reduction towards pre diabetic levels seen in diabetic animals treated with PM+OS for only 15 days. The powerful renal and hepatoprotective effect of PM+OS mixture finds validation from the reported noticeable reduction in these serum markers on treatment of diabetic rats with either PM or OS (Narendhirakannan et al., 2006; Gayathri and Kannabiran, 2008).

In conclusion, streptozotocin or alloxan induced diabetes in rats is a well established model of Type I insulin dependent diabetes mellitus. Glycaemic dysregulation, metabolic alterations with dyslipidemia, oxidative stress and hepatic and renal functional impairment are characteristic manifestations, which
need to be addressed to in the search for an effective anti diabetic therapy. Herbal preparations are ideal candidates of choice and in this context, the present combination of PM and OS provides compelling evidences for a holistic efficacy in ameliorating all diabetic manifestations/dysregulations.