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Biochemical Evaluation of Mitochondrial Protective Effect of *Terminalia chebula* Studied in STZ-Induced Diabetic Rats

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Abstract: Impairment of mitochondrial function is intrinsically related with diabetes. Treatment strategies that focus on enhancing mitochondrial function are the important options for diabetes management. The present study was aimed to evaluate the protective effect of *Terminalia chebula* (*T. chebula*) fruits on mitochondrial damage in STZ-induced diabetic rats. Oral administration of ethanolic extract of the fruits (200 mg kg^{-1} body weight day^{-1}) for 30 days significantly increased the activities of mitochondrial enzymes in diabetic rats. Transmission Electron Microscopic (TEM) observations also provided an additional strength to the prophylactic activity of fruit extract. Thus it may be concluded that the presence of biologically active ingredients in the fruit extract of *T. chebula* potentiate its anti-diabetic properties by regulating the mitochondrial enzymes.

Key words: *Terminalia chebula*, mitochondrial enzymes, transmission electron microscopy

INTRODUCTION

Mitochondrion is now gaining importance in diabetes because of its central role as a regulator of energy balance (Wallace, 1999). It is well established that mitochondrial function is required for normal glucose-stimulated insulin release from pancreatic β-cells. Recent Magnetic Resonance Spectroscopy (MRS) studies of humans suggest that more subtle defects in mitochondrial function might play a role in the pathogenesis of insulin resistance in diabetes, which is the most common metabolic disease in the world (Lowell and Shulman, 2005).

Mitochondrion generates most of the energy in animal cells through oxidative phosphorylation, a process in which electrons are passed along a series of carrier molecules called the electron transport chain. These electrons are generated from NADH (reduced nicotinamide adenine dinucleotide), which is produced by oxidation of nutrients such as glucose and are ultimately transferred to molecular oxygen. The electron transport chain consists of four respiratory enzyme complexes arranged in a specific orientation in the inner mitochondrial membrane. The passage of electrons between these complexes releases energy, stored in the form of a proton gradient across the membrane and is then used by ATP synthase to make ATP from ADP and phosphate (Saraste, 1999).

Disruption of mitochondrial functions has been implicated in more than 40 known diseases, including diabetes, cancer, atherosclerosis, ischemic heart disease and neurodegenerative disease such as Alzheimer's disease and Parkinson's disease (Schon, 2000). Given the critical role that mitochondria play in the citric acid cycle (TCA), the Electron Transport Chain (ETC), oxidative phosphorylaton, fatty acid oxidation and amino acid metabolism, it is not surprising that mitochonidrial dysfunction often have an associated metabolic component (Maassen *et al*., 2004).

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The observation that reduced the rates of ATP synthesis in subjects with a family history of diabetes occur before the onset of impaired glucose tolerance indicates the significance of mitochondrial dysfunction in diabetes progression (Petersen *et al*., 2004).

In long term hyperglycemic state, the generation of super oxide ion $O_2^{\bullet -}$ increases in the liver mitochondria. An increase in free radical generation is the most important mechanism leading to mitochondrial membrane damage (Song, 1993). Chronic exposure to free radicals can result in oxidative damage to mitochondrial and cellular protein, lipids and nucleic acid and also inactivates the iron-sulfur (Fe-S) centers of ETC complexes I, II and III and TCA cycle aconitase resulting in the shutdown of mitochondrial energy production (Wallace, 1999).

The biological activity of various plant extracts have been screened for medicinal use (Srinivasan, 2005). Furthermore, plant sources have been evaluated for developing natural anti-oxidants that may be involved in anti-ageing and anti-wrinkling care (Ravi *et al*., 2004). Many indigenous natural phenolic compounds have been reported to retard the oxidation process in their natural environment and in products to which they have been mixed (Surveswaran *et al*., 2007). Natural anti-oxidants occur in all higher plants and in all parts of the plant (Chanwitheesuk *et al*., 2005). Typical compounds that possess anti-oxidative activities include tocopherols, flavonoids, cinnamic acid derivatives, coumarins, phenylpropanoids, tannians and triterpenes. Recent studies indicate that the compounds with anti-oxidative and free radical scavenging activities can inhibit mutagenesis and carcinogenesis in addition to retarding ageing (Larkins and Wynn, 2004).

Terminalia chebula (Combretaceae), commonly known as chebulic myrobalan, is found in abundance in North India and southwards up to Decca table at 1000-3000 feet. Formation of fruits occurs during November to January. The fruits are, known for their medicinal value in Ayurveda. *T. chebula* is reported to promote digestive power, heal wounds, ulcers, cure local swelling, anemia, diabetes, chronic and recurrent fever. The fruits are astringent, purgative, laxative, gastroprotective and used in asthma, piles and cough (Chatterjee and Pakrasi, 2000). In view of its reputation, *T. chebula* has also found to use in several polyherbal preparations, most notably in 'Triphala'- which is an important formulation in the Ayurvedic pharmacopea prescribed to cure liver and kidney dysfunctions (Chatterjee and Pakrasi, 2000). It is also a constituent of HP-1, a polyherbal hepatoprotective drug (Tasaduq *et al*., 2003). *T. chebula* has been reported to exhibit a variety of biological activities, such as free radical-scavenging ability (Cheng *et al*., 2003), anti-diabetic (Sabu and Kuttan, 2002), anti-cancer (Saleem *et al*., 2002), anti-mutagenic (Kaur *et al*., 2002) and anti-viral (Ahn *et al*., 2002) activity. Recently, we have reported the anti-diabetic activity of *T. chebula* fruits on STZ-induced experimental diabetes (Senthilkumar *et al*., 2006). In this present study, we have evaluated the protective effect of *T. chebula* on mitochondrial damage in STZ- induced diabetic rats.

MATERIALS AND METHODS

Plant Material

Fresh mature *T. chebula* fruits were collected from a tree in Kolli Hills, during the month of November to January 2006, Namakkal District, Tamil Nadu, India. The plant was identified and authenticated by Dr. V. Kaviyarasan, CAS in Botany, University of Madras and a voucher specimen was deposited at the herbarium of Botany, University of Madras.

Preparation of *T. chebula* Fruit Extract

Dried fruits were powdered in a pulverizer and stored at 5°C until further use. One hundred gram of the powder was extracted with petroleum ether (60-80°C) to remove lipids. It was then filtered and the filtrate was discarded. The residue was extracted with 95% ethanol by Soxhlet extraction.

The ethanol was evaporated in a rotary evaporator at 40-50°C under reduced pressure. The yield of the extract was 8.5 g/100 g.

Animals

Adult male albino rats of Wistar strain weighing approximately 150 to 180 g were procured from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. They were acclimatized to animal house conditions, fed with standard rat feed supplied by Hindustan Lever Ltd., Bangalore, India. All the animal experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee guidelines (Approval No. 01/030/04).

Induction of Experimental Diabetes

The animals were fasted overnight and diabetes was induced by a single intraperitoneal (i.p) injection of a freshly prepared solution of Streptozotocin (55 mg kg^{-1} body weight) in 0.1 M cold citrate buffer pH 4.5 (Sekar *et al*., 1990). The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. Control rats were injected with citrate buffer alone. After a week's time for the development of diabetes, the rats with moderate diabetes having glycosuria and hyperglycemia (blood glucose range above 250 mg dL^{-1}) were considered as diabetic and used for the drug treatment. The fruit extract in aqueous solution was administered orally through a gavage at a concentration of 200 mg kg^{-1} body weight rat^{-1} day^{-1} for 30 days.

Experimental Design

The animals were divided into four groups comprising of six animals in each group as follows:

Group I : Normal control rats.
Group II : Diabetic control rats.
Group III : Diabetic rats given *T. chebula* fruit extract (200 mg kg^{-1} body weight day^{-1} rat^{-1}) in aqueous solution orally for 30 days.
Group IV : Diabetic rats administered with glibenclamide (600 μg kg^{-1} body weight day^{-1} rat^{-1}) in aqueous solution orally for 30 days (Vijayakumar *et al*., 2006).

Biochemical Assays

After 30 days of treatment, the rats were fasted overnight and sacrificed by cervical dislocation and the blood was collected for the estimation of blood glucose (Sasaki *et al*., 1972). The activity of aspartate aminotransferase (AST) alanine transaminase (ALT) and alkaline phosphatase (ALP) was assayed by the method of King and Armstrong (1988).

Portions (100 mg) of liver tissue were homogenized in 5 mL ice-cold 0.25 M sucrose solution to isolate the mitochondria according to the method of Johnson and Lardy (1967). A 10% (w/v) homogenate was prepared in 0.25 M sucrose solution and centrifuged at 600 x g for 10 min. The supernatant fraction was decanted and centrifuged at 15,000 x g for 5 min. The resultant mitochondrial pellet was then washed and resuspended again in 0.25 M sucrose. Mitochondrial protein was estimated by the method of Lowry *et al*. (1951). The activity of isocitrate dehydrogenase was assayed by the method of King (1965). The activity of α-ketoglutarate dehydrogenase was assayed by the method of Reed and Mukkerjee (1969), the activity of succinate dehydrogenase was assayed according to the method of Slater and Bonner (1952). The enzyme activity of malate dehydrogenase was assayed by the method of Mehler *et al*. (1948). The activity of NADH dehydrogenase was determined by the method of Minakami *et al*. (1962). The activity of cytochrome-C-oxidase was assayed by the method of Wharton and Tzagoloff (1967).

Electron Microscopy Studies

For electron microscopic examination of liver, primer fixation was made in 3% glutaraldehyde in sodium phosphate buffer (200 mM, pH 7.4) for 3 h at 4°C. Materials were washed with same buffer and post fixed in 1% osmium tetroxide and in sodium phosphate buffer (pH 7.4) for 1 h at 4°C. Tissue samples were washed with same buffer for 3 h at 4°C and were dehydrated in graded ethanol series and were embedded in Araldite. Sixty to ninety nanometer sections (60-90 nm) were cut on an LKBUM4 ultra microtome using a diamond knife and sections were mounted on a copper grid and stained with uranyl acetate and Reynolds lead citrate. The grids were examined under a Phillips EM201C transmission electron microscope.

Statistical Analysis

All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) test; p-values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as the mean±Standard Deviation (SD) for six animals in each group.

RESULTS

The Level of Blood Glucose Control and Experimental Groups of Rats

The diabetic rats exhibited a significant increase in blood glucose level when compared with control rats (Table 1). Upon oral administration of *T. chebula* extract and glibenclamide a reference anti-diabetic drug, the levels were found to be similar to those of normal rats with the effect being more pronounced in the group of rats treated with *T. chebula* extract alone.

The Level of Plasma Insulin Control and Experimental Groups of Rats

A marked decrease in the level of plasma insulin was observed in diabetic rats when compared with control rats (Table 2). Treatment with *T. chebula* fruit extract and glibenclamide reversed these levels to near normal levels while, the effect was more pronounced in the group of rats treated with *T. chebula*.

Table 1: Levels of blood glucose in control and experimental groups of rats

Groups	Blood glucose (mg dL^{-1}) 15 days	Blood glucose (mg dL^{-1}) 30 days
Control	87.53±4.53	88.18±5.12
Diabetic control	278.12±19.18*[a]	283.27±21.53*[a]
Diabetic + *T. chebula*	189.53±6.15*[b]	94.49±9.43*[b]
Diabetic + Glibenclamide	194.67±7.16*[c]	106.31±8.21*[c]

Values are given as mean±SD for groups of six animals each. *Values are statistically significant at $p<0.05$. Statistical significance was compared with in the groups as follows: [a]Diabetic rats were compared with control rats; [b]*T. chebula* treated diabetic rats were compared diabetic rats; [c]Glibenclamide treated diabetic rats were compared with diabetic rats

Table 2: Levels of plasma insulin in control and experimental groups of rats

Groups	Plasma insulin (μU mL^{-1}) 15 days	Plasma insulin (μU mL^{-1}) 30 days
Control	17.12±1.13	17.58±1.42
Diabetic control	6.11±0.98*[a]	5.83±0.88*[a]
Diabetic + *T. chebula*	11.85±1.23*[b]	16.94±1.65*[b]
Diabetic + Glibenclamide	10.21±1.12*[c]	16.13±1.68*[c]

Values are given as mean±SD for groups of six animals each. *Values are statistically significant at $p<0.05$. Statistical significance was compared with in the groups as follows: [a]Diabetic rats were compared with control rats; [b]*T. chebula* treated diabetic rats were compared diabetic rats; [c]Glibenclamide treated diabetic rats were compared with diabetic rats

The Activities of Aspartate Transaminase, Alanine Transaminase and Alkaline Phosphatase Control and Experimental Groups of Rats

Table 3 depicts a significant increase the activities of serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) in STZ-induced diabetic rats when compared with control rats. Treatment with *T. chebula* extracts (group III) and glibenclamide restored the activities of liver marker enzymes to near normalcy (group IV).

The Activities of Liver Mitochondrial Enzymes, Isocitrate Dehydrogenase, α-ketoglutarate Dehydrogenase, Succinate Dehydrogenase, Malate Dehydrogenase, NADH Dehydrogenase and Cytochrome-C-Oxidase in Control and Experimental Groups of Rats

Table 4 depicts the activities of liver mitochondrial enzymes like isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, NADH dehydrogenase and cytochrome-C-oxidase. The activities of liver mitochondrial enzymes were markedly lowered in diabetic rats (Group II) when compared with control rats (Group I). *T. chebula* therapy significantly enhanced the activities of these enzymes in Group III animals when compared to Group II diabetic rats.

Electron Microscopic Sections of Liver Mitochondria in the Control and Experimental Group of Rats

Electron microscopic sections of liver in the control and experimental group of rats are presented in Fig. 1-4. Figure 1 shows the normal architecture of liver mitochondria in the control rats. STZ-induced rats Fig. 2 presented abnormal architecture of mitochondria, increased areas of vacuolation an indicator of odema, which may be due to the presence of increased number of electron dense particles (calcium particles). Diabetic rats treated with *T. chebula* extract showed near normal architecture (Fig. 3). Similar observations were also observed in diabetic rats treated with glibenclamide (Fig. 4).

Table 3: Levels of serum aspartate transaminases, alanine transaminases and alkaline phosphatase in control and experimental groups of rats

Groups	Aspartate transaminases ($IU^X L^{-1}$)	Alanine transaminases ($IU^X L^{-1}$)	Alkaline phosphatase ($IU^y L^{-1}$)
Control	72.28±7.51	22.12±2.83	72.15±5.95
Diabetic control	108.31±9.63*[a]	48.51±5.96*[a]	115.12±9.51*[a]
Diabetic + *T. chebula*	77.45±8.13*[b]	25.34±3.10*[b]	76.31±6.13*[b]
Diabetic + Glibenclamide	79.81±8.75*[c]	27.51±3.57*[c]	79.21±7.47*[c]

Values are given as mean±SD for groups of six animals each. *Values are statistically significant at $p<0.05$. Statistical significance was compared with in the groups as follows: [a]Diabetic rats were compared with control rats; [b]*T. chebula* treated diabetic rats were compared diabetic rats; [c]Glibenclamide treated diabetic rats were compared with diabetic rats. U^x-μmol of pyruvate liberated h^{-1}; U^y-μmol of phenol liberated min^{-1}

Table 4: Activities of liver mitochondrial enzymes in control and experimental groups of rats

Groups	Control	Diabetic control	Diabetic + *T. chebula*	Diabetic + Glibenclamide
Iso-citrate dehydrogenase	648.17±34.15	488.31±28.22*[a]	627.15±43.19*[b]	615.41±45.62*[c]
α-ketoglutarate dehydrogenase	175.11±12.34	113.57±9.67*[a]	170.33±13.75*[b]	168.22±14.20*[c]
Succinate dehydrogenase	38.01±2.16	22.13±1.43*[a]	35.17±2.52*[b]	33.11±2.98*[c]
Malate dehydrogenase	349.22±22.15	251.12±15.24*[a]	330.47±25.16*[b]	325.31±21.70*[c]
NADH-dehydrogenase	23.57±1.37	15.8±1.22*[a]	22.36±1.68*[b]	21.11±1.18*[c]
Cytochrome-C-oxidase	6.97±0.81	4.41±0.67*[a]	6.83±0.95*[b]	6.45±0.73*[c]

Values are given as mean±SD for groups of six animals each. *Values are statistically significant at $p<0.05$. Statistical significance was compared with in the groups as follows; [a]Diabetic rats were compared with control rats; [b]*T. chebula* treated diabetic rats were compared diabetic rats;[c] Glibenclamide treated diabetic rats were compared with diabetic rats. The enzyme activities are expressed as Iso-citrate dehydrogenase-nmoles of α-ketoglutarate h^{-1} mg^{-1} protein; α-ketoglutarate dehydrogenase-μmoles of ferrocyanide h^{-1} mg^{-1} protein; Succinate dehydrogenase-μmoles of succinate oxidised min^{-1} mg^{-1} protein; Malate dehydrogenase, NADH-dehydrogenase-μmoles of NADH oxidized min^{-1} mg^{-1} protein; Cytochrome-C-oxidase-O.D.$\times 10^{-2}$ min^{-1} mg^{-1} protein

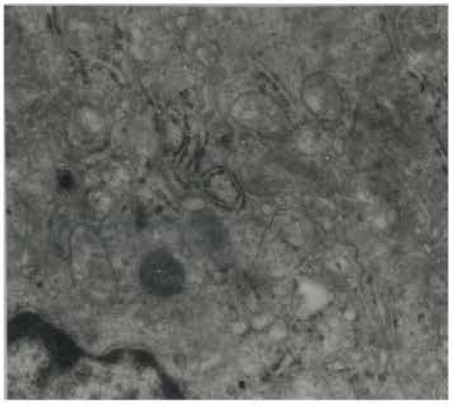

Fig. 1: Control group shows normal architecture

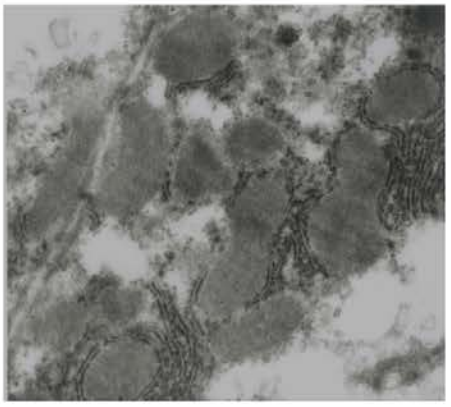

Fig. 2: STZ-induced diabetic group of rats

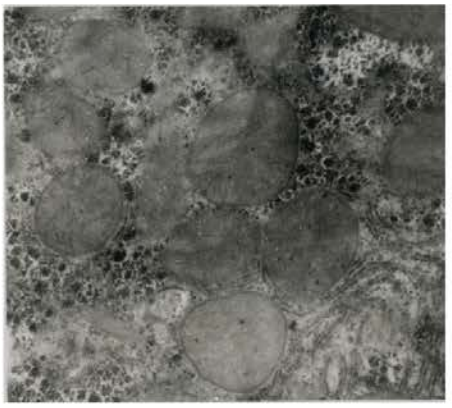

Fig. 3: Electron micrograph of mitochondria in the diabetic rats given in *T. chebula*

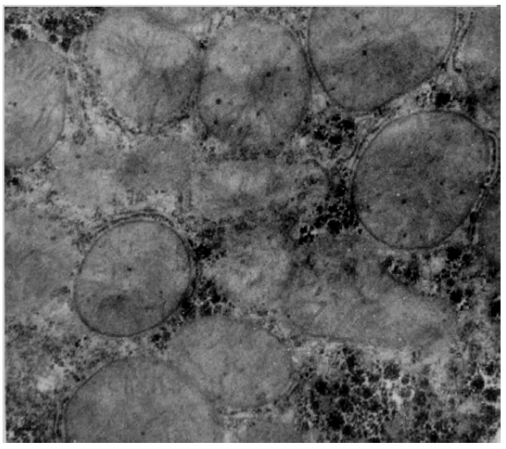

Fig. 4: Electron micrograph of mitochondria rats given glibenclamide

DISCUSSION

The present study showed that the oral administration of *T. chebula* extract decreased the blood glucose levels in STZ induced diabetic rats. We have reported that, the administration of *T. chebula* fruit extract to STZ-induced diabetic rats optimized the activities of carbohydrate metabolizing enzymes and thus maintained blood glucose levels (Senthilkumar *et al*., 2006). Treatment of *Momordica charantia* seed extract to mildly STZ-diabetic rats resulted in the activation of β-cells and granulation returns to normal, giving insulinogenic effect (Sekar *et al*., 2005). *T. chebula* may bring about its hypoglycemic action through stimulation of surviving β-cells of islets of langerhans to release more insulin. This was clearly evidenced by the increased level of plasma insulin in diabetic rats treated with *T. chebula*. Since the percentage fall in plasma glucose levels was different in models with varying intensity of hyperglycemia it implies that the anti-hyperglycemic effect of a plant is dependent upon the dose of diabetogenic agent and therefore on the degree of β-cell destruction (Grover *et al*., 2000). The presence of biologically active ingredients such as ellagic acid, 2, 4-chebulyl-β-D-glucopyranose, chebulinic acid, casuarinin, chelanin and 1, 6-di-*O*-galloyal-β-D-glucose in the *Terminalia chebula* might have been responsible for its medicinal properties (Lee *et al*., 2005). In this context, a number of other plants have also been reported to elicit an antihyperglycaemic effect and a stimulatory effect on insulin release (Ravi *et al*., 2004).

Liver is the most vital organ in the metabolism of drugs and other toxic substances. Liver cell destruction shows its effects mostly as impairment in the liver cell membrane permeability, which results in the leaking out of tissue contents into the blood stream. In STZ-diabetic rats, the activity of pathophysiological enzymes such as AST, ALT and ALP was significantly increased in serum when compared to control rats. Supporting our findings, it has been found that the liver was necrotized in STZ-diabetic rats. Therefore, the increase of the activity of AST, ALT and ALP in serum is mainly due to the leakage of these enzymes from the liver cytosol into the blood stream (Tawata *et al*., 2000; Mansour *et al*., 2002) which gives an indication on the hepatotoxic effect of streptozotocin. On the other hand, administration of *T. chebula* extract and glibenclamide to STZ-diabetic rats reduced AST, ALT and ALP activity towards its normal values. However, *T. chebula* extract was more effective than glibenclamide.

Mitochondria are of importance for cell viability due to the nature of its function. It plays a central role in the regulation of intracellular Ca^{2+} especially during toxic insult or stress (Carafoli, 1987) and mitochondrial phosphorylation provides approximately 95% of cellular energy needs

(Erecinska and Wilson, 1982). Numerous toxic compounds that target mitochondrial injury have been investigated as a potential initiating factor in various organ toxicities caused by chemicals (Wong *et al*., 2000). Since mitochondrial oxidative phosphorylation plays a central role in the maintenance of cellular energy supply (Burcham and Harman, 1991), the deleterious effect of xenobiotics on mitochondrial respiration may have serious consequences for the viability of the cell.

In the present study, the observed decreases in the activities of mitochondrial enzymes in liver of the diabetic rats. In Insulin Dependent Diabetes Mellitus (IDDM) various agents like Interleukin-1 beta (IL-β), interferon gamma, tumor necrosis factor alpha (TNF-α), alloxan and streptozotocin-could operate by forming free radicals that could attack the mitochondrial damage (Gerbitz, 1992). The increased production of free radicals in mitochondria may damage β-cells, which are known to be very sensitive to free radicals (Oexle *et al*., 1994). Also, a decrease in oxygen consumption and respiratory ratio were observed in the mitochondria of diabetic rats (Puckett and Reddy, 1979). A similar decrease in the activities of citric acid cycle enzymes were also observed by Sener *et al*. (1990). Furthermore, decrease in the activities of pyruvate dehydrogenase and malate dehydrogenase and increase in NAD^+/NADH ratio were reported in alloxan-induced diabetic rats (Obrosova and Stevens, 1999). Administration of *T. chebula* extracts and glibenclamide increased the activities of isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and succinate dehydrogenase in the diabetic rats. Our results are also inline with the previous report (Panneerselvam and Govindaswamy, 2002).

Mitochondrial respiratory chain is the main site of Reactive Oxygen Species (ROS) production, especially at the level of NADH dehydrogenase and cytochrome-C-oxidase complexes (Ide *et al*., 1999). In the present study, we have found a moderate decrease in the activities of respiratory chain enzymes in the liver tissue of STZ-induced diabetic rats. A decrease in the activities of respiratory chain enzymes may promote leakage of electrons from the mitochondrial inner-membrane-associated with electron transport complexes contributing to the increased mitochondrial ROS production in the tissues of STZ-induced diabetic rats. The increased mitochondrial NAD^+/NADH ratio may be related to inhibited activity of the mitochondrial NADH-dehydrogenase (Ide *et al*., 1999). Treatment of *T. chebula* fruit extract increased the activities of NADH dehydrogenase and cytochrome-C-oxidase in the diabetic group of rats. The pathological change in the liver mitochondrial enzymes was evident by the transmission electron microscopic studies.

In conclusion, impaired activities of the respiratory chain enzymes can therefore be attributed to the increased intra-mitochondrial oxidative stress during diabetes. *T. chebula* treatment to diabetic rats significantly improved the activity of mitochondrial enzymes in liver probably by controlling the diabetes induced oxidative stress.Further comprehensive chemical and pharmacological investigations are in progress to elucidate the exact mechanism of protection of mitochondrial enzymes by *T. chebula* during STZ-induced diabetes.

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