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Research Article

HEPATO-PROTECTIVE EFFECTS OF *PIMPINELLA TIRUPATIENSIS* EXTRACT ON CYTOSOLIC AND MITOCHONDRIAL ENZYMES AGAINST STREPTOZOTOCIN (STZ) -INJECTED PATHOGENIC DIABETIC RATS

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ABSTRACT

Objective: The study was conducted to evaluate the anti hyperglycemic effect of *P. tirupatiensis* on altered blood glucose levels, water, food intake, cytosolic and mitochondrial enzymes in control and STZ- treated diabetic rats.

Methods: The anti hyperglycemic effect of *P. tirupatiensis*was determined by various methods like blood glucose levels, water, food intake, cytosolic and mitochondrial enzymes.

Results: On oral administration of *P. tirupatiensis* at a dose of 0.750 g/kg body weight per day to diabetic rats for 30 days; resulted in a significant ($p<0.01$) reduction in the levels of blood glucose, water and food intake whereas a significantly ($p<0.01$) increase in the activities of hepatic cytosolic and mitochondrial marker enzymes such as glucose- 6-phosphate dehydrogenase (G6PD), succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and glutamate dehydrogenase (GDH) along with significant ($p<0.01$) reduction in the activity of hepatic cytosolic enzyme lactate dehydrogenase (LDH) in diabetic treated rats. Furthermore, protection against body weight loss of diabetic animals also observed.

Conclusion: In this study, we concluded that *P. tirupatiensis*extract could lower the blood glucose levels as well as improve activities of cytosolic and mitochondrial enzymes in streptozotocin (STZ) - injected pathogenic diabetic rats.

Keywords: Diabetes, Mitochondrial function markers, *P. tirupatiensis*, Rat.

INTRODUCTION

Diabetes mellitus is one of the five leading causes of death worldwide, and the prevalence of this disease is rapidly increasing worldwide. According to the World Health Organisation (WHO), nearly 347 million people worldwide suffer from diabetes and more than 80% diabetic people live in low and middle-income countries. WHO projected those deaths due to diabetes will be double between 2005 and 2030 [1-2].

Chronic hyperglycemia among diabetes patients is associated with long term damage and dysfunction of vital organs, which leads to the failure of organs like eyes (retinopathy), nerves (neuropathy), kidneys (nephropathy), heart and blood vessels [3].

The major organ like liver is severely damaged during diabetes. Which plays a pivotal role in glucose and lipid homeostasis [4]. Widely varieties of synthetic antidiabetic drugs are available in the market, however they were reported to be associated with various side effects. The focus has been shifted towards phytomedicine in diabetic management owing to their safety, efficacy and low side effects [5].

P. tirupatiensis Bal. &Subr. (Family Apiaceae; local name, kondakothimera) is a rare and endemic medicinal plant and restricted to the Seshachalam hills of the Eastern Ghats, India [6]. Various pharmacological activities like antimicrobial antidiabetic, cardioprotection, hepatoprotection, nephroprotection, and antioxidant of *P. tirupatiensis* were reported in animal models [7-10].

Therefore, this study was aimed to evaluate the extract of *P. tirupatiensis* on cytosolic and mitochondrial enzymes and also whether extract of *P. tirupatiensis* can control the physiological parameters and revert the altered Cori and Krebs cycles in streptozotocin (STZ) - induced pathogenic rats.

MATERIALS AND METHODS

Collected the plant materials

P. tirupatiensis was collected from Tirumala Hills of Chittoor district, Andhra Pradesh, India during on October and the plant material was taxonomically identified and authenticated by the concerned herbarium officer, Voucher specimen (1533) was deposited in the Department of Botany, Sri Venakateswara University (SVU), Tirupati, Andhra Pradesh, India.

Preparation of extract

P. tirupatiensis tuberous root was dried in the shade and made into fine powder that powder was used for the extraction using ethyl acetate. The powder (500g) was soaked in ethyl acetate in different glass jars for 2 days at room temperature and the solvent was filtered.

This was repeated three to four times until the extract gives no coloration. The extract was distilled and concentrated under reduced pressure in the Rotary Evaporator (Model no-HS-2005V) and finally freeze dried by lyophilizer (Lyodel).

Selection of animals

Wistar strain male albino rats (n = 30), weighing 130± 10 g and six months age were used in this study. All the rats were maintained in the polypropylene cages (six rats per cage), at an ambient temperature of 25 ± 2 0C with 12-h-light/12-h-dark cycle. Rats were allowed for the free access to standard chow (Hindustan Lever Ltd., Bangalore, India) and water ad libitum during the study.

The study was approved by the University Animal Ethical Committee and experiments were performed according to the regulations for the care and use of laboratory animals and its resolution number; 09 (ii)/a/CPCSCA/IAEC/07-08/SVU/Zool/ dated 26/6/08.

Chemicals and reagents

All the chemicals used in the current study were Analar Grade (AR) and purchased from the following scientific companies: Sigma (St. Louis, MO, USA), Fisher (Pittsburgh, PA, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), and Qualigens (Mumbai, India).

Induction of diabetes

Streptozotocin (STZ) solution (40mg/ml) was freshly prepared in 0.1M citrate buffer (pH 4.5) and 1ml/kg b.w of STZ solution was injected by intraperitoneally [11]. STZ is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, so rats were treated with 20% glucose (5-10 ml) orally after 6 h of injection for the next 48 hours to prevent hypoglycemia. Neither death nor any other adverse effect was observed at the tested concentration throughout the study. After one week, rats with diabetes (i.e., high blood glucose levels, 200-300 mg/dL) that exhibited glycosuria and hyperglycemia were selected for the experiment.

Experimental design and treatment

Thirty rats were randomly divided into five equal groups and treated as follows;

i. Group I: Normal control rats

ii. Group II: Diabetic control rats, treated with vehicle alone

iii. Group III: Diabetic rats treated with 0.750 g/kg of *P. tirupatiensis* extract

iv. Group IV: Normal rats treated with 0.750 g/kg of *P. tirupatiensis* extract

v. Group V: Diabetic rats were given glibenclamide 20 mg/kg body weight

P. tirupatiensis extract was dissolved in a vehicle containing 0.9% NaCl, 0.2% Tween-20, in 97.2% distilled water. Animals were received by oral gavage twice a day for a period of one month continuously. After treatment, animals were fasted for 12 h, anaesthetized between 8:30 a.m. and 9:30 a.m. using ketamine (24 mg/kg body weight, intramuscular injection). Hepatic tissues was immediately dissected out, washed in ice-cold saline solution to remove the blood, The sample was sliced into pieces and homogenized in cold phosphate buffer solution (pH 7.0) to given a 10% homogenate (w/v). The homogenates were centrifuged at 5000 g for 10 min at 0 ^{o}C and the supernatants were taken for further biochemical evaluation.

Biochemical estimation

Body weight, food and water intake of all groups of animals were monitored on a daily basis for one month at a fixed time. Fixed amount of rat chow and fluid was given to each rat and refilled the next day. The blood glucose levels were measured with an Accu Check Glucometer (Roche, Switzerland) with a small drop of blood from tail of each rat on the glucometer strip [12-13]. Activities of selected cytosolic enzymes were assayed: glucose-6-phosphate dehydrogenase (G6PD) activity was assayed by the method of Lohr and Waller, [14], and lactate dehydrogenase (LDH) activity was monitored by the method of Nachlas et al., [15] as modified by Prameelamma and Swami, [16] with slight modifications. Mitochondrial enzymes including succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) were also assayed by the modified version of Nachlas et al., [15]. The activity of another important enzyme, glutamate dehydrogenase (GDH), was determined by the methodof Lee and Lardy, [17]. All enzymatic assays in this study were performed using the crude homogenate of liver.

Histopathological studies

Liver slice fixed in Bouins solution, were dehydrated by upgrading from 30% to 100% in alcohol and then xylene each for 1 h, followed by embedding in wax at 60 ^{0}C Paraffin blocks of the tissue slice were sectioned to 5 im thickness. The sections were then stained in

hematoxylin and eosin (H&E) following the earlier described methods of Michaele et al., [18].

Statistical analysis

The results were expressed as mean ± SEM of six rats per group and the statistical significant was evaluated by one-way analysis of variance (ANOVA) using the SPSS (version 15.0) program followed by LSD. Values were considered statistically significant when ($p < 0.01$).

RESULTS

A significant reduction in body weight was observed in diabetic rats when compared to control rats ($P < 0.01$). Rise in body weight was observed in *P. tirupatiensis* extract treated diabetic rats when compared to control diabetic rats. Food intake was significantly ($P < 0.01$) augmented in diabetic rats as compared with normal rats. On the other hand, water intake was significantly increased in diabetic rats as compared to normal rats. Treatment with extract of *P. tirupatiensis* brought down food and water intake in diabetic rats as compared with diabetic control rats (**Table.1**).

The blood glucose levels in STZ-injected diabetic rats were drastically increased from the baseline. This increase of blood glucose was almost three-fold higher even after 30 days compared to the control rats. However, it was found that the elevated blood glucose levels in diabetic rats reduced significantly ($P < 0.01$) after 30 day *P. tirupatiensis* administration. Glibenclamide, which has been used as a standard diabetic drug to compare the beneficial effects of *P. tirupatiensis* extract, showed significantly decline in blood glucose levels and this was almost equal to the normal control rats (**Table 2**).

The activities of hepatic G6PD were found to be significantly ($P < 0.01$) decreased while, hepatic LDH was significantly ($P <0.01$) increased in STZ-injected diabetic rats when compared to normal control rats. However, diabetic rats treated with *P. tirupatiensis* for 30 days resulted in significant marked recovery of the above parameters, which was almost similar with glibenclamide treatment. No change was observed in rats treated with extract and control rats (**Fig. 1 and 2**).

The activities of the mitochondrial marker enzymes such as SDH, GDH and MDH were significantly ($P < 0.01$) dropped in the STZ - injected diabetic rats. This study demonstrated that the reduced mitochondrial marker enzymes activities in diabetic rats were improved by *P. tirupatiensis* extract treatment.

This augmented mitochondrial marker enzymes in *P. tirupatiensis* treated diabetic rats were similar with that of glibenclamide-induced augmentation. We also found increased mitochondrial marker enzymes with *P. tirupatiensis* alone treatment compared to the normal control group (**Figure 3, 4 and 5**).

Table 1:Effect of *P. tirupatiensis* extract on changes in food, water intake and body weight

Groups	Water intake (ml/per day)		Food intake (ml/per day)		Body weight (g)	
	Before	After	Before	After	Initial	Final
Normal control	90 ± 4.7	89 ± 4.5	17.2 ± 2.1	15.99 ± 1.7	185 ± 2.1	200 ± 3.1
Diabetic control	180 ± 8.1	195 ± 2.1 Ψ	49.1 ± 6.9	65.1 ± 7.2 **ψ**	170 ± 0.14	106 ± 2.37 **ψ**
Normal +*P. tirupatiensis*	91 ± 6.2	90 ± 6.1	18.2 ± 4.2	16.1 ± 2.1	183± 1.19	229 ± 1.2
Diabetic +*P. tirupatiensis*	170 ± 7.0	154 ± 8.2 **ψ #**	33.11 ± 1.3	29.19 ± 6.4 **ψ #**	173 ± 2.89	187 ± 1.9 **ψ #**
Diabetic + glibenclamide	169 ± 4.3	145 ± 2.1 **ψ #**	31.99 ± 3.4	26.21 ± 2.9 **ψ #**	180 ± 1.3	194 ± 9.1 **ψ #**

All the values are Mean ± SEM of six individual observations; Values are significant compared to normal control (ψP<0.01) and diabetic control (ψ #P<0.01).

Fig. 6. (1) Shows control rat liver normal hepatocytes (H) with prominent nucleus (N) and sinusoids (S). The liver of the STZ -induced diabetic rats as observed under the light microscope is demonstrated in **Fig. 6(2**) and indicates distortion in the arrangement of cells with congestion (C) and cellular swelling (SC) and perivascular infiltration (PVI). **Fig. 6(3)** Shows cytoarchitecture of liver of *P. tirupatiensis* aqueous extract treated diabetic rats, which include slight congestion (SC), and hepatocytes (H) restored towards normalcy.

Whereas **Fig. 6 (4)** Shows *P. tirupatiensis* aqueous extract treated control rats with no changes. **Fig. 6 (5)** Shows cytoarchitectural changes in glibenclamide treated diabetic rats. Which include normal hepatocytes, (H) nucleus (N) and sinusoids (S). *P.tirupatiensis*aqueous extract administered to the diabetic rats effectively prevented the cellular damages such as disturbed arrangement of the hepatocytes in the liver tissue of the diabetic rats.

Table 2: Effect of *P. tirupatiensis*extractson blood glucose levels in normal control and diabetic rats

Groups	Blood glucose mg/ dl	
	Initial	**Final**
Normal control	84±0.9	85±2.43
Diabetic control	353±1.07	389±3.97 ψ
Normal +*P. tirupatiensis*	87±0.12	83±1.39
Diabetic + *P. tirupatiensis*	365±1.8	104±3.58 ψ #
Diabetic + *glibenclamide*	337±1.75	100±1.15 ψ #

All the values are Mean ± SEM of six individual observations; Values are significant compared to normal control (ψP<0.01) and diabetic control (ψ #P<0.01).

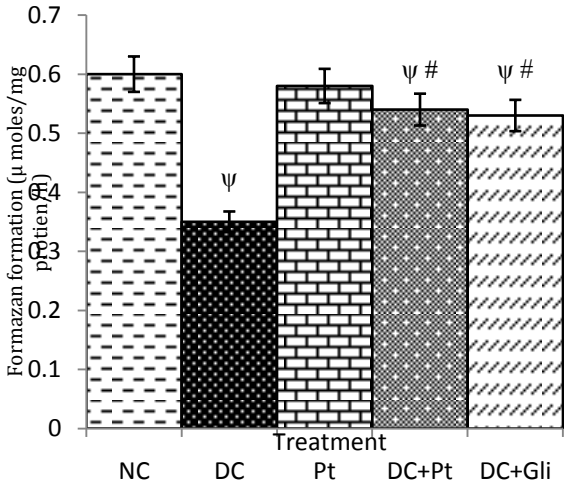

Fig. 1: Effect of oral administration of *P. tirupatiensis* on G6PD activity in normal and diabetic rats. All values are significant compared to normal control (NC, ψP< 0.01) and diabetic control (DC, #P < 0.01).

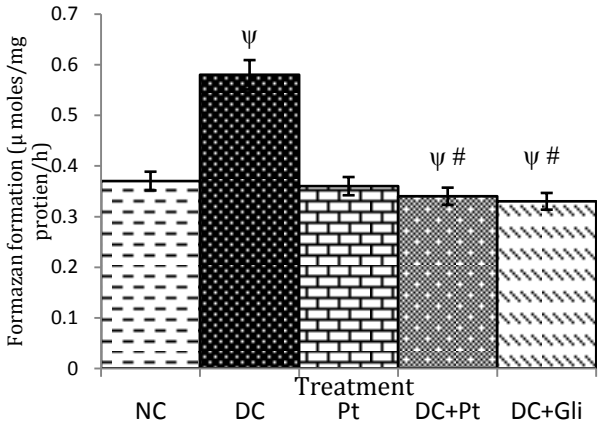

Fig. 2: Effect of oral administration of *P. tirupatiensis* on LDH activity in normal and diabetic rats.All values are significant compared to normal control (NC, ψP< 0.01) and diabetic control (DC, #P < 0.01).

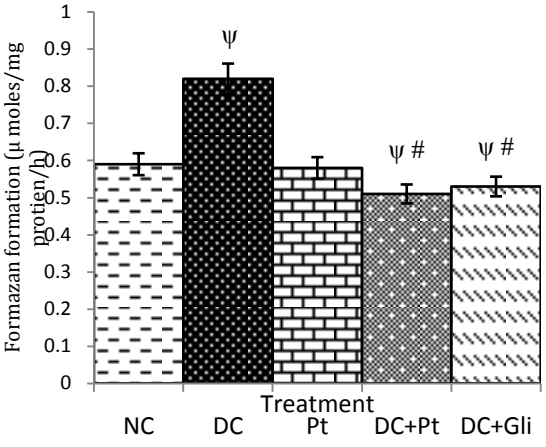

Fig. 3: Effect of oral administration of *P. tirupatiensis* on SDH activity in normal and diabetic rats. All values are significant compared to normal control (NC, ψP< 0.01) and diabetic control (DC, #P < 0.01).

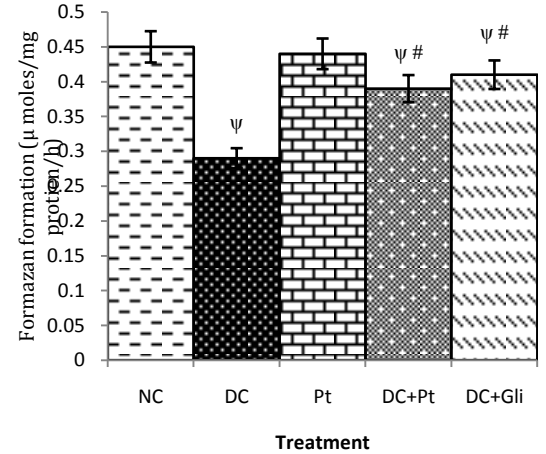

Fig. 4: Effect of oral administration of *P. tirupatiensis* on GDH activity in normal and diabetic rats. All values are significant compared to normal control (NC, ψP< 0.01) and diabetic control (DC, #P < 0.01).

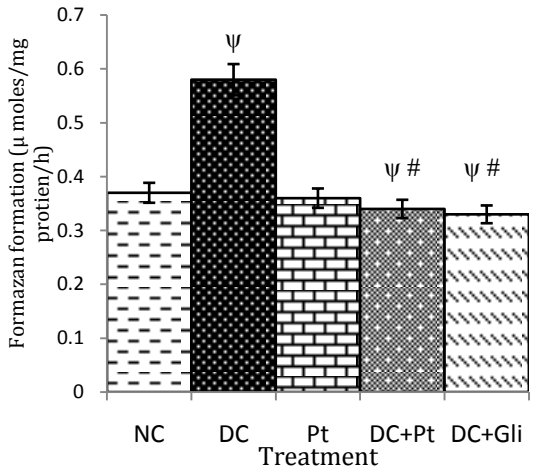

Fig. 5: Effect of oral administration of *P. tirupatiensis* on MDH activity in normal and diabetic rats. All values are significant compared to normal control (NC, ψP< 0.01) and diabetic control (DC, #P < 0.01).

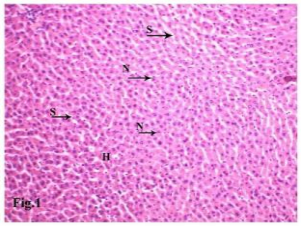

Fig. 6(1): Control rat liver showing normal hepatocytes (H) with prominent nucleus (N) and sinusoids (S).

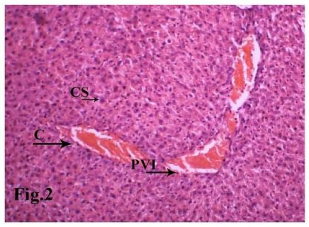

Fig. 6 (2): Distortion in the arrangement of cells with congestion (C) and cellular swelling (SC) and perivascular infiltration (PVI)

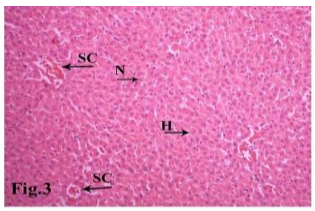

Fig. 6 (3): *P. tirupatiensis* aqueous extract treated diabetic rat liver shows slight congestion (SC) and hepatocytes (H)

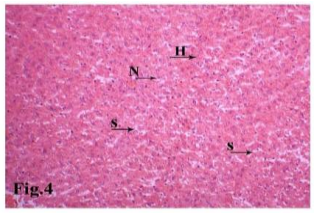

Fig. 6 (4):*P. tirupatiensis* aqueous extract treated control rats with no changes in the liver architecture

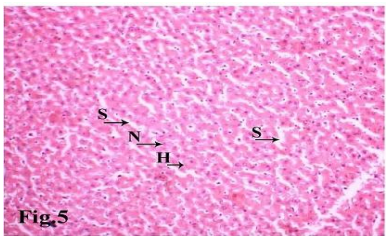

Fig. 6 (5):Cytoarchitectural changes in glibenclamide treated diabetic rats. Which include normal hepatocytes, (H) nucleus (N) and sinusoids (S).

DISCUSSION

Cytosolic and mitochondrial enzymes play a crucial role in maintenance of favorable physiological conditions in the cell. A fluctuation in these enzyme activities in diabetes leads to severe physiological malfunctions in the tissues. In this investigation, we found that the diminished mitochondrial enzymes activities in STZ-injected pathogenic diabetic rats were significantly reversed to normal enzyme activity levels by 30 days of *P. tirupatiensis* oral supplementation.

In the present study, elevated blood glucose levels in STZ-induced diabetic rats confirmed abnormalities of glucose levels, which might be due to destruction of pancreatic β-cells by streptozotocin[19]. Our results clearly showed that extract of *P. tirupatiensis* effectively dropped the blood glucose levels in diabetic rats when compared with normal control rats. Predictable decrease in blood glucose levels by glibenclamide treatment indicates the efficiency of antidiabetic properties of the drug. In previous reports the diabetic animals exhibited polyuria, increased water and food intake, dehydration, weight loss and muscle wasting, excessive hair loss and scaling, diarrhea, and cataracts [20-21]. Reduced in body weight of diabetic rats is due to catabolism of fats and proteins. Due to insulin insufficiency the protein content is reduced in muscular tissue by proteolysis [22-23]. Further, the present study suggests that reduced body weight while augmented food and water intake are of significance in the pathogenesis of diabetic complications and the level of body weight, food and water intake are regulated to near normalcy by the treatment with extract of *P. tirupatiensis*.

Glucose-6-phosphate dehydrogenase (G6PD) catalyses the first reaction of the pentose phosphate pathway and is an important site of metabolic control [24] is has been traditionally thought G6PD dehydrogenase is typical “house keeping” enzymes that is regulated solely by the ratio of NADPH and NADP. The activity of glucose-6-phosphate dehydrogenase is also regulated through alternative splicing[25] in response to hormonal and nutritional cues such as glucose and lipids. In present analysis reduced activity of G6PD was noticed in liver of pathogenic diabetic rats. Similar to our finding results; previous reports also demonstrated lower glucose-6-phosphate dehydrogenase activity in diabetic condition [26]. This suggested reduced conversion of glucose-6-phosphate dehydrogenase to 6-phosphogluconate leading to reduced formation NADPH and HMP shunt. This cycle is an alternate source of energy impairment of glycolytic and Krebs cycle pathways. Inhibition of oxide reductase in the mitochondria results in the decreased energy supply for normal metabolic functions, thus, increases oxidative stress leading to diabetic complications [27]. In this study, augmented G6PD activity with *P. tirupatiensis* receiving for 30 days to the diabetic rats may help to overcome diabetes complications.

Lactate dehydrogenase (LDH) is a key of anaerobic glycolysis and catalyses the reversible oxidation of lactate to pyruvate in the terminal step of glycolysis. The reaction catalyzed by lactate dehydrogenase interlinks anaerobic and aerobic oxidation. Lactate dehydrogenase activity is found to be altered by insulin, glucose, NADH, as well as increases in mitochondrial membrane potential, cytosolic free ATP and cytosolic free Ca2+ [28]. The decreased activity of lactate dehydrogenase in tissues could be important to ensure that a high proportion of both pyruvate and NADH, supplied by glycolysis, is subsequently oxidized by mitochondria [29]. This excessive pyruvate is converted to lactate for which lactate

dehydrogenase is needed and therefore the activity of lactate dehydrogenase may be increased due to less insulin availability in diabetes. Increased lactate dehydrogenase activity in diabetic rats has been reported by [30]. The results of the present study showed that the hepatic lactate dehydrogenase activity in the STZ - injected diabetic rats was significantly augmented when compared to normal control. *P. tirupatiensis* treatment to the diabetic rats for 30 days resulted in reduced lactate dehydrogenase activity. The confined lactate dehydrogenase activity by *P. tirupatiensis* was parallel with the antidiabetic drug glibenclamide treatment.

The decrease in succinate dehydrogenase (SDH) activity due to the STZ - injected oxidative stress condition indicates reduction in the conversion of succinate to fumarate resulting in depressed in oxidative metabolism mitochondria. During the diabetic stress condition diversion of phosphoenolpyruvate leads to increased formation of fumarate resulting in product inhibition of SDH [31], the decrease in the activities of SDH in tissues of diabetic rats can be associated with enzyme dysfunction due to activation of lipid peroxidation. This may be due to excess production of free radicals to counter these toxic effects. In diabetic rats supplementation with *P. tirupatiensis* succinate dehydrogenase activity was increased when compared to untreated pathogenic diabetic rats. There are several reports on the reduction of oxidative stress by herbal medicines [32] and also plants have the capacity in normalizing the levels of lipid peroxidation. Hence by normalizing the levels of lipids the mitochondrial enzymes may become to normal level more or less in diabetic rats treated with *P. tirupatiensis* treatment. Increase in succinate dehydrogenase activity in *P. tirupatiensis* treated rats indicates better utilization of energy yielding intermediates by TCA cycle thus, suggesting increased mitochondrial oxidative potential and energy synthesis, utilization of carbohydrates and fats as substrates.

Malate dehydrogenase (MDH) is plays an important role in the TCA cycle as SDH. It has been demonstrated that several mitochondrial soluble NAD^+dependant dehydrogenases including malate dehydrogenases, are specifically associated with NADH ubiquinone oxidoredutase[33]. Remarkable decrease in hepatic MDH activity in diabetic rats indicates irregularity in the TCA cycle and ultimately affects other mitochondrial enzymes. Decrease in MDH activity in diabetic rats suggests decreased utilization of malate. Reduced levels of TCA cycle intermediates in diabetic stress condition may be responsible for the decrease in MDH activity. STZ - induced diabetic stress condition depressed expression of genes involved in carbohydrate and energy metabolism through effects on known pathways such as glycolysis, TCA cycle and oxidative phosphorylation [34]. However, *P. tirupatiensis* treatment of diabetic rats showed improved MDH activity. This may be due to reduced oxidative stress and increase the activities of mitochondrial enzymes.

Glutamate dehydrogenase GDH is homohexameric mitochondrial matrix enzyme that catalyses the reversible oxidative deamination of glutamate to α-ketoglutarate plus free ammonia using either NAD or NADP as a co-factor. The present study reveals that the activity of hepatic GDH was significantly reduced with STZ - induced pathogenic diabetic rats. Our findings are similar with the previous studies also demonstrated lower GDH activity in diabetic hepatic tissues [30]. The decrease in GDH activity may be due to disturbances in energy metabolism, impairment of glutamate and activation of lipid peroxidation in the renal tissues [35]. However, diabetic rats treated with *P. tirupatiensis* of 30 days exhibited increased hepatic glutamate dehydrogenase. The augmented hepatic glutamate dehydrogenase activity might be to synchronization of energy metabolism and elevation of glutamate in the cells by *P. tirupatiensis* supplementation. The repaired activities of mitochondrial enzymes and glutamate by *P. tirupatiensis* treatment confirmed the protective role of *P. tirupatiensis* against the STZ - induced diabetes complications.

CONCLUSION

In conclusion, our study suggests that extract of *P. tirupatiensis* may have useful effects in diabetes mellitus that holds the hope of new generation of antidiabetogenic drugs. Further research is required to find out the exact mechanism of this extract for its antidiabetogenic effect and to identify the bioactive compounds responsible for this effect.

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