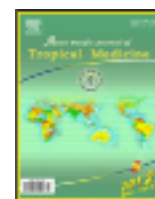




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Protective effect of tannins from *Ficus racemosa* in hypercholesterolemia and diabetes induced vascular tissue damage in rats

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ABSTRACT

Objective: To evaluate the protective effect of tannins from *Ficus racemosa* (*F. racemosa*) on the lipid profile and antioxidant parameters in high fat meal and streptozotocin induced hypercholesterolemia associated diabetes model in rats. **Methods:** The crude tannin fraction was separated from the acetone (70% v/v) bark extract of *F. racemosa*. Oral administration of tannin fraction (TF) (100 & 200 mg/kg body weight) to rats fed with high fat meal for 30 days (4% cholesterol, 1% cholic acid, 0.5% egg albumin) and injected with streptozotocin (35 mg/kg *i.p.* in citrate buffer on 14th day). **Results:** The administration of TF significantly reverse the increased blood glucose, total cholesterol, triglycerides, low density lipoprotein and also significantly restored the insulin and high density lipoprotein in the serum. In addition tannins significantly restored the activity of antioxidant enzymes such as superoxide dismutase, catalase and decreased the, glutathione peroxidase, and glutathione, thereby restoring the antioxidant status of the organs to almost normal levels. **Conclusions:** The results of this study show that two different doses of tannin supplementation had a favorable effect on plasma glucose and lipid profile concentrations. It also had an influence on attenuating oxidative stress in diabetic rats.

1. Introduction

Diabetes is a common metabolic disease characterized by abnormally high plasma glucose levels, leading to major complications, such as diabetic neuropathy, retinopathy and cardiovascular diseases[1,2]. Type 2 diabetes mellitus (T2DM) is a heterogeneous disorder characterized by a progressive decline in insulin action (insulin resistance), followed by the inability of beta cells to compensate for insulin resistance (pancreatic beta cell dysfunction). Insulin resistance is a characteristic metabolic defect that pre-cedes overt beta cell dysfunction and is primarily associated with resistance to insulin-mediated glucose disposal at the periphery and compensatory hyperinsulinemia. The beta cells normally compensate insulin resistance by secreting more amounts of insulin to maintain the glucose homeostasis. In the course of time, however, this beta cell function gets impaired leading to deterioration in glucose homeostasis

and subsequent development of impaired glucose tolerance and frank diabetes[3,4]. Although diet and exercise are the first steps toward achieving treatment goals of diabetics, 90% of patients with T2DM cannot maintain long-term glycemic control with diet and exercise alone. Thus, antihyperglycemic drugs are necessary for the treatment of T2DM. Presently available oral hypoglycemic agents exhibit several side effects. Therefore, there is a need for more effective oral antihyperglycemic agent, particularly those that normalize both insulin and glucose levels. A wide array of plants and its active principles, with minimal side effects, provide an alternate therapy for T2DM. Moreover, the plant kingdom represents a largely unexplored reservoir of biologically active compounds.

Growing epidemiological study suggests that the consumption of fruits, vegetables and few medicinal herbs decreased the incidence of diabetes associated with hyperlipidemia[5]. Recent research and plethora of literature suggest that cardio protective medicinal herbs and its extract namely *Magifera indica*, *Terminalia arjuna*, *Semicarpus anacardium*, *Curcuma longa*, *Zinger*

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officinalis and *Plumbago zeylanica* in *in-vitro* and *in-vivo* models of cardiovascular degeneration[6–10]. *Ficus racemosa* (*F. racemosa*) is widely used in Ayurvedic medicine in India, mostly as fruit and bark decoction to treat uncontrolled diabetes, hyperlipidemia and inflammatory joint diseases[11–15]. This *F. racemosa* has high tannin content and few reports have shown anti-cancer, gastroprotective, anti-inflammatory, free radical scavenging effect of extracts of *Ficus*[16–19]. Nevertheless, the effect of the tannins as main phytoconstituents of stem bark in diabetes associated with hypercholesterolemia has not investigated. The present study was to investigate the effect of tannins in high fat meal and streptozotocin (STZ) induced diabetes in rats. In this investigation we have measured the effect of tannins in serum insulin, blood glucose, lipid profile level in high fat diet and STZ treated rats. Also we have measure the oxidative stress markers and antioxidant status in heart, liver and kidney.

2. Materials and methods

2.1. Collection of plant material and authentication

F. racemosa bark was collected from Walajabad District, Chennai. Tamilnadu. They were identified and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai, Tamil Nadu, the voucher specimen no: Parc/2008/229 has been deposited at the herbarium unit of the Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels University, Chennai.

2.2. Separation of tannins from *F. racemosa* L

The barks of *F. racemosa* were shade dried and coarsely powdered. Total tannins were separated from crude acetone (70% v/v) extract as described by McCallum *et al*[20]. Briefly, the powdered material (1 kg) was extracted with acetone in water (70% v/v) (2 500 mL) by cold maceration. The acetone extract was filtered and saturated with sodium chloride (saturated NaCl) to salt out acetone and the upper solvent phase was removed. This acetone phase was then extracted with three successive 250 mL portions of the deionized water containing 0.1% ascorbic acid to prevent auto-oxidation. Excess of acetone in the aqueous portion is removed *in vacuo* at 25 °C. To the aqueous portion was treated with an equal volume of water then extracted with three successive portions of petroleum ether (40–60 °C) to remove any lipid material and ethyl acetate. Finally the remaining aqueous phase containing crude tannins was collected and freeze dried. The yield of the crude tannin fraction (TF) was found to be 15% w/w. The tannin fraction was subjected to qualitative chemical test and thin layer chromatography studies and showed positive test for tannins.

2.3. Thin layer chromatographic studies (TLC) of tannin fraction

Pre coated silica gel GF254 Plate 15 cm×20 cm (E. Merck,

Mumbai, India) was used as the stationary phase. The tannin fraction was dissolved in ethanol. This fraction was applied by means of a Linomat IV sample applicator to the plates about 1 cm above the edge. The chromatogram was developed up to 10 cm with Toluene: Ethyl acetate: GAA: Formic acid (50:50:10:10) as the solvent system in a CAMAG twin trough chamber. The developed TLC plate was observed under UV-light. From the thin layer chromatographic studies, the presence of various tannins was observed with R_f values between 0.10 and 0.87. The R_f value of tannins present in bark of *F. racemosa* was compared with literature values of tannins and gave a good agreement[21,22]. Further the tannin fraction was labeled as TF.

2.4. Chemicals

Streptozotocin from Sigma, USA. Cholic acid is purchased from Loba chemie. Pvt Ltd Mumbai. Cholesterol was purchased from SISCO Research Laboratories Pvt Ltd, Mumbai, India. Egg yolk powder was purchased from Himedia laboratories Pvt Ltd, Mumbai, Adrenaline bi tartrate was purchased from Sisco Research Laboratories Pvt Ltd, Mumbai. Thiobarbituric acid (TBA) from SRL, Mumbai, other solvents and chemicals were purchased from Qualigens.

2.4.1. DPPH scavenging assay

The effect of TF on DPPH radicals was estimated as described by Lim *et al*[23] with minor modification. In brief, 2 mL DPPH in methanol (3.6×10^{-5} M) was added to 50 μ L of various concentrations of fraction (10–100 μ g/mL). The mixtures were vortexed for 15 s and left to stand for 30 min at 37 °C. The decrease in the absorbance at 515 nm was continuously recorded on a spectrophotometer for 15 min at room temperature. All determinations were performed in triplicate. The DPPH scavenging activity (decrease in absorbance at 515 nm) of the fraction was plotted against time.

2.4.2. Nitric oxide scavenging assay

The nitric oxide scavenging assay was carried out as described by Sreejayam *et al*[24]. Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of tannin fraction dissolved in methanol and incubated at 25 °C for 30 min. Then, 1.5 mL of the incubation solution was removed and diluted with 1.5 mL modified Griess reagent. The absorbance was measured at 546 nm.

2.5. Animals

Male wistar rats (150–200 g) were used for this investigation. Animals were maintained under standard laboratory conditions and had free access to feed and water *ad libitum*. Experimentation on animals is approved by Institutional Animals Ethics Committee, School of Pharmaceutical Sciences, Vels University.

2.6. Induction hyperlipidemia with type-2 diabetes

Animals were treated with modified high fat meal (HFM)

for 30 days. The high fat diet is freshly prepared every day and the method of preparation is described earlier by Devi *et al*[25]. Control animals were provided with normal pellet chow (Lipton, India). After 3 days on high fat diet, animals were fasted overnight and diabetes was induced by injecting STZ (Sub diabetogenic dose–35 mg/kg in 0.1 mol/L citrate buffered saline, pH 4.5, injected intraperitoneally)[26].

2.7. Animal grouping and drug administration

Animals were divided into five groups. Group 1 (n=6) served as control animal treated with 0.9% saline. Group 2 (n=6) served as high fat diet fed diabetic animal treated with 0.9% saline. Group 3 (n=6) served as high fat diet fed diabetic animals treated with metformin. Group 4 (n=6) served as high fat diet fed diabetic animals treated with TF 100 mg/kg in normal saline. Group 5 (n=6) served as high fat diet fed diabetic animals treated with TF 200 mg/kg in normal saline.

2.7. Biochemical estimation

2.7.1. Estimation of blood glucose, insulin and lipid profiles

Blood glucose level was determined by one touch horizon blood glucometer using one drop of blood collected from tail vein. At the end of 30th day animals (n=3) were sacrificed by euthanasia and blood was collected. Lipid profiles, total cholesterol, lactate dehydrogenase (LDH), creatinine phospho kinase (CPK) and uric acid were measured in plasma by using standard bio chemical kit (Auto analyser). Organ like heart, liver and kidneys were isolated, weighed and homogenized with ice cold phosphate buffer (pH 7.2) in Teflon glass homogenizer. The homogenate was centrifuged at 1000 rpm 4 °C for 15 min. Protein was estimated by the method Lowry *et al*[27]. The supernatant was used for estimation of oxidative stress markers by Sagu *et al*[28], Ohawa *et al*[29], and antioxidants and Beers and Seizers[30].

2.7.2. Histopathological examination

After 30 days of STZ and STZ+TF treated animals were euthanized. The pancreas dissected out quickly, fixed in

10% formalin and 10 μ m thick sections were taken. The sections were processed and stained in 0.1% Hematoxylin and Eosin. The stained sections were observed under a binocular light microscope and photographed. Quantitative scoring of histopathological examination was performed according to (Block and Schwarz, 1996) method with slight modifications.

2.8. Statistical analysis

For *in-vivo* experiments values are represented by mean ± SEM. The mean values are analyzed by one way ANOVA followed by Dunnett's test. The P<0.05 was considered as statistically significant.

3. Results

3.1. Effect of TF from F. racemosa L on DPPH radical scavenging assay

Figure 1a depicts the free radical scavenging capacity of TF fraction using DPPH generated radical in *in-vitro*. It was observed that increase in the % inhibition of free radicals has observed in increasing concentration of TF. The IC₅₀ value of TF was found to be 53.98 μ g/mL and the R² linear regression value was found to be 0.990 1. The TF was compared with the standard quercetin IC₅₀=26.93 μ g/mL; R²=0.998 7.

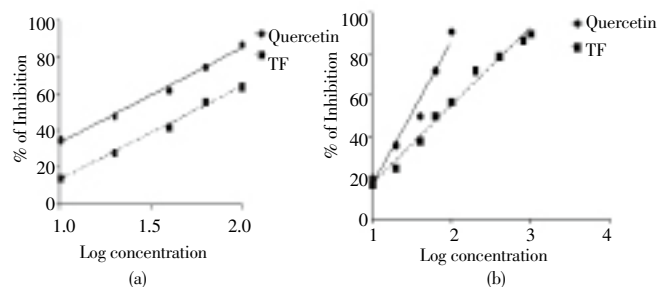


Figure 1. Effect of TF from F. racemosa L in DPPH(a) and NO(b) radical scavenging assay.

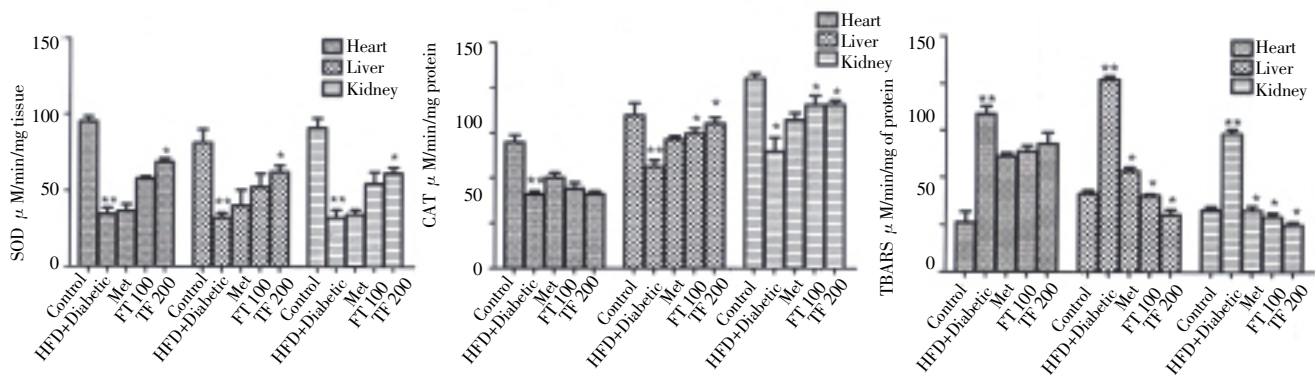


Figure 2. Effect of TF on SOD(a), CAT(b) and TBARS(c) level of heart, liver and kidney of HFM fed diabetic rats. *P<0.05 treatment vs. diabetic; **P<0.01 control vs. diabetic.

3.2. Effects of TF fractions from *F. racemosa* L on nitric oxide scavenging

Figure 1b depicts the ability of TF fraction to quench NO radicals *in-vitro*. The results indicates that TF of *F. racemosa* exhibited IC₅₀ and R² values of 54.14 μg/mL, 0.9978, respectively compared with standard quercetin IC₅₀ value of 13.36 μg/mL and R² value of 0.9939.

3.3. Anti-diabetic effect of TF on high fat meal treated diabetic rats

The anti-diabetic effect of tannins from *Ficus* has shown in Table 1. HFM and STZ treated diabetic rats treated with tannin fractions of *F. racemosa* had significant ($P<0.05$) decrease in reducing blood glucose levels at 7th Day as compared with saline treated HFM + STZ rats. The similar significant ($P<0.05$) anti-diabetic effect was noted at 14th day as well as at the end of the experiment at 30th day as compared with HFM + STZ treated diabetic control (349.16 ± 3.31) mg/dL. The anti diabetic effect of tannins in reducing the blood glucose level at 30th day was comparable to that of metformin.

3.4. Anti-hyperlipidemic effect TF on high fat meal treated diabetic rats

Table 2 depicts the effect of tannins fractions on total cholesterol and lipid profiles of the high fat meal fed diabetic rats. Administration of HFM significantly ($P<0.01$) increase the total cholesterol (TC), low density lipoprotein (LDL), and triglycerides (TG) with significant decrease ($P<0.01$) in high density lipoprotein (HDL) level compared with normal rats. Administration tannins significantly ($P<0.05$) protected the rats against HFM induced hyperlipidemia as observed by decrease in the TC and LDL level. In addition tannins significantly increased the HDL level respectively. However, the effects of tannins on TG levels were insignificant. There

is a significant increase in CPK and LDH level was observed in HFM treated diabetic rats compared with normal rats. Attenuation of CPK and LDH level was observed in HFM treated diabetic rats fed with tannin fraction. However there is no effect was observed in uric acid level (data not shown).

3.5. Effect of TF on super oxide dismutase (SOD) level of heart, liver and kidney of HFM fed diabetic rats

It is observed from the Figure 2a that significant ($P<0.01$) depletion in superoxide dismutase level (SOD) in heart, kidney and liver of HFM fed diabetic rats as compared with non diabetic control rats. Per oral administration of tannins significantly ($P<0.05$) increased the SOD levels in heart and kidney of the HFD treated diabetic rats as compared with vehicle treated hyperlipidemic diabetic rats. The effect was dose dependent.

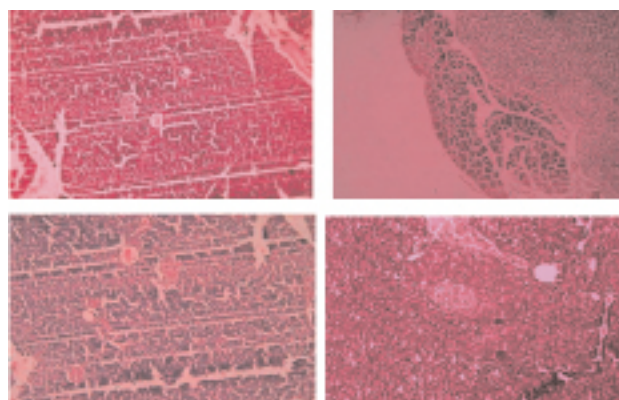


Figure 3. Effect of TF at high dose on histopathological result.

a: pancreas showing normal acini with islets of β-cells; b: pancreas shows atrophic acini and vascular degenerative changes with reduction in islet β-cell size; c: TF 100 mg/kg treated pancreas showing markedly proliferative stages of (hyperplastic) islets β-cells; d: TF 200 mg/kg treated pancreas showing initial regenerating & preserved islet cells.

Table 1

Effect of TF in blood glucose level of the high fat diet treated diabetic rats.

Group	7th day (mg/dL)	14th day (mg/dL)	30th day (mg/dL)
Control	101.50 ± 6.80	100.33 ± 5.12	99.83 ± 5.28
HFM+STZ	312.33 ± 18.27*	406.50 ± 25.62*	394.16 ± 3.31*
Metformin	179.50 ± 8.40#	111.66 ± 3.92#	111.00 ± 4.41#
TF 100	186.33 ± 18.17#	151.66 ± 9.27#	130.50 ± 3.13#
TF 200	259.83 ± 49.28#	161.16 ± 14.68#	113.16 ± 4.60#

* $P<0.05$ control vs. diabetic. # $P<0.05$ treatment vs. diabetic.

Table 2

Effect of TF in lipid profile, LDH, CPK and uric acid level of HFM fed diabetic rats.

Groups	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)	CPK (IU/mL)	LDH (IU/mL)
Control	85.66 ± 8.48	22.00 ± 1.73	57.66 ± 2.33	67.66 ± 3.48	211.50 ± 17.01	75.50 ± 8.20
HFM + STZ	285.40 ± 18.70*	151.66 ± 3.17*	29.33 ± 2.90*	77.01 ± 1.73*	312.40 ± 22.40*	145.80 ± 9.30*
Metformin	158.66 ± 12.11**	142.23 ± 4.16**	34.14 ± 1.52	74.00 ± 3.51	276.00 ± 21.50**	97.50 ± 7.80**
TF 100	167.33 ± 16.17**	40.66 ± 7.71**	38.70 ± 2.08**	79.00 ± 2.08	255.50 ± 26.80**	86.20 ± 8.90**
TF 200	144.66 ± 7.70**	35.33 ± 2.72**	43.13 ± 2.08**	76.66 ± 3.75	220.20 ± 20.60**	79.40 ± 9.20**

* $P<0.05$ control vs. diabetic; ** $P<0.01$ treatment vs. diabetic.

3.6. Effect of TF in catalase (CAT) in heart, liver and kidney of HFM fed diabetic rats

Figure 2b shows the significant ($P < 0.01$) decrease in the catalase (CAT) level was observed in heart, liver and kidney of HFM fed diabetic rats as compared with non-diabetic control rats. TF at two different doses significantly ($P < 0.05$) increase the catalase level of the insulin dependent liver tissue and non-Insulin dependent tissue kidney and heart.

3.7. Effect of TF in TBARS level of heart, liver and kidney of HFM fed diabetic rats

Figure 2c depicts the effect of TF on thiobarbituric acid reactive substances (TBARS) levels in vital organs of the rats fed with HFM and streptozotocin. Significant ($P < 0.05$) increase in the TBARS was observed. High fat diet treated diabetic rats heart, liver and kidney has compared with non-diabetic control a animal group. Administration of metformin, different doses of tannin fraction are significantly ($P < 0.05$) decrease the elevated TBARS level in insulin dependant liver and non insulin dependant kidney of the high fat diet treated diabetic rats compared with saline treated high fat treated diabetic animals.

3.8. Histopathology

The effect of TF at high dose on histopathological findings on the pancreas shown in Figure 3a–d. It is observed that diabetogenic agent streptozotocin produced lesion in the pancreatic islets as viewed by very scanty islets with acinar tissue. Treatment with insulin has decreased the degree of lesions as indicated by partial intact pancreatic cells with acini. However attenuation of pancreatic degeneration was observed in high fat diet treated diabetic animals treated with tannin fraction 200 mg/kg.

4. Discussion

In the present study emphasized the protective effect of tannins separated from the stem bark of *F. racemosa* Linn in high fat diet/streptozotocin treated diabetic rats. The present study is the first biochemical inspection to show the anti-hyperglycemic and hypolipidemic effect of tannins present in *Ficus* bark in animal model of type II diabetes associated with hyperlipidemia. Ever-growing epidemiological and recent clinical reports suggest that the high prevalence of cardiovascular diseases like coronary artery diseases associated with hypercholesterolemia and diabetes^[31–33]. Hyperlipidemia is associated with profound alterations in the plasma lipid and lipoprotein profile and with an increased risk of coronary heart disease^[34–36]. The liver and some other tissues participate in the uptake, oxidation and metabolic conversion of free fatty acids, synthesis of cholesterol and phospholipids and secretion of specific classes of plasma lipoproteins. Lowering of blood glucose levels and serum

lipid levels through dietary or drug therapy seems to be associated with a decrease in the risk of vascular disease and related complications^[37–39]. Many medicinal herbs from Indian system of medicine have been shown to have hypoglycemic and hypolipidemic properties^[40,41]. Evidence is presented to show that, chronic administration of tannins from *Ficus* affect glucose level and plasma lipid lowering properties in diabetic animals. It is observed from the data that increase in plasma cholesterol and lipid profile levels were observed in high fat fed/STZ treated diabetic rats. Our study results demonstrated that crude tannin fractions from *Ficus* bark was controlled hyperglycemia by significantly reducing blood glucose level in diabetic rats. TF extracts (100 & 200 mg/kg) fails to show euglycemia (Data not shown) on 48th hrs after STZ injection whereas it has decreased blood glucose levels on 7th, 14th and 30th day.

Ingestion of tannins normalized TC, LDL level in plasma, suggesting that tannins affect fatty acid catabolism in the liver possibly by controlling the hydrolysis of lipoproteins and their selective uptake and metabolism by different tissues.

There is a clear link between hyperglycemia and active oxygen/nitrogen species in experimental and clinical types of diabetes^[42]. Accumulation of reactive oxygen species (ROS) due to oxidative stress is also instrumental in the expression of cell death as ROS can easily react with and oxidize vital cellular components such as lipids, proteins and DNA^[43]. The vital organs are particularly susceptible to the effects of ROS due to its poly unsaturated integrity and modest antioxidant defense^[44]. Experimental studies have indicated the potential use of exogenous antioxidants for prevention and treatment of diabetes mellitus. Plant derived anti-oxidant treatment has been reported to reduce the development of diabetic complications such as retinopathy, cataract formation, neuropathy, vascular complication and nephropathy. Another important factor determining the level and composition of serum and tissue lipids is lipid peroxide (LPO) associated with cellular membranes. During diabetes an increased oxidative stress in certain tissues may lead to a rise in the rate of LPO^[45–52]. The formation of the lipid peroxide product, malonaldehyde (MDA), was measured in tissue and serum as an index for increased LPO in diabetic rats, but with the exception of kidney, there was no appreciable increase in the liver. MDA formation was actually decreased in diabetic rats.

In this study we investigated the effect of tannins supplementation on preventing oxidative damage in high fat diet treated diabetic rats. SOD and CAT are enzymes that protect tissues from the effects of free radicals and lipid peroxides, and the activities of both SOD and CAT increase after free-radical-mediated injury and lipid peroxidation^[53,54]. CAT is a heme protein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals^[55–58]. Therefore, reduction in the activity of these enzymes (SOD, CAT) may result in a number of deleterious effects due to the accumulation of superoxide anion radicals and hydrogen

peroxide. The results of our study show that SOD and CAT activities as well as MDA concentrations in liver, heart, and kidney homogenates did differ between the experimental and control groups. These results suggest that tannin supplementation for 30 days to diabetic rats had antioxidant effect on reducing oxidative stress. In conclusion, the results of this study show that two different doses of tannin supplementation had a favorable effect on plasma glucose and lipid profile concentrations. It also had an influence on attenuating oxidative stress in diabetic rats. The high dose tannin administered group showed a tendency to have better chronic glycemic control than did the low dose treated tannin groups. In addition, larger amounts of tannin supplementation may have beneficial effects on reducing plasma TC, and LDL levels. Further characterization of active tannins in *Ficus*, such as phenolics or related analogues is warranted and studies are in progress to isolate, identify and characterize such active components.

Conflict of interest statement

We declare that we have no conflict of interest.

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