

Protective role of *Trigonella foenum graceum* extract against oxidative stress in hyperglycemic rats

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Abstract. – Background and Objectives: “In all things of nature there is something of the marvelous.” In the present study the anti-hyperglycemic and anti-oxidative potential of aqueous extract of *Trigonella foenum graceum* (TFG), a traditional medicinal herb was assessed in liver and WBC of alloxan induced diabetic rats. Free radicals can cause oxidative damage, which is balanced by the antioxidants. This has been implicated in aging, and diseases such as diabetes and other chronic conditions.

Material and Methods: TFG extract was administered orally [500 (LM) and 1000 mg/kg body weight (HM)] for six weeks. The effect of TFG on blood glucose were studied and the levels of lipid peroxidation [MDA (Malondialdehyde)] and antioxidant enzymes [SOD (Superoxide dismutase), GPx (Reduced Glutathione peroxidase)] were estimated and compared with standard drugs glibenclamide and insulin.

Results: Treatment with TFG, insulin and glibenclamide resulted in significantly reduced blood glucose in LM (8.71%) and HM (3.87%) in comparison with normal controls. There was a significant decrease in lipid peroxidation in liver and white blood cells (WBC) in both low and high doses [liver LM (49%), HM (57.25%)], [WBC LM (54.28%), HM (62.5%)] and increase in antioxidant enzymes SOD [liver LM (33.59%), HM (58.7%)] [WBC LM (44.9%) HM (58.7%) and GPx [Liver LM (58.55%), HM (40.20%)], [WBC LM (55.46%), HM (56.4%)] when compared to diabetic controls.

Discussion: Potency of TFG in restoring several parameters to normal values is comparable to glibenclamide, though not as efficient as insulin, an indication of its antihyperglycemic and antioxidant effect.

Key Words:

Diabetes, Alloxan, Antioxidant enzymes, MDA, Oxidative Stress, *Trigonella foenum graceum* (TFG).

Introduction

Diabetes Mellitus (DM) is a complex metabolic disorder that involves abnormalities in both insulin secretion and its action in peripheral tissue. DM has become a global epidemic and a major health concern in both developing and developed countries. As a disease, it kills more individuals on a per annum basis than AIDS and breast cancer combined¹. There is increasing evidences from various scientific investigations that diabetic complications involving kidney, heart, nerves are due to oxidative stress and subsequent weakening of antioxidant defense system².

Modern diabetes medicines, even though offer a variety of effective treatment options, can also have several side effects^{3,4}. Owing to its high cost and need for lifelong treatment, many of economically weak patients, may go untreated. In this context, there is increasing interest worldwide for herbal medicines that could considerably reduce economic and clinical toll of DM⁵.

Trigonella foenum graceum (TFG) leaves are consumed widely in India as a green, leafy vegetable and are a rich source of calcium, iron, β -carotene, thiamine, niacin, vitamin C and vitamin K⁶. In our present investigation, TFG has been chosen for testing antihyperglycemic and antioxidant property⁷. With regard to glycemic control capacity of TFG leaf extract, scientific investigations are very few when compared to seed⁸. Our present work aims at understanding antioxidant activity of TFG leaf in male albino wistar rats. After 6 weeks of oral supplementation, different parameters such as body weight (b.w.), fasting

blood glucose (FBS), antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) and lipid peroxidation were measured.

Materials and Methods

Collection and Authentication of Plant Material

TFG was collected from local market of Bangalore (Karnataka) during the month of August – November and were authenticated at the Regional Research Centre (RRC), Bangalore. A specimen was deposited in the Herbarium of the regional research centre (RRCBI/2136). Plant material was shade dried at temperature less than 40°C.

Preparation of Aqueous Extract⁹

The dried plant material was grounded into a moderately coarse powder using domestic electric grinder. The powdered drug was boiled with sixteen parts of water for a period of 15 minutes. It was filtered hot through muslin cloth. The filtrate was evaporated under reduced pressure and dried to obtain 6.8% of aqueous extract.

Animals

Male *Wistar albino* rats (140-160 g) were housed under standard laboratory conditions of light and dark cycles of 7:00 am to 7:00 pm, temperature of 25% ± 2C and 75% ± 1% relative humidity. The animals were given standard rat pellet (Lipton India Ltd., Bangalore) and tap water *ad libitum*. The study protocol was approved by Maharani Lakshmi Ammanni College Ethical Committee, clearance from Ethical Committee (1368/ac/10/CPCSEA), Bangalore.

Acute Toxicity Test

Swiss albino mice (25-30 g) of either sex were divided into 5 groups of 10 each. Animals were fasted overnight but there was free access to water prior to experiment. Aqueous extract of *TFG* leaf at different dose levels (5.0, 6.0, 7.0, 8.0, 9.0 g/kg b.w.) was administered once to experimental groups. The volume of each administered dose was 1 ml. The mice were observed for 24 hours, mortality was recorded and median lethal dose LD₅₀ (Median lethal dose) was determined using the arithmetic method of Karber modified by Aliyu and Nwude (1982)¹⁰.

The acute toxicity (LD₅₀) was calculated using the formula:

$$LD_{50} = LDy - \frac{1}{n} \sum (Dd \times Md)$$

where:

LDy = highest dose and

n = number of animals per group (n = 10),

Dd = Dose difference,

Md = Mean dead

Alloxan induced Wistar Albino Rat Model for Diabetes Mellitus

DM was induced by administering alloxan (150 mg/kg b.w. i.p) in 50 mM PO₄ buffer (pH 7.0) in a volume of 1 ml/kg. After two days of alloxan injection, animals showing the fasting blood glucose level more than 220-260 mg/dl were considered as diabetic^{11,12}. After a period of one week induced animals were used for experimental purposes.

Experimental Groups

Male rats were randomly allotted into 6 groups of six animal (n=6) each.

Group 1 served as normal control and received distilled water (NL).

Group 2 served as DM control and received distilled water (DC).

Group 3 DM animals treated orally with aqueous extract of *TFG* leaf at a dose of 0.5 g/kg/day (LM).

Group 4 DM animals treated orally with aqueous extract of *TFG* leaf at a dose of 1 g/kg/day (HM).

Group 5 DM animals treated with glibenclamide at a dose of 600 µg/kg/day (DG).

Group 6 DM animals treated with insulin (Insuzen, Biocon, Bangalore, India) at a dose of 2 U/kg/day by *i.p.* route (DI).

The drug treatment was carried out every day morning with the help of 16 guage ball tipped feeding needle for a period of six weeks¹³. FBS determined after 1 week and 6th week of drug treatment. Body weight was determined every day. After 6 weeks of drug treatment antioxidant content in WBC and liver were evaluated.

Estimation of Blood Glucose¹⁴

Blood samples were withdrawn by end tail vein cutting method from overnight fasted ani-

mals and blood glucose level was determined by using one touch electronic glucometer ACU check (Apollo Pharmacy, Bangalore, India).

Estimation of Antioxidant Activity in White Blood Cells (WBC) and Tissue

Collection of WBC¹⁵

Separation of WBC has been done by gradient method.

Gradients

10% Ficoll (24.00 g) in 240 ml of distilled water and adding 100 ml of 34% isopaque[®] (sodium metrizoate) to Ficoll solutions. The Ficoll-isopaque gradients were sterilized by autoclave at 121°C for ten min. Subsequently, the gradients were adjusted to the pH of 8 with 1 N sodium hydroxide and the solutions were kept at 40°C.

Separation of WBC

One ml blood from each of the six rats was drawn into a heparinized and siliconized syringe (20 i.u./ml) which was then supported in an upright position at ambient room temperature for 20 min to allow the erythrocytes to sediment. The leukocyte rich plasma was expressed through a bent needle into a 15 ml siliconized tube. The leukocyte rich plasma was thoroughly mixed and equally distributed into siliconized, conical, 10 ml tubes, containing 3 ml of the gradient. In addition, a sample of the leukocyte rich plasma was taken in order to count the total white and red cells as well as doing a differential count. Subsequently the tube with gradient overlaid with leukocyte rich plasma was spun for 25 min at 800 × g at 20°C. Some of the white blood cells formed a band at the plasma Ficoll-isopaque interface in the gradient or sedimented on the bottom of the tube. Each band of cells was removed separately with a Pasteur pipette and placed in a siliconized, conical tube. Only the cells from the surface of the bottom layer were collected and used for the further experiment.

Collection of Tissue

Rats were sacrificed by an overdose of anesthetic ether. The liver was immediately excised and chilled in ice cold 0.9% sodium chloride. The tissue was kept at -80°C until processed. The tissue was homogenized in 50 mM phosphate buffer pH (7.0) containing 0.1 mM EDTA to obtain a

5% homogenate for the malondialdehyde (MDA) tests. SOD was assayed in the supernatant obtained after centrifugation of the 5% homogenate at 600 × g for 10 min at 4°C (RV/FM, super spin, Plastocraft, India)¹⁶. The supernatant was collected and used for analytical procedures.

Enzyme Assays

Glutathione Peroxidase (GPx, EC 1.11.1.9)

GPx activity was measured at 37°C by the method of Flohe and Gunzler¹⁷. The reaction mixture consisted of 500 µl of phosphate buffer, 100 µl of 0.01 M reduced glutathione (GSH), 100 µl of 1.5 mM NADPH and 100 µl of GR (0.24 U). 100 µl of tissue extract was added to the reaction mixture and incubated 37 °C for 10 min. Fifty micro litres of 12 mM t-butyl hydro-peroxide was added to 450 µl of tissue reaction mixture and measured at 340 nm for 180s in a spectrophotometer (ELICO, Model BL 192, Andhra Pradesh, India). A molar absorptivity of 6.22 × 10³ M/cm was used to determine enzyme activity. One unit of activity is equal to mM NADPH oxidized per min per mg protein.

Total Superoxide Dismutase (SOD, E.C. 1.15.1.1)

SOD was measured by the method of Misra and Fridovich¹⁸. 0.05 M carbonate buffer (pH 10.2) containing 0.1 mmol EDTA and 30 mmol epinephrine in 0.05% acetic acid were added to the tissue extract and changes in activity were measured at 480 nm for 4 min. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equivalent to one unit and is expressed in terms of units/mg protein.

Lipid Peroxidation (LPO)

Malondialdehyde (MDA) was determined by the method of Okhawa et al¹⁹ using 1, 1, 3, 3-tetramethoxy propane as standard. In brief, 8.1% SDS (sodium dodecyl sulfate) was added to the tissue homogenate and incubated for 10 minutes at room temperature (RT), followed by boiling with 20% acetic acid and 0.6% thiobarbituric acid for 60 minutes in a water bath. On cooling, butanol: pyridine (15: 1 v/v) was added and centrifuged at 600 × g for 5 min. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed in terms of nM /mg protein.

Total Protein Estimation

The total protein concentration was estimated by Lowry et al.²⁰ using BSA as standard. Briefly, to 1ml of the supernatant, 5 ml of alkaline copper sulphate was added and incubated for 10 min at RT. Following this 0.6 ml of 1:1 diluted Folin Catecholamine reagent was added and incubated for 30 min at RT. Absorbance was read at 660 nm against reagent blank.

Statistical Analysis

Data were expressed as means \pm SE comparison between different groups was done using one-way ANOVA followed by Turkey's multiple comparison test's <0.05 was considered to be statistically significant²¹.

Results

Acute Toxicity Test

LD₅₀ of the aqueous extract of *TFG* was found to be 8.40 gm/kg body weight in mice.

Effect on Fasting Blood Glucose and Body Weight

The body weight and glucose concentrations of rats fed *TFG* and alloxan are presented in Table I. There was an increase in the body weight in the normal control rats (19.88%) and rats treated with 500 mg/kg extract, LM (8.71%) and 1000 mg/kg, HM (3.87%), while there was a decrease in the body weights of rats treated with alloxan, DC (-11%). Glucose concentrations remained unchanged in the NL group at the end of

the experiment. In rats treated with alloxan, DC there was a significant increase in blood glucose level compared to NL. *TFG* leaves at the doses of 500 mg, LM and 1000 mg/kg, HM significantly attenuated the alloxan-induced elevated blood glucose concentration by 51.3% and 69.8%, respectively. The standard drug glibenclamide similarly reduced the glucose level in alloxan-treated rats by 70.7%.

After administration of *TFG* extract for a period of six weeks, the antioxidant enzymes, GPx levels were determined experimental rats are given in Table II. The result shows that GPx level significantly increased 58.55% in lower and 40.20% with higher dose in liver and significantly increased 55.46% in lower and 56.4% with higher dose in WBC as compared with that of DC group.

Figure 1 and 2 shows the activities of SOD in liver and WBC of normal and experimental rats. During diabetes, the activities of these enzymes were reduced in WBC and liver of diabetic rats. *TFG* leaf extract supplementation increased the activities of SOD in the tissues of experimental rats. *TFG* extract at both doses showed significant effects in liver. The effect of 1000 mg/kg is more effective in liver as compared to WBC. There was no significant effect seen in between WBC of lower dose of *TFG* and DG group.

Figure 3 and 4 shows the concentration of MDA in liver and WBC in normal and experimental rats. Diabetic animals showed a significant increase in MDA. Supplementation of *TFG* lowered the lipid peroxidation in LM and HM groups significantly.

Table I. Effect of supplementation of *TFG* extra on FBC and B.W. (body weight) in diabetic rats.

Group	Blood glucose (mg/dL)		Body weight (gm)	
	Initial	Final	Initial	Final
NL	107.3 \pm 1.5	105.7 \pm 2.8 ^a	181.0 \pm 12.3	217.5 \pm 12.0 ^a
DI	106.9 \pm 3.2	103.6 \pm 2.1 ^b	189.3 \pm 10.7	215.5 \pm 14.9 ^a
DC	396.9 \pm 2.7	394.6 \pm 6.4 ^a	184.0 \pm 11.6	163.5 \pm 10.7 ^b
LM (0.5 g/kg)	394.5 \pm 4.3	190.6 \pm 7.10 ^a	167.5 \pm 12.1	182.1 \pm 11.3 ^c
HM (1.0 g/kg)	395.4 \pm 2.3	119.2 \pm 7.10 ^a	180.5 \pm 13.0	187.5 \pm 8.3 ^d
DG	397.1 \pm 2.1	116.1 \pm 5.2 ^f	192.5 \pm 10.4	182.5 \pm 11.5 ^{ef}

NL = Normal; DC = Diabetic Control; DG = Diabetic Glibenclamide; DI = Diabetic Insulin; LM = Low *TFG* (0.5 g/kg b.w.); HM = High *TFG* (1.0 g/kg b.w.). Values are means \pm S.E. (n=6 animals/groups) in experimental animals. Result were considered significant between the groups at $p < 0.001$. In a comparative studies with DC, those are not sharing the same letter (a-f) are significantly different.

Table II. Effect of supplementation of TFG extra on GPx in liver and W.B.C. in diabetic rats.

Group	GPx (mM/mg protein)		No of experimental animals (N)
	Liver	W.B.C.	
NL	18.78 ± 2.027 ^a	10.33 ± 1.628 ^a	6
DI	18.09 ± 1.726 ^f	9.9 ± 0.9265 ^f	6
DC	9.959 ± 1.151 ^b	5.903 ± 0.2168 ^b	6
LM (0.5 g/kg)	15.79 ± 0.6765 ^c	9.177 ± 0.7745 ^c	6
HM (1.0 g/kg)	13.95 ± 1.937 ^d	9.238 ± 0.2308 ^d	6
DG	15.55 ± 1.742 ^{ce}	9.15 ± 0.487 ^{ce}	6

Values are means ± S.E. (n=6 animals/groups) in experimental animals. Results were considered significant between the groups at $p < 0.001$. In a comparative studies with DC, those are not sharing the same letter (a-f) are significantly different.

Discussion

In DM, insulin insufficiency or tissue resistance impairs carbohydrate, protein, lipid metabolism resulting in hyperglycemia. The same has been elucidated by numerous biochemical and molecular studies conducted in the last century²². Elevated blood glucose level causes damage of blood vessels with an eventual coronary artery disease, stroke, nephropathy, neuropathy, etc. In the present studies, male *Wistar* rats developed diabetes after receiving a single dose of alloxan, a known β -cytotoxin that damages insulin secreting β -cells of islets²³.

Increased blood glucose level of diabetic rats was brought down to normal level after supplementing the animals with *TFG* leaf extract. The mechanism by which *TFG* exerts its effect is not clear but it could be similar to that of sulphonylurea, where K^+ ATP channel closes which opens Ca^{++} channel resulting in the influx of Ca^{++} ions that act as second messengers for insulin secretion²⁴. *TFG* leaf extract may exhibit its anti-hyperglycemic effect by enhancing Na^+-K^+ ATPase activity of translocation of GLUT4 to membrane, there by increasing glucose uptake by the cell²⁵. Novel amino acids of seed extract, 4-hydroxyisoleucine, has displayed insulin tropic and anti-

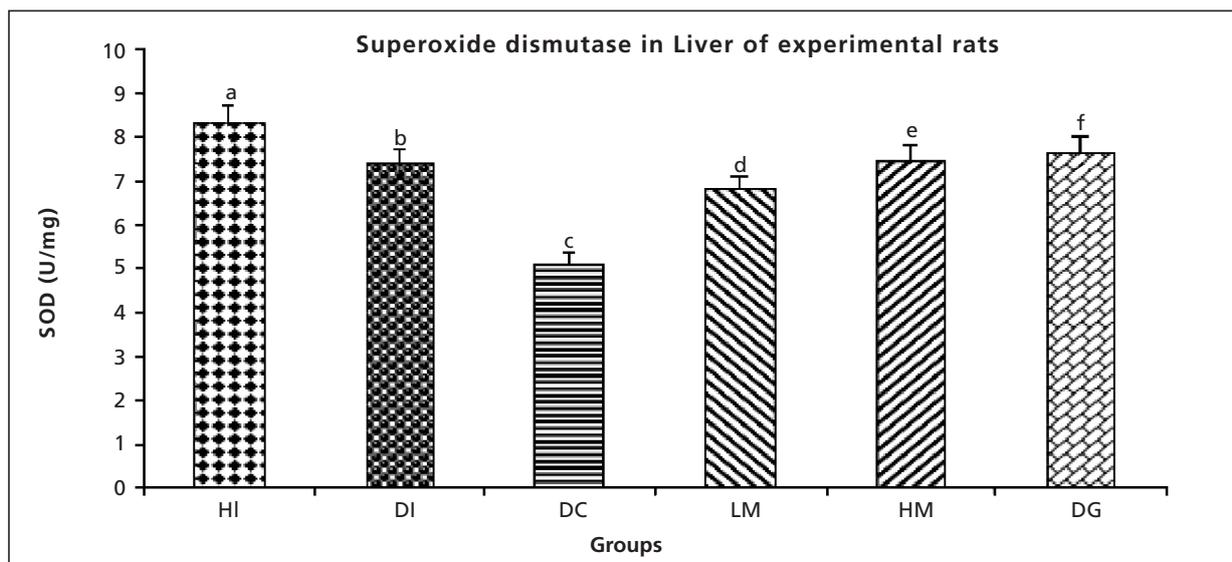


Figure 1. Values are means + S.E. (n=6 animals/groups) in liver of experimental animals. Results were considered significant between the groups at $p < 0.001$. In a comparative studies with DC, those are not sharing the same letter (a-f) are significantly different.

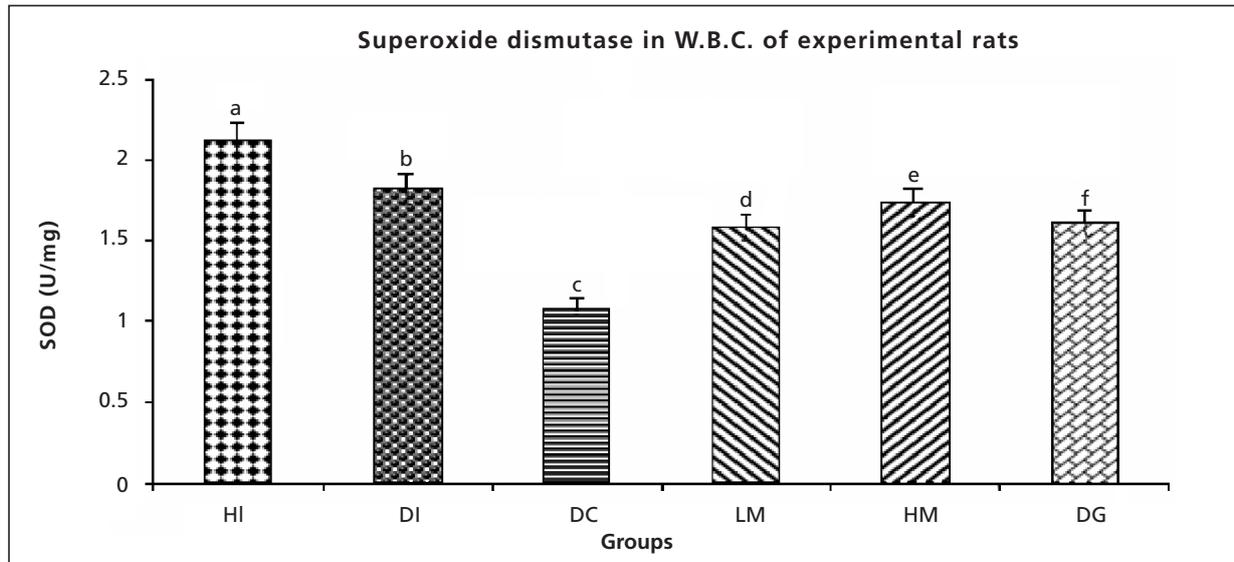


Figure 2. Values are means + S.E. (n=6 animals/groups) in W.B.C. of experimental animals. Results were considered significant between the groups at $p < 0.001$. In a comparative studies with DC, those are not sharing the same letter (a-f) are significantly different.

hyperglycemic property both *in vivo* and *in vitro* animal studies²⁶. Steroidal saponine of *TFG* seed has been shown to possess antihyperglycemic property and serum cholesterol and triglyceride lowering effect^{27,28}. Several earlier investigations have reported antioxidant property of *TFG* seed

but not much on leaf extract²⁹. Our present investigation has shown a increase in SOD and GPx, two major free radical scavenging enzymes in rats treated with *TFG* extract. Previous studies have also indicated reduced activity of SOD, GPx and accumulation of free radicals in liver

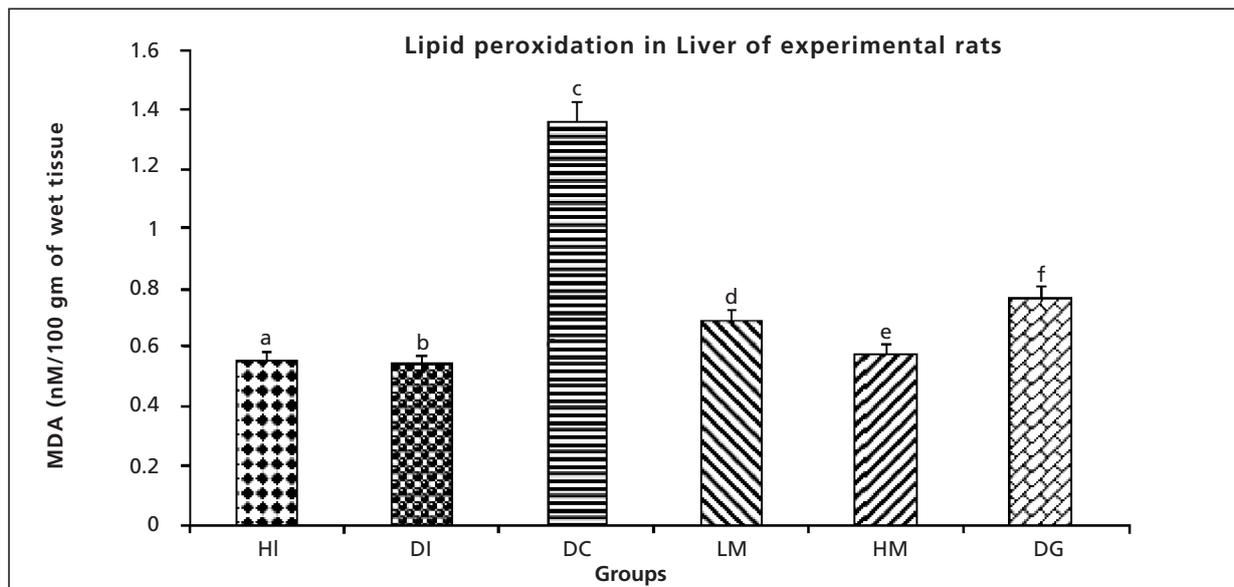


Figure 3. Values are means + S.E. (n=6 animals/groups) in liver of experimental animals. Results were considered significant between the groups at $p < 0.001$. In a comparative studies with DC, those are not sharing the same letter (a-f) are significantly different.

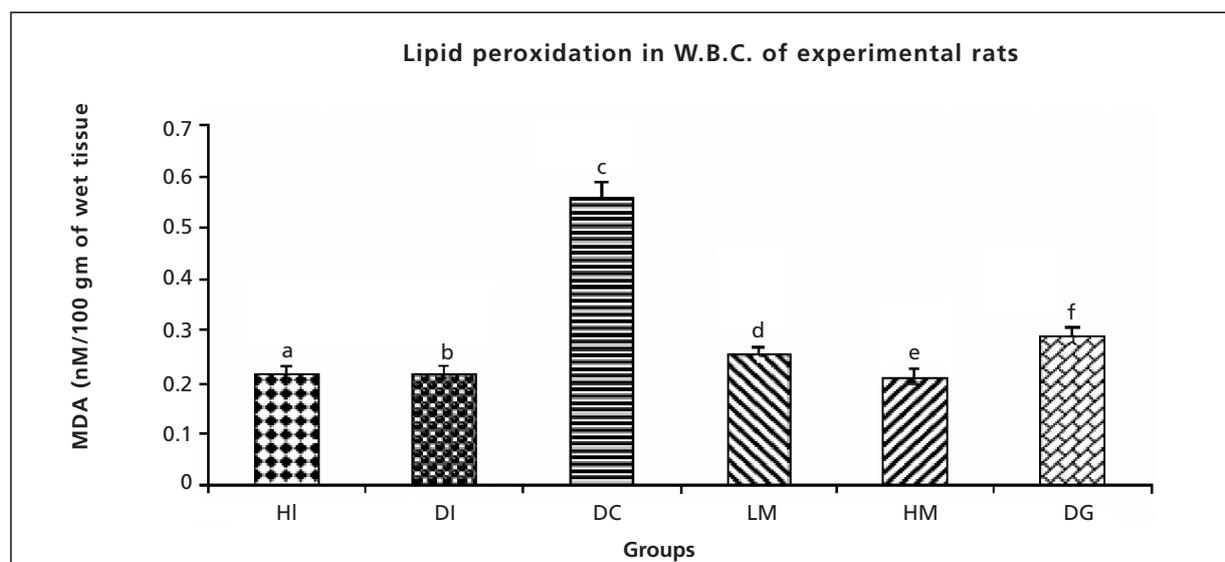


Figure 4. Values are means + S.E. (n=6 animals/groups) in W.B.C. of experimental animals. Results were considered significant between the groups at $p < 0.001$. In a comparative studies with DC, those are not sharing the same letter (a-f) are significantly different.

and kidney of diabetic animals³⁰. Over production of free radicals could be due to increased auto oxidation of blood glucose that is at a higher level in DM. Higher oxidative stress in diabetic animals necessitates more GSH utilization hence its decreased level in DM condition^{31,32}. Glutathione system (GPx, GSH, Gred), a major antioxidant defense against H₂O₂ and lipid peroxides, observed to be at decreased level in liver and kidney of streptozotocin (STZ) induced rats after 3 weeks in the liver of diabetic rats^{33,34}.

Several workers have reported increased GPx when experimental animals were subjected to oxidative stress (OS) and then treated with herbal extract containing antioxidants³⁶. Protection rendered by GPx against OS is demonstrated by Tanakashi et al using adenoviral technique to over express GPx in beta cells. SOD, an important anti-oxidant required to sustain life in aerobic conditions has shown reduced activity in several organs (liver, kidney, brain, spleen) of STZ treated rats after 4 months³⁷⁻³⁹. Similarly our studies have shown an increase in antioxidant enzyme levels such as SOD and GPx which scavenge the free radicals and so inturn decrease glucose level and complications associated with DM. Decreased activity of SOD in DM is attributed to glycation of SOD caused because of increased blood glucose level. Treating of laboratory diabetic rats with *TFG* leaf extract for 6 weeks

elevated SOD to normal levels in the present investigation, a clear indicator of antioxidant property of *TFG*. MDA, a 3 Carbon chain aldehyde is often used as a biomarker for lipid peroxidation. High levels of MDA in diabetic rats and decreased level in treated groups is observed in our experiment which is supported by many other workers⁴⁰.

Conclusion

In conclusion, the administration of *TFG* leaf extract has an extremely beneficial role in overcoming the occurred adverse effects of diabetes, which is probably through its excellent antioxidant properties and highly nutritional values.

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