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Research article

Tribulus terrestris fruit extract improves antioxidant defense in female reproductive tract: A comprehensive study in diabetic rats

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Key words: *Tribulus terrestris*, ethanolic fruit extract, antioxidant potential, female reproductive tract, diabetes.

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Abstract

Objectives: The role of reactive oxygen species (ROS) within female reproductive system is complex and oxidative stress can contribute to infertility, given the fact that diabetes mellitus strongly affects fallopian tubes, ovaries and uterus. *Tribulus terrestris* (family-Zygophyllaceae) is known for medicinal value and fruit is rich in saponins, flavonoids and antioxidants. In this study the antioxidant potential of *Tribulus terrestris* fruit (TTF) on diabetic female reproductive tract is assessed. **Material and methods:** Wistar strain, female rats were induced diabetes by STZ (45 mg/kgbw) and supplemented with graded doses of TTF ethanolic extract (50-250 mg/kgbw) for 30-days to measure the counter effects. Studies were directed to evaluate diabetes caused changes in blood glucose, body weight and antioxidant enzyme activities in female reproductive tract upon exposure of phytoextracts. **Results and Conclusions**: Normalcy in body weight and blood glucose levels was evident upon TTF supplementation on and beyond 19th-day of extract exposure and among supplemented doses 200 mg/kgbw found to be efficient to quench the free radicals and ameliorated the status of antioxidant enzymes viz., SOD, CAT, GPx, GST and GSH. TTF extract in 200 mg/kgbw dose improves endogenous antioxidant defense system, thereby recommended for therapeutic use as an alternative to current modalities for the management of diabetes.

Introduction

Diabetes mellitus (DM), an endocrine metabolic disorder of multiple etiologies is characterized by chronic hyperglycemia with impaired carbohydrate, fat and protein metabolism resulting from abnormal insulin production/action. DM ranks fourth in prevalence worldwide among the diet-related non-communicable chronic diseases [1]. The global incidence of DM reached 387 million in 2014 and expected to increase to 592 million by 2035 [2]. Scientific evidences confirmed that chronic hyperglycemia causes increased production of reactive oxygen species (ROS) in all tissues through glucose autoxidation and protein glycosylation resulting in elevated oxidative stress and diabetic complications [3-5]. In addition, repercussions of oxidative stress in the pathogenesis of DM have been confirmed not only to generate oxygen free radicals but also alter endogenous antioxidant enzymes and the formation of lipid peroxides that cause infertility issues in females. Although a number of pharmaceutical agents are beneficial in treating the symptoms of the DM, their usage may cause unwanted side-effects. Apart from dietary restrictions and allopathic medication, phytochemicals originating from plants are shown to be of great help in minimising the risk of side effects caused by medication as well as progression of disease. A scientific validation of several plant species has proven the efficacy in reducing the hyperglycemia caused complications and restoring the integrity of pancreatic β-cells. Though several plants used in traditional medicine have gained importance, many remain to be scientifically investigated.

Tribulus terrestris (TT) known as Gokhru, a perennial creeping herb of family Zygophyllaceae and is being used for generations as folk medicine to treat several ailments by tribal. The fruit of this herb has wonderful aphrodisiac potential in male rats and work has been explored by previous studies (Hemalatha and Rajeshwari Hari, 2015; EI-Tantawy and Hassanin, 2007) [6, 7]. In our previous study we have reported presence of saponins and flavonoids in the fruit extract of TT [8]. In this study an attempt has been made to assess the ameliorative efficacy of *Tribulus terrestris* fruit (TTF) excract on hyperglycemia caused oxidative stress indices in discrete regions of female reproductive tract as no attempt has been made till date on female rats.

Experimental

Materials and methods

Chemicals

Streptozotocin (STZ), trichloroacetic acid (TCA), nicotinamide adenine dinucleotide (NADH), 1-chloro- 2, 4-dinitrobenze (CDNB), 5, 5 -dithio-bis (2-nitrobenzoic

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acid) (DTNB) and epinephrine were obtained from Merck India Ltd, Mumbai.

Plant material and extraction

Collected *Tribulus terrestris* fruits from Bangalore University campus were cleaned and shade dried for 25-30 days at room temperature, crushed to fine powder and subjected to exhaustive 70% ethanol extraction using soxhlet apparatus and the filtrate stored. The authentication of *Tribulus terrestris* fruit was confirmed by 'Taxonomists Internal Committee' (TIC), Department of Botany, Bangalore University wide herbarium maintained No: BZD 01 during 2013-14.

Animals

Experiments were conducted with strict compliance to ethical principles and guidelines formulated by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and performed in accordance with the Institutional Animal Ethical Committee (IAEC) of Bangalore University, Bengaluru, India (CPCSEA No. 402, File No. 25/525/2009 dated 23.03.2011).Three-month old female albino rats Wistar strain (*Rattus norvegicus albinus*), weighing 180–200 g were procured from Sri Raghavendra Enterprises, Bengaluru and animals were housed in a polyethylene cages, acclimatized to standard laboratory conditions of 12-12 hr light and dark cycle, temperature 25±2ºC and with standard feed pellet and to water *ad libitum*.

Experimental design

Induction of experimental diabetes

Diabetes was induced to female rats by intraperitoneal injection of freshly prepared streptozotocin (STZ) at a dose of 45 mg/kgbw dissolved in 0.1M citrate buffer (pH 4.5). Three days-post STZ administration, blood samples were drawn from tail vein and glucose levels were tested and rats confirmed diabetic when their fasting blood glucose levels were more than 200 mg/dL were selected for the study and made into three groups and number of animals in each group was restricted to six (n=6). To check the dose response and efficacy of phytoextracts on hyperglycemia, five grades of ethanolic extract of TTF prepared in 1ml having 50, 100, 150, 200 and 250 mg/kgbw/day for 30 days were given to diabetic animals (group III) using oral gavage tube from 5th day of STZ administration and blood glucose levels were measured on 5th, 10th, 15th and 30th day of TTF exposure by tail vein puncture from overnight fasted animals. Control and experimental group (I, II and III) animals were euthanized by spinal dislocation under 1% pentobarbital sodium (0.4 ml/100gbw) anesthesia and reproductive tissues viz., uterus, ovary and oviduct were isolated and homogenized in requisite buffers. Upon centrifugation, the aliquots were used to determine following biochemical parameters connected to oxidative stress.

In vivo antioxidant assays

The biochemical estimations were performed spectrophotometrically using Jenway-6405(UV/VIS) Spectrophotometer by adopting suitable methods as indicated.

Lipid peroxidation (LPO)

Lipid peroxidation product was estimated by measuring thiobarbituric acid reactive substance (TBARS) using the method given by Niehaus and Samuelsson (1968) [9]. The pink chromogen produced by the reaction of the thiobarbituric acid with malondialdehyde (MDA), a secondary product of lipid peroxidation was estimated at 535 nm. Results are expressed as µmol MDA formed/mg protein.

Superoxide dismutase (SOD)

SOD activity was assayed by measuring the inhibition of epinephrine auto-oxidation as described by Misra and Fridovich (1972) [10]. The absorbance was recorded at 480 nm for 60s. Results are expressed as µmol/min/mg protein.

Catalase (CAT)

CAT activity was measured as described by Aebi (1984) [11]. The rate constant of hydrogen peroxide (H_2O_2) decomposition was monitored by measuring the decrease in absorbance at 240 nm for 60s. Results are expressed as µmol H_2O_2 consumed/mg protein.

Glutathione peroxidase (GPx)

GPx activity was estimated by measuring the oxidation of NADPH as described by Lawrence and Burk (1972) [12] and change in absorbance was measured at 340 nm. Results are expressed as nmol NADPH oxidized/min/mg protein.

Glutathione-S-transferase (GST)

GST activity was estimated by the method of Habig *et al.*, (1974) [13] by following the increase in absorbance at 340 nm using 1-chloro-2, 4-dinitrobenze (CDNB) as substrate. The assay conducted by monitoring the appearance of conjugated complex of CDNB and GSH, viz., 2, 4-dinitrophenyl glutathione at 340 nm. Results are expressed as µmol 2,4-dinitrophenyl glutathione formed/ min/mg protein.

Reduced glutathione (GSH)

Reduced glutathione content was determined using 5, 5' -dithio-bis (2-nitrobenzoic acid) (DTNB) as fluorescent reagent according to the method given by Ellman (1959) [14]. GSH levels were monitored at 412 nm and results are expressed as µmol GSH/mg protein.

Protein assay
Protein content was estimated by the method of Lowry *et al.*, (1961) [15], using bovine serum albumin (BSA) as standard.

Statistical analysis
Results are shown as mean ± SEM of six measurements. One-way Analysis of Variance (ANOVA) with post hoc test was performed for the inter-group comparisons using Bonferroni test at probability (P) value 0.05 level of significance by SPSS software 20.0. Graphs were plotted using 'Origin Pro' software 8.0.

Results and discussion

Results
Blood glucose levels and body weight
Among TTF supplemented doses, only 200 mg/kgbw dose curbed the elevated blood glucose levels significantly (P<0.05) on day 19th of administration and further remained constant till day 30th suggesting the effective dose required to normalize the hyperglycemia, while the experimental rats gained body weight suggesting anabolic effect (Figure 1 and 2).

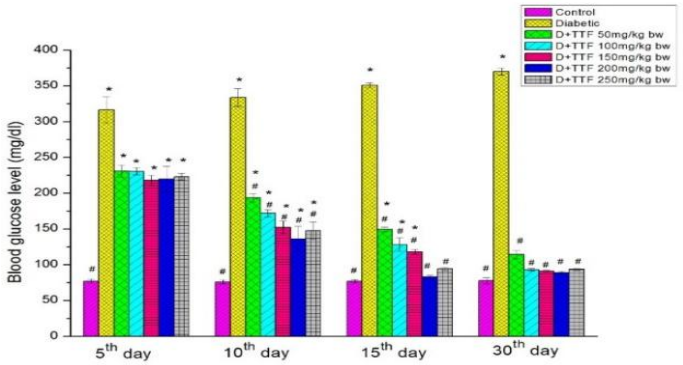


Figure 1. Effect of *Tribulus terrestris* fruit (TTF) extract (50-250 mg/kgbw/day) on blood glucose levels (mg/dl) in STZ induced diabetic female rats. Values are mean ± SEM (n=6), significant at P<0.05 as compared to * normal control rats; # diabetic (D).

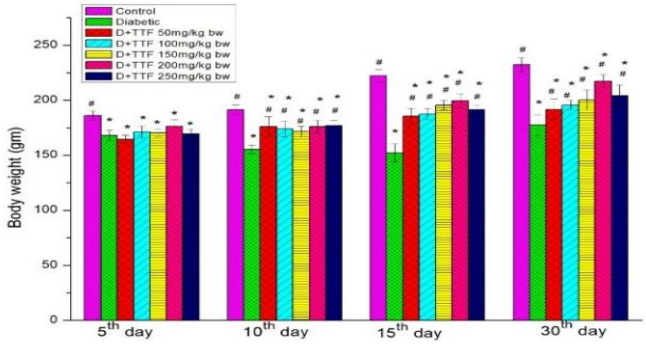


Figure 2. Effect of *Tribulus terrestris* fruit (TTF) extract (50-250 mg/kgbw/day) on body weight (gm) in STZ induced diabetic female rats. Values are mean ± SEM (n=6), significant at P<0.05 as compared to * normal control rats; # diabetic (D).

In vivo antioxidant activity
LPO
In diabetic rats, a significant (P<0.05) increase in MDA levels was apparent in discrete functional tissues studied. In comparison, uterus and ovary had high elevations in MDA content than oviduct. Among TTF supplemented doses, only 200 mg/kgbw dose curbed high MDA levels while a higher rate of amelioration was evident in ovary than uterus and oviduct (Figure 3a).

SOD activity
Diabetes caused decrements in SOD activity was evident in rat functional tissues studied. In comparison, oviduct had high suppressions than uterus and ovary. Among TTF supplemented doses, 150 and 200 mg/kgbw showed high efficacy in normalizing the affected SOD levels in oviduct followed by uterus and ovary (Figure 3b).

CAT activity
There was a marked decrease in CAT activity in all the functional tissues studied as a consequence of induced diabetes. Uterus and ovary showed highest decrements in CAT activity than oviduct. Among TTF supplemented doses, only 200 mg/kgbw dose exhibited maximum amelioration in uterus followed by ovary and oviduct (Figure 3c).

GPx activity
With regard to GPx activity, differential response was evident in functional tissues of female reproductive tract upon STZ exposure. Uterus showed higher supressions in GPx activity followed by ovary and oviduct. Among TTF supplemented doses both, 150 and 200 mg/kgbw showed high efficacy in normalizing the affected GPx levels in oviduct and ovary than uterus (Figure 3d).

GST activity
A significant (P<0.05) decrease in the GST activity was observed in diabetic rat reproductive tissues and uterus affected to a greater extent than the ovary and oviduct in STZ treated rats. Among TTF supplemented doses, only 200 mg/kgbw dose augmented the inhibitions found in the activity levels of GST in oviduct and ovary, however uterus showed considerable amelioration by TTF supplementation (Figure 3e).

GSH levels
In diabetic rats, oviduct was severely affected by exhibiting higher suppressions in GSH levels than uterus and ovary. A dose-dependent augmentation in GSH was evident upon TTF extract supplementation in all the tissues studied, however the effect was more in oviduct followed by ovary and uterus (Figure 3f).

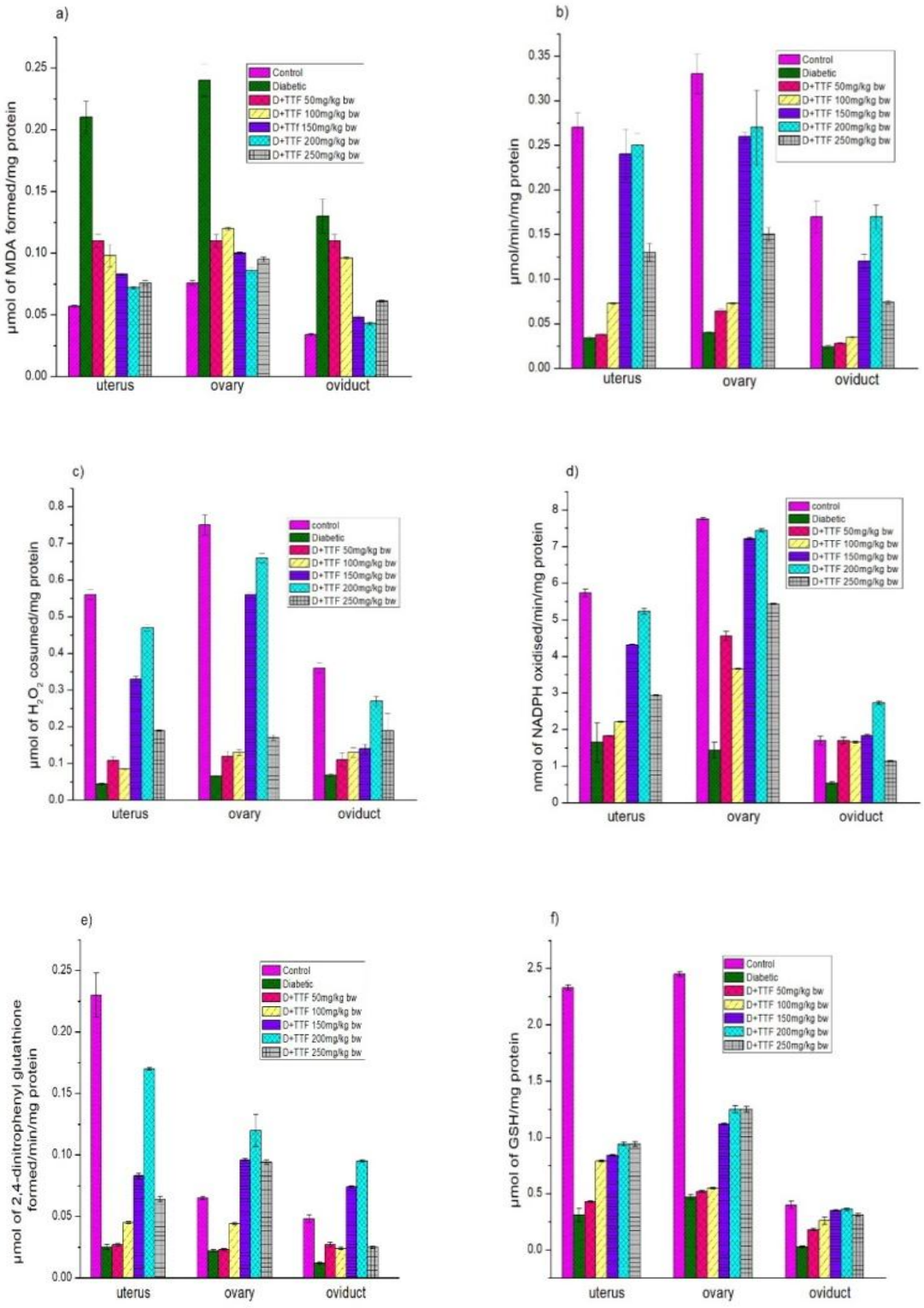


Figure 3. Effect of *Tribulus terrestris* fruit (TTF) extract (50-250 mg/kgbw/day) on oxidative stress indices a) LPO b) SOD c) CAT d) GPx e) GST f) GSH content in uterus, ovary and oviduct of STZ induced diabetic rats (D). Values are mean ± SEM (n=6); significant at P<0.05 compared to control.

Discussion

Streptozotocin, a natural diabetogenic agent is known to induce permanent diabetes in animal models by damaging pancreatic β-cells that stops insulin production [16]. In this study, STZ administration to rats caused elevation of plasma glucose levels which in turn found to be responsible for the induction of oxidative stress in female reproductive tract by generating more oxygen free radicals. Thus the impaired glucose metabolism alters the functions of several organs and tissues in the body. Further, STZ administration caused a significant increase in the lipid peroxidation product MDA; its accumulations in turn aggravate mitochondrial oxidative damage to cause changes in oxidative indices. Resultantly a significant decrease in SOD, CAT, GSH levels were evident in discrete areas of female reproductive tract. Increased blood glucose levels, glucose-oxidation, non-enzymatic glycation of proteins and their subsequent degradation cause unbalanced free-radical generation, altered antioxidant defence system and increased lipid peroxidation due to increased lipolysis in absence of insulin thereby decrements in enzymatic and non-enzymatic antioxidants witnessed in reproductive tract which in turn may cause altered levels of LH and FSH [17], faulty ovulation, tubal and diseases [18-19]. By employing different antioxidant assays such as DPPH, superoxide and hydrogen peroxide radical scavenging ability, in our previous communication [8] we have reported that the total phenolic and flavonoid contents of TTF extract is good source of antioxidants and helps to curb free radicals such as ROS/RNS thereby highly recommended as substitute to handle oxidative damage. In this study TTF extract was subjected in various doses to STZ treated female rats to check the ameliorative efficacy in discrete reproductive tract. In response, the elevated glucose levels were significantly lowered in a dose-dependent manner and restored the serum glucose levels to normal in diabetic rats and results are in accordance with other reports [7-20]. The mechanism of hypoglycemic action of TTF extract may be either by increasing the peripheral utilization of glucose or by stimulating the secretion of insulin by the remaining intact pancreatic β-cells after the destruction of the pancreas or insulin-like action of the extract [21]. The major chemical constituents of TTF extract being steroidal saponins and tribulusamide A and B [22] and saponins highlight its hypoglycemic effect by inhibiting α-glucosidase activity in rats [23]. Supplementation of TTF extract to STZ-induced diabetic rats showed a protective effect against weight loss, possibly controlling glycemic level and this effect were characterized by an increase in body weight and results are in accordance with previous reports [24-25]. Elevated serum glucose levels promote overproduction of superoxide (O_2-), H_2O_2 radicals and ROS. Since ROS and oxidative stress are associated as important physiological and pathological mediators in many reproductive disorders and involved in the modulation of an entire spectrum of physiological reproductive functions such as the formation of the endometrium, oocyte maturation, ovarian steroidogenesis, corpus luteal function and luteolysis [26-27]. Hyperglycaemia mediated advanced glycation of intracellular antioxidant enzymes results in hyper susceptibility to the elevated oxidative stress due to lowered anti-oxidative protection and considered to play a vital role in posing adverse effects of diabetes. Further, it is well established from the findings of Hala *et al.*, (2013) [28] that the fruit extract having compositional analysis of the aqueous infusions of two oligosaccharides including polyphenols as main components and a stereoisomer of di-p-coumaroylquinic acid in aerial parts wherein authors reported strong DPPH radical scavenging activity from this component. In our previous communication [8] we have reported strong DPPH radical scavenging activity from fruit extract of this plant; thereby presence of active derivatives of 4,5-di-p-coumaroylquinic acid, offer protection by suppressing the DPPH radical to diabetic rats.

LPO

Studies suggest that the tissue content having a relatively high concentration of easily peroxidizable fatty acids and increased activities of enzymes like fatty acyl coenzyme, co-enzyme A oxidize due to hypoinsulinemia which initiates the oxidation of fatty acids resulting in lipid peroxidation [29]. In the present study, elevated LPO in uterus, ovary, and oviduct tissues indicate an increase in oxygen free radicals in diabetes could be due to augmented blood glucose levels, which upon auto-oxidation generate free radicals and oxidative stress that may cause extensive cellular damage unless it is arrested by certain protective agents. The protective actions of TTF extract found to be effective against increased MDA and among supplemented doses, only 200 mg/kgbw curbed the high MDA levels while a higher rate of amelioration was evident in ovary than uterus and oviduct (Figure-3a) indicating differential sensitivity among tissues.

SOD activity

Superoxide dismutase is one of the most important enzyme(s) in the enzymatic antioxidant defense system. It removes superoxide anion by converting it to hydrogen peroxide and thus diminishing the toxic effect caused by this radical. The altered balance of the antioxidant enzymes with a decrease in SOD activity may be due to increased production of superoxide radicals by the auto-oxidation of glucose and non-enzymatic glycation. In comparison, oviduct had high suppressions than uterus

and ovary and this depicts the inactivation of the enzyme by superoxide anions and their association with reproductive tract. Among TTF supplemented doses, 150 and 200 mg/kgbw showed high efficacy in normalizing the affected SOD levels in oviduct followed by uterus and ovary (Figure-3b) this could be due to differential ability of tissues in handling the radical effects.

CAT activity

Catalase being effective antioxidant enzyme widely distributed in the animal tissues and it decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [30]. The decreased activity of this enzyme in reproductive organs may lead to deleterious effects as a result of superoxide and hydrogen peroxide assimilation. In the present study, the inhibited CAT activity found to be augmented significantly by administration of TTF extract especially at 200 mg/kgbw dose.

GPx activity

GPx plays a crucial role in H_2O_2 catabolism and cells lacking or deficits in its content found to be susceptible to oxidative stress [31]. In the present study, induction of diabetes caused a remarkable decrease in the GPx activity in uterus, ovary and oviduct tissues indicating the extent of cellular damage caused by oxidative stress and inability of enzyme to check it. These alterations caused could be due to their difference in cell types, composition, function and sensitivity. Noticeable increase in GPx activity was observed in all the functional tissue samples of diabetic rats upon TTF supplementation at a dose of 200 mg/kgbw.

GST activity

This enzyme is involved in the binding, transport, and detoxification as well as cellular defence. Increased free radicals in reproductive tract might have enforced GST detoxification resultantly decreased its activity to a significant level. Supplementation of TTF extract, especially a dose of 200 mg/kgbw, found in restoring the GST activity and has brought the enzyme level near to normal by controlling the free radical generation during diabetes.

GSH levels

Reduced glutathione, a tripeptide, non-enzymatic biological antioxidant presents in the reproductive organs and it plays an important role in detoxification process and metabolism as a co-factor or as a substrate for enzymes and as an antioxidant agent in protecting the tissue from oxidative stress.GSH also protects cellular proteins against reactive oxygen species generated by diabetes induction. A significant ($P<0.05$) decrease as found in the level of GSH in discrete areas of reproductive tract indicates the extent of damage caused to antioxidant enzymes and results are in accordance with earlier reports [32]. Elevated GSH levels in uterus, ovary and oviduct samples upon TTF supplementation may help in offering protection to cellular proteins against oxidation through glutathione redox cycle.

Conclusion

Uncontrolled diabetic state cause disruptions in antioxidant balance and bring impairments in female reproductive tract by inducing oxidative stress. In a nutshell, the results demonstrate that the total phenolic and flavonoid contents of TTF extract are good source of antioxidants. In addition, presence of active derivatives of 4,5-di-p-coumaroylquinic acid, offer protection to diabetic rats and exogenous supplementation of TTF extract especially at 200 mg/kg bw dose found effective and encounter free radicals as well as reactive oxygen species by strengthening cellular antioxidant defence and improve reproductive functional ability in diabetics.

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Conflicts of Interest

There are no conflicts of interest.

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