Journal of Advanced Zoology

ISSN: 0253-7214

Volume ***44*** *Issue* ***-01 Year 2023*** *Page* ***63:78***

COLD STRESS INDUCED MODULATIONS ON ANTIOXIDANT STATUS OF STZ INDUCED DIABETIC FEMALE RAT REPRODUCTIVE SYSTEM: A PROTECTIVE ROLE OF *TRIBULUS TERRESTRIS* FRUIT EXTRACT

Dakshayini P N[1*], Madhusudan S[2], Bhushanam M[3] , Mahaboob Basha P [4] and Arun Jyothi Mathias[5]

[1, 3]Associate Professor, Department of Zoology, Maharani's Science College for Women, Maharani Cluster University, Palace Road, Bangalore, Karnataka*

[2]Associate Professor, Department of Biotechnology, Maharani's Science College for Women, Maharani Cluster University, Palace Road, Bangalore, Karnataka[2,]

[4]Professor, Department of Zoology, Jnanbharathi Campus, Bangalore University, Bangalore

[5]Professor, Department of Microbiology, Maharani's Science College for Women, Maharani Cluster University, Palace Road, Bangalore, Karnataka

****Corresponding Author***

E-Mail: dakshayinipn@gmail.com

Article History

Received:14 Jun2023

Revised:05 Aug2023

Accepted:26 Aug 2023

ABSTRACT: Diabetes mellitus induced oxidative stress could lead to the impairment of female reproductive antioxidant defense mechanism and low temperature exposure leads to oxidative stress as well. There is a positive relationship between hypothalamic noradrenergic neuronal activity and blood glucose concentrations under stressful conditions. Treatment of female reproductive disorders using herbal remedies is gaining popularity in human medicine. In light of this, the study was designed to investigate the effectiveness of TTF ethanol extract in STZ-induced diabetic female rat reproductive system exposed to cold stress and to evaluate their therapeutic potential for the treatment of cold stress induced modulations on antioxidant status of DM. Diabetic rats exposed to cold stress by housing in an acute cold stress apparatus at 4±2 °C for 3hrs per day for 7 days later they were supplemented with TTF extract at a dose of 200mg/kgbw. To check the efficacy of TTF on oxidative stress indices in uterus, ovary and oviduct, the animals were sacrificed on the 19th day of TTF extract exposure and subjected to in vivo biochemical and hormonal assays. The findings of this study confirm the deleterious effect of diabetes and cold stress on the reproductive organs weight, antioxidant defense system and hormones of female reproductive organs viz uterus, ovary and oviduct. Supplementation of TTF (200mg/kgbw) extract to diabetic rats exposed to cold stress, the serum glucose levels decreased significantly ($P<0.05$) and significantly increased the weights of reproductive organs and serum oestrogen, FSH and LH levels when compared with the intoxicated control groups. Diabetes toxicity and cold stress in the female reproductive organs may result in disruption of oxidants and antioxidants balances, which provides a strong coupling of altered equilibrium processes and loss of energy capacity to meet an oxidation challenge. Moreover, exposure to diabetes and cold stress can increase the effects of oxidative stress. Exogenous supplementation of TTF extract has been found to counter free radical generated oxidative stress and to facilitate reduction of the toxic effects induced by diabetes and cold stress, there by strengthening the cellular antioxidant defense and improved reproductive functioning ability.

KEYWORDS: Diabetes, Cold stress, Antioxidants, Hormones, *Tribulus terrestris frui*

CC License CC-BY-NC-SA 4.0

INTRODUCTION

Diabetes mellitus (DM), a chronic metabolic and endocrine disorder affecting many people around the world and the number of diabetic subjects expected to increase by 592 million in year 2035[1]. DM is characterized by chronic hyperglycaemia due to abnormal insulin secretion or insulin receptor or post-receptor events affecting metabolism of carbohydrate, protein, and fats[2]. Moreover, as a consequence of hyperglycaemia, abnormally high levels of free radicals and decline of antioxidant defence systems have also been reported[3]. Chronic hyperglycaemia evokes oxidative stress by a variety of mechanisms including increased advanced glycation end-products formation, polyol pathway flux, glucose autoxidation and mitochondrial superoxide overproduction that eventually results in diabetes complications including reproductive disorders. DM induced oxidative stress which known to be involved in the normal human physiological functions that could lead to the impairment of reproductive antioxidant defense mechanism. STZ induced diabetes, which causes severe insulinopenia, have revealed that both male and female rats with uncontrolled diabetes display a profound hypogonadotropic state, characterized by low basal levels of gonadotrophins and sex steroids, reduced luteinizing hormone (LH) pulsatility and defective gonadotrophin responses to gonadectomy[4-8]. In diabetic females, disruption of positive-feedback effects of estradiol, delayed or absent preovulatory LH surges and anovulation are observed[4].

Stress as an adaptive response of the body, produces a wide range of biochemical and behavioural manifestations to respond to a threat[9-11] and is known to alter the physiological homeostasis of the organism and complex mechanisms contributing to the breakdown in adaptation processes[12]. It has been postulated that stress is involved in etiopathogenesis of a variety of diseases like depression and anxiety, immunosuppression, endocrine disorders, and male potency[13]. Minehiro Gotoh et al.[14] findings support the idea that hypothalamic noradrenergic neurons play an important role in the sympathoadrenal hyperglycaemic response to stressful stimuli and elevation of plasma glucose[15]. Moreover, studies have been proved that low temperature exposure leads to oxidative stress[18] and there is a significant positive relationship between hypothalamic noradrenergic neuronal activity and blood glucose concentrations under stressful conditions[16,17]. Hans Selye[19], suggested that chronic exposure to stressors increases Hypothalamus-Pituitary-Adrenal (HPA) axis activity and concomitantly reduces Hypothalamus-Pituitary-Gonadal (HPG) axis activity, and this antagonistic relationship between glucocorticoids and gonadal hormones has been consistently observed. Cold stress increased ovarian norepinephrine (NE) levels and induced ovarian function alterations, which led to polycystic ovarian morphology in rats[20-22] and also induced the formation of follicular cysts and follicles with hyper- thecosis alongside increased plasma estradiol and testosterone levels, irregular estrous cyclicity, and reduced ovulation[23].

Plants often contain substantial amounts of antioxidants, and suggest that antioxidant action may be an important property of plant medicines associated with diabetes. The increasing interest in various medicinal plants and their bioactive compounds has led to increased attention to their safety and efficacy in the treatment of diabetes and also alternative treatment of female reproductive disorders using herbal remedies is gaining popularity in human medicine. *Tribulus terrestris* L. (TT) from Zygophyllacea family, is an annual herb that grows worldwide, especially in the subtropical regions, and is used in traditional medicines in India, China, South Africa, Bulgaria and other countries against sexual impotency, edema,

abdominal distention, and cardiovascular diseases. The main constituents of TT include saponins, diosgenins, alkaloids and amides. In modern pharmacological studies, reported that *Tribulus terrstris* fruit (TTF) ethanol extract was found to be effective in treating streptozotocin-induced hyperglycemia[24,25]. Further studies also disclosed that TT improved reproductive function, including increased concentration of hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol, with significant effect on both ovarian and uterine activities, thereby improving reproductive performance, libido and ovulation[26,27]. Even though preliminary studies disclosed that TT extract has a luteinizing effect in treated immature female rats and enhances puberty[28], there is limited information regarding the effects of TT on the female reproductive system, and also lack of literature supporting the antioxidant potential and oxidative status on consumption of the TTF extract in diabetic subjects exposed to low varied temperatures. In light of this, the study was designed to investigate the effectiveness of TTF ethanol extract in STZ-induced diabetic female rat reproductive system exposed to cold stress and to evaluate their therapeutic potential for the treatment of cold stress induced modulations on antioxidant status of DM.

MATERIALS AND METHODS

Plant material and extraction

Tribulus terrestris fruits were cleaned and shade dried for around 25-30 days at room temperature and then crushed to fine powder. The plant sample was subjected to exhaustive 70% ethanol extraction using soxhlet apparatus and the extract was stored in an airtight container.

Animals

Experiment was 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, Bangalore, India. Three-month-old female albino rats Wistar strain (Rattus norvegicus albinus), weighing 180–200g were procured from Sri Raghavendra Enterprises, Bangalore. Animals were housed in a polyethylene cages and acclimatized to standard laboratory conditions of 12-12hr light and dark cycle, temperature 25±2 ºC and with standard feed pellet and free access to water *ad libitum.*

Induction of experimental diabetes mellitus

Diabetes mellitus was induced in rats by the intraperitoneal injection (volume of 1ml/kg body weight) of freshly prepared streptozotocin (STZ) at a dose of 45 mg/kg body weight dissolved in 0.1 M citrate buffer solution of pH 4.5[29]. Three days after the STZ injection, the blood was withdrawn from the tail vein, and the glucose level was determined. Rats were diabetic when their fasting blood glucose levels were more than 200 mg/dL.

To check the dose response on hyperglycemia, five grades of TTF ethanol extract in 1ml having 50,100,150,200 and 250mg/kg body weight/day for 30 days were given in the pilot study to diabetic animals using oral gavage tube on 5th day of STZ administration respectively. To assess the anti-hyperglycemic effect of TTF extract, blood was collected by tail vein puncture from overnight fasted animals and the blood glucose levels were measured on 5th, 10th, 15th, and 30th days of TTF extract exposure.

Induction of cold stress

To induce cold stress, rats were housed in an acute cold stress apparatus (Colton BOD incubator) at 4±2 °C for 3hrs per day for 7 days on a 12-h light/12-h dark cycle with a built-in heater and cooler that could be controlled by self-timer.

Supplementation of extract

Among the examined doses through pilot study (50-250mg/kg bw/day), the most effective dose of TTF extract was 200mg/kg, to check the efficacy of TTF extract on oxidative stress indices in uterus, ovary and oviduct, the animals were sacrificed after 19 days of TTF exposure as the extract has shown anti-hyperglycemic effect on the 19th day of TTF extract exposure.

Experimental Design

Rats were divided into seven groups: Group I— control animal group was kept at the laboratory room temperature; Group II—induced diabetes; Group III—exposed to cold stress at 4±2 ºC; Group IV—diabetic rats exposed to cold stress at 4±2 ºC; Group V—induced diabetes plus supplemented with TTF; Group VI—exposed to cold stress at 4±2 ºC plus supplemented with TTF; Group VII —diabetic rats exposed to cold stress at 4±2 ºC plus supplemented with TTF. The body weight of all the animals was recorded during and before the termination of experiment and rats were sacrificed, the blood samples were collected and used for the reproductive hormone analysis. Dissected reproductive organs viz uterus, ovary and oviduct tissues were washed in ice-cold saline, patted dry. The tissue homogenates were made by using appropriate buffer and supernatant was stored at a temperature of -20 ºC and used for biochemical assays.

***In vivo* Biochemical assays**

The biochemical estimations were performed spectrophotometrically using Jenway-6405(UV/VIS) Spectrophotometer by the following methods;

Lipid peroxidation (LPO) product was estimated by measurement of thiobarbituric acid reactive substances using the method of Niehaus WG & Samuelsson B[30]. The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde (MDA), a secondary product of lipid peroxidation, was estimated at 535 nm.

Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of epinephrine auto-oxidation as described by Misra HP & Fridovich I[31]. The absorbance was recorded at 480 nm for 60s.

Catalase (CAT) activity was measured as described by Aebi H[32], and the decomposition of hydrogen peroxide (H_2O_2) was monitored by measuring the decrease in absorbance at 240 nm for 60s.

Glutathione peroxidase (GSH-Px) activity was determined by the method of Lawrence RA & Burk RF[33], by measuring the oxidation of NADPH at 340 nm.

Glutathione-S transferase (GST) activity was estimated by the method of Habig WH et al.[34], by following the increase in absorbance at 340 nm using 1-chloro-2,4-dinitrobenze as a substrate.

Reduced Glutathione Reduced glutathione (GSH) content was determined by the method of Ellman GL[35], on the development of yellow color while adding DTNB to compounds containing sulphydryl groups. GSH levels were monitored at 412 nm.

Protein content was estimated by the method of Lowry OH et al.[36], using bovine serum albumin as a standard.

Hormonal Assay

Serum concentrations of follicle stimulating hormone (FSH) and luteinising hormone (LH) were measured by radioimmuno assay method estrogen and progesterone by chemiluminescence with reagent kit provided by Thyrocare Technologies Ltd, Mumbai, India.

The values are expressed as mean ± standard deviation (SD) with the percent (%) change shown in parenthesis and the % recovery made by the antioxidant against the toxicity level or activity of diabetes and cold stress without addition of antioxidant calculated using the formula,

$$\text{Recovery} = \frac{[\%\ \text{change from positive control} - (\%\ \text{change from the positive control} + \text{Antioxidant supplement}) \times 100]}{\%\ \text{change from positive control}}$$

Statistical Analysis

The results are expressed as mean ± SD of six observations (n = 6) in each group. Differences between treatment groups were assessed by one-way analysis of variance (ANOVA) using the SPSS software package for windows version 20.0. Post hoc testing was for inter-group comparisons using Bonferroni test at probability (P) value 0.05 level of significance.

RESULTS

The findings of this study confirm the deleterious effect of diabetes and cold stress on the body and organs weight, antioxidant defense system and hormones of female reproductive organs viz uterus, ovary and oviduct.

Supplementation of TTF extract to diabetic rats, the serum glucose levels decreased significantly ($P<0.05$) on the 19th day of extract administration and further remained constant till 30th days of TTF exposure, indicating the anti-hyperglycemic properties of the fruit extract of TT, as the substance with anti-hyperglycemic properties would be effective in the management of diabetes (Table 1). Statistical analysis reveal that the serum glucose levels reduced at a dose of 200mg/kg body weight suggesting the ameliorative role of TTF on the 19th day of exposure in extending protection to diabetic animals, compared to other doses. Hence, the present study demonstrates that TTF extract of 200mg/kg body weight dosage was found to be an effective dose.

Table 1- Effect of *Tribulus terrestris* fruit ethanol extract of different doses (50-250mg/kg bw/day) on blood glucose level (mg/dl) in STZ induced diabetic female rats during the experimental period of 30 days. [Values are expressed as mean ± SD (n=6) from each group]

Groups	5th day	10th day	15th day	30th day
Control	77±3.22$^{\#}$	76±2.68$^{\#}$	77±2.25$^{\#}$	77.66±4.13$^{\#}$
STZ	316.33±18.11* (-310.3)	333.66±12.40* (-338.9)	351± 3.09* (-355.8)	370 ±5.08$^{\#}$ (-380.5)
STZ +TTF 50	231±8.53* (+26.9)	193.33±5.95$^{*\#}$ (+42)	149.33±3.14$^{*\#}$ (+57.4)	114.33±5.75$^{\#}$ (+69.1)
STZ +TTF 100	230.66±5.08* (+27)	172±5.04$^{*\#}$ (+48.4)	128±9.46$^{*\#}$ (+63.5)	93±1.78$^{\#}$ (+74.8)
STZ +TTF 150	218±7.32*	152±8.80$^{*\#}$	118±3.22$^{*\#}$	91.33±1.36$^{\#}$

	(+31)	(+54.4)	(+66.3)	(+75.3)
STZ +TTF 200	219.66±17.89* (+30.5)	136.33±17.76*# (+59.1)	83±2.36# (+76.3)	89±1.78# (+75.9)
STZ +TTF 250	223±4.64* (+29.5)	147.66±11.80*# (+55.7)	94.33±2.25# (+73.1)	93.66±1.36# (+74.6)

STZ= Streptozotocin; TTF= *Tribulus terrestris* fruit.
P<0.05 as compared to * normal control rats; # diabetic control. Values in parenthesis indicate the % change & recovery; '+'sign indicates increase and '-'indicates decrease over the controls

Result showed a significant (P<0.05) decrease in body weight gain in all the diabetic groups and increase in cold stressed group was noted, while upon supplementation of TTF extract (200mg/kg body weight) a significant (P<0.05) increase on the body weight gain in diabetic and co-exposed rats was observed when compared with the normal control group is shown in Table 2.

Table 2-Effect of *Tribulus terrestris* fruit ethanol extract (200mg/kg bw/day) on body weight (gm) of diabetic female rats exposed to cold stress (4±2 °C) during the experimental period of 30 days. [Values are expressed as mean ± SD (n=6) from each group]

Group	5th day	15th day	30th day
Control	191.42 ± 4.5	222.32 ± 5.7	232.36 ± 6.3
STZ	185.37 ± 3.9*	152.10 ± 8.2*	177.31 ± 9.6*
STZ + TTF	175.91 ± 5.8*	199.41 ± 6.4*	211.42 ± 5.8*
CS	191.33 ± 3.9	208.11 ± 8.2	227.30 ± 9.6
CS + TTF	205.29 ± 5.8	219.42 ± 4.9	229.21 ± 6.5
STZ + CS	163.21 ± 2.8*	132.21 ± 7.4*	117.43 ± 6.9*
STZ+CS+TTF	169.94 ± 4.6*	148.40 ± 5.4*	178.62 ± 5.5*

STZ= Streptozotocin; CS= Cold stress; TTF= *Tribulus terrestris* fruit.
P<0.05 as compared to * control female rats.

Induction of diabetes and cold stress to rats during the experimental period induced significant (P < 0.05) decrease in weights of reproductive organs viz uterus, ovary and oviducts, whereas rats treated with TTF extract significantly increased the weights of reproductive organs when compared to STZ induced diabetes and co-exposed control groups. No significant changes observed in cold stressed alone when compared to normal control rats and the results are shown in the form of organo somatic index (Table 3).

Table 3 -Effect of *Tribulus terrestris* fruit extract (200mg/kg/bw/day) on organo somatic index in diabetic female rat reproductive system of exposed to cold stress (4±2 °C). [Values are expressed as mean ± SD of six observations]

Groups	Uterus	Ovary	Oviduct
Control	0.27±0.003	0.033±0.005	0.012±0.002
STZ	0.11±0.003*	0.021±0.003*	0.009±0.001*
STZ + TTF	0.15±0.013*	0.023±0.001*	0.010±0.001
Cold stress	0.26±0.004	0.030±0.001	0.012±0.005
Cold stress + TTF	0.25±0.005	0.026±0.005	0.012±0.005

STZ + Cold stress	0.085±0.009*	0.017±0.019*	0.005±0.007*
STZ + Cold stress + TTF	0.12±0.001*	0.023±0.006*	0.009±0.041*
STZ= Streptozotocin; TTF= *Tribulus terrestris* fruit. P<0.05 as compared to * normal control rats.			

In vivo Biochemical assays

MDA level significantly (P<0.05) increased in all the functional tissues in the STZ induced diabetes and cold stress control group and while TTF significantly reduced (P<0.05) the level of MDA when compared with the diabetic and cold stress group. The one-way ANOVA indicated a significant interaction between diabetes toxicity and cold stress treatment performed at 4±2ºC temperature and the exacerbated MDA levels were noticed at diabetes was greatest % than at cold stress in all the functional tissues studied (Fig. 1).

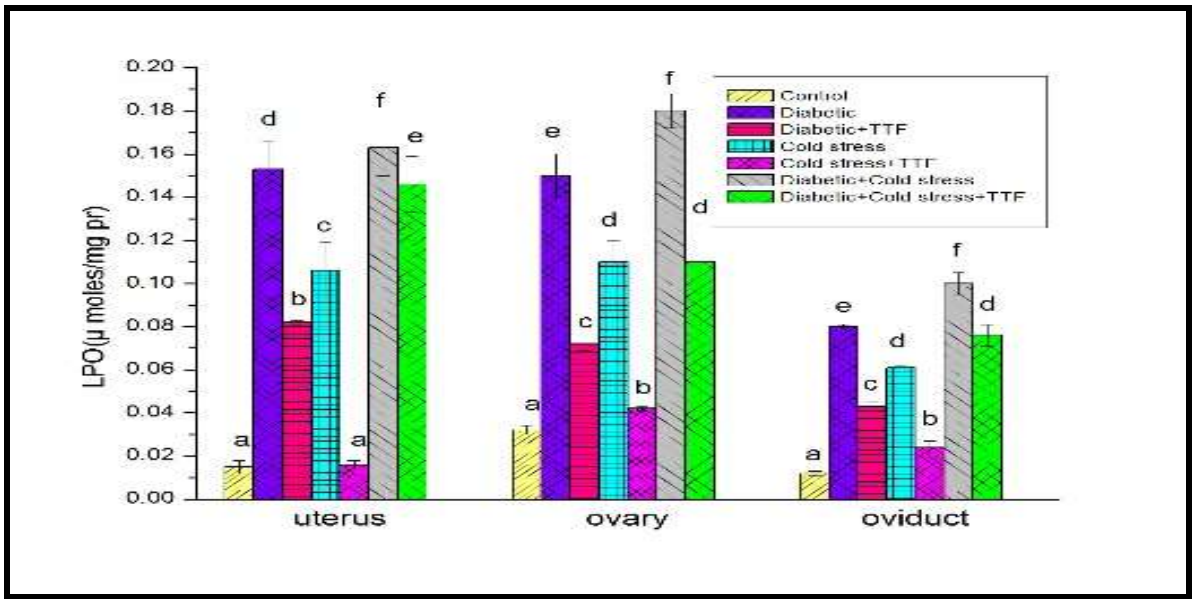


Fig. 1- Effect of *Tribulus terrestris* fruit ethanol extract on lipid peroxidation (LPO) levels in reproductive system (uterus, ovary & oviduct) of STZ induced diabetic female rats exposed to cold stress (4±2 °C). Values are mean ±SD of six observations and a,b,c,d….. letters denote significantly different from control values by one-way ANOVA(P<0.05).

In this study, diabetes and cold stress co-exposure induced decrement in SOD activity was evident in all the functional tissues; however, ovary was severely affected than other tissues studied, showed the greatest decrease in activity (-89.7%). The one-way ANOVA indicated an interaction between diabetes-induced toxicity and cold stress resulting in a significant decrease in SOD levels. In general diabetes and cold stress alone also significantly decreased the SOD levels in all the functional tissues studied (Fig. 2).

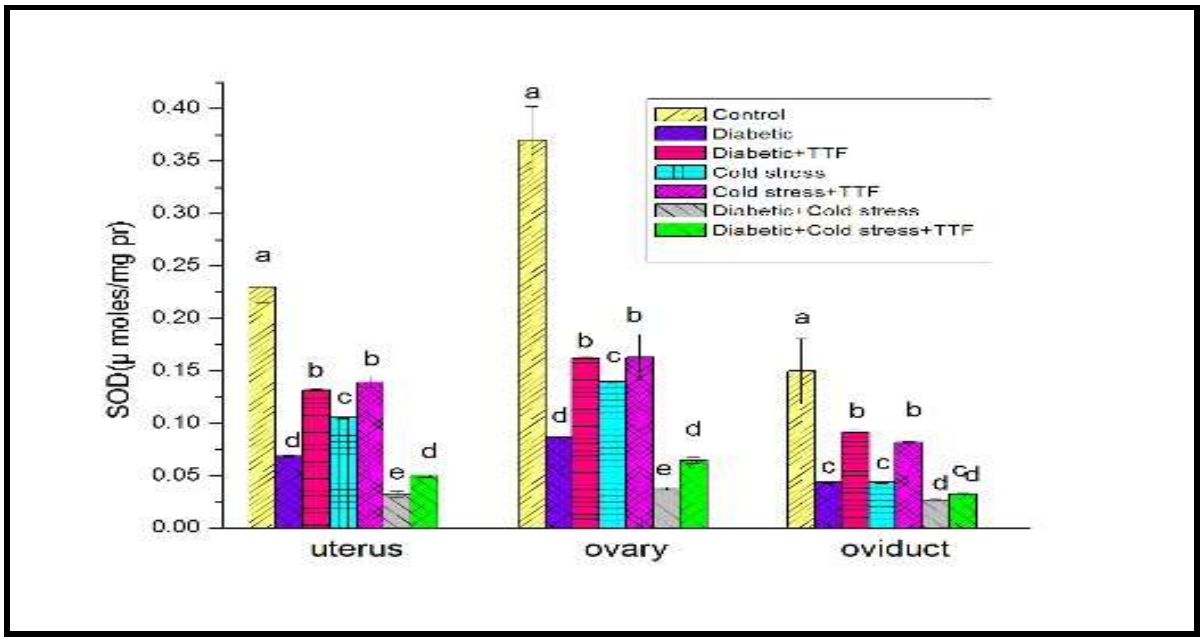


Fig. 2- Effect of *Tribulus terrestris* fruit ethanol extract on SOD antioxidant enzyme activity level in reproductive system (uterus, ovary & oviduct) of STZ induced diabetic female rats exposed to cold stress (4±2 °C). Values are mean ±SD of six observations and a,b,c,d….. letters denote significantly different from control values by one-way ANOVA(P<0.05).

There was a marked decrease in CAT activity as a consequence of severe diabetes. Among the three organs studied in the female rats, the uterus showed the greatest decrease in the activity of CAT (-86.0%) and cold stress treatment at low temperature also significantly decreased CAT levels in uterus while the changes were mild in ovarian and oviduct tissues. The one-way ANOVA indicated an interaction between diabetes toxicity and cold stress treatment performed at low temperature resulting in a significant decrease in CAT levels in all functional tissues studied (Fig. 3).

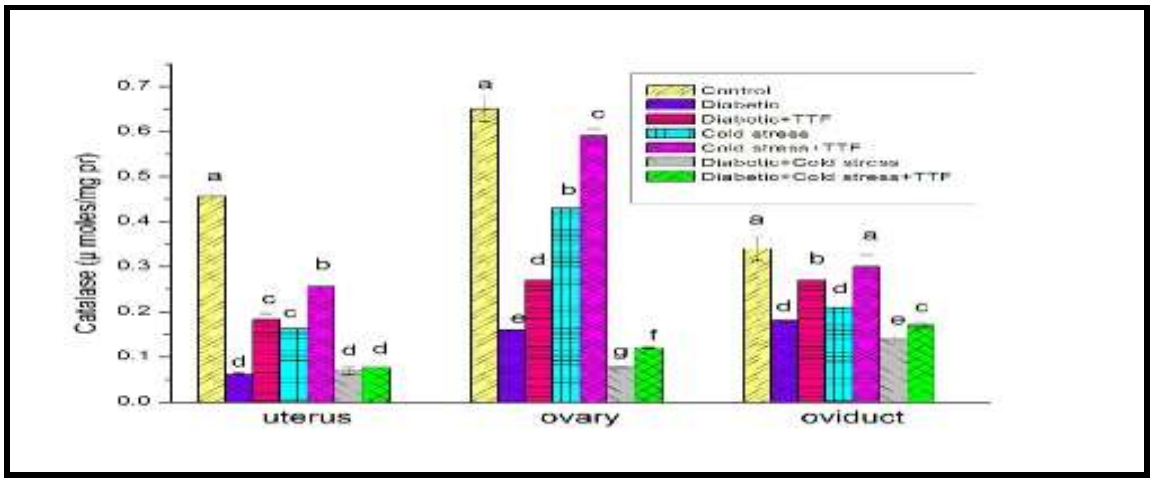


Fig. 3- Effect of *Tribulus terrestris* fruit ethanol extract on Catalase (Cat) antioxidant enzyme activity level in reproductive system (uterus, ovary & oviduct) of STZ induced diabetic female rats exposed to cold stress (4±2 °C). Values are mean ±SD of six observations and a,b,c,d….. letters denote significantly different from control values by one-way ANOVA(P<0.05).

Differential toxic responses were observed with regard to GSH-Px levels upon diabetic and cold stress induction and results indicate significant suppression in GSH-Px activity in all the functional tissues studied. The one-way ANOVA showed interactive effects of diabetic toxicity and cold stress treatment resulting in a significant decrease in GSH-Px activity levels leading to decrease of GSH levels. In general, co-exposure of diabetes and cold stress showed

adverse effect to higher extent in uterus (-69.5%), ovary (-61.9%) and oviduct (-69.8%) than the diabetes and cold stress alone in all the functional tissues studied (Fig. 4).

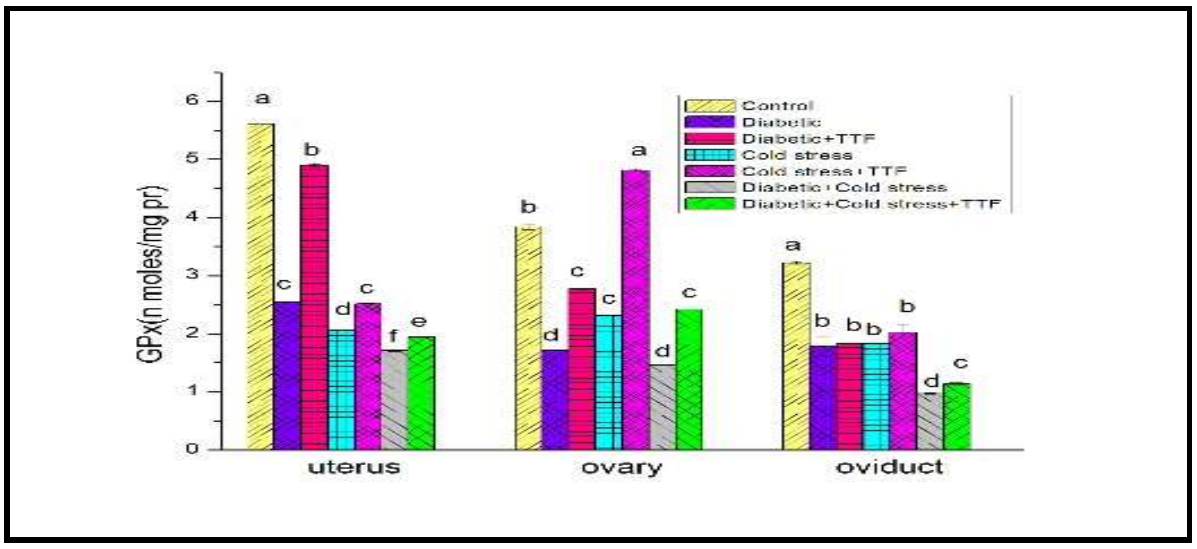
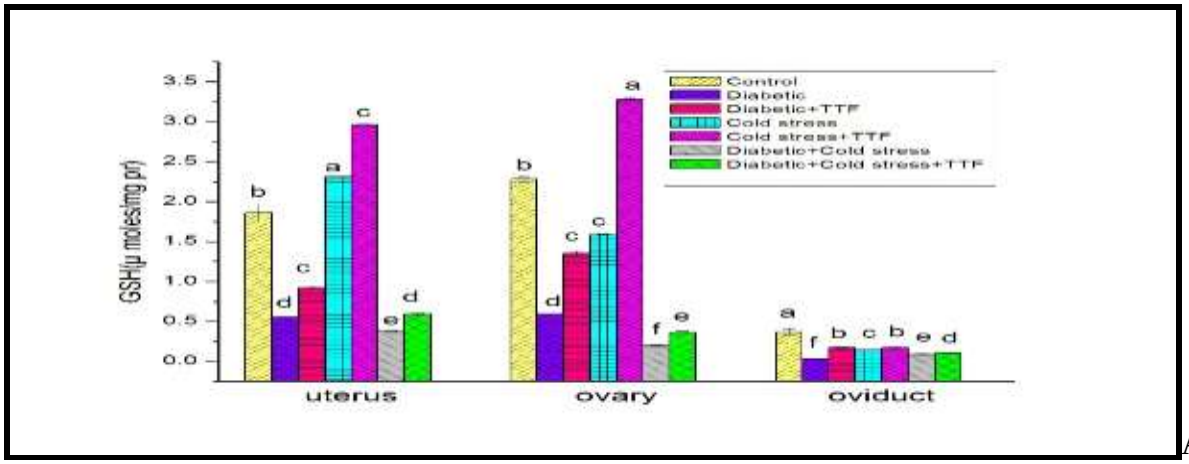


Fig. 4- Effect of *Tribulus terrestris* fruit ethanol extract on GSH-Px (GPx) antioxidant enzyme activity level in reproductive system (uterus, ovary & oviduct) of STZ induced diabetic female rats exposed to cold stress (4±2 °C). Values are mean ±SD of six observations and a,b,c,d….. letters denote significantly different from control values by one-way ANOVA(P<0.05).

One-way ANOVA indicated significant decrease in GSH levels in all the functional tissues studied as a consequence of co-exposure of diabetes and cold stress and among the three tissue studied uterus and ovarian tissues were affected to a higher extent (-79.5%) and (-91.2%) respectively. Cold stress significantly increased the GSH levels in the uterus (+24.1%), while in general, diabetes decreased the GSH levels in all the functional tissues studied (Fig. 5).



A

Fig. 5- Effect of *Tribulus terrestris* fruit ethanol extract on GSH antioxidant enzyme activity level in reproductive system (uterus, ovary & oviduct) of STZ induced diabetic female rats exposed to cold stress (4±2 °C). Values are mean ±SD of six observations and a,b,c,d….. letters denote significantly different from control values by one-way ANOVA(P<0.05).

significant (P<0.05) decrease in the GST activity was noticed in all the functional tissues studied upon co- exposure to diabetes and cold stress (Fig. 1). In general, uterus and ovary tissues were affected to a greater extent (-79.3%) and (-67.2%) respectively than the oviduct in diabetic group while cold stress significantly increased the GST activity (+62.5%) in oviduct when compared to control (Fig. 6).

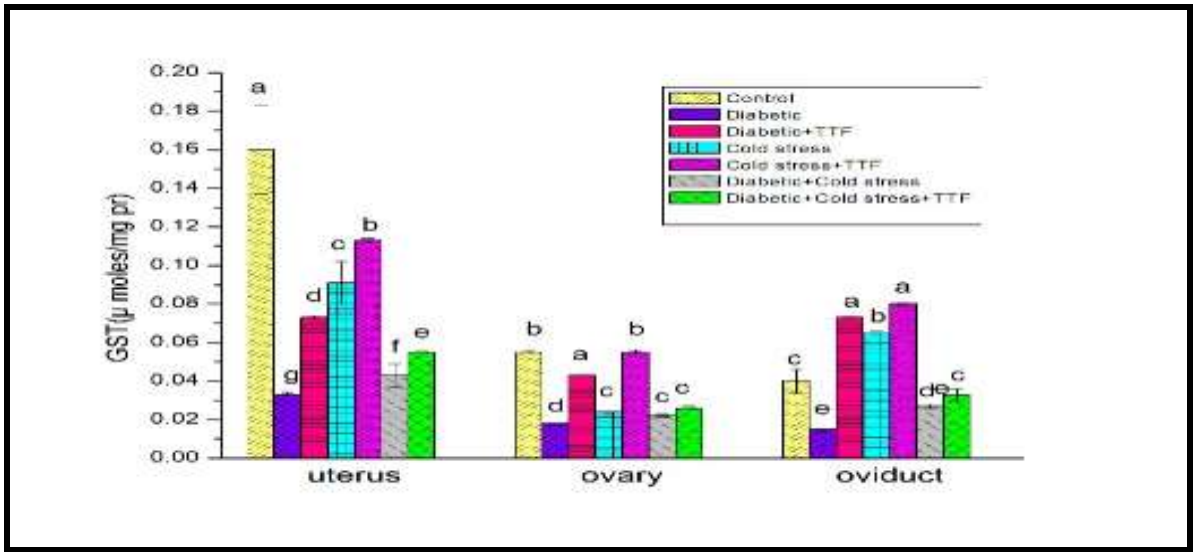


Fig. 6- Effect of *Tribulus terrestris* fruit ethanol extract on GST antioxidant enzyme activity level in reproductive system (uterus, ovary & oviduct) of STZ induced diabetic female rats exposed to cold stress (4±2 °C). Values are mean ±SD of six observations and a,b,c,d….. letters denote significantly different from control values by one-way ANOVA(P<0.05).

Dietary antioxidant supplementation proved to be effective in restoring oxidative damage evidenced by diminishing elevated MDA levels and enhancing the inhibited activities of SOD, CAT, GSH-Px, GSH and GST expressed in terms of % recovery against the MDA level and enzyme activities induced by diabetes and cold stress. In uterus, the minimum ameliorative effect of supplementation was observed with TTF extract on the level of LPO (+46.4%) and an enzyme activities maximum with CAT (+190.4%) and GST (+121.2%) and followed by SOD (+91.3%), GSH-Px (+92.9%) and GSH (+67.2%) in diabetic group. The maximum ameliorative effect of supplementation was observed with TTF extract on the level of LPO (+84.9%) and an enzyme activities moderate with CAT (+57.0%) and minimum activities with SOD (+31.1%), GSH-Px (+21.8%) and GSH (+28.1%) GST (+24.1%) in cold stressed group.

The minimum ameliorative effect of supplementation was witnessed with TTF extract on the level of LPO (+7.0%) and an enzyme activities maximum with SOD (+54.5%) and GSH (+27.9%) in co-exposed group. In ovary, the minimum amelioration occurred with the supplementation of TTF on the LPO level (+52.0%) and an enzyme activities maximum with GST (+138.8%) and GSH (+128.8%) and followed by moderate enzyme activities with SOD (+68.7%), CAT (+57.0%) and GSH-Px (+61.9%) in diabetic group. The moderate amelioration occurred with the supplementation of TTF on the LPO level (+61.8%) and an enzyme activities maximum with GST (+129.1%), GSH-Px (+108.2%) and GSH (+106.2%) and followed by minimum enzyme activities with CAT (+37.2%) and SOD (+16.4%) in cold stressed group.

The moderate amelioration occurred with the supplementation of TTF on the LPO level (+38.8%) and an enzyme activities maximum with GSH (+80.0%), SOD (+71.0%), GSH-Px (+65.7%) and CAT (+53.8%) and followed by minimum enzyme activity with GST (+18.1%) in co-exposed group. In the oviduct, moderate amelioration occurred with TTF on the LPO level (+46.2), and maximum enzyme activities on GSH (+400%) and GST level (+386.6%) and minimum enzyme activities on SOD (+106.8%), CAT (+50%) and least was found in GSH-Px (+2.8%) in diabetic group. In the oviduct, the moderate amelioration occurred with TTF on the LPO level (+60.6), and maximum enzyme activities on SOD (+86.3%), and minimum enzyme activities on CAT (+42.8%), GSH-Px (+10.3%), GSH (+21.4%) and GST

level (+23.0%) in cold stressed group. In the oviduct, minimum amelioration occurred with TTF on the LPO level (+24.0) and as well as in all the enzyme activities SOD (+22.2%), CAT (+21.4%) GSH-Px (+17.5%) GSH (+32.5%) and GST level (+22.2%) in co-exposed group.

Induction of diabetes and cold stress to rats during the experimental period significantly decreased serum oestrogen, FSH and LH levels when compared with the normal control group. Pre-treatments of diabetes-intoxicated and cold stress induced rats with TTF extract significantly (P<0.05) increased serum oestrogen, FSH and LH levels when compared with the intoxicated control groups (Table 4).

Table 4-Effect of *Tribulus terrestris* fruit ethanol extract (200mg/kg bw/day) on hormone levels in STZ induced diabetic female rats exposed to cold stress (4±2°C). [Values are expressed as mean ± SD (n=6) from each group]

Groups	FSH (mIU/ml)	LH (µg/dl)	Estradiol (pg/ml)
Control	0.47 ± 0.01	0.19 ± 0.02	35.62 ±3.58
STZ	0.37±0.03* (-21.2)	0.11±0.01* (-42.10)	17.87±0.56* (-49.8)
STZ + TTF	0.39±0.11* (+86.4)	0.15±0.02* (+36.36)	24.46±0.61* (+36.8)
Cold stress	0.40±0.04 (-23.4)	0.15±0.01* (-21.05)	29.93±0.42* (-15.9)
Cold stress + TTF	0.44±0.04 (+27.7)	0.17±0.05 (+13.33)	32.8±1.38 (+9.58)
STZ + Cold stress	0.31±0.03* (+2.1)	0.15±0.03* (-21.05)	15.26±0.08* (-57.1)
STZ + Cold stress + TTF	0.35±0.05* (+6.2)	0.12±0.03* (-20.00)	21.76±0.29* (+3.2)

STZ= Streptozotocin; TTF= *Tribulus terrestris* fruit. P<0.05 as compared to * normal control rats. Values in the parenthesis indicate the % change & recovery; '+'sign indicates increase and '-'indicates decrease over the controls.

DISCUSSION

Increased glucose-oxidation, non-enzymatic glycation of proteins and their subsequent degradation cause unbalanced free-radical generation in diabetes Indeed, hyperglycaemia mediated advanced glycation of intracellular antioxidant defence enzymes results in hyper-susceptibility to the elevated oxidative stress due to lowered anti-oxidative protection. In the present study the elevated glucose level in all the experimental rats was significantly lowered upon TTF supplementation and may be through inhibition of α-glucosidase as well as by its antidiabetic effects and the results are in accordance with Lamba et al.[24]. It was also reported that saponin has the hypoglycemic effect and the fraction of it inhibited the activity of α-glucosidase in small intestines in rats and retarded the increase in postprandial blood glucose level in rats[37,38]. Our results indicated a decrease in body and reproductive organs weight of the diabetic and co-exposed rats in comparison to the normal control rats. The decrease in body and reproductive organs weight was as a result of loss of tissue proteins and muscle mass in diabetes[39]. It is known that glycosuria causes a significant loss of calories for every gram of glucose exerted and most likely, this loss results in severe weight loss in spite of increased appetite, particularly when it is coupled with muscle and adipose tissue due to excessive breakdown of the protein. In this study, supplementation of TTF extract at a 200mg/kg bw dosage level in STZ-induced diabetes intoxicated and co-exposed rats produced a significant protective effect against reproductive organs functional tissues toxicity

and this effect characterized by increased weights of body and reproductive organs viz., uterus, ovary and oviduct, improved follicular quality and quantity. These findings are in accordance with those previously reported[40,27]. TT extract contents such as saponins (disgenin) and sterol (ß-siosterol, stigma sterol) which contain phytoestrogen and the metabolites of phytoestrogen exert an estrogenic effect on central nervous system which induces estrous and stimulates cell division and growth of genital tract of female animals[41]. On the other hand, in contrast Martino- Andrade *et al.*[42], who reported that low dose levels of TT purified extract given to castrated female rats for 28 days was unable to stimulate endocrine sensitive tissues such as uterus and vagina.

Elevated systemic glucose level promotes overproduction of superoxide radical (O_2•-) and H_2O_2 and ROS are associated as important physiological and pathological mediators in many reproductive disorders[43-45]. ROS is involved in the modulation of an entire spectrum of physiological reproductive functions such as oocyte maturation, ovarian steroidogenesis, corpus luteal function and luteolysis. Generation of free radicals, LPO and altered antioxidant systems are considered to play a vital role in posing toxic effects of diabetes and in addition, cold stress activate hypothalamic-pituitary-adrenal axis, subsequently release corticosterone from the adrenal cortex into the bloodstream[46] that in turn accelerates the generation of free radicals. Diabetes pose the threat of hypothermia, because they increase body temperature decline that accompanies cold exposure. Increased oxidative stress has also been directly linked to oxidation of cellular macromolecules that may called injury to the reproductive organs or induce a variety of adverse cellular responses. A high rate of oxygen consumption coupled with low potential of reproductive organs to prevent oxidative stress may be the main triggering factor for their enhanced release of ROS during diabetic and cold stress exposure. In the presence of free radicals, diabetes and cold stress induces reproductive toxicity by biphasic action where, it behaves as an oxidant and the other as an inhibitor of antioxidant enzyme systems. In the present study, elevated LPO in the uterus, ovary and oviduct samples of STZ diabetic and cold stressed alone and co-exposed rats indicated an increase in oxygen free radicals in diabetes and cold stress primarily could be due to augmented blood glucose level, which upon auto-oxidation generate free radicals and secondarily, the effects of the diabetogenic agent, STZ and cold stress. Studies suggest that the tissue content having relatively high concentration of easily peroxidizable fatty acids and increased activities of enzymes like fatty acyl coenzyme, co-enzyme A oxidise due to hypoinsulinemia initiate the β-oxidation of fatty acids resulting in lipid peroxidation. In the present study, the elevated levels of LPO in the uterus, ovary and oviduct appear to be due to diabetes and cold stress induced generation of free radical oxidative stress that may cause extensive cellular damage unless it is arrested by certain protective agents. The present study evidenced the protective actions of TTF against diabetes and cold stress induced oxidative stress in functional tissues of reproductive system and such protection by TTF methanol extract in diabetic male mice was observed[47] and similar results were also understood on administration of Simploxa racemosa and triphala in cold restraint stressed rats[48,49].

The altered balance of the antioxidant enzymes with a decrease in SOD and CAT activities in STZ diabetic and cold stressed condition, may be due to increased production of superoxide and H_2O_2 by the auto-oxidation of the glucose and non-enzymatic glycation and this depicts the inactivation of the enzymes by superoxide anions. These enzymes are suggested to play an important role in maintaining physiological levels of oxygen and H_2O_2 by accelerating the

dismutation of oxygen radicals and by eliminating the organic peroxides and hydro peroxides generated from accidental exposure to STZ.

GSH-Px plays a crucial role in H_2O_2 catabolism[50]. Distribution of cells lacking GSH-Px coincided with those tissues that found to be more susceptible to oxidative stress. In the present study, diabetes and cold stress exposure caused a remarkable decrease in the levels of GSH-Px activity in uterus, ovary and oviduct tissues indicating the extent of cellular damage caused by oxidative stress and the inability of GSH-Px to check it. These alterations caused could be due to their difference in cell types, composition, function and sensitivity. Noticeable increase in GSH-Px content in all the functional tissue samples on TTF administration to experimental animals may help in protecting the cellular damage against oxidative stress.

GSH plays an important role in the detoxification and metabolism as a co-factor or as a substrate for enzymes and as an antioxidant agent to protecting the tissue for oxidative stress. In the present study decreased GSH levels in diabetic and co-exposed rats' uterus, ovary and oviduct may be due to the enhanced GST activity and increased GSH levels in cold stressed uterus. Elevated GSH content in uterus, ovary and oviduct samples on TTF administration to diabetic, cold stressed and co-exposed animals may help in offering protection to cellular proteins against oxidation through glutathione redox cycle and further help in detoxifying ROS generated during diabetes and cold stress exposure.

GST an enzyme involved in the binding, transport and detoxification, and cellular defence. Increased free radicals in STZ diabetic and cold stressed rats' uterus and ovary may have enforced GST detoxification thereby increasing its activity to a significant level. The beneficial role of TTF, especially a dose of 200mg/kg body weight, helps in ameliorating the oxygen free radicals, may have brought the enzyme levels nearly to normal and may help to control free radical generation during diabetes and cold stress exposure. The anti-oxidant effect of TTF extract was similar to that previously demonstrated[51] and the anti-oxidant activity of TTF extract was attributed to the presence of active derivatives of 4,5-di-p-coumaroylquinic acid, which were isolated from the fruits and proved to exhibit a potent anti-oxidant effect[52].

The mechanism(s) underlying the protective effect of TTF against reproductive toxicity induced by diabetes and cold stress in rats could be attributed to its potent anti-oxidant property, so reducing oxidative stress in the reproductive organs and improving reproductive function by increased release of estradiol, FSH and LH serum levels. The results are partially in agreement with Adaay MH & Mosa AR[27], where an obvious increase was noticed in FSH and LH levels but a decrease in estradiol level was detected in normal control mice and Sato *et al*.[53] reported that systemic and local IGF-I play a major role in estrogen effect on growth and epithelial proliferation of mouse uterus. Dehghan A et al.[54], inferred that TT have a luteinizing effect which may relate to its gonadotropin-like activity and also, it can efficiently resume ovarian activity. Moreover, Gauthaman K & Adaikan PG[55], reported that protodioscin a steroidal saponin upregulate the levels of testosterone and leuteinizing hormone in male rats. The present study suggests that the food substances having flavonoids and polyphenolic compounds might be effective antioxidants for human health and in prevention of reproductive organs against oxidative stress and degeneration.

In conclusion, diabetes toxicity and cold stress in the female reproductive organs may result in disruption of oxidants and antioxidants balances, which provides a strong coupling of altered equilibrium processes and loss of energy capacity to meet an oxidation challenge. Moreover, exposure to diabetes and cold stress can increase the effects of oxidative stress. Exogenous supplementation of TTF extract has been found to counter free radical generated oxidative stress and to facilitate reduction of the toxic effects induced by diabetes and cold stress, there by strengthening the cellular antioxidant defense and improved reproductive functioning ability.

ACKNOWLEDGEMENTS

This study was supported by UGC-SWRO, Bangalore in the form of Faculty Improvement Programme (FIP) to Dakshayini P N.

REFERENCES

1. International Diabetes Federation, *Diabetes Atlas*. (Brussels, Belgium), 2013, 6th Edition.
2. American Diabetes Association. Diagnosis and classification of diabetes mellitus, *Diabetes Care*, 34(Suppl 1) (2011) 62.
3. Maritim AC, Sanders RA & Watkins JB. Diabetes, oxidative stress, and antioxidants: A Review. *Journal of biochemical and molecular toxicology*, 17 (2003) 24.
4. Katayama S, Brownscheidle CM, Wootten V, Lee JB & Shimaoka K, Absent or delayed preovulatory luteinizing hormone surge in experimental diabetes mellitus, *Diabetes*, 33 (1984) 324.
5. Dong Q, Lazarus RM, Wong LS, Vellios M, Handelsman DJ & Pulsatile LH, Secretion in streptozotocin-induced diabetes in the rat, *J Endocrinol*, 131 (1991) 49.
6. Valdes CT, Elkind-Hirsch KE, Rogers DG & Adelman JP, The hypothalamic-pituitary axis of streptozotocin-induced diabetic female rats is not normalized by estradiol replacement, *Endocrinology*, 128 (1991) 433.
7. Kienast SG, Fadden C & Steger RW, Streptozotocin-induced diabetes blocks the positive feedback release of luteinizing hormone in the female rat, *Brain Res Bull*, 32 (1993) 399.
8. Steger RW & Rabe MB, The effect of diabetes mellitus on endocrine and reproductive function, *Proc Soc Exp Biol Med*, 214 (1997) 1.
9. Chrousos GP, Stressors, stress, and neuroendocrine integration of the adaptive response, The 1997 Hans Selye Memorial lecture. *Annals of the New York Academy of Sciences*, 851 (1998) 311.
10. Szabo S, Hans Selye and the development of the stress concept: special reference to gastroduodenal ulcerogenesis, *Annals of the New York Academy of Sciences*, 851 (1998) 19.
11. Tache Y & Brunnhuber S, From Hans Selye's Discovery of biological stress to the identification of corticotropin-releasing factor signalling pathways: Implication in stress-related functional bowel diseases, *Annals of the New York Academy of Sciences*, 1148 (2008) 29.
12. Chrouses GP, Philip W & Gold MD, The concept of stress system disorders, *JAMA*, 267(1992) 1244.
13. Goel RK & Bhattacharya SK, Gastroduodenal mucosal defense and mucosal protective agents, *Indian J. Exp. Bio*, 29 (1991) 71.
14. Gotoh M, Tajima T, Suzuki Y, Ikari H, Iguchi A, Kakumu S & Hirooka Y, Swimming stress that causes hyperglycemia increases in vivo release of noradrenaline, but not acetylcholine, from the hypothalamus of conscious rats, *Brain Research*, *780*(1) (1998) 74.

15. Angelogianni P & Gianoulakis C, Ontogeny of the beta-endorphin response to stress in the rat: Role of the pituitary and the hypothalamus, *Neuroendocrinology,* 50 (1989) 372.
16. Smythe GA, Grunstein HS, Bradshaw J E, Nicholson MV & Compton PJ, Relationships between brain noradrenergic activity and blood glucose, *Nature,* 308 (1984) 65.
17. Smythe GA, Pascoe WS & Storlien LH, Hypothalamic noradrenergic and sympathoadrenal control of glycemia after stress, *Am. J. Physiol,* 256 (1989) E231.
18. Dhanalakshmi S, Srikumar R, Manikandan S, Parthasarathy NJ & Devi RS, Antioxidant property of triphala on cold stress induced oxidative stress in experimental rats. *J Health Sci,* 52 (2006) 843.
19. Selye H, The general adaptation syndrome and the diseases of adaptation, *J. Clin. Endocrinol,* 6 (1946) 117.
20. Paredes A, Galvez A, Leyton V, Aravena G, Fiedler JL, Bustamante D & Lara HE, Stress promotes development of ovarian cysts in rats: The possible role of sympathetic nerve activation, *Endocrine,* 8 (1998) 309.
21. Dorfman M, Arancibia S, Fiedler JL & Lara HE, Chronic intermittent cold stress activates ovarian sympathetic nerves and modifies ovarian follicular development in the rat, *Biology of reproduction,* 68 (2003) 2038.
22. Greiner M, Paredes A, Araya V & Lara HE, Role of stress and sympathetic innervation in the development of polycystic ovary syndrome, *Endocrine,* 28 (2005) 319.
23. Bernuci MP, Szawka RE, Helena CV, Leite CM, Lara HE & Anselmo-Franci JA, Locus coeruleus mediates cold stress-induced polycystic ovary in rats, *Endocrinology*, *149*(6) (2008) 2907.
24. Lamba HS, Bhargava CS, Thakur MAYANK & Bhargava SHILP, A-Glucosidase and aldose reductase inhibitory activity in vitro and anti-diabetic activity in vivo of *Tribulus terrestris* L.(Dunal), *Int J Pharm Pharm Sci*, *3* (2011) 270.
25. El-Tantawy WH & Hassanin LA, Hypoglycemic and hypolipidemic effects of alcoholic extract of *Tribulus alatus* in streptozotocin-induced diabetic rats: A comparative study with *T. terrestris* (Caltrop), *Indian J. Exp. Bio,* 45 (2007) 785.
26. Tomova M, Gyulemetova R, & Steroidsapogenine VI, Furostanol bisglykosid aus *Tribulus terrestris* L, *Planta Med,* 34 (1978) 188.
27. Adaay MH & Mosa AR, Evaluation of the effect of aqueous extract of *Tribulus terrestris* on some reproductive parameters in female mice, *J. mater. environ. sci, 3*(6) (2012) 1153.
28. Esfandiari A, Dehghan A, Sharifi S, Najafi B & Vesali E, *J. Anim Veter Advances,* 10 (2011) 883
29. Siddique O, Sun Y, Lin JC & Chien YW, Facilitated transdermal transport of insulin, *J Pharm Sci,* 76 (1987) 341.
30. Niehaus WG & Samuelsson B, Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation, *European Journal of Biochemistry*, *6*(1) (1968) 126.
31. Misra HP & Fridovich I, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, *Journal of Biological Chemistry*, *247*(10) (1972) 3170.
32. Aebi H, Catalase, in methods in enzymatic analysis, edited by HU Bergmeyer (Academic Press, New York) 3 (1983) 276.
33. Lawrence RA & Burk RF, Glutathione peroxidase activity in selenium-deficient rat liver, *Biochemical and Biophysical Research Communications*, *71*(4) (1976) 952.
34. Habig WH, Pabst MJ, Jakoby WB, Glutathiones-transferases. The first enzymatic step in mercapturic acid formation, *J. Biol. Chem*, *249*(22) (1974) 7130.
35. Ellman GL, Tissue sulfhydrl groups. *Arch Biochem Biophys*, 82 (1952) 70.

36. Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein measurement with the folin phenol reagent, *J Biol Chem*, *193*(1) (1951) 265.
37. Li M, Wang Y & Tian C, Hypoglycemic effects of saponins from *Tribulus terrestris*, *Zhaong Yao Cai*, 25 (2002) 420.
38. Zhang SJ, Qu WJ & Zhong SY, Inhibitory effects of saponins from *Tribulus terrestris* on a-glucosidase in small intestines of rats. *Zhonqquo Zhong Yao Za Zhi*, 31 (2006) 910.
39. Mishra B, Pancholi SS, Deshmukh AB & Panjwani D, Preclinical investigations of a novel dose regimen based on the combination of pioglitazone and gymnema sylvestre extract, *Mol Clin Pharmacol*, 2 (2012) 20.
40. Abid NN, *Effect of crude aqueous extract of tribulus terrestris on some parameters of fertility in albino mice*, M.sc. thesis, Institute of embryo researches and infertility treatment, Al-Nahrain University, Baghdad, Iraq, 2010.
41. Lieberman SJ, *Clin Endocrinol Metab*, 81 (1996) 850.
42. Martino- Andrade AJ, Morais RN, Spercoski KM, Rossi SC, Vechi MF, Golin M, Lombardi NF, Greca CS & Dalsenter PR, *J. Ethnopharmacol*, 127 (2010) 165.
43. Behrman HR, Kodaman PH, Preston SL & Gao S, Oxidative stress and the ovary, *J. Soc Gynecol Investing*, 8 (2001) S40.
44. Suzuki T, Sugino N, Fukaya T, Sugiyama S, Uda T, Takaya R, Yajima A & Sasano H, Superoxide dismutase in normal cycling human ovaries: Immuno-histochemical localization and characterization. *Fertil Steril*, 72 (1999) 720.
45. EI Moutassim S, Guerin P & Menezo Y, Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. *Mol Hum Reprod*, 5 (1999) 720.
46. Owens MJ & Nemeroff CV, Philosophy and pharmacology of corticotrophin releasing factor, *Pharmacol Rev*, 91 (1991) 425.
47. Shalaby MA & Hammouda, Assessment of protective and anti-oxidant properties of tribulus terrestris fruits against testicular toxicity in rats. *Journal of Intercultural Ethnopharmacology*, *3*(3) (2014) 113.
48. Saraswathi CD, Gupta1 SK & Sreemantula S, Protective effect of symplocos racemosa bark on cold restraint stress induced reproductive changes in female rats, *Journal of Natural Products*, (2012) 5251.
49. Dhanalakhmi SK, Srikumar R, Manikandam S, Parthasarathy NJ & Devi RS, Antioxidant property of triphala on cold stress induced oxidative stress in experimental rats, *J. Health Sci*, 52 (2006) 843.
50. Eaton JW, Catalase, glutathione peroxidase and hydrogen peroxidase, *Journal of Lab Clin Med*, 118 (1991) 3.
51. Sailaja KV, Shivaranjani VL, Poornima H, Rahamathulla SM & Devi KL, Protective effect of *Tribulus terrestris* L. fruit aqueous extracton lipid profile and oxidative stress in isoproterenol induced myocardial necrosis in male albino wistar rats, *EXCLI Journal*, 12 (2013) 373.
52. Hammoda HM, Ghazy NM, Harraz FM, Radwan MM, ElSohly MA & Abdallah II, Chemical constituents from *Tribulus terrestris* and screening of their antioxidant activity. *Phytochemistry*, Aug 31 (92) (2013) 153.
53. Sato T, Wang G, Hardy MP, Kurita T, Cunha GR & Cooke PS, *Endocrinol*, 143 (2002) 2673.
54. Dehghan A, Esfandiari A & Bigdeli SM, Alternative treatment of ovarian cysts with *Tribulus terrestris* extract: A rat model, *Reproduction in Domestic Animals*, *47*(1) (2012).
55. Gauthaman K & Adaikan PG, Effect of *Tribulus terrestris* on nictinamide adenine dinucleotide phosphate-diaphorase activity and androgen receptors in rat brain, *J. Ethnopharmacol*, 96 (2005) 127.