AMELIORATION OF LEAD ACETATE-INDUCED TESTICULAR TOXICITY BY *TRIBULUS TERRESTRIS* ROOT EXTRACT AND VITAMIN C IN SWISS ALBINO MICE

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ABSTRACT

The aim of the present study was to access the efficacy of Tribulus terrestris root extract in reducing lead-induced changes in mice testes. Animals exposed to lead acetate showed significant increase in testicular LPO and acid phosphatase activity, however a significant decrease in GSH and alkaline phosphatase activity was observed. Serum testosterone, FSH and LH levels were suppressed in lead treated group compared with the control. Histopathological examination of testes in lead-treated animals showed gross damage within the seminiferous tubules. These influences of lead acetate were prevented by concurrent daily administration of T. terrestris root extract to some extent. The antioxidant potential of T. terrestris root extract was carried out by the DPPH and reducing power assays. Phytochemical screening of the plant was also done. The results thus led us to conclude that administration of T. terrestris root extract significantly protects against lead-induced oxidative stress.

Keywords: Antioxidant Potential, Biochemical Parameters, Histopathology, Lead Acetate, Tribulus Terrestris

I. INTRODUCTION

Reproductive hazards from metal exposure in males are one of the fastest growing areas of concern in toxicology today [1]. Lead is an environmental pollutant and metabolic poison with a variety of toxic effects, among which is its adverse influence on reproduction [2,3]. Toxicity is manifested in male reproductive system by deposition of lead in testes [4,5], epididymis, vas deferens, seminal vesicle and seminal ejaculate. Lead also has an adverse effect on sperm count and retarted the activity of alive sperm [6]. Motility and prolonged latency of sperm melting both in exposed person and experimental animals were observed after lead exposure [7]. Occupational exposure to lead showed hypogonadism and decreased serum testosterone, with a reproductive and endocrine impact on hypothalamic-pituitary-testicular axis in rabbits [8,9].

Recent studies have proposed that one possible mechanism of lead toxicity is the disturbance of pro-oxidant and oxidant balance by generation of reactive oxygen species (ROS) [10,11]. This can evoke the oxidative damage of critical biomolecules such as lipids, proteins and DNA. It has been reported that lead exposure has a dose-response relationship with changes in antioxidant enzyme levels and their activities [12].

Tribulus terrestris is a flowering plant in family Zygophyllaceae, native to warm temperate and tropical regions

of the Old World in southern Europe, southern Asia, throughout Africa and Australia. *T. terrestris* has long been used as a tonic and aphrodisiac in Unani system of medicine. It has been used in India and Pakistan as a treatment for impotence and as a stimulant to enhance sexual drive and performance [13]. Clinical studies showed *T. terrestris* improved reproductive function, including increased concentration of hormones such as estradiol, with testosterone being very slightly influenced, thereby improving reproductive function, libido and ovulation [14,15].

The present investigation was carried out to evaluate the efficacy of the root extract of *T. terrestris* against lead acetate induced oxidative stress in the testes of Swiss albino mice.

II. MATERIAL AND METHODS

The proposed experiment was conducted to observe the lead induced toxicity in mice testes by observing some biochemical parameters, serum hormonal levels (Testosterone, FSH, LH) and histopathology and its modulation by *T. terrestris* root extract and vitamin C (as positive control).

1.1 Animals and Treatment

Random-bred, male Swiss albino mice (7-8 weeks) were used for the experiment. These animals were maintained in the animal house at a temperature of 24±3°C, relative humidity of 50%±15% and normal photoperiod (12 hr light and 12 hr dark). Animals were housed in polypropylene cages and fed standard mice feed (Hindustan Lever Ltd., India). Tap water was provided to the animals *ad libitum* and tetracycline was given as a preventive measure against infections once in a fortnight. The ethical committee of Department of Zoology, University of Rajasthan, Jaipur (India) has approved to carry out the experimental protocol.

1.2 Chemicals

Lead acetate was procured from Central Drug House (India). All other chemicals used in the study were of analytical reagent and obtained from SD fine chemicals (India), HIMEDIA (India).

2.3 Experimental Plant

The plant *Tribulus terrestris* (roots) were collected locally in the month of July and August and were identified in the herbarium of Botany Department, University of Rajasthan, Jaipur as an RUBL20825 variety. Shade dried *T. terrestris* roots were ground to a fine powder, the powder was then distilled in soxhlet apparatus (for 36 hours using Double Distilled Water) at 40°C. The remaining material was dried in oven at a temperature of 36°C and used for the study.

2.4 Preparation of Aqueous Extract of *T. Terrestris* Roots

The animals were administered *T. terrestris* root extract dissolved in DDW orally by oral gavage up to 30 days (100, 400, 800 mg/kg body weight) and reduced glutathione (GSH) and lipid peroxidation (LPO) contents were measured in the liver. The optimum dose selection of *T. terrestris* root extract was decided on the basis of minimum LPO and maximum GSH level in the liver tissue. Among the doses 800 mg/kg b.wt. was selected for the study.

2.5 Antioxidant Potential of *T. Terrestris* Root Extract

Antioxidant potential of *T. terrestris* root extract was determined by the DPPH radical scavenging activity by the method of Blois [16] and reducing power by the method of Oyaizu [17]. The phytochemical screening of the plant was done by the method of Njoku [18].

2.6 Experimental Design

Adult Swiss albino male mice were divided into four groups of 25 mice each and following experiments were designed.

Group I (Control group): Normal Control (received DDW as vehicle).

Group II (Heavy metal treated group): Freshly dissolved lead acetate in 0.1 ml double distilled water was given subcutaneously only once at a dose of 10mg/kg body weight. This day was considered as day zero and the experiment was continued for 30 days.

Group III (*T. terrestris* + Lead acetate + *T. terrestris*): *T. terrestris* root extract was given at a selected dose level (800mg/kg body weight) for 7 days and on the 7^{th} day just after 30 minutes of *T. terrestris* root extract administration lead acetate was given only once. Then from the next day (considered as day 1^{st}) *T. terrestris* was given continuously for 30 days. The total experimental period was of 37 days.

Group IV [Vitamin C + Lead acetate + Vitamin C (positive control group)]: Vitamin C was administered at a dose level of 100 mg/kg body weight for 7 days and on the 7^{th} day just after 30 minutes of vitamin C administration lead acetate was given only once. Then from the next day (considered as day 1^{st}) vitamin C was given continuously for 30 days. The total experimental period was of 37 days.

Autopsy intervals: The animals from the above groups were autopsied at various intervals i.e. 1, 3, 7, 15 and 30 days.

2.7 Preparation of Testis Homogenate

Testes were sliced into pieces and homogenized in 10 times its volume of tris-HCl (0.2 M, pH 7.3) at 1–4°C to give 10% homogenate (w/v). The homogenate was centrifuged at 10,000 rpm for 15–20 min at 4°C. The resulting supernatants were separated and used for various biochemical estimations as depicted below:

2.8 Biochemical Assays

Lipid peroxidation (LPO) was estimated by thiobarbituric acid reaction with malondialdehyde (MDA), as described earlier by Okhawa *et al* [19]. Glutathione (GSH) content was determined by the method of Moron *et al* [20]. Acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were determined by the method of Fiske and Subbarow [21].

2.9 Serum Hormonal Assay

Serum testosterone, FSH and LH level were assessed by ELISA by the method of Wilke [22].

2.10 Histopathological Analysis

Excised testes were fixed in Bouin's fixative for 24 hrs. The fixed tissue was further processed by standard method and sections were cut at 5 µ and stained with Haematoxyline and Eosine to observe quantitative and qualitative assessment of various cell types in the testis according to the method of Clermont and Leblond [23].

2.11 Statistical Analysis

Data obtained from the above study were analyzed statistically by analysis of variance one-way (ANOVA) and the level of significance was set at $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

III. RESULTS

78.27% inhibition on 100µg/ml concentration of DPPH free radical was observed of the root extract of *T. terrestris* which was lesser than the antioxidant potential of the standard ascorbic acid having 93.54% inhibition on the same concentration of DPPH free radical (Fig.1). Almost significant ($P<0.05$) increase was found in the

reducing power of root extract on increasing the concentration of root extract but it was lesser than the standard ascorbic acid reducing power (Fig.2). These results showed the anti-oxidant potential of the root extract of *T. terrestris.* Phytochemical screening of the plant showed the presence of tannins, saponins, steroids and cardiac glycosides (Table 1).

The level of lipid peroxidation was normal in the untreated mice control group from the day 1^{st} to 30^{th}. But a significant ($P<0.01$) increase was observed in LPO level from day $1^{st}$to day 30^{th} in lead treated group II. After the treatment with *T. terrestris* root extract (group III) a significant decrease ($P<0.01$) was observed from 7^{th} day to 30^{th} day. In group IV after administration of vitamin C, the level of lipid peroxidation was decreased significantly ($P<0.01$) at all autopsy intervals (Fig.3). GSH level was almost significantly ($P<0.05$) decreased in lead treated group as compared to normal control group on the 1^{st} and 3^{rd} day and a highly significant ($P<0.001$) decrease was observed on 7^{th}, 15^{th} and 30^{th} day. After administration of *T. terrestris* root extract a significant ($p<0.01$) increase was observed during all autopsy intervals. Administration of vitamin C (group IV) also shows a highly significant ($P<0.001$) increase in GSH level from day 1^{st} to day 30^{th} during the whole experimental period (Fig.4).

Alkaline phosphatase activity was decreased significantly ($P<0.001$) in lead treated group II as compared to normal control group I. After *T. terrestris* root extract administration alkaline phosphatase activity was significantly ($P<0.01$) increased up to a certain level. Vitamin C treatment also showed highly significant ($P<0.001$) increase in alkaline phosphatase activity up to 30 days of experiment (Fig.5). It has been seen that acid phosphatase activity was significantly ($p<0.01$) increased after lead acetate treatment as compared to normal control group. But after the treatment with *T. terrestris* root extract and vitamin C (group III and group IV) the activity was significantly ($P<0.01$) decreased from day $7^{th}$to 30^{th} day and after 30 days of treatment the values were almost similar to normal values (Fig.6).

Serum testosterone, FSH and LH levels were observed in all the groups after 30 days of experiment and it was found that in lead treated group highly significant ($P<0.001$) decrease was observed as compared to normal control group. After administration of *T. terrestris* root extract and vitamin C, highly significant ($P<0.001$) increase was observed. It was also found that *T. terrestris* root extract was more protective than vitamin C in testosterone, FSH and LH Level increment (Table 2).

Testicular Histopathology

In the testes of the normal control mice (group I) seminiferous tubules gave healthy appearance with all germ cell population. In group II animals, which were poisoned with lead showed deformities in the testis architecture with a serious damage within the seminiferous tubules. Seminiferous tubules were shrunken and basement membrane had a wavy appearance. Broken sperm heads in the lumen were observed. The interstitial cells of Leydig were also reduced and their characteristics tendency of clumping together to form groups was also reduced. All these features were suggestive of atrophy of the testes.

In group III (*T. terrestris* root extract) and group IV (Vitamin C treated), a partial recovery was observed as compare to lead treated group. Sperms in seminiferous tubules lumen were more or less normal. Leydig cells in interstitial spaces were also increased up to a certain level. Wavy appearance of basement membrane was ameliorated by treatment (Fig.7).

IV. DISCUSSION

In the present study we investigated the efficiency of *T. terrestris* root extract and vitamin C against the lead intoxicated Swiss albino mice model. Our results clearly demonstrated that exposure of an adult male Swiss albino mice to lead acetate can seriously alter the testes histoarchitecture. It is evident from the results of the present investigation that supplementation of *T. terrestris* aqueous root extract and vitamin C with lead acetate protected animals to some extent from toxic effect of lead in general and oxidative stress in particular.

DPPH radical has been widely used to test the radical scavenging ability of various natural products and has been accepted as a model compound for free radicals originating in lipids [24]. *T. terrestris* aqueous root extract showed a concentration dependent antiradical activity by scavenging DPPH radical with an IC_{50}value of 39.85 µg/ml compared with reference standard ascorbic acid (IC_{50} : 33.16 µg/ml), the scavenging effect was lesser. Lower IC_{50} value indicates greater antioxidant activity.

The reducing power determined in the present study depends on the redox potential of the root extract. The *T. terrestris* aqueous root extract exhibited a concentration dependent increase in reducing power, when compared with ascorbic acid the reducing power was less.

In the present study the phytochemical screening of the aqueous root extract of *T. terrestris* showed the presence of tannins, saponins, steroids and cardiac glycosides. It has been previously reported that *T. terrestris* contains biologically rich compounds as steroids, saponins, flavonoids, alkaloids and unsaturated acids [25]. Several workers have been reported that tannins have antimicrobial, antibacterial and anti-inflammatory properties [18,26]. The presence of saponins have been reported to be responsible for the tonic and stimulating activities observed in Chinese and Japanese Medical herbs [27]. Steroids are generally found in all plants and these have importance in pharmacy due to their relationship with such compounds as sex hormones [28]. Glycosides have been known to lower blood pressure and cardiac action [29].

The present study demonstrates that 30 days after administration of lead acetate in mice, a highly significant decrease was found in serum levels of FSH, LH and testosterone. Some authors suggest that a principal mechanism of action of lead toxicity is at the level of the hypothalamic-pituitary axis or a combined defect involving the gonad and hypothalamic-pituitary sites [30,31]. The impairment of spermatogenesis appeared to be as a consequence of the decline of testosterone in serum of lead acetate treated mice since androgen is clearly essential to the gametogenesis [31,32]. Various studies suggest an interaction of heavy metal with the hypothalamo-hypophysis axis controlling spermatogenesis [33]. These products may also interact directly with Sertoli and Leydig cells, responsible for testicular production of proteins involved in the transport and the production of testosterone, respectively. But the concurrent daily administration of *T. terrestris* root extract and Vitamin C in group III and group IV upto 30 days leads to an increase in serum testosterone, FSH and LH levels. It has been already reported that *T. terrestris* is a natural stimulant of Luteinizing hormone (LH) which signals the body to produce more of its own testosterone [34,35].

T. terrestris contains three groups of active phytochemicals: Dioscin, protodioscin and diogenin. These substances have been found to improve the percentage of free testosterone level for men and they affect pregnenolone, progesterone and estrogen [25]. *T. terrestris* works by stimulating the anterior pituitary gland to release LH, which is responsible for stimulating the testes to produce testosterone. It has been observed by the scientists that *T. terrestris* significantly elevates the level of several hormones; Testosterone, Luteinizing Hormone and Follicle Stimulating Hormone [14,25].

The review of literature suggest that some metals, such as lead, cadmium, arsenic and mercury can affect male reproductive functions including sperm morphology [36] and spermatogenesis [37]. In the present study our results clearly demonstrates that exposure of an adult mice to lead acetate exhibited disordered arrangement of germ cells, a decrease spermatogenic layer in the seminiferous tubules, structural defects in spermatids and sperms in the lumen which were similar to earlier findings which indicated that lead altered testes histology [38].

Lead acetate treatment reduced the thickness of epithelium and of seminiferous tubule diameter as a consequence of the action of lead in the reduction in numbers of spermatogonia and spermatocytes [39]. Atrophication of seminiferous tubules and the reduction in the number of Leydig cells in the Pb-treated group was also reported by Shan *et al* [40]. Lead causes lipid peroxidation by generation of ROS and thus the generated ROS react with the polyunsaturated fatty acids-rich spermatozoa and results in peroxidation which finally leads to destruction in spermatozoa [41,42].

Daily administration of *T. terrestris* root extract (group III) and Vitamin C (group IV) upto 30 days after lead acetate treatment reveals that alteration in histology of testes and abnormalities of sperm due to the toxic effects of lead was minimized to some extent. As a possible mechanism, it could be stated that *T. terrestris* root extract have a recovery role on lead-acetate mediated toxicity by inducing an antioxidant effect against the oxidative stress.

In the present study, increase in LPO, acid phosphatase (ACP) and decrease in GSH level and alkaline phosphatase activities has been observed after lead acetate treatment. Lipid peroxidation, a basic cellular deteriorative change, is one of the primary effects induced by oxidative stress and occurs readily in the tissues due to the presence of membrane rich in polyunsaturated, highly oxidizable fatty acids [42,43]. Yiin and Lin [44] demonstrated a significant enhancement of malondialdehyde (MDA) when lead was incubated with linolic, linolenic and arachidonic acid. These initial studies for the first time and subsequent studies demonstrated that lead exposed animals showed increased lipid peroxidation or decrease in antioxidant defense mechanism [45,46].

The intrinsic mechanism underlying lead-induced oxidative damage to membranes is associated with changes in its fatty acid composition [47]. The fatty acid chain length and unsaturation are the determinant for membrane susceptibility to peroxidation, and lead induced arachidonic acid elongation might be responsible for the enhanced lipid peroxidation of the membrane [48,49].

After the treatment of *T. terrestris* root extract (group III) and Vitamin C (group IV) LPO level was decreased upto a certain level during 30 days of experiment.

GSH is one of the most important compounds, which helps in the detoxification and excretion of heavy metals. In our present study a marked decrease was observed in GSH level after lead exposure. The intracellular levels of oxidized glutathione (GSSG) increase from metabolism of H_2O_2 by glutathione peroxidase and decrease from export of GSSG from the cell and from glutathione reductase and NADPH-mediated reconversion of GSSG to GSH [50]. Because of the low concentrations of GSSG relative to GSH, small increase in the oxidation of GSH to GSSG results in increase ROS and H_2O_2 production.

Lead is known to deplete GSH level which results in the excess formation of GSH from cysteine via the γ-glutamyl cycle but GSH is usually not effectively supplied, if depletion continues because of chronic metal exposure. Several enzymes in antioxidant defense systems may protect the imbalance between pro-oxidant and

antioxidant but unfortunately most of the enzymes contain sulfhydryl groups at their active site hence become inactive due to direct binding of lead to sulfhydryl group [51,52].

Overall, these inhibitory effects of lead on various enzymes would probably result in impaired antioxidant defenses by cells and render cells more vulnerable to oxidative stress. After the treatment with *T. terrestris* root extract and Vitamin C, GSH level was improved. In this study, administration of lead acetate showed elevation in testicular acid phosphatase activity and a significant ($p<0.01$) decrease in testicular alkaline phosphatase activity which reflects testicular degeneration, which may likely be a consequence of suppressed testosterone and indicative of lytic activity [53].

Administration of *T. terrestris* root extract (group III) and Vitamin C (group IV) significantly improves the enzyme activities after lead exposure. Vitamin C is a major antioxidant that scavenges the aqueous ROS by very rapid electron transfer that inhibits lipid peroxidation and increased the activity of GSH and other antioxidant enzymes [54].

V. CONCLUSION

In conclusion, we would like to state that *T. terrestris* root extract and vitamin C plays a protective role against lead induced toxicity in mice testes. This protective effect of *T. terrestris* root extract and vitamin C suggests the antioxidant and radical scavenging activity of the plant extract and vitamin C.

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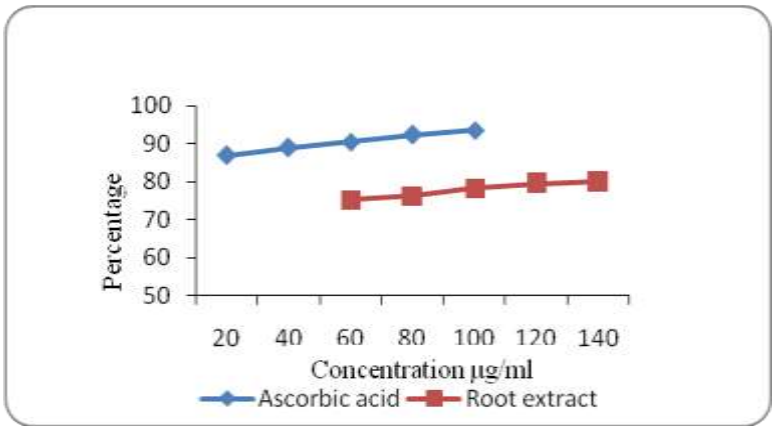

Fig.1. DPPH % inhibition of the *T. terrestris* root extract. The curve was obtained by plotting various concentrations of the *T. terrestris* root extract and ascorbic acid against percent inhibition of DPPH radical. 78.27% (IC_{50}=39.85 µg/ml) inhibition of *T. terrestris* root extract and 93.54% (IC_{50}=33.16 µg/ml) inhibition of standard ascorbic acid on 100 µg/ml concentration of DPPH free radical were observed.

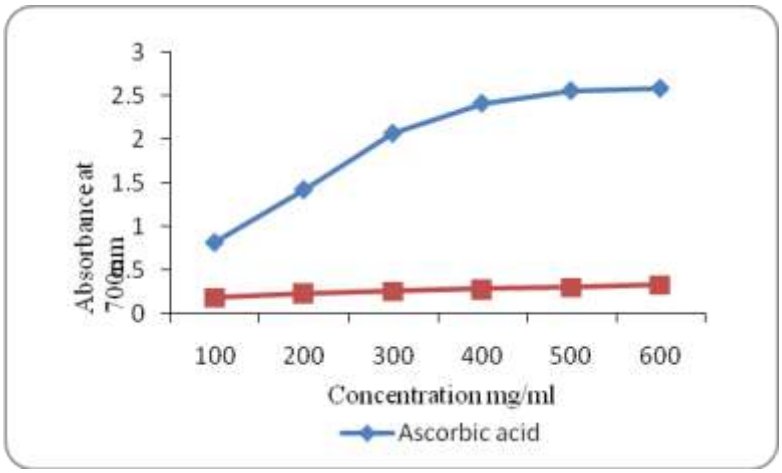

Fig.2. Determination of reducing power of the *T. terrestris* root extract and ascorbic acid. A concentration dependent increase in the antioxidant activity of *T. terrestris* root extract and ascorbic acid was observed.

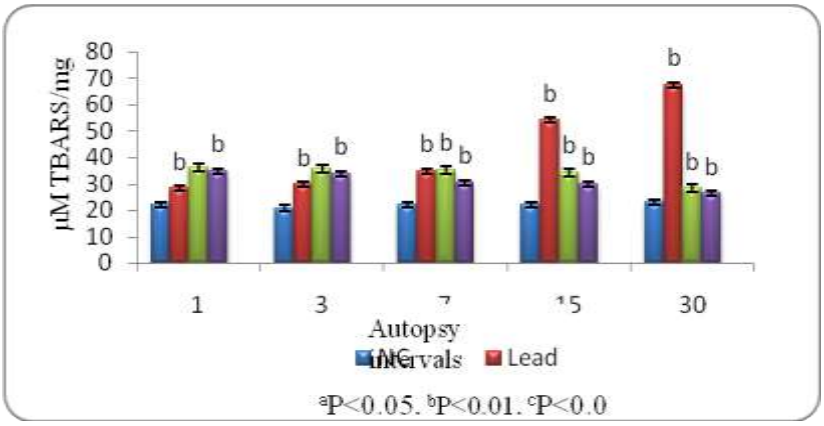

Fig.3. Effect of *T. terrestris* root extract and vitamin C (positive control) on LPO level (µM TBARS/mg protein) against lead-induced toxicity in testes of Swiss albino mice.

NC= Normal Control (Group I), Lead= Lead acetate treated (Group II), Pb+Root= Lead + Root extract treated (Group III) and Pb+Vit C= Lead + vitamin C treated (Group IV). Group II was compared with Group I and Group III and Group IV were compared with Group II statistically.

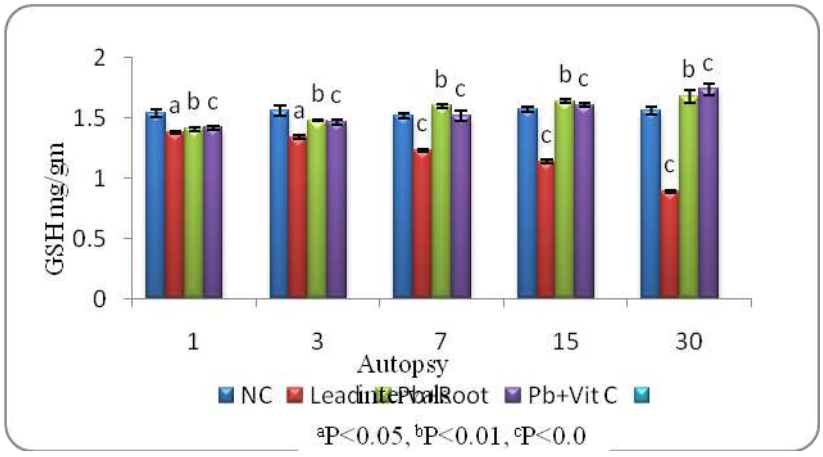

Fig.4. Effect of *T. terrestris* root extract and vitamin C (positive control) on GSH level (mg/gm tissue) against lead-induced toxicity in testes of Swiss albino mice.

NC= Normal Control (Group I), Lead= Lead acetate treated (Group II), Pb+Root= Lead + Root extract treated (Group III) and Pb+Vit C= Lead + vitamin C treated (Group IV). Group II was compared with Group I and Group III and Group IV were compared with Group II statistically.

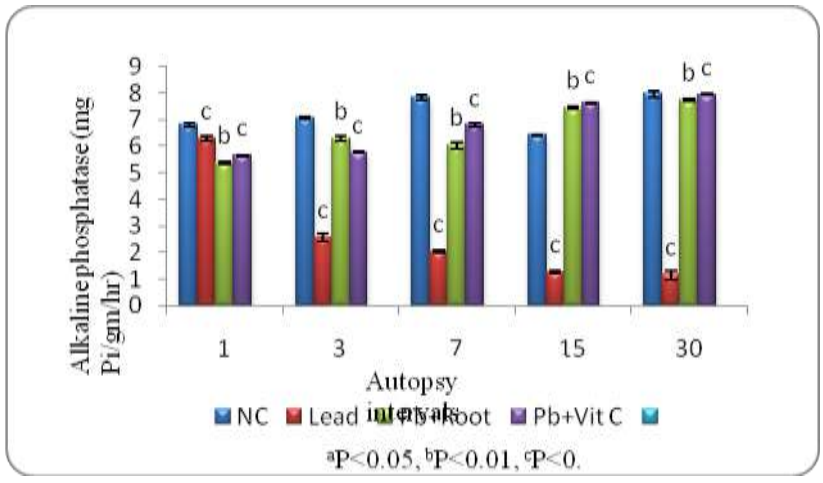

Fig.5. Effect of *T. terrestris* root extract and vitamin C (positive control) on alkaline phosphatase activity (mg Pi/gm/hr) against lead-induced toxicity in testes of Swiss albino mice.

NC= Normal Control (Group I), Lead= Lead acetate treated (Group II), Pb+Root= Lead + Root extract treated (Group III) and Pb+Vit C= Lead + vitamin C treated (Group IV). Group II was compared with Group I and Group III and Group IV were compared with Group II statistically.

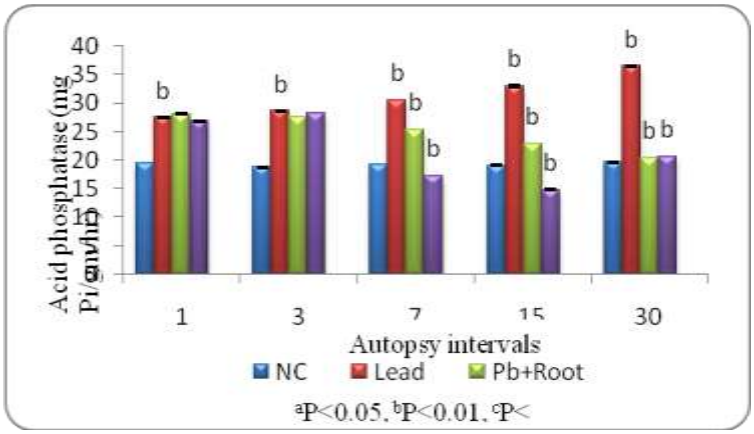

Fig.6. Effect of *T. terrestris* root extract and vitamin C (positive control) on acid phosphatase activity (mg Pi/gm/hr) against lead-induced toxicity in testes of Swiss albino mice.

NC= Normal Control (Group I), Lead= Lead acetate treated (Group II), Pb+Root= Lead + Root extract treated (Group III) and Pb+Vit C= Lead + vitamin C treated (Group IV). Group II was compared with Group I and Group III and Group IV were compared with Group II statistically.

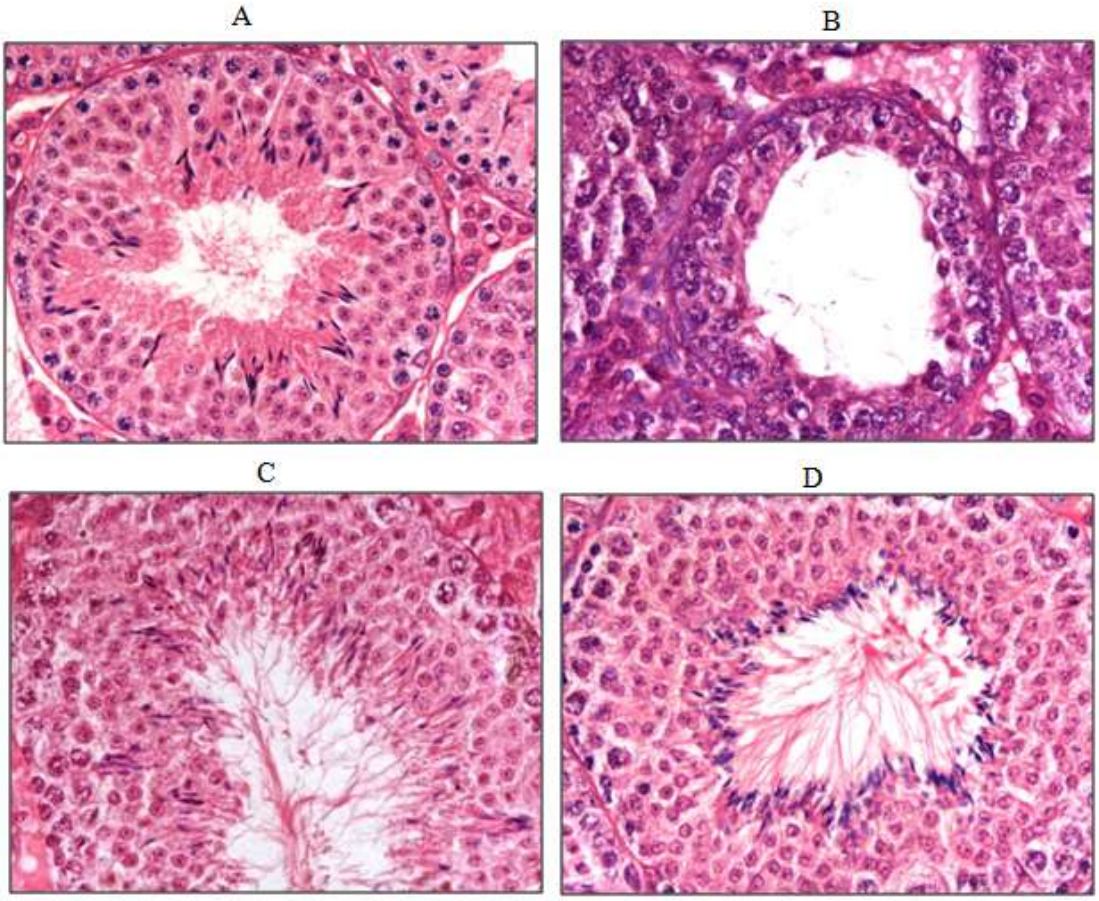

Fig.7. Haematoxyline and Eosin stained testes section of (A) normal mice testes showing active spermatogenesis with Spermatogonia A & B, Primary and Secondary spermatocytes and Spermatids (40x), (B) lead acetate treated group showing total cessation of spermatogenesis (40x), (C) *T. terrestris* root extract treated group (40x) and (D) vitamin C treated group showed almost normal histoarchitecture (40x).

Table 1. Phytochemical screening of the *T. terrestris* root extract

Phytochemical constituents	*T. terrestris* root extract
Phenolic compounds	-
Tannins	+
Saponins	+
Steriods	+
Phlobatannins	-
Terpenoids	-
Flavonoids	-
Cardiac Glycosides	+

+ = presence of constituent, - = absence of constituent

Table 2. Prophylactic efficacy of *T. terrestris* root extract on Testosterone, FSH and LH level in testes of lead-intoxicated mice

Parameters	Normal Control (Untreated) Group I Double Distilled Water	Treated Mice Group		
		Group II Lead acetate	Group III Lead acetate + *T. terrestris* aqueous root extract	Group IV Lead acetate + vitamin C (Positive control group)
Testosterone (ng/ml)	2.14±0.026458	1.60±0.052915[c]	2.3±0.027285[c]	1.9±0.031798[c]
FSH (mIU/ml)	6.2±0.026034	4.2±0.032146[c]	6.4±0.026458[c]	7.4±0.045826[c]
LH (mIU/ml)	7.9±0.053645	7.4±0.020817[c]	9.4±0.055076[c]	9.7±1.020065[c]

Significance level is set as [a]P<0.05, [b]P<0.01, [c]P<0.001.