Journal of New Technology and Materials (JNTM)
J. New Technol. Mater.
Vol. 08, N°02 (2018)70-76

OEB Univ. Publish. Co.

Effect of alkaloids extract of peganum harmala seeds on histo-function of rat's testes

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Received date: Jan. 03, 2018; revised date: Oct. 18, 2018; accepted date: Nov. 12, 2018

Abstract

The Peganum harmala plant is commonly used as traditional herbal medicine in many countries including Algeria. The aim of this study was to investigate the effect of alkaloids extract of P. harmala seeds on histo-function of rat'stestes. Twenty adult Albinos rats were divided equally into four groups, and treated intraperitoneally for 30 days: Group I served as control, received water. Group II, group III, and Group IV received daily a single dose of 40, 80, and 120 mg/kg body weight of alkaloids P. harmala extract respectively. Harmine and harmaline were the components identified in total alkaloids seeds extract by using high performance liquid chromatography (HPLC) method. Body weight, testes relative weights, and testosterone serum levels decreased significantly in group III (P< 0.05) and group IV (P<0.01), whereas levels of LH hormone decreased significantly only (P<0.01) in group IV. The histological alterations were seen in group III and IV,but they were severe in group IV.They mainly included stop of spermatogenesis, deformation in seminiferous tubules structure, damage to leydig cells, and absence of Sertoli cells.In conclusion, P. harmala alkaloids induced testicular toxicity at higher doses, and disturbed the function of hypothalamic pituitary testis axis, which caused infertility.

Keywords: Peganum harmala;HPLCofAlkaloids;β-carboline; Reproductive hormones; Rat testis histology

1. Introduction

Medicinal plants were well known to contain many effective therapeutic compounds. Therefore, the uses of these types of plants for traditional medicine have increased in recent years, especially among peoples of developing countries.*Peganum harmala* L. is one of the most used plants in Algerian folk medicine. People used seeds for delayed menstruation and abortion, leaves to relief pain of joint and rheumatism, and boiled solution of leaves to wash children's clothing against jaundice. In addition, seeds and aerial parts are used as anti-inflammatory agents.*P. harmala* L. is a wild and perennial plantthat belongs to the family Zygophylaceae. It is widely distributed in the Mediterranean region (North Africa), Central Asia, America and Australia [1]. In Algeria, it grows spontaneously on the edges of roads, in arid and rocky areas, and sandy soils [2]. *P. harmala* is an important source of natural products, such as essential oils, tannins, alkaloids, anthraquinones, coumarins, flavonoids, sterols and terpenoids [3]. These compounds are of great importance in pharmacology [4], and recently *P. harmala* capsules have shown to relieve symptoms associated with urinary tract pain in men with benign prostatic hyperplasia [5]. Moreover,many studies reported that alkaloidsof type β-carboline constituted the main active components in seeds of the plant [6].These chemical were represented by harmine, harmaline, harmol and harmalol, which are responsible for many biological and pharmacological activities [7], like hypothermic [8], antinociceptive [9],antitumoral activity, cytotoxicity [10, 11],inhibition of monoamine oxydase (MAO) [12] and myeloperoxidase[13] enzymes,Antidepressant [14], antibacterial and antiviral[15, 16]. The plant is not always benefit to human and animals. It can cause various complications and induced toxicity if it's consumed excessively [17]. Some studies *in vivo* showed that *P. harmala*alkaloids had toxic effects on rat embryos, leading to fetal mortality, decreasing fetal body weight, enhancingskeletal anomalies and abortion [1,18].

We previously found that the alkaloids of the *P.harmala* plant grown in Algeria are moderately toxic products and lethal dose [LD_{50}] was 350 mg / kg body weight [3]. Since the toxicity of *P.harmala* was mainly related to presence of alkaloids in seeds of plant, and there is no studies on their effects on male reproduction function. Therefore we are interested in study of sub-chronic toxicity of the alkaloids of *P.harmala* seeds from Eastern Algerian on histology and function of adult rat testes.

2. Materials and methods

2.1. Plant collection and identification

Seeds of *P. harmala* were collected from the Harmalia region (South-East of Ain M'lila, Algeria) in September 2013. The plant was identified according to the encyclopedia of plants [2].

2.2. Chemicals

Harmine (Aldrich-286044) and Harmaline (Aldrich-51330) were purchased from sigma Aldrich, while, other chemicals and reagents including HPLC grade solvents were purchased from Merck, Darmstadt, Germany.

2.3. Total alkaloid extraction

Total alkaloids were extracted according to the method described by Balbaa *et al.* [19]. 100 g of dried seeds of *P. harmala* were macerated in ethanol (70%, v/v), evaporated to one fifth of the initial volume by rotary evaporator. The ethanol extract was subsequently dissolved in 20 ml of hydrochloric acid (0.1N) and filtered. Then, it was extracted twice with 20 ml of chloroform, and then treated twice with 10 ml of hydrochloric acid (0.1 N). The aqueous layer was adjusted to pH= 9 with ammonia (NH_3;0.1 N) andextracted three times with chloroform. Finally, total alkaloids were obtained by evaporation of chloroform extract.

2.4. Analyses of alkaloids by High performance liquid chromatography(HPLC)

Qualitative determination of harmala alkaloids was performed by high Performance Liquid Chromatography (HPLC). The LC system is consisted of a Shimadzu Prominence model LC-20ATpump, a degasys DG-20A3 and a UV-SPD-20A Prominence UV/VIS detector. Separation was carried out using an EC 125/4.6 Nucleosil 100-5 C18 Nautilus column. We followed the method described by Kartal *et al.* [6] with some modifications.The mobile phase composition was Isopropyl alcohol - Acetonitrile–Water -Formicacid (100:100:300:0.1) (v/v/v/v)and pH adjusted 8.3 with triethylamine. The flow rate is 1.5 ml/min under room temperature (28° C). The extract of the total alkaloids of the seeds and the standards harmine and harmaline are solubilized in methanol grade HPLC [20]. All extracts were filtered through a 0.45 µM polypropylene filter and the injection volume was 10µl. Absorbance detection was set at 330 nm. The chromatographic run time was 10 minutes.

2.5. Animals

Twenty sexually mature *Wistaralbino* rats weighing 161.55±3.00 g were used for the present study.The animals were housed in plastic cages(5 animals /cage).Before experimentation, animals were acclimatized for two weeks at a temperature of 24± 2°c, and under natural photoperiod conditions (12 h light: 12 h dark cycle), with free access to standard pellets dietprovided by National livestock food board (Bejaia, Algeria), and water *ad libitum*. All experimental procedures were approved by the ethics and regulations of animal experiments of the University.

2.6. Experimental design

Animals were randomly divided into four groups, each group contained five rats, and animals were treated for 30 days intraperitoneally: The first group (group I) served as control, received water. The second group (group II) received daily a single dose of 40 mg/kg body weight (bw) of alkaloids*P. harmala*extract. The third group (group III) received daily a single dose of 80 mg/kg bw of alkaloids *P. harmala* extract, and finally the forth group (group IV) received daily a single dose of 120 mg/kg bw of alkaloids *P. harmala* extract. All the animals were euthanized by chloroform and sacrificed after 24h of the last treatment. Then, blood and testes were collected for hormones assay and histological studies respectively, In other hand, the relative weight of testis (%) was calculated as grams per 100 grams body weight (absolute testes weights/bw of rat on the day of sacrifice) X100.

2.6.1. Blood and hormone assays

Blood samples were collected from abdominal aorta of each animal, then blood samples were centrifuged at 3000 rpm for 10 min. Serum was separated and stored at - 20°C until were used. The serum concentration of testosterone and LH is measured by the kit Mini Vidas, Biomerieux Diagnostic, Automated Immunoassay Analyzer. The assays were conducted according to the standard manufacturer's protocol.

2.6.2. Histological studies

For histological studies, testes were dissected immediately from sacrificed rats, cleaned from the adherent fat, weighed (absolute organ weight), fixed in Bouin solution for 24 h,dehydrated in ethyl alcohol, cleared in xylol, and then embedded in paraffin.Sections of 5-µm thickness were stained with hematoxylin and eosin (H&E) and examined under light microscope. Photomicrographs of the desired sections were obtained for further observations.

2.7. Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M). Student's "t" test was used to compare between the mean values of paired groups, using Statisticasoftware (Version 5.1, StatSoft France, 1997).

Values were considered statistically significant when P<0.05.

3. Results and discussion

3.1. HPLC analysisof the alkaloids of Peganum harmala

Using known standards of harmaline and harmine (Fig.1 and Fig. 2), the HPLC analysis of the alkaloids of the seeds showed the existence of these two alkaloids. The retention times for harmine and harmaline were observed to be 2.70 and 4.40 min respectively (Fig. 3). The total time of analysis was less than 10 minutes.

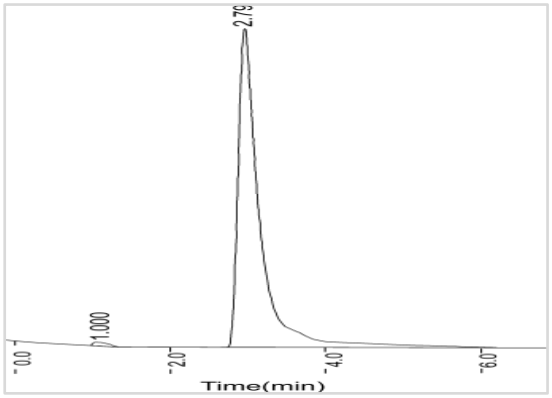


Figure 1. Chromatogram of methanolic sample of harmine (2.79min)

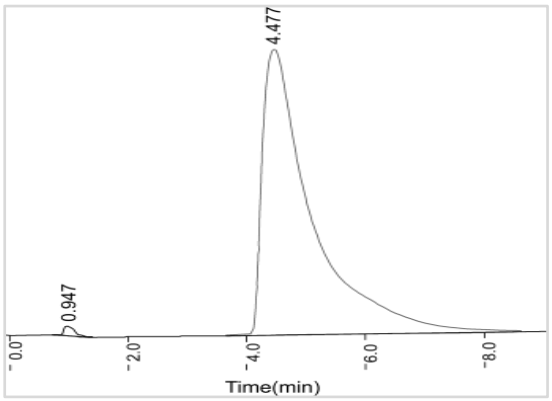


Figure 2. Chromatogram of methanolic sample of harmaline (4.47min)

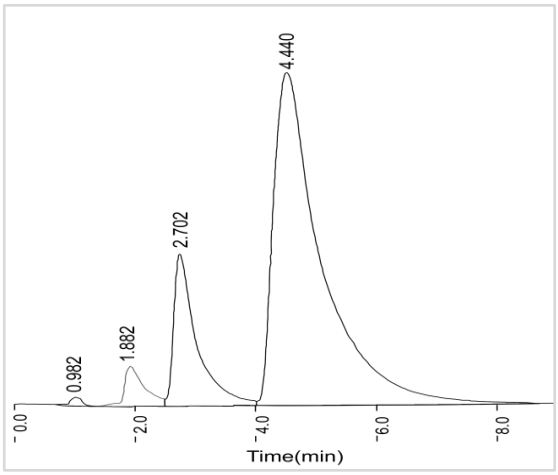


Figure 3. Chromatogram of *P.harmala* seeds extract: harmine (2.70min) and harmaline (4.44 min)

3.2. Effect of treatment on body and testes weights

The effect of administration of alkaloids extract of *P. harmala* on body weight of male *Wistar albino* rats during 30 days were presented in Table 1. Final body weights increased in all groups compared to their initial body weights, but the increase in treated groups with 80 and 120 mg /kg bw were significantly (15.68 %; P< 0.05, 18.70 %; P< 0.01) lower than those of control group (24.21%). While body weights did not increased significantly when rat were treated with low dose of alkaloids extract of the plant (40 mg/kg bw).

Table 1. Effect of different concentrations of P. harmala alkaloids seeds extracts on body and testes weights of male rats after 30 days of treatment

Parameters	Group I (control)	Group II (40 mg/ kg bw)	Group III (80 mg/kg bw)	Group IV (120 mg/kg bw)
Initial bodyweight(g)	162 ± 2.90	162.41 ± 2.80	160.92 ± 3.412	160,38 ± 2.95
Final body weight(g)	201.76± 5.863	199.24 ± 2.59^{NS}	191.02 ± 5.77*	185.53 ± 6.04**
increase in weight %	24.21	22.67	18.70	15.68
Absolute testis weight	2.11 ± 0. 16	1.86 ± 0.40^{NS}	1.67 ± 0.27*	1.36 ± 0.25**
Relative weight of testis (g/ 100g bw)	1.04 ± 0.05	0.93 ± 0.18^{NS}	0.87 ± 0.12*	0. 76 ± 0.10**

Values are given as mean ±S.E.M. (n=5).
Significant at $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, and NS (non significant) vs. control group.

The decrease in rat body weights might be due to diarrhea and anorexia observed during experimentation, because of the anticholinergic effects of β-carboline alkaloids; leading to digestive disorders [21].

These β-carboline alkaloids were represented mainly by harmine and harmaline as found by HPLC analysis (Fig. 3).Similarly[22] noticed a significant decrease in body weights of rabbits compared to control group when treated intramuscularly with chloroformic extract of *P.harmala*.In the same manner of body weights changes, absolute and relative testes weights in group III and group IV revealed a significant decrease in comparison to control group (Table 1). Similar results were reported by EL-Dawairi and Banihani [23]and Ramaiyan Dhanapal [24]. The decrease in both types of testes weight could be explained by testicular damage at the histological level as seen by the reduction in height of germinal epithelium of seminiferous tubules, cell degeneration, and finally by stopping spermatogenesis (Fig. 8 and Fig. 9). This decrease in relative weight of testes was also related to decrease in serum levels of testosterone found in this study (Fig.4), where the production of this hormone became insufficient to maintain gonad weight [25]. It's well known that testosterone is necessary for maintaining the normal growth of testes.

3.3. Effect of treatment on serum levels of testosterone

Treatment of different concentrations of *P.harmala* alkaloids seeds extract induced significant decrease in serum testosterone in group III (3.63± 0.05 ng/ml; P< 0.05) and group IV (2.62 ± 0.049 ng/ml; P <0.001) in comparison with control group of rats (3.72± 0.01 ng/ml) as observed in Fig. 4.

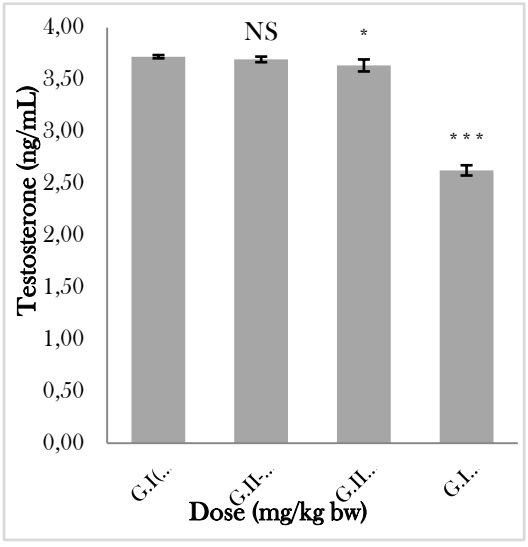


Figure 4. Effect of different concentrations of *P.harmala* alkaloids seeds extract on serum testosterone levels after 30 days of treatment. Valueswere given as mean ±S.E.M. (n=5). Significant at *P <0.05, **P<0.01, ***P<0.001, and NS (non significant) vs. control.

The possible mechanism of decrease in this hormone was degeneration or decrease in numbers of functional leydig cells; site of testosterone production as shown by histological studies. The functional efficiency of leydig cells was disturbed in uptake of cholesterol in accordance to these histological findings, where these cells had decreased in numbers and had scanty cytoplasm. A decrease in testosterone levels was also due to decrease in luteineizing hormone (LH) secretion in rats treated with 120 mg/kg bw of *P. harmala* alkaloids (Fig. 5). It's well known that LH binds to leydig receptors to induce cholesterol uptake and to stimulate steroidogenesis, which led to increase in testosterone production. Moreover, β-carboline alkaloids were shown to have high-affinity type II ligand for steroidogenic cytochromes *P*450, viz. CYP17 in rat testicular microsomes [26], which might be competitively inhibited testosterone production.

3.4. Effect of treatment on serum levels of LH

LH serum levels declined only significantly (2.90±0.079 mIU/ml; P< 0.01) in high dose treated animals (120 mg /kg bw; group IV) compared to control ones (3.47± 0.24 mIU/ml). The other two groups of treated rats (Group II and group III) did not show significant variations in their LH serum concentrations in comparison with control group (Fig. 5). This decrease could be explained by the negative effect of alkaloids on function of gonadotropic cells in anterior pituitary gland responsible for LH production. In other hand, alkaloids might influence the function of hypothalamic cells responsible for LH production, thus resulting in disturbance of hypothalamus pituitary axis.

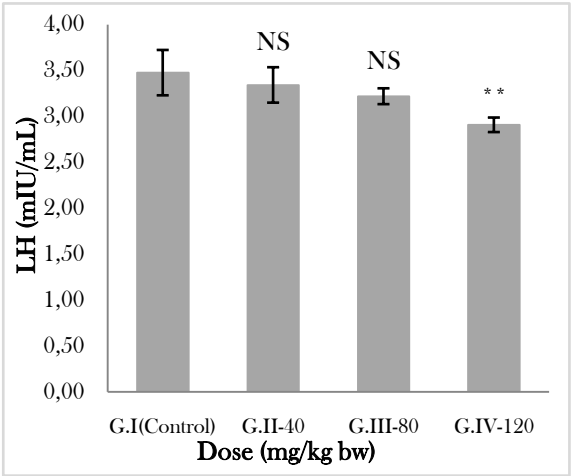


Figure 5. Effect of different concentrations of *P. harmala* alkaloids seeds extract on serum LH levels after 30 days of treatment. Values were given as mean ±S.E.M. (n=5). Significant at *P <0.05, **P <0.01, ***P <0.001, and NS (non significant) vs. Control.

3.5. Effect of treatment on testis histology

For histological studies, all rats in the control group showed normal histological pattern (Fig. 6 a–b).

Seminiferous tubules were rounded or oval contoured by regular basement membrane, contained the different stages of the normal development of germ cells, narrow lumen filled with spermatozoa, and the interstitium contained a delicate loose connective tissue and leydig cells.

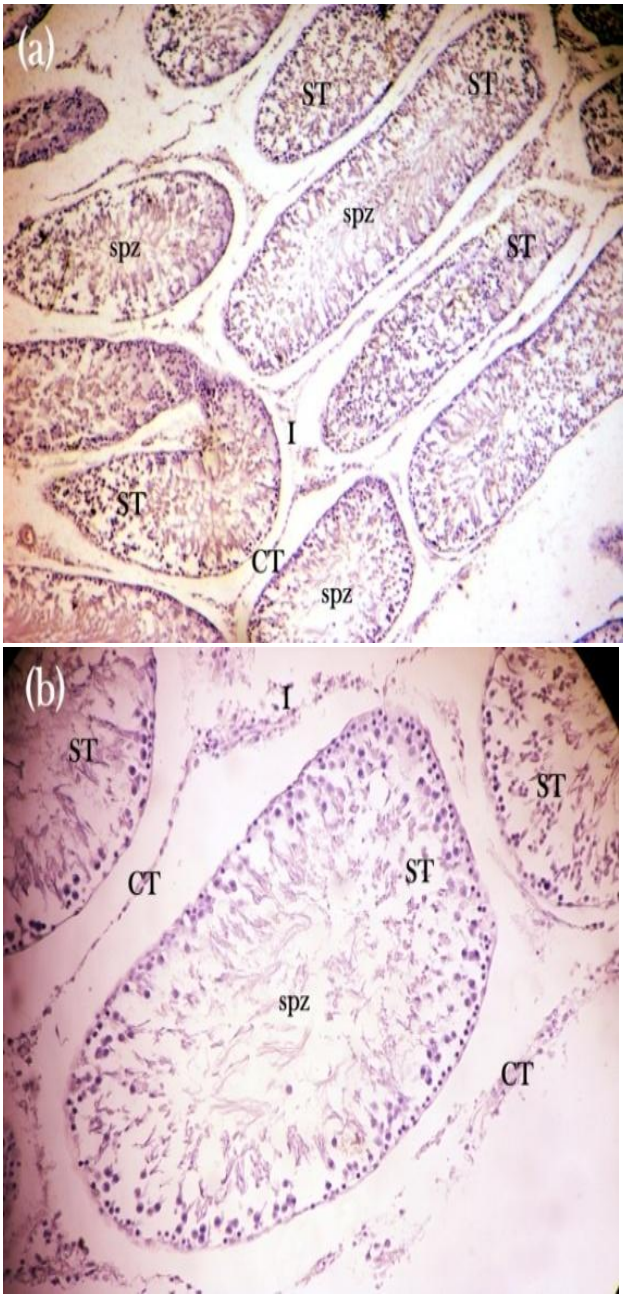


Figure 6. Photomicrographs of control rat testis (a and b) showing seminiferous tubules (ST) with regular contour, and containing different types of germ cells with narrow lumen filled with spermatozoa (spz). Interstitium (I) contained connective tissue (CT), and leydig cells (a: x100; b ×400, H & E).

The results of the effect of alkaloids extract of *P. harmala* on spermatogenesis in rats were presented in Figs. 7, 8 and Fig. 9. Haematoxylin and Eosin stained section of 30 days treated animals with 40 mg/kg alkaloids extract of *P. harmala* seeds was presented in Fig.7.

It revealed also normal histology of testis and seminiferous tubules, where all germ cell lines were visible (spermatogonia, primary spermatocytes and spermatids, and spermatozoa).

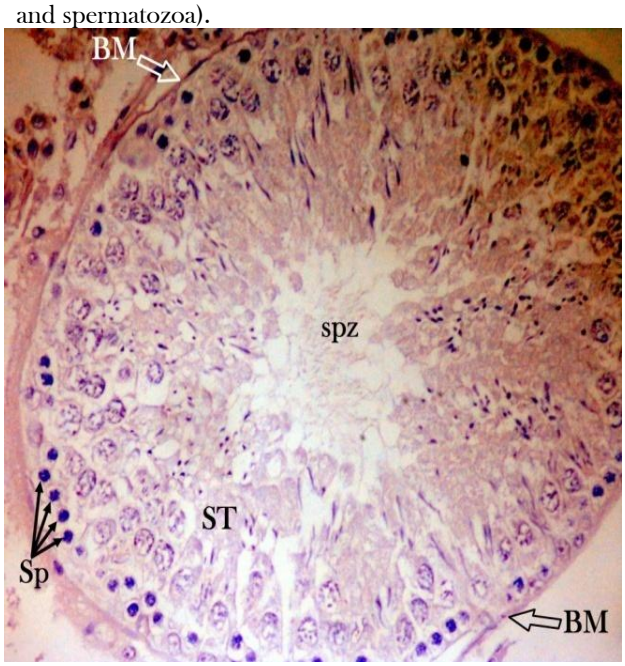
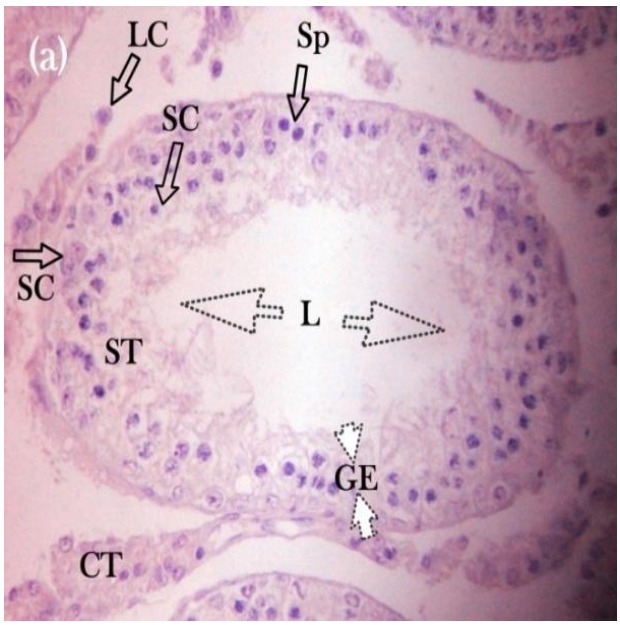


Figure 7. Photomicrograph of rat testis treated with 40mg/kg of alkaloids extract of *P. harmala* seeds showing normal structure seminiferous tubules (ST), and all stages of spermatogenesis and spermatozoa (spz) as seen in control group. Detachment of basement membrane (BM)in some places (×400, H & E)

Testes of rats administered with 80 mg /kg bw (Fig. 8) showed many histopathological changes. These changes included disturbance of the intertubular connective tissue



surrounding seminiferous tubules, and contained leydig cells with scanty cytoplasm and deeply stained nuclei, with

decrease in their numbers. The seminiferous tubules had reduced germinal epithelium with only spermatogonies and the presence of fewer Sertoli cells, and as a consequence an increase in lumen of seminiferous.

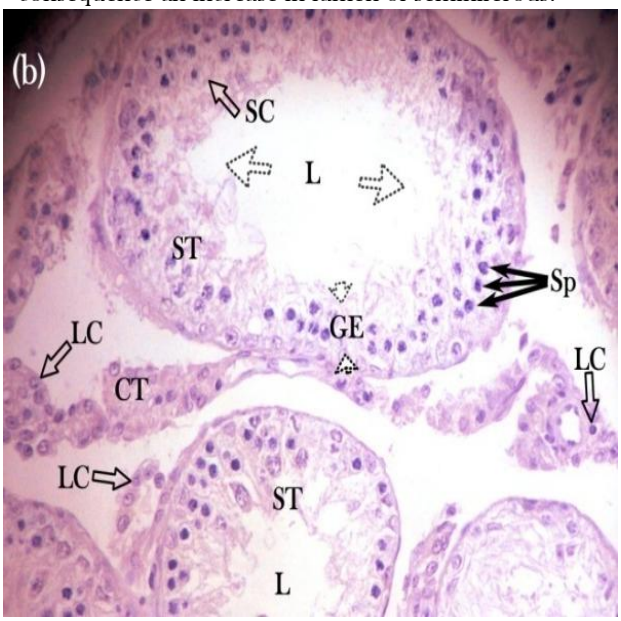


Figure 8. Photomicrographs of rat testis administered with 80mg/kg of alkaloids extract of *P. harmala* seeds (a and b) showing a decrease in width of germinal epithelium (GE) with only spermatogonies (Sp) and an increase in lumen (L) of seminiferous tubules (ST) with disturbance in interstitium, Sertoli cells (SC) were also presentleydig cell (LC) had scanty cytoplasm with deeply stained nuclei (×400, H & E)

The histopathological alterations seen in group III were increased in rats testes with 120 mg /kg of alkaloids extract of *P. harmala* seed (Fig.9; group IV). Cases of acute atrophy of the sperm tubes structure and of interstitial tissue were observed, with complete absence of sperm in the tube lumen, the degeneration of Sertoli and spermatogenic cells. Interstitum between seminiferous tubules was disturbed, and basement membrane was thickened in some places. Complete degeneration of the spermatogenic epithelial cells, and an increase in numbers of degenerated leydig cells were observed

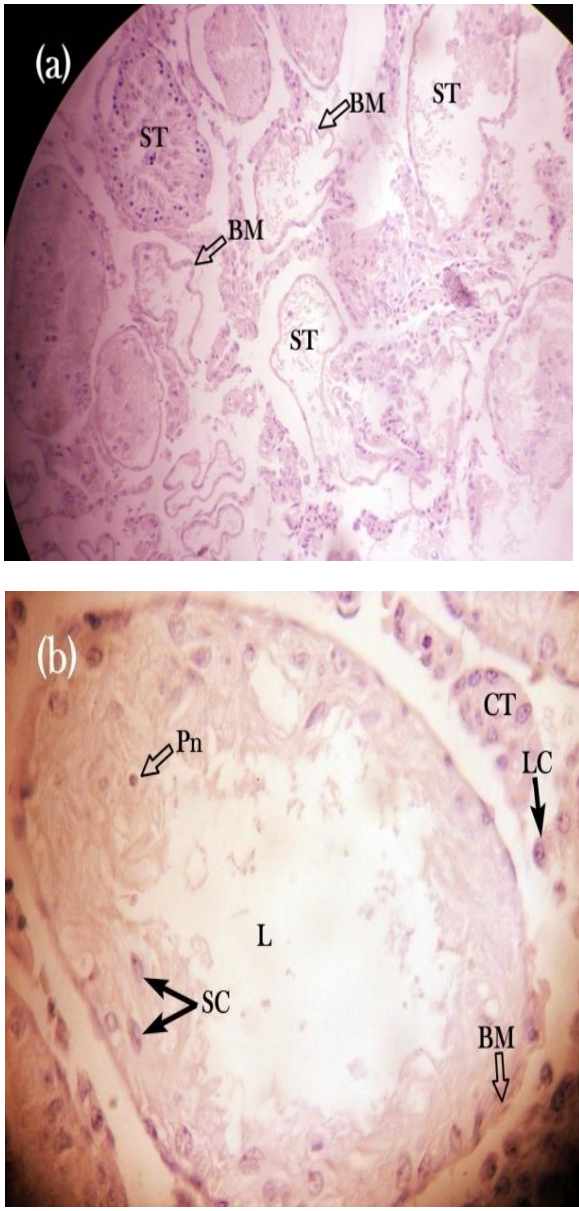


Figure 9. Photomicrographs of rat testis administered with 120mg/kg /day of alkaloids extract of *P. harmala* seeds (a and b) showing acute atrophy of the sperm tubes structure, Most of them had lost their normal shape; with totally stop of spermatogenesis as seen by total absence of sperm in lumen tube (L), complete degeneration of the spermatogenic epithelial series, and degeneration of sertoli cells (SC). Pyknotic nucleus (Pn) and rupture of the intertubular connective tissue (CT) wereseen with increase in degeneration of leydig cells. The basement membrane (BM) was disrupted and thick-ened, leydig cels (LC) with scanty cytoplasm were seen (a: x100; b ×400, H & E)

Thearrested spermatogenesis and cell degeneration observed in male rats treated with high doses of alkaloids compared to the control group, correlated to decrease in testosterone levels found in this study (Fig. 4), which was necessary for spermatogenesis. Other studies, reported

that the morphine alkaloids could affect all stages of sperm formation[27]and had anti-fertility activity [28; 29].

The histo-toxicity of alkaloids seen on testes was also dose dependent, and these effects included mainly the stop of spermatogenesis, cells degeneration, and lost of seminiferous tubules structure. The mechanism of action of alkaloids might be explained by inducing cell death in humans via autophagy and not by apoptosis [30], or by cytotoxic activity of against human tumor cell lines as reported by Cao *et al.* [31]. Moreover, Lamchouri *et al.* [10]had also revealed in vitro that alkaloids of the *P.harmala* plant possessed toxic and anti proliferative effects on several murine tumor cell lines.

4. Conclusion

In this study, harmine and harmaline were identified with HPLC method as two natural components of total alkaloids of *P.harmala* seeds extract. We concluded that the seed extract of this plant when administered at higher doses to adult male albino rats caused decrease in the body weights and relative testes weights, toxic degenerative changes of testis tissue, arrest of spermatogenesis, decrease in production of reproduction hormones represented by testosterone and luteinizing hormone (LH).

Therefore,*P.harmala* alkaloids induced testicular toxicity at high doses, and disturb the function of hypothalamic pituitary testis axis, which caused infertility. The study suggests that people should pay attention in using this plant as traditional medicine, and drugs from seeds of this herb must be tested for their effects on reproduction.

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