Effect of neem oil on the structure and function of the mature male albino rat testes

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Abstract

This study was undertaken to observe the effect of neem oil, on the microscopic structure of testes in mature male albino rats and the associated changes in the serum levels of male reproductive hormones. The animals were divided in different groups as A_1 = treated males at low dose (0.6 mL of neem oil/animal), A_2 = treated males at high dose (1.2 mL of neem oil/animal), A_3 = controls for group A_1 (corresponding dose of peanut oil) and A_4 = controls for A_2 (corresponding dose of peanut oil). Animals were kept under observation for a period of six weeks. At the end of this period animals were anesthetized, blood was removed by cardiac puncture and sacrificed. Testes were removed and fixed in10% formolsaline for microscopy and methanol for HPLC purpose. The microscopic sections of the testes from animals treated at high dose have revealed significant decrease in the seminiferous tubular diameter and number of spermatozoa. Interstitial spaces are shown to be wide with possible decrease in the number of Leydig cells. These changes are associated with significant alteration in the serum levels of male reproductive hormones and presence of higher concentrations of active neem compounds in testicular tissue.

Keywords: antifertility, seminiferous tubule, sterility, testes, testosterone.

1 Introduction

Neem, Azadirachta indicia A. Juss is widely prevalent and highly esteemed wonder tree of Indian subcontinent, (SARKAR, BOSE, LASKAR et al., 2007) belongs to family Meliacae. The environmental compatibility of neem products, lack of resistance development to them, their harm less nature against non target organisms and lack of toxicity, all have significantly enhanced the integrated use of neem in medicine. It is a fast growing plant of tropical and sub tropical countries, usually reaches a height of 15-20 m.

It has been shown to posses anti fungal, anti worm, (FUJIWARA, TAKEDA, OKIHARA et al., 1982, 1984), anti-inflammatory (RAVIDHAR, ZHANG, TALWAR et al., 1998, FUJIWARA, TAKEDA, OKIHARA et al., 1982, 1984) insecticidal, (SCHUMUTTERER, ASCHER and REMBOLD 1981; SCHMUTTERER and ASCHER, 1984, 1987), Jacobson (1989), Randhawa and Parmar (1993), bactericidal (ARA, SIDDIQUI, FAIZI et al., 1989), Antifertility (JACOBSON, 1995) and immuno stimulating (van der NAT, KLERX, van DIJK et al., 1987), immuno modulating potential (SARKAR, BOSE, LASKAR et al., 2007).

Antifertility potential of neem has been reported by various researchers but most of the work has been done in the scientific laboratories of India. Naqvi (1998) referring the history and importance of this plant has mentioned that Sadhus of India used to chew neem leaves to suppress their libido during their meditation.

This study, have evaluated the effect of neem oil treatment on the microscopic structure of the testes, it's effect on serum concentration of male reproductive hormones and the quantitative analysis of the active neem components in testicular tissue by using HPLC.

2 Materials and method

The present study included forty eight (48) mature male albino rats of weight between 150-200 g. The animals were obtained from the Animal House of Baqai Medical University, Karachi. The strain of albino rats was obtained from Jinnah Post Graduate Medical Institute Karachi where it was originally obtained from Charles River Laboratory, Brooklyn, Massachusetts, USA and was cross bred. The animals were kept in experimental room for one week prior to the commencement of study, for acclimatization to experimental conditions with 12 hours light and dark cycle. The animals were fed at laboratory chow and water ad libitum.

Groups

- A_1 = low dose treated males = 12 rats, A_2 = high dose treated males = 12 rats.
- A_3 = controls for A_1 = 12 rats, A_4 = controls for A_2 = 12 rats.

Twelve animals were administered with low dose (LD) 0.6 mL/animal (MUKERJEE and TALWAR, 1996) of neem oil and were assigned as group A_1. Another twelve animals were administered with high dose (HD) 1.2 mL /animal of neem oil and were assigned as group A_2. Corresponding number of animals were administered with corresponding doses of pea nut oil and were assigned as group A_3 and A_4.

They acted as control groups for A_1 and A_2 respectively, (MUKERJEE and TALWAR, 1996). All animals were administered with single oral dose between 10-12 hours. The animals were kept under observation for a period of six weeks.

At the end of experiment, the animals were anesthetized by deep ether anesthesia, blood is removed from heart by cardiac puncture (ALLAN, HAYWOOD, SOJI et al., 2001) for hormonal analysis and sacrificed (INAUWA and WILLIAMS, 1995). One of the testes from each animal removed and fixed in 10% formolsaline (BANCROFT, 1990, RONING and KANTOMA, 1988) for microscopy and the other testes from each animal removed and placed in methanol and water, 1:1 composition for HPLC purpose.

2.1 Microscopy

Testes which were fixed in formal saline (BANCROFT, 1990, RONING and KANTOMA, 1988) blocked in Paraffin wax cut horizontally at 5 µ thickness and stained with hemotoxyline and eosin (BANCROFT, 1990). Stained horizontal sections were observed under light microscope for microscopic changes and micrometry.

2.2 Hormonal analysis

Blood removed from the animals by intracardiac method was centrifuged at 2000 rpm (Revolution per minute) to separate the serum for the measurement of FSH, LH, and Testosterone.

The quantitative determination of hormones was done by using Enzyme Immunoassay Method (ELISA). The Kits used for this purpose were provided as follows:

- for Luteinizing hormone

By Equipar Srl via G.Ferrari 21/N-21047 saronno (va) italy

- for testosterone

By Biocheck, Inc, 323 vintage park Dr. Forster City, CA 94404

- for FSH

By Pishtazteb Diagnostics - Nº 1855, 13th Alley, Simaye Iran.

2.3 Quantitative analysis of neem compounds by high performance liquid chromatography (HPLC) method

a) Cleaning procedure of samples for HPLC:

For determination of residual components of neem oil, the tissues were ground in Teflon Pyrex tissue ground at 500 rpm, by speed control homogenizer. Then the neem oil components were subjected to following procedure.

b) Extraction of neem oil

Neem oil may bind with fat present in tissues. Therefore extraction by using Rota vapor was necessary.

Soxhlation method: For the extraction of neem compounds from samples, Holden and Marsden (1969) method was used. A known quantity of samples (One gm sample) was macerated with Na_2SO_4 (anhydrous sodium sulphate) and was transferred into a thimble made of filter paper. The thimble was then placed in the extractor which was fitted to the bolt head flask containing 170 mL of n-hexane, which was then fitted with condenser connected to the tap water for cooling. The apparatus was then placed on a water bath. The process of extraction was carried out for three hours during which all the fat contents have been extracted with the solvent. Better recoveries were noticed by this method. The fat extracted solvent was then reduced to about 1.o mL in rotavapor. For complete recovery of Neem Compounds the column chromatography (Sorption) was employed and the material was passed 3 to 4 times through the columns of alumina (HOLDEN and MARSDEN, 1969) and silica (KADOUM, 1968).

c) Quantitative analysis of neem compounds HPLC method

HPLC has been used for the separation of compounds by using a packed column (Zorbax™ NH_2), a polar bound phase with particle size of 7 µm in diameter. The columns were packed to uniform bed density by using a high pressure slurry loading techniques. This column was used with fractionated n-hexane as mobile phase with a flow rate of 0.5 mL/minute. A UV detector was used at a wavelength of 250 nm, pressure 20,265,004.8 pascles and absorbance 0.32 with chart speed 2.5 mm/min, for the detection of peaks of neem compounds. The purified samples of different concentrations were made and then 10 µL of the samples were injected by special chromatographic syringe, in the HPLC apparatus, attached with a chart recorder on the basis of retention time (RT) with the standards peaks, The area of each peak was calculated to quantify the detection of different compound residues in the samples. Concentrations of the compounds are given in the Table 4

2.4 Statistical analysis of results

In the present study the data was subjected to student's t test for statistical analysis.

3 Results

3.1 Seminiferous tubular diameter in testes (µm) AT 10X

3.1.1 Low dose group

The mean seminiferous tubular diameter at low dose treated male rats was 180.25 µm ± 5.50 µm while the seminiferous tubular diameter in control animals was 220.21 µm ± 6.60 µm at power 10, when measured under the microscope (Table 1, Figure 2). This difference in seminiferous tubular diameter is significant statistically ($p < 0.05$).

3.1.2 High dose group

The mean seminiferous tubular diameter at high dose treated male rats was 150.50 µm ± 6.80 µm while the seminiferous tubular diameter in control animals was 220.21 µm ± 6.60 µm at power 10, when measured under the microscope (Table 1, Figure 1, 3). This difference in seminiferous tubular diameter is highly significant statistically ($p < 001$).

3.2 Microscopic changes in testes

Seminiferous tubular diameter of the rats treated at high dose has decreased as compared to control rats. (Figure 1, 3). There is evident reduction in height of the lining cells in the tubules of these animals. Interstitial spaces between the seminiferous tubules are shown to be widened with a decrease in the number of Leydig cells in the sections from animals

Table 1. Comparison of seminiferous tubular diameter.

Group	Diameter (µm)	Statistical Significance	
		P Value	Remark
Treated at Low Dose	180.25 ± 5.50	(<.05)	Significant
Treated at High Dose	150.50 ± 6.80	(<.001)	Highly Significant
Control Group	220.21 ± 6.60		

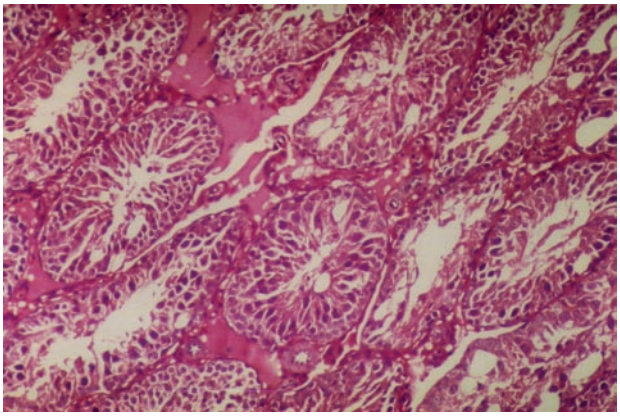

Figure 1. Photomicrograph of a 5 µm thick horizontal section of the testes from a control group male rat, showing seminiferous tubules, spermatozoa with in tubular lumen and interstitial space with Leydig cells.

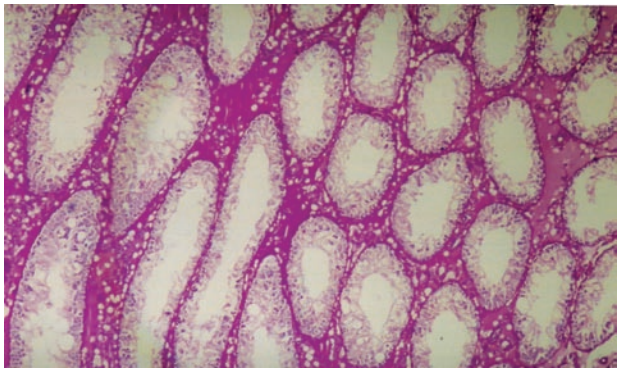

Figure 2. Photomicrograph of a 5 µm thick horizontal section of the testes from a male rat treated at low dose showing seminiferous tubules, few of them showing spermatozoa and interstitial spaces with Leydig cells.

treated at high dose. Most of the seminifeorus tubular lumen does not show any spermatozoa (Figure 3).

At low dose treatment the microscopic features of testes does not show any major change. Seminiferous tubules are showing intact germinal epithelial cells and within lumen spermatozoa are also seen (Figure 2).

3.3 Male reproductive hormones

a) At low dose

3.3.1 Follicle stimulating hormone (FSH)

Mean serum FSH concentration in treated male rats at low dose was 13.7 + 1.43 (Iu/L) while the FSH concentration in control rats was 13.2 + 3.16 (Iu/L). The FSH level in

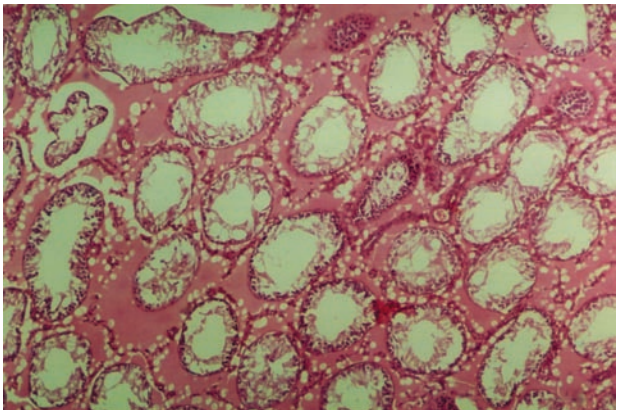

Figure 3. Photomicrograph of a 5 µm thick horizontal section of the testes from a male rat treated at high dose showing seminiferous tubules with narrow lumens, reduced height of the tubular lining cells and wider interstitial spaces as compared to Figura 1 and 2.

treated males at low dose is about 4% higher but statistically non significant ($p > 0.05$) when compared to control males of this group (Table 2)

3.3.2 Luteinizing hormone (LH)

Mean serum LH Concentration in orally treated male rats at low dose was 5.09 + 0.09 (Iu/L) while the LH concentration in control rats was 5.08 + 0.19 (Iu/L). There is no statistically significant difference ($p > 0.05$) in the LH levels, in treated males at low dose and control males of this group (Table 2) .

3.3.3 Testosterone

Mean serum testosterone concentration in treated male rats at low dose was 3.76 + 0.22 (ng/mL) while the testosterone concentration in control rats was 3.81 + 0.24 (ng/mL). The Testosterone level in treated males at low dose is about 3% lower and statistically non significant ($p > 0.05$) when compared to control males of this group (Table 2).

b) At high dose

3.3.4 Follicle stimulating hormone (FSH)

Mean serum FSH concentration in treated male rats at high dose was 13.00 + 0.89 (Iu/L) while the FSH concentration in control rats was 13.53 + 0.54 (Iu/L). The FSH level in treated males at high dose is about 4% lower and statistically non significant ($p > 0.05$) when compared to control males of this group (Table 3).

3.3.5 Luteinizing hormone (LH)

Mean serum LH Concentration in orally treated male rats at high dose was 3.67 + 0.21 (Iu/L) while the LH concentration in control rats was 5.86 + 0.54 (Iu/L). The LH

Table 2. Comparison of serum concentrations of reproductive hormones in males treated at low dose.

Hormone	Treated at low dose	Control group	Statistical significance	
			P Value	Remark
FSH (lu/l)	13.7 ± 1.43	13.2 ± 3.16	(>.05)	Non significant
LH (lu/l)	5.09 ± 0.09	5.08 ± 0.19	(>.05)	Non significant
Testosterone (ng/mL)	3.76 ± 0.22	3.81 ± 0.24	(>.05)	Non significant

FSH = Follicle Stimulating Hormone; LH = Leuteinizing Hormone; Iu/l = International Unit per litre; ng/mL = Nanogram per mm.

Table 3. Comparison of serum concentrations of reproductive hormones in males treated at high dose.

Hormone	Treated at high dose	Control group	Statistical significance	
			P Value	Remark
FSH (lu/l)	13.0 ± 0.89	13.53 ± 0.54	(>.05)	Non significant
LH (lu/l)	3.67 ± 0.21	5.86 ± 0.54	(<.05)	Significant
Testosterone (ng/mL)	3.49 ± 0.10	5.12 ± 0.17	(<.001)	Highly significant

FSH = Follicle Stimulating Hormone; LH = Leuteinizing Hormone; Iu/l = International Unit per litre; ng/mL = Nanogram per mm.

Table 4. Neem oil extracted with metahnol, Chromatography analysis.

S. No	Group	Time (Minutes)	Area of peak	Quantity per sample / concentration
	Standard			
	Azadirachtin	7.630	19959	1.99 µg /10 µl
	Azadirachtinin	8.917	85729	8.57 µg /10 µl
	Male albino rats			
	Male rats treated as low dose			
1	Azadirachtin	7.945	4693	0.4 µg /10 µl
	Azadirachtinin	9.397	7110	0.7 µg /10 µl
	Male rats treated as high dose			
2	Azadirachtin	7.282	16826	1.7 µg /10 µl
	Azadirachtinin	9.022	17237	1.7 µg /10 µl

level in treated males at high dose is about 37.37% lower and statistically significant ($p < 0.05$) when compared to control males of this group (Table 3).

3.3.6 Testosterone

Mean serum testosterone concentration in treated male rats was 3.49 + 0.10 (ng/mL) while the testosterone concentration in control rats was 5.12 + 0.17 (ng/mL). The testosterone level in treated males at high dose is about 32.23% lower and statistically highly significant ($p < 0.001$) when compared to control males of this group (Table 3).

3.4 Quantitative analysis of neem compounds

HPLC chromatographic recordings have shown consistent peaks for two compounds present in neem oil (Table 4) these compounds are Azadirachtin and Azadirachtinin.

Standard peaks recorded for these compounds were observed at following concentrations

a) Azadirachtin peak recorded at 7.00 ± 0.6 minutes with 1.99 µg/10 µL concentration per sample
b) Azadirachtinin peak recorded at 9.00 ± 0.7 minutes with 8.57 µg/10 µL concentration per sample

3.4.1 At low dose treatment

Testicular sample have shown

- peak for Azadirachtin 0.4 µg/10 µL conc. per sample
- peak for Azadirachtinin 0.7 µg/10 µL conc. per sample

3.4.2 At high dose treatment

Testicular sample have shown

- peak for Azadirachtin 1.7 µg/10 µL concentration per sample
- peak for Azadirachtinin 1.7 µg/10 µL concentration per sample

4 Discussion

At low dose treatment the levels of FSH are slightly higher while the levels of LH and Testosterone are not any different in treated and control animals. These hormonal levels reflect that the neem oil treatment in male, at low dose, does not alter the male reproductive hormones. Animals treated at high dose have shown the lower levels of all male reproductive hormones including FSH, LH and testosterone as compared to control male rats. These hormonal levels are very much correlate to the microscopic changes (Figure 3) in the tests which have shown decrease in the diameter of seminiferous tubules (Table 1) widening of interstitial spaces with an evident decrease in the number of leydig cells. Most of the tubules are not showing spermatozoa in the lumen (Figure 3).

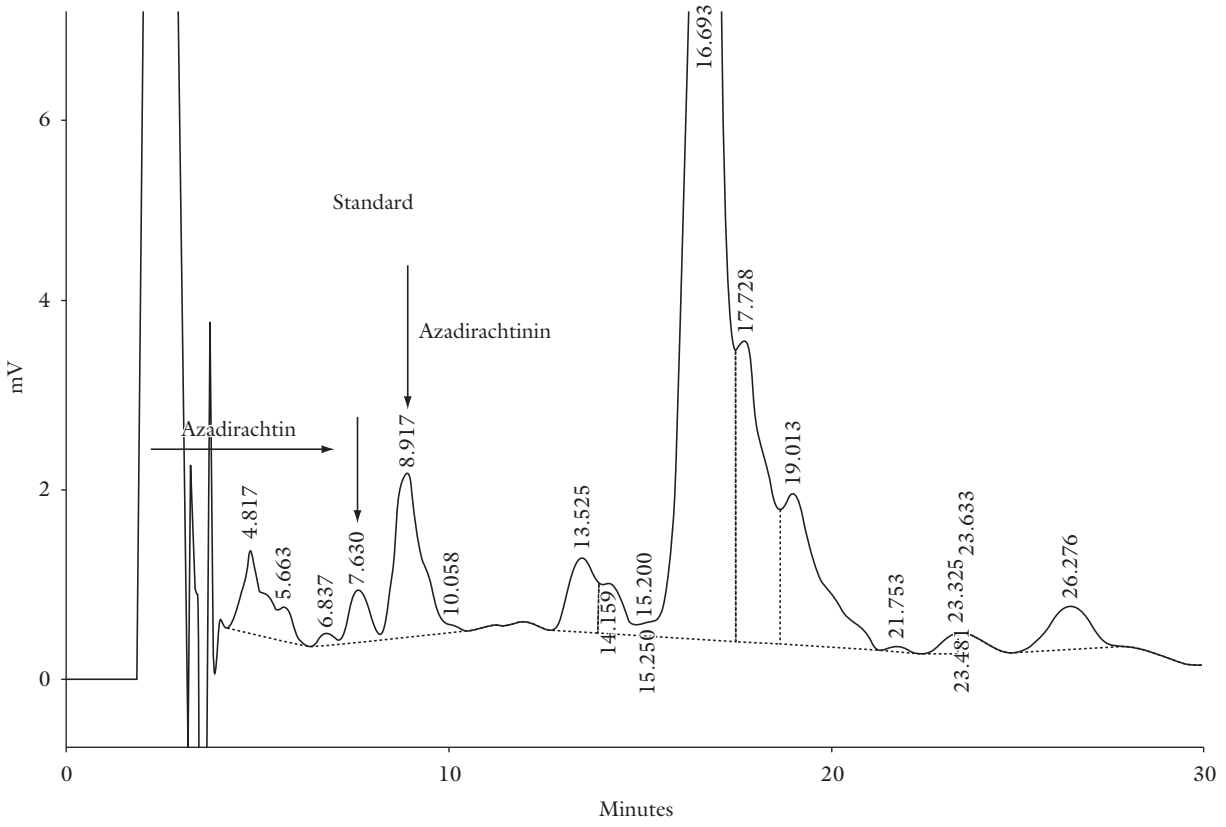

Plate I. High performance liquid chromatogram standard.

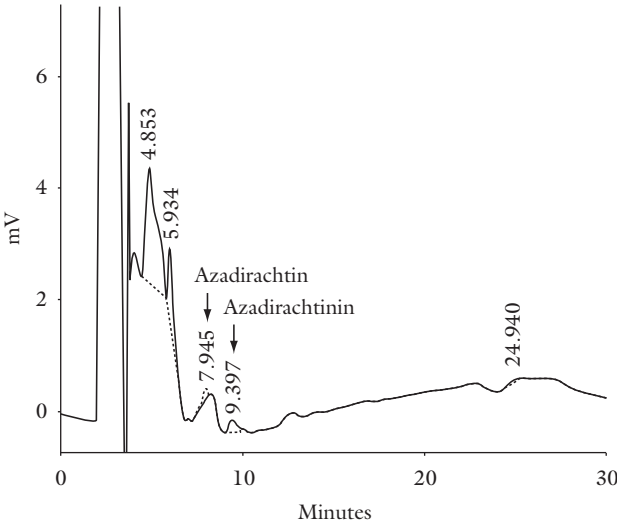

Plate II. High performance liquid chromatogram testes from low dose group.

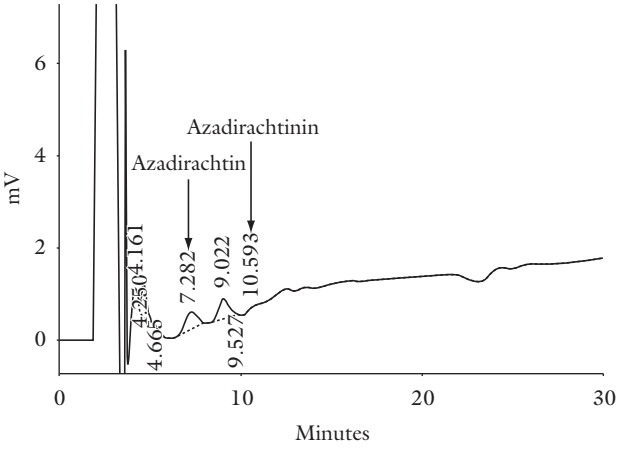

Plate III. High performance liquid chromatogram testes from high dose group.

These effects of neem oil at high dose in male rats may be because of various possibilities. Lower testosterone levels may result in delayed maturation of spermatozoa, or lower FSH levels though not significant statistically may have affected the Sertoli cell function present in the seminiferous tubules resulting in disturbed facilitatory function of these cells which is essential for the maturation and release of spermatozoa in the tubular lumen (SADLER, 1990). Gustafson and Shemesh (1976) while studying the changes in plasma Testosterone levels during the annual reproductive cycle of hibernating bat has suggested that a testosterone surge is required to stimulate the accessory glands fully. Akinloye, Abatan, Alaka et al. (2002), in their study has suggested that decrease number of leydig cells in interstitial space is respon-

sible for decrease production of testosterone known to be responsible for normal testicular architecture.

Raji, Udoh, Mewoyeka et al. (2003), after administering neem stem bark ethanol extract intraperitoneally have reported a dose dependent decrease in the level of testosterone and LH but did not find any change in FSH level. Parshad, Singh, Gardner et al. (1994) has also reported a significant decrease in serum testosterone level after the oral administration of crude neem extract in male wistar rats for 10 weeks.

In the present study, at low dose treatment hormonal levels have not shown any significant change. However at high dose they have been reduced significantly, except FSH which has shown a decrease though statistically non significant.

Quantitative analysis done by HPLC method has shown the presence of two active neem oil components, Azadirichtin and Azadirichtinin , in the testicular tissue. Table 4, Plate 1 to 3 shows the time, peak area and concentration of the neem oil components which have been calibrated by standard peaks of the two compounds.

Comparing the concentrations of Azadirachtin in treated males rats, it is evident that high dose treated rats have 425% higher concentration of Azadirachtin in testes at high dose. Similarly the concentration of

Azadirachtinin is about 242% higher in treated males at high dose.

Presence of high concentrations of active neem components in the testicular tissue with significant changes in microscopic structure and serum levels of reproductive hormones after a single oral dose suggests that neem oil has a potential to accumulate or concentrate in testicular tissue and proposes a dose dependent sterility potential for male albino rats.

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