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PHARMACOLOGY

ASPECTS OF THE ANTIANDROGENIC/ANTIFERTILITY PROPERTY OF AZADIRACHTIN-A FROM *AZADIRACHTA INDICA* LEAVES IN MALE ALBINO RATS: EFFECT ON THE BIOCHEMICAL AND CAUDA EPIDIDYMALSPERM PARAMETERS

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Abstract

Technical azadirachtin, a major component of *A. indica* leaves, has low acute/subchronic toxicity and non-mutagenic/teratogenic in mammalian species along with minimal disruption to the ecosystem. The aim of the present study is to examine the dose dependent effect of azadirachtin-A on biochemical, sperm functional parameters and fertility performance in treated groups. Wistar strain male albino rats were administered subcutaneously with graded concentrations of azadirachtin-A (0.5, 1.0 and 1.5 mg in suspension of 50% DMSO, respectively / kg body weight) followed by maintaining suitable controls for 24 days. Five animals from each group were used for fertility test. 24 hrs after the last dose, the control and treated animals were sacrificed; reproductive organs were then used for biochemical analysis and cauda epididymal plasma for sperm analysis. No significant differences in their body weight were observed. However, at high dose level of 1.5 mg/kg body weight, there was a general decrease in reproductive organs weights, altering in biochemical parameters and reduction in the sperm functional parameters with increased abnormal sperms. Furthermore, fertility performance test showed 30% even at high dose of azadirachtin-A treated for 24 days. In this study such high dose effects may have resulted from the deficiency in the level of circulating androgen, probably due to androgen deficiency consequent to the anti-androgenic property of azadirachtin-A and using this compound, in effective dose manner, may be a potential candidate as a contraceptive agent for the induction of infertility in humans by means of phytochemical approach.

Keywords: Azadirachtin-A, Reproductive organs, Biochemical parameters, Sperm analysis, Fertility and albino rats

Introduction

Azadirachta indica A.Juss (Syn: Melia Azadirachta L, Meliaceae family), commonly known as neem, is an important medicinal plant cultivated throughout India and Burma. This plant is extensively used as an astringent, antiperiodic, antispirochaetal, antiprotozoal; leprosy and bronchitis; for healing ulcers in urinary passages; for chronic fever and many other disorders (Bhakuni *et al.,* 1990). It has been reported that the crude oral administration of *A.indica* leaves exhibit as antispermatic, antiandrogenic (Aladakatti & Nazeer Ahamed, 2005a, b) and several such effects appear reversible (Joshi *et al.,* 1996). Study from Khillare and Shrivastav (2003) have been demonstrated and confirmed that the lyophilized *A.indica* leaves extract is carbohydrate in nature with spermicidal activity on rat spermatozoa. Recently, it has been shown that oral administration of lyophilized *A.indica* leaf powder to male rats produced dose-related effects on biochemical parameters of testis and epididymis due to changes in the chemical composition of testis and the epididymis and probably due to a deficiency in the level of circulating androgen in the male rats; these results indirectly reflect the antiandrogenic property of the lyophilized *A.indica* leaf powder (Aladakatti *et al.,* 2010).

Neem product has been widely used in agriculture as an alternative to synthetic pesticides (Anon, 1992). A number of chemical components have been isolated from neem plant extract. Many of the secondary compounds of the neem have been identified (Van der Nat *et al.,* 1991), purified and some have been tested for their effects on mammals. These include for example azadirachtin, nimbolide and nimbinin, solannin, deacetylazadirchtinol, nimbin, nimbidinin and meliantriol all being biologically active compounds of neem. Azadirachtin (Fig) is a triterpenoid of the class of limonoids, found in three species, the trees *Azadirachta indica* (Schmutterer, 2002, Rutales: Meliaceae), *A. excelsa* (Schmutterer *et al.,* 2002), and *A. siamensis* (Sombatsiri *et al.,* 2002).

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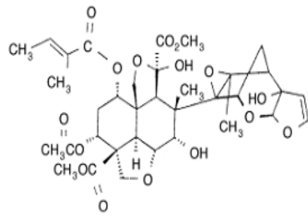
Fig. Chemical structure of azadirachtin



It is chemically interesting because of its complex structure and the challenge its synthesis provided; and moreover, the chemistry, environmental behaviour and biological effects of azadirachtin have been reviewed (Sundaram, 1996). Although, technical azadirachtin, molecular weight of 720.2, has low acute/subchronic toxicity and non-mutagenic/teratogenic in mammalian species, with LD_{50} greater than 5000 mg/kg in the rat (Raizada *et al.,* 2001), consequently the possibility of future hazards of neem products therefore should not be ignored (Anon, 1992). A recent wide scale use of azadirachtin in agriculture, the population in general and vulnerable groups such as virgin or pregnant women in particular may be exposed continuously throughout several generations. In order to establish their potential use, though various experimental studies reported by using this plant sources on biochemical and sperm parametric studies are available, it is very much required to evaluate the antiandrogenic/antifertility nature of the azadirachtin-A from the neem leaves. Based on the current available data, the present experimental study has therefore been designed to study the effect of azadirachtin –A on the androgen dependent reproductive organs at a dose dependent manner. The present work mainly deals with the determination of some of the androgen dependent biochemical parameters like estimation of protein, free sugar content, acid phosphatase (ACP), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in the reproductive organs, sperm functional parameters and fertility in both the control and treated rats.

Materials and Methods

Animals: Colony bred healthy adult male albino rats (Wistar strain) weighing 200 g were utilized for experiments. All animals were proven fertility and obtained from the rat colony maintained in the department. They were housed at a temperature of 22 $\pm$ 2^0 C and exposed to 12-12 h of daylight and maintained on a standard rat pellet diet and water was given *ad libitum*. The animals were acclimatized to the laboratory conditions before conducting experiments and the care of the laboratory animals was taken as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations.

Chemicals: Reagents were obtained as follows: Technical Azadirachtin-A (from SPIC Ltd., Chennai, India). All other reagents were from either Sigma or Hi Media Lab Pvt. Ltd. (Mumbai, India).

Treatment: In this study, the concentration of azadirachtin-A was used in vivo, i.e., 0.5, 1.0 and 1.5 mg / kg body weight were calculated as described from the studies of Glinsukon et al. (1986). The different concentrations of azadirachtin-A dissolved in 50% Dimethyl sulfoxide (DMSO) and administered to rats by subcutaneous injection using a micro-syringe. Forty male rats were divided into four groups of 10 animals each. The rats in group A were administered with 50% DMSO (vehicle control; 5 mL / kg body weight), while the rest treatment three groups (B, C, and D) were administered with graded doses of azadirachtin-A of 0.5, 1.0 and 1.5 mg/kg body weight, respectively. The DMSO and the graded doses of azadirachtin-A were administered subcutaneously on daily for 24 days. Five animals from each group were used for fertility test. Twenty-four hours after the last dose, the control and treated animals were sacrificed by cervical dislocation. The testis, epididymis of caput and cauda, vas deferens, seminal vesicle and ventral prostate were dissected out, blotted free of mucus and weighed to the nearest milligram used for biochemical and sperm functional parameters.

Biochemical analysis

50-100 mg of each tissue was quantitatively homogenized in 1 ml of 0.1 M Tris-Hcl buffer (pH 7.2), 0.1 M phosphate buffer (pH 7.2) or distilled water and then centrifuged at 8000 g for 15 minutes at 4°C. The supernatants were collected and used for various biochemical analysis using Hitachi.U.V. Vis. Spectrophotometer.

i) Estimation of total protein: The total protein level in the testis was estimated by the method of Lowry *et al* (1951) using Bovine Serum Albumin as standard. The OD of the resultant colour was read at 660nm and expressed as mg protein per gm wet tissue.

ii) Estimation of total free sugar: The total free sugar content of the tissue was estimated following the method of Folin and Wu (Oser, 1965). The O.D. was recorded at 420 nm and expressed as mg sugar per gm wet tissue.

iii) Estimation of acid phosphatase (ACP) and alkaline phosphatase (ALP): The enzyme assays were carried out according to the method described by Andersch and Szezypinski (1947). The O.D. of the resultant colour was read on a colorimeter at 400nm and both phosphatase

activities were expressed in terms of m Moles of P-nitrophenol formed per hour per gram protein.

iv) Estimation of lactate dehydrogenase (LDH): LDH levels in the testis were determined by the method of King (1965) .The intensity of the colour was measured at 440 nm and expressed as μ Mole/gm/hr.

Sperm analysis

For the standard sperm analysis, the cauda epididymis from each animal was chopped into phosphate buffered glucose saline (PBGS: Composition: Nacl / 50 mM / l, Na_2 HPO_4 200 m M / l, glucose 200 mM/l, KH_2PO_4 26 mM/l). The debris was removed and the epididymal suspension was used for sperm analysis. The following observations were made: (i)Total number of sperms per ml; (ii) Total number of motile sperms per ml; (iii)Normal and abnormal sperm count (relative percentage); and (iv)Forward velocity of the sperm i.e., μm/sec.

The total sperm count was calculated by the method of Besley *et al.,* (1980), using Neubauer haemocytometer. To increase the accuracy of sperm count, the epididymal plasma was diluted with a spermicidal solution, prepared by dissolving 5 g of $NaHCO_3$ and 1 ml of 40 % formaldehyde in 100 ml of normal saline. A twenty times dilution was made by using W.B.C pipette, which was thoroughly mixed and one drop was added to both sides of Neubauer haemocytometer. The sperms were allowed to settle down in the haemocytometer by keeping them in a humid chamber for one hour. The sperm count was done in R.B.C counting 5 major squares designated E_1, E_2, E_3, E_4 and a central E_5. Each square is 1 mm long, 1 mm wide and 0.1 mm of height. The total volume represented by each major square E is thus 0.1 mm^3 or 10^{-4} mm. The total number of sperms were counted in all the major squares and calculated as follows.

$$\text{Total no. of sperms / ml plasma} = \frac{\text{Total no. of sperms per square (X)}}{\text{Total volume per square } (10^{-4})} \times \text{dilution factor (20)}$$

Similarly the total number of motile sperms was calculated, using PBGS instead of spermicidal solution.

The relative proportion of the normal and abnormal sperms were calculated by smear preparation according to the method of Bauer *et al.,* (1974). Equal volume of cauda epididymal plasma and 5 % sodium bicarbonate were taken in a centrifuge tube, mixed well and centrifuged for 5 minutes at 4000 g. The supernatant was discarded and to the precipitate 5 ml of normal saline was added, mixed well and centrifuged again. The procedure was repeated 2 to 3 times and a clear precipitate was obtained. To the final precipitate few drops of normal saline were added, mixed thoroughly and a smear was prepared on a clean slide. The smear was dried at room temperature, fixed by heating it over the flame for two to three seconds. Then the smear was flushed with 95 % alcohol, drained and dried. It was stained in Ziehl Neelson's Carbol Fuchsin diluted with equal volume of 95% alcohol for 3 minutes and counter stained with 1:3 (v/v) aqueous solution of Loeffer's methylene blue for 2 minutes. After staining, the smear was rinsed in water and dried in air. The abnormal sperms included categories like double tailed, detached head, detached tail, mid piece bending and irregular head. The relative proportion of the normal and abnormal sperms was from the smear and expressed in terms of percentage.

To assess the forward velocity of sperms, the method of Ratnasoorya (1984) was adopted. The epididymal plasma was suspended in PBGS, cleared the tissue debris and a clear solution was used for the assessment of average forward velocity of sperms. The assessment was made under light microscope, fitted with a movable mechanical stage and a calibrated ocular micrometer, at 400 X magnification. A drop of sperm suspension was transferred to a clean glass slide and the initial place and time of each sperm was recorded. The time taken for forward movement of sperm from the initial place within microscopic field was recorded using a stop watch. The procedure was repeated for 10 spermatozoa in each sample and the average forward velocity of sperm was calculated and expressed as μm / sec.

Fertility test

To assess the fertility rate with reference to the number of implantations, the remaining five males per group were paired with female of proven fertility exhibiting regular estrous cycles and were separately housed with the B,C, D of azadirachtin-A treated rats and left overnight. The appearance of sperm in the vaginal smear next morning confirmed the mating and is considered as day 1 of the pregnancy. After 8 days, the females were laparotomized and the number of implantations were recorded.

Statistical analysis

Results are expressed as the mean value ± standard error of mean (S.E.M.). All percentage data were subjected either to Student's *t*-test or one-way analysis of variance (ANOVA) followed by multiple t-tests for analysis.

Results

Body and organ weights in azadirachtin-A treated rats: The body weight of the rats did not differ due to the dose dependent treatment of azadirachtin-A. In 0.5 mg / kg body weight rats (group B), the weights of testis and other accessory structures remain unchanged excluding seminal vesicle and ventral

prostate (P ≤ 0.05) when compare to controls. Whereas, groups C and D (1.0 mg and 1.5 mg / kg body weight), exhibited a significant decrease in the weight of ventral prostate (P ≤ 0.01 and P ≤ 0.001 respectively). Testis and seminal vesicle of 1.5 mg / kg treated shown significant decrease (P ≤ 0.01) in their weights. However, there were decrease (P ≤ 0.05) in the weights of epididymis (both caput and cauda) and vas deference in 1.0 mg and 1.5 mg treated groups when compare to controls (group A, Table.1).

Table 1: Dose dependent effect of azadirachtin-A on the body weight (g) and reproductive organs weights (mg) of albino rats

Group	Body weight	Testis	Epididymis		Vas deferens	Seminal vesicle	Ventral prostate
			Caput	Cauda			
Group A Phosphate buffer saline (PBS, 5 mL kg^{-1} BW)	205.00 ± 3.76	695.05 ± 2.57	137.28 ± 2.87	116.51 ± 2.04	168.88 ± 3.26	601.33 ± 7.26	381.92 ± 12.58
Group B Azadirachtin- A (0.5 mg / kg^{-1} BW)	211.85 ± 2.48	684.18 ± 3.73	138.86 ± 1.31	115.39 ± 3.93	173.72 ± 2.14	629.71 ± 5.23	372.71 ± 5.23
Group C Azadirachtin- A (1.0 mg / kg^{-1} BW)	205.57 ± 3.22	670.27 ± 2.03*	136.53 ± 2.20	112.55 ± 3.76*	161.43 ± 3.12*	589.65 ± 4.92*	366.35 ± 11.92**
Group D Azadirachtin- A (1.5 mg / kg^{-1} BW)	195.83 ± 2.45	657.10 ± 4.12**	132.60 ± 2.33*	108.35 ± 2.49*	152.23 ± 4.02*	551.21 ±12.34**	335.21 ±10.27***

Data are mean ± S.E.M. of 5 replicates
* Significant difference at $p < 0.05$ level, when compared with control group
**Significant difference at $p < 0.01$ level, when compared with control group
***Significant difference at $p < 0.001$ level, when compared with control group

Androgen dependent biochemical parameters in azadirachtin-A treated rats: Biochemical composition of protein, free sugar content, ACP, ALP and LDH in the testis, epididymis (caput and cauda), vas deferens, seminal vesicle and ventral prostate were observed in the control and dose dependent concentration of azadirachtin-A treated rats.

Protein content in testis and other reproductive organs: The protein content in testis was reduced in 1.5 mg (group D) of azadirachtin-A / kg body weight treated rats (P ≤ 0.05) and in 0.5 mg (Group B) and 1.0 mg (Group C) treated rats, there was no difference in the protein content when compare to controls (group A, 37.30 ± 1.08 mg/g, Table.2). In epididymis of group D, the protein content of cauda was significantly reduced (P ≤ 0.01) and caput (group D) and cauda (group C) exhibited reduction in protein content (P ≤ 0.05). However, in group B and caput of group C, there was no difference in the protein content when compare to controls of both caput and cauda (46.93 ± 1.34 mg/g and 36.42 ± 2.19 mg/g respectively, Table.3). In vas deference, the protein content was significantly reduced (P ≤ 0.01) in group D, reduction in group C (P ≤ 0.05) and no difference in group B when compare to controls (group A, 46.53 ± 2.16 mg/g, Table.4). In seminal vesicle, the protein content was significantly reduced (P ≤ 0.01) in groups C and D and no difference in group B when compare to controls (group A, 50.49 ± 1.04 mg/g, Table.5). In ventral prostate, the protein content was highly significant (P ≤ 0.01) in group D and significantly reduced (P ≤ 0.01) in group C and no difference in group B when compare to controls (group A, 70.65 ± 1.05 mg/g, Table. 6).

Table 2: Dose dependent effect of azadirachtin-A on various biochemical analysis of the Testis of albino rats

Group	Protein (mg/g)	Sugar (mg/g)	Acp (mM/g/hr)	Alp (mM/g/hr)	LDH (μM/g/hr)
Group A Phosphate buffer saline (PBS, 5 mL kg $^{-1}$ BW)	37.30 ± 1.08	0.60 ± 0.03	2.96 ± 0.06	1.22 ± 0.04	268.28 ± 4.92
Group B Azadirachtin- A (0.5 mg / kg $^{-1}$ BW)	36.36 ± 1.10	0.76 ± 0.02	2.80 ± 0.03	1.78 ± 0.11	286.35 ± 4.91*
Group C Azadirachtin- A (1.0 mg / kg $^{-1}$ BW)	34.91 ± 0.88	1.38 ± 0.03	2.08 ± 0.03	3.60 ± 0.04*	373.89 ± 4.74**
Group D Azadirachtin- A (1.5 mg / kg $^{-1}$ BW)	30.90 ± 0.96*	2.30 ± 0.02*	1.51 ± 0.05*	4.22 ± 0.03**	519.93 ± 4.85***

Data are mean ± S.E.M. of 5 replicates
* Significant difference at $p < 0.05$ level, when compared with control group
**Significant difference at $p < 0.01$ level, when compared with control group
***Significant difference at $p < 0.001$ level, when compared with control group

Table 3: Dose dependent effect of azadirachtin-A on various biochemical analysis of the caput and cauda of epididymis of albino rats

Group	Protein (mg/g)		Sugar(mg/g)		Acp(mM/g/hr)		Alp (mM/g/hr)		LDH (μM/g/hr)	
	Caput	Cauda	Caput	Cauda	Caput	Cauda	Caput	Cauda	Caput	Cauda
Group A Phosphate buffer saline (PBS, 5 mL kg $^{-1}$ BW)	46.93± 1.34	36.42± 2.19	0.62 ± 0.03	0.72± 0.02	10.91± 0.06	8.01± 0.06	1.01± 0.05	1.25± 0.04	302.3± 4.87	374.4± 4.91
Group B Azadirachtin- A (0.5 mg / kg $^{-1}$ BW)	46.65± 1.40	33.74± 1.17	0.93± 0.02	1.71± 0.03*	9.86± 0.03	7.17± 0.05	1.58± 0.03	2.40± 0.02*	318.7± 4.80	544.3± 4.54**
Group C Azadirachtin- A (1.0 mg / kg $^{-1}$ BW)	45.01± 1.11	29.65± 1.20*	1.15± 0.03*	2.63± 0.02**	8.58 ± 0.03*	6.10± 0.03*	2.20± 0.05*	2.98± 0.02*	422.0± 5.06**	679.7± 4.78***
Group D Azadirachtin- A (1.5 mg / kg $^{-1}$ BW)	40.66± 1.57*	23.71± 1.19**	1.98± 0.03*	4.11± 0.03***	6.50 ± 0.05**	4.28± 0.05**	3.80± 0.02**	3.64± 0.03**	567.7± 4.77***	773.8± 4.65***

Data are mean ± S.E.M. of 5 replicates
* Significant difference at $p < 0.05$ level, when compared with control group
**Significant difference at $p < 0.01$ level, when compared with control group
***Significant difference at $p < 0.001$ level, when compared with control group

Table 4: Dose dependent effect of azadirachtin-A on various biochemical analysis of the Vas deferens of albino rats

Group	Protein (mg/g)	Sugar (mg/g)	Acp (mM/g/hr)	Alp (mM/g/hr)	LDH (μM/g/hr)
Group A Phosphate buffer saline (PBS, 5 mL kg $^{-1}$ BW)	46.53 ± 2.16	1.86 ± 0.02	4.89±	1.03 ±	363.7±
Group B Azadirachtin-A (0.5 mg / kg $^{-1}$ BW)	45.49 ± 2.22	2.80 ±	4.58±	0.96 ±	510.5±
Group C Azadirachtin-A (1.0 mg / kg $^{-1}$ BW)	42.41 ± 2.24*	3.17 ±	4.18±	1.08 ±	671.9±
Group D Azadirachtin-A (1.5 mg / kg $^{-1}$ BW)	40.64 ± 2.14**	3.76±	3.94±	1.12 ±	729.8±

Data are mean ± S.E.M. of 5 replicates
* Significant difference at $p < 0.05$ level, when compared with control group
**Significant difference at $p < 0.01$ level, when compared with control group
***Significant difference at $p < 0.001$ level, when compared with control group

Table 5: Dose dependent effect of azadirachtin-A on various biochemical analysis of the Seminal vesicle of albino rats

Group	Protein (mg/g)	Sugar (mg/g)	Acp (mM/g/hr)	Alp (mM/g/hr)	LDH (μM/g/hr)
Group A Phosphate buffer saline (PBS, 5 mL kg $^{-1}$ BW)	50.49 ± 1.04	0.66 ±	4.43 ±	1.18 ±	356.6±
Group B Azadirachtin- A (0.5 mg / kg $^{-1}$ BW)	49.51	2.40 ±	4.10 ± 0.06	1.40 ±	618.4±
Group C Azadirachtin- A (1.0 mg / kg $^{-1}$ BW)	45.48	3.67 ±	3.86 ±	2.27 ±	759.8±
Group D Azadirachtin- A (1.5 mg / kg $^{-1}$ BW)	41.75	4.17 ± 0.02**	3.38 ±	3.62 ±	823.7± 4.75***

Data are mean ± S.E.M. of 5 replicates
* Significant difference at $p < 0.05$ level, when compared with control group
**Significant difference at $p < 0.01$ level, when compared with control group
***Significant difference at $p < 0.001$ level, when compared with control group

Table 6: Dose dependent effect of azadirachtin-A on various biochemical analysis of the Ventral prostate of albino rats

Group	Protein (mg/g)	Sugar (mg/g)	Acp (mM/g/hr)	Alp (mM/g/hr)	LDH (μM/g/hr)
Group A Phosphate buffer saline (PBS, 5 mL kg $^{-1}$ BW)	70.65 ± 1.05	0.76 ±	5.54 ±	1.05 ±	372.3 ± 4.89
Group B Azadirachtin- A (0.5 mg / kg $^{-1}$ BW)	68.99 ± 0.87	2.16 ±	4.66 ±	1.18 ±	644.3 ± 4.86**
Group C Azadirachtin- A (1.0 mg / kg $^{-1}$ BW)	62.99 ± 0.80**	3.85 ±	4.32 ±	1.84 ±	719.8 ± 4.72***
Group D Azadirachtin- A (1.5 mg / kg $^{-1}$ BW)	55.76 ± 0.91***	5.57 ±	3.18 ±	2.30 ± 0.03*	817.8 ± 4.84***

Data are mean ± S.E.M. of 5 replicates
* Significant difference at $p < 0.05$ level, when compared with control group
**Significant difference at $p < 0.01$ level, when compared with control group
***Significant difference at $p < 0.001$ level, when compared with control group

Sugar content in testis and other reproductive organs: The free sugar content in testis was increased 1.5 mg (group D) of azadirachtin-A / kg body weight treated rats (P ≤ 0.05) and in 0.5 mg (Group B) and 1.0 mg (Group C) treated rats, there was no difference in the sugar content when compare to controls (group A, 0.60 ± 0.03 mg/g, Table.2). In epididymis of groups D and C, the sugar content of cauda was increased with highly significant and significant (P ≤ 0.001, P ≤ 0.01 respectively) and increased (P ≤ 0.05) in group B. However, sugar content of caput increased in groups C and D (P ≤ 0.05) and no difference in group B when compare to controls of both caput and cauda (0.62 ± 0.03 mg/g and 0.72 ± 0.02 mg/gm respectively, Table.3). In vas deference, the sugar content was increased in groups D and C (P ≤ 0.05) and no difference in group B when compare to controls (group A, 1.86 ± 0.02 mg/g, Table.4). In seminal vesicle, the sugar content was significantly increased (P ≤ 0.01) in groups C and D and increased in group B when compare to controls (group A, 0.66 ± 0.03 mg/g, Table.5). In ventral prostate, the sugar content was increased highly significant (P ≤ 0.001) in group D and significantly in (P ≤ 0.01) in group C. whereas it increased in group B (P ≤ 0.05) when compare to controls (group A, 0.76 ± 0.02 mg/g, Table.6).

Acid phosphatase concentration (ACP) in testis and other reproductive organs: In testis, the ACP activity was reduced in 1.5 mg (group D) of azadirachtin-A / kg body weight treated rats (P ≤ 0.05) and in 0.5 mg (Group B) and 1.0 mg (Group C) treated rats, there was no difference in the activity when compare to controls (group A, 2.96 ± 0.06 mM/g/hr, Table.2). In epididymis of group D, the activity of caput and cauda was significantly reduced (P ≤ 0.01) and reduction in the activity of both caput and cauda of group C (P ≤ 0.05). However, in group B, there was no difference in the activity when compare to controls of both caput and cauda (10.91 ± 0.06 mM/g/hr and 8.01 ± 0.06 mM/g/hr respectively, Table.3). In vas deference, the activity was reduced in groups C and D (P ≤ 0.05) and no difference in group B when compare to controls (group A, 4.89 ± 0.05 mM/g/hr, Table.4). In seminal vesicle, the activity was reduced significantly in group D (P ≤ 0.01) and reduction in the group C (P ≤ 0.05). However, there was no difference in group B when compare to controls (group A, 4.43 ± 0.06 mM/g/hr, Table.5). In ventral prostate, the activity was significant in group D (P ≤ 0.01) and no difference in groups B and C when compare to controls (group A, 5.54 ± 0.03 mM/g/hr, Table.6).

Alkaline phosphatase concentration (ALP) in testis and other reproductive organs: In testis, the ALP activity was significantly increased in 1.5 mg (group D) of azadirachtin-A / kg body weight treated rats (P ≤ 0.01). However, there was a gradual increase in 1.0 mg (Group C, P ≤ 0.05) and no difference in the activity of group B when compare to controls (group A, 1.22 ± 0.04 mM/g/hr, Table.2). In epididymis of group D, the

activity of caput and cauda was significantly increased ($P \leq 0.01$) and exhibited increased activity in group C and cauda of group B ($P \leq 0.05$). However, caput of group B, there was no difference in the activity when compare to controls of both caput and cauda (1.01 ± 0.05 mM/g/hr and 1.25 ± 0.04 mM/g/hr respectively, Table.3). In vas deference, this activity exhibited absolutely no difference in all groups when compare to controls (group A, 1.03 ± 0.05 mM/g/hr, Table.4). In seminal vesicle, the activity was significantly increased ($P \leq 0.01$) in group D and a gradual increase group C ($P \leq 0.05$). Whereas no difference in group B when compare to controls (group A, 1.18 ± 0.05 mM/g/hr, Table.5). In ventral prostate, the activity was increased in groups C and D ($P \leq 0.05$) and no difference in group B when compare to controls (group A, 1.05 ± 0.04 mM/g/hr, Table.6).

Lactate dehydrogenase concentration (LDH) in testis and other reproductive organs: The LDH activity in testis was increased highly significant in 1.5 mg (group D) of azadirachtin-A / kg body weight treated rats ($P \leq 0.001$). Whereas significantly increased in 1.0 mg (Group C) and gradual increased in 0.5 mg (Group B) when compare to controls (group A, 268.28 ± 4.92 µM /g/hr, Table.2). In epididymis of group D, the activity of both caput and cauda was increased highly significant ($P \leq 0.001$) and cauda of group C exhibited highly significant ($P \leq 0.001$). However, in caput of group C and cauda of group B revealed increase their LDH activity with significant ($P \leq 0.05$). However, caput of group B, there was no difference in the activity when compare to controls of both caput and cauda (302.3 ± 4.87 µM /g/hr and 374.4 ± 4.91 µM /g/hr respectively, Table.3). In vas deference, the activity was increased highly significant in groups C and D ($P \leq 0.001$) and increased with significantly in group B ($P \leq 0.01$) when compare to controls (group A, 363.7 ± 4.82 µM /g/hr, Table.4). In seminal vesicle, the activity was increased highly significant in groups C and D ($P \leq 0.001$)and increased with significantly in group B ($P \leq 0.01$) when compare to controls (group A, 356.6 ± 4.80 µM /g/hr, Table.5). In ventral prostate, the activity was increased highly significant in groups C and D ($P \leq 0.001$) and increased with significantly in group B ($P \leq 0.01$) when compare to controls (group A, 373.3 ± 4.89 µM /g/hr, Table.6).

Sperm parameters and fertility in azadirachtin-A treated rats: Analysis of sperm parameters, such as total sperm count, total number of motile sperms, forward velocity of the sperm and percentage of abnormal sperm of the cauda of epididymal plasma were carried out in the control and all azadirachtin-A treated animals. The control rats (Group A) showed 56.20 X 10^4 total number of sperms / ml epididymal fluid, 51.80 X 10^4 number of motile sperms / ml epididymal fluid with a speed of 127.2 µm / sec and 11.20 % of abnormal sperms were recorded (Table.7) Where as in the treated animals (Groups B, C & D), the abnormal sperms in cauda epididymal plasma of albino rats are doses dependent. With a dose of 0.5 mg of azadirachtin-A / kg body weight treated rats (Group.B), the animals showed no difference in the total sperm count (53.60 X 10^4 / ml), total number of motile sperms (50.60 X 10^4 / ml), forward velocity of the sperm (128.2 µm / sec) and the percentage of abnormal sperms (11.40%). In 1.0 mg treated rats (Group.C), the animals showed a significant decrease ($P \leq 0.01$) in forward velocity of sperm (105.4 µm / sec) and the percentage of abnormal sperms (23.20%). Whereas, there were decrease ($P \leq 0.05$) in the total sperm count (49.20 X 10^4 / ml) and total number of motile sperm (44.20 X 10^4 / ml). However, in maximum dose of 1.5 mg of azadirachtin-A / kg body weight treated rats (Group.D), the animals showed highly significant decrease ($P \leq 0.001$) in the in forward velocity of sperm (90.60 µm / sec) and the percentage of abnormal sperms(41.60%). In addition, there were significant ($P \leq 0.01$) decrease in the total sperm count (37.60 X 10^4 / ml) and total number of motile sperm (30.60 X 10^4 / ml) when compare to control animals (Group A).

Discussion

Besides its therapeutic efficacies, neem has already established its potential as a source of naturally occurring insecticide, pesticide and agrochemicals. Furthermore, evaluation of safety aspects of different parts of neem and neem compounds along with commercial formulations are also taken into consideration. Though, systematic scientific knowledge on neem are reported so far, it is also necessary and essential for adequate safety evaluation of those derived preparations in order to develop a potential drug without side effects by means of phytotherapy research. Reports from the earlier subchronic toxicity study of azadirachtin in rats for 90 days did not show any adverse effects, as evidenced by organ to body weight ratio, clinical enzyme assay, histopathological and hematological parameters (Raizada *et al.,* 2001); further, at the tested doses, it did not induce fetal death or any malformations (Srivastava and Raizada , 2007). This experiment was designed to study primarily the technical azadirachtin (purity 95%) on androgen dependent biochemical, sperm functional status and antifertility performance in rats following its subcutaneous injection after dissolved in 50% DMSO at three concentrations.

Table 7: Dose dependent effect of azadirachtin-A on various sperm parameters of cauda epididymal plasma in albino rats

Group	Sperm count (Total No. x 10^4/ml)	Motile sperm (Total No. x 10^4/ml)	Forward velocity (µm/sec)	Abnormal sperms (%)
Group A Phosphate buffer saline (PBS, 5 mL kg $^{-1}$ BW)	56.20 ± 3.07	51.80 ± 2.91	127.2± 3.07	11.20 ± 3.07
Group B Azadirachtin-A (0.5 mg / kg $^{-1}$ BW)	53.60 ± 4.80	50.60 ± 4.80	128.2 ± 4.80	11.40 ± 4.16
Group C Azadirachtin-A (1.0 mg / kg $^{-1}$ BW)	49.20 ± 4.90*	44.20 ± 4.90*	105.4 ± 4.90**	23.20 ± 4.90**
Group D Azadirachtin-A (1.5 mg / kg $^{-1}$ BW)	37.60 ± 4.80**	30.60 ± 4.80**	90.6 ± 3.07***	41.60 ± 4.80***

Data are mean ± S.E.M. of 5 replicates
* Significant difference at $p < 0.05$ level, when compared with control group
**Significant difference at $p < 0.01$ level, when compared with control group
***Significant difference at $p < 0.001$ level, when compared with control group

Table 8: Dose dependent effect of azadirachtin-A on the implantations of female rats mated with treated male rats

Group	No. of Implantations
Group A Phosphate buffer saline (PBS, 5 mL kg $^{-1}$ BW)	10.66 ± 0.95
Group B Azadirachtin- A (0.5 mg / kg $^{-1}$ BW)	9.50 ± 0.67
Group C Azadirachtin- A (1.0 mg / kg $^{-1}$ BW)	8.75 ± 0.56
Group D Azadirachtin- A (1.5 mg / kg $^{-1}$ BW)	7.35 ± 0.47*

Data are mean ± S.E.M. of 5 replicates.
* Significant difference at p < 0.05 level, when compared with control group

Administration of high dose of azadirachtin (1.5 mg/kg body weight) for 24 days has shown that the body weight of rats did not altered other than a decrease in the mean testis, seminal vesicle and ventral prostate organs weights. However, low doses of azadirachtin (0.5 and 1.0 mg/kg body weight have not produced any such changes in rats. It is known that monitoring body weight provides information on the general health of animals, which is important to the interpretation of reproductive effects (US EPA, 1996). The present data show that the reduction in the testis, seminal vesicle and ventral prostate organs weights at high dose may be due to the decrease in serum testosterone concentration. The functional status of male accessory reproductive organs can be accurately evaluated by determining the chemical composition of seminal plasma (Turner and Bagnara, 1976). Hence, present investigation gives a clue that change in the chemical composition of the testis and other reproductive organs in treated groups is probably due to a change in the circulating androgen level. As testosterone plays a major if not sole role in the maintenance of structural integrity and functional activities of the accessory sex organs and reduction in accessory sex organ weight is a reflection of decreased testosterone level in the blood (Gupta *et al.,* 2007; Patil *et al.,* 2010).

In the present study, a decrease in the protein and ACP activity and an increase in total free sugar and the activities of ALP and LDH in the testis and other reproductive organs like caput and cauda of epididymis, vas deferens, seminal vesicle and ventral prostate on maximum dose treatment with technical active constituent azadirachtin-A reveals the antiandrogenic property as above said biochemical parameters are androgen sensitive. These observations are similar to those found in studies (Gupta *et al.,* 2007; Parandin *et al.,* 2008; Kuang *et al.,* 2009; Aladakatti *et al.,* 2010) and reported that the structural and functional integrity of the reproductive organs depends on the circulating level of the androgen, and any small change in the androgen level results in the reduction in the biochemical parameters of organs leading to reduction of fertility.

It has been reported that protein level is directly correlated with the secretory activity of the testis and accessory glands, which in turn depends on the androgen levels (Jones, 1977).The most pronounced general metabolic action of the androgen is the promotion of protein anabolism (Steinberger, 1971); the reduction in protein content observed in the present study may be attributed to the reduction in secretory activity of the testis and other organs because of the androgen deprivation effect which indirectly indicates the anti-androgenic property of these active constituents. It is generally known that any interference in the normal reproductive physiology would result in decrease of carbohydrate metabolizing enzymes (Aruldhas *et al.,* 1982a & b). Any such decrease in the levels of such enzymes would result in under utilization of sugar and hence its accumulation in the target organs (Verma *et al.,* 1980; Joshi *et al.,* 1996; and Aladakatti *et al.,* 2010). Taking into consideration the above reports, it can be presumed that an increase in the total free sugar content of testis and other organs of azadirachtin-A treated rats may be due to a decrease in the carbohydrate metabolizing enzymes, resulting in accumulation of sugar in the target organs.

Both ACP and ALP are sensitive functional indicators of the reproductive status of animal and directly correlated with the sperm count. Both are concerned with the hydrolysis of phosphomonoesters releasing free phosphorus and thus responsible for the secretory activity of target organs (Mann *et al.,* 1981). In present findings, a decline in the ACP and increase in ALP activity in the testis and other reproductive organs may possibly due to the decrease in serum testosterone and stimulatory glucocorticoids as well as degeneration of testicular germ cells (Ghosh *et al.,* 1990). This agrees with the observations of aqueous extract of *Chromolaena odoratum* and lyophilized *A.indica* leaf powder in rats (Yakubu *et al.,* 2007; Aladakatti *et al.,* 2010). In the present study, increase in LDH level observed in the testis and other reproductive organs of treated animals suggests the elevation of the substrate lactate level as LDH is one of the key enzymes in Embden-Mayeroff pathway of carbohydrate metabolism and the low activity of the enzyme has been used as a marker for active spermatogenesis. According to Sinha *et al.* (1995) and Pant and Srivastava (2003), increase in LDH activity level has a direct effect on testicular functions such as sperm count, its production and sperm morphology. In this study a considerable elevation in the epididymal and other organ's LDH activity of the treated rats indicate the production of substrate lactate, suggesting a switch over from aerobic to anaerobic type of respiration or altered physiological/metabolic activity which may have a definite influence on androgen-regulated glycolytic enzyme activities in the male accessory organs, thereby indirectly affecting the secretory activities of these tissues.

In the present study of 1.5 mg/kg body weight treatment resulted in reducing sperm count, motility and sperm speed in a significant manner. It has been shown that androgens are essential for survival and motility of spermatozoa in the rat epididymis, cauda region appears to be the most favourable site. It is likely that any contraceptive agent that affects sperm motility would influence spermatozoa indirectly through disruption of epididymal epithelial cell function or act directly on the spermatozoa by affecting their enzymes

(Cooper and Young, 1999). A significant increase in the abnormal sperm count and inhibition of sperm forward movement in high dose treated rats suggests that this azadirachtin-A's target is within the internal milieu of the epididymis or alterations in the epididymal epithelium in the cauda epididymis (Aladakatti *et al.,* 2001; Ghodesawar *et al.,* 2004). As a result of previous observations of *A. indica* leaf powder treated rats (Aladakatti *et al.,* 2001; Ghodesawar *et al.,* 2004), it indicate that it is less likely that one of the active constituents of *A.indica* leaves, i.e., azadirachtin-A affects the sperm motility by altering the epididymis itself. Although, the effect of 1.5 mg / kg body weight of azadirachtin-A, significant increase in the abnormal sperm count and inhibition of sperm forward movement suggest that this azadirachtin-A's target is within the internal milieu of the epididymis and the significant alterations in the epididymal epithelium in the cauda epididymis in *A. indica* leaf powder treated rats (Aladakatti *et al.,* 2001; Ghodesawar *et al.,* 2004) indicate that it is less likely that azadirachtin-A affects the sperm motility by altering the epididymis itself.

It was suggested that these compounds cause androgen depletion at the target level, particularly in the cauda epididymis thereby affecting physiological maturation of the sperm (Chinoy *et al.,* 1995). The observations made in this study are supported from studies of flavonoid-rich seed extracts of *Vitex negundo* (Das *et al.,* 2004); methanol extract of *Dendrophthoe falcate* (Gupta and Kachhawa, 2007); aqueous extract of *Peganum harmala* (El-Dwairi and Banihani, 2007); and methanol subfraction of *Carica papaya* seeds (Manivannan *et al.,* 2009). The inhibition of fertility by 30% in azadirachtin-A seemed to depend on the graded doses of subcutaneous administration for 24 days. The male fertility potency resulting after the treatments, could indicate that for a 100% non-successful contraceptive effect, it is apparently necessary that rats be subjected to further treatment with high doses or increasing duration of treatment. As in this study, it has been shown that known graded doses of azadirachtin-A with time period (24 days), some of the androgen dependent biochemical parameters exhibited considerable changes which in turn reduced sperm count, motility and sperm speed due to androgen depletion at the target level, particularly in the cauda epididymis thereby affecting physiological maturation of the sperm (Chinoy *et al.,* 1995). This study suggests that azadirachtin-A, at effective concentration and route administration, may be a potential use as an antifertility drug with the advantage of antifertility activity and without toxicity.

In conclusion, subcutaneously administration with graded concentrations of azadirachtin-A (0.5, 1.0 and 1.5 mg in suspension with 50% DMSO, respectively/kg body weight) to male rats produced effects at maximum dose on biochemical parameters of reproductive organs, sperm functional parameters and fertility potency. It can be suggested that a change in the chemical composition of the reproductive organs in the present study is probably due to a deficiency in the level of circulating androgen; these results indirectly reflect the antiandrogenic property of the azadirachtin-A. Further, sperm functional and fertility observations, it can be suggested that changes in sperm parameters may be a general disturbance of proteins and alteration in the epididymal milieu probably due to androgen deficiency consequent upon the antiandrogenic /antifertility quality of azadirachtin-A. Based on the present findings, it can be concluded that the active constituent azadirachtin-A from *A.indica* leaves which probably affect the androgen synthesis and thus exhibit antiandrogenic effects on androgen sensitive target glands and such effects changes in the androgen sensitive biochemical and sperm parameters clearly indicate some dwindling in the androgenic status of the treated rats.

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