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Implication of reproductive endocrine malfunction in male antifertility efficacy of *Azadirachta indica* extract in rats

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Summary

The effects of the ethanol extract of *Azadirachta indica* stem bark on body and organ weights, sperm morphology, counts and viability, serum levels of testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) were studied in albino rats. Intraperitoneal administration (i.p) of the extract for ten weeks caused significant dose-dependent decreases in weights of the testis, epididymis and seminal vesicles but an increase in that of the adrenal gland. Sperm counts, morphology and viability were adversely affected in the extract treated rats. Rats that received 150 $mgkg^{-1}$ b.w. *Azadirachta* extract were unable to impregnate female rats throughout the duration of the study. However, these female rats conceived and sired physically normal litters about four weeks after cohabitation with untreated male rats. *Azadirachta indica* produced dose-dependent reduction in serum testosterone and LH but no change in FSH levels. Most of the changes produced in this study were restored in recovery experiments.

Keywords: *Antifertility, A.indica, gonadrotrophins testosterone, antimalaria.*

Résumé

Les effets de l'extrait d'éthanol d'Azadirachta indica (la tige et l'écorce) sur corps et la pèse de l'organe, la morphologie du sperme, les comptes et la viabilité, le niveau de sérum de testostérone, l' hormone du luteinizing (LH), et la follicule qui stimule l'hormone (FSH) ont été étudiés dans les rats albinos. L'administration Intra-peritoneal (i.p) de l'extrait pour dix semaines ont causé des baisses dose dépendantes considérables dans poids des testicules, epididymies et vésicules séminales mais une augmentation dans cela de la glande surrénale. Les comptes du sperme, la morphologie et la viabilité ont été affectés de façon défavorable dans les rats traités de l'extrait. Des rats qui ont reçu 150. l'extrait Azadirachta de mgkg-1 b.w sont incapable de féconder des rats féminins pendant la durée de l'étude. Cependant, ces rats féminins ont conçu et ont engendré des litières physiquement normales approximativement quatre semaines après cohabitation avec les rats virils non traités. Les Azadirachta indica ont produit la réduction dose dépendante dans testostérone du sérum et LH mais aucun changement dans les niveaux FSH. La plupart du changement produit dans cette étude a été restauré dans les expériences de la récupération.

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Introduction

Azadicrachta indica A. Juss (Meliaceae) is an Indian plant now naturalized and grown in West Africa. It is called neem, mangosa tree or "*dongoyaro*" in Nigeria and is reputed to have several medicinal values. Different parts of the plant have been used as antimalarial, antiviral and antibacterial agents [1].

Many antimalarial agents possess antifertility actions [2,3,4]. Currently, interest in the activities of *A. indica* on reproduction is growing. While Prakash *et al* [5], reported that *A. indica* oil did not possess oestrogenic, antioestrogenic or progestational activity in rats, Bardhan *et al* [6] found that the oil extracted from the seeds of the plant has a good spermicidal activity in rhesus monkey. This study further revealed that pre- and post- coital application of the oil intravaginally prevented pregnancy in rhesus monkey. The long term and reversible blocking in fertility with no apparent effects on teratogenicity and ovarian functions in female Wistar rats, by neem oil, has also been reported [7]. A single intra-vas application of the plant oil resulted in infertility with a block of spermatogenesis without affecting testosterone production, whereas oral administration of *A. indica* extract decreases serum testosterone levels [8].

In view of this potential importance of *A. indica* as a male antifertility and its increasing use as an anitmalarial agent, more rigorous studies on its effects on male reproductive functions have become imperative. There is no report on the effect of the stem bark extract of this plant on gonadotrophin secretion and sperm functions *in vivo*.

In this paper we report the effects of *A. indica* stem bark extract on sperm functions, serum levels of gonadotrophins and testosterone in male albino rats.

Materials and methods

Plant material: The stem bark of *Azadirachta indica*, A. juss (Neem) was collected from the Sports Centre of the University of Ibadan in October 1999. The specimen was examined and identified by Mr. A. E. Ayodele of Botany Department of the University, where voucher specimen (No 22239) was deposited.

Preparation of extract: The air-dried pulverized stem bark (200g) of the plant was exhaustively extracted with absolute ethanol by means of a soxhlet apparatus and the extract was evaporated *in vacuo*. The residue was processed to give 15g (7.5% yield) of brown-greenish solid crude extract, which was stored in a refrigerator for the study. Fresh solution of the extract was prepared in peanut oil when required. Phytochemical screening (9) of this extract confirmed presence of alkaloids, flavonoids and tarpenes but gave negative results for starch granules and calcium oxalate.

Animals: Wistar strain albino rats (120-150g, initially) obtained from the central animal house, College of Medicine, University of Ibadan were used for the study. The animals kept in wire mesh cages were acclimated to laboratory condition (12D: 12L cycles; 24 ± 1°C) and had free access to food and water *ad libitum*. Each rat was certified fertile by isolated mating technique before inclusion in the study.

Study Protocol: Thirty rats divided into six equal groups were used as follows: Group 1 rats received peanut oil (vehicle for the extract) and served as the control; group 2 rats received 50 $mgkg^{-1}$ b.w; group 3, 100 $mgkg^{-1}$ b.w; group 4, 150 $mgkg^{-1}$b.w- of *A.indica* extract; group 5 rats received 3.33$mgkg^{-1}$ b.w ovine luteinizing hormone (oLH) only (i.p) and group 6 rats received 150 $mgkg^{-1}$ b.w *A. indica* extract plus 3.33 $mgkg^{-1}$ oLH (i.p) weekly for ten weeks. Another batch of 30 rats divided into six equal groups were treated as above. They were however allowed a recovery period of ten weeks during which the animals were not treated with the drugs.

After six weeks of the experiment, untreated female rats (each weighing about 200g) of proven fertility and at estrus were cohabited with male rats placed on *A. indica* extract, at a ratio of 2 males to 3 females.

At the end of each experiment, rats were killed by exsanguinations under ether anesthesia (4). The seminal vesicles, epididymis, testes, thyroid, adrenal and pituitary glands were removed and weighed. The serum levels of LH, FSH and testosterone were measured by validated radioimmunoassay (RIA) technique (4). The respective intra– and interassay variations were 10.41% and 10.23% for testosterone; 10.30% and 9.80% for LH and 9.60% and 10.10% for FSH. The RIA reagents used were those of the World Health Organization Matched Reagent Program, while oLH was supplied by NIADDK-NIH (USA). All solvents and mineral acids used were of the analytical grade.

Sperm Analysis: Epididymal sperm counts, motility and morphology were determined, essentially as described by Moss *et al.* (14). Briefly, epididymal fluid was collected via retrograde perfusion through the *vasa deferentia*. A small cut was made in the cauda epididymis at the junction of visible change from small to large tubule diameter. A known volume of phosphate buffered saline (PBS, pH 7.4) was back flushed using a blunt-tipped needle inserted into the severed end of the vas deferens.

Progressive sperm motility was done immediately after the collection of the semen. Two drops of the semen were placed on the microscope slide with two drops of warm 2.9% sodium citrate. It was then covered with a cover slip and examined under the microscope using x 40 objective with reduced light. Sperm viability was done using the Eosin/Nigrosin stain while sperm morphology was carried out by means of the Walls and Ewas stain.

Testicular Histology: Immediately after weighing, the testes were fixed in 10% formal saline for histological analysis. The preserved testes were then passed through routine histological procedures. The slides were stained with haematoxylin and eosine.

Statistical Analysis: Statistical analysis was performed using student's *t*-test and ANOVA. The significance of difference was accepted at $P<0.05$. Data are presented as mean ± standard error of the mean (M±SEM).

Results

Effects of A. indica on body and organ weights

Intraperitoneal administration *A. indica* to the animals for ten weeks did not appear to cause any significant change in their body weights when compared with the control. The same trend was observed during the recovery period. As shown in Table 1, the extract appears to produce a dose dependent decrease in the weight of most reproductive organs in these rats. There was no significant difference between most of the other organ (except adrenal gland) weights when the test groups were compared with the control, during treatment and recovery periods (Table 2). The weights of adrenal gland increased as the dose of the extract administered was increased. The increase in weight, which appeared more noticeable

Table 1: Reproductive organ weights of rats after *A. indica* treatment

Treatment Group (g) (n=5)	Final Body weight (g)	Testis (g)	Epididymis (g)	Seminal vesicle
Control (Peanut oil)	203.30±23.60	1.16±0.22	0.49±0.07	0.52±0.23
	(230.11±25.20)	(1.20±0.04)	(0.50±0.00)	(0.54±0.06)
50 mg kg^{-1} b.w *A. indica*	185.00±15.17	1.05±0.19	0.52±0.14	0.31±0.12*
	(185.00±18.00)	(0.97±0.16)	(0.38±0.03)	(0.32±0.09)
100 $mgkg^{-1}$ b.w *A.indica*	187.10±5.10	1.10±0.20	0.48±0.03	0.28±0.06*
	(196.10±25.30)	(1.22±0.17)	(0.37±0.02)	(0.40±0.12)
150 $mgkg^{-1}$ b.w *A. indica*	179.00±8.00	0.96±0.16*	0.35±0.10*	0.23±0.05*
	(196.10±26.70)	(0.98±0.18)	(0.35±0.09)	(0.29±0.06)
3.33 $mgkg^{-1}$ b.w oLH	205.12±2.70	1.17±0.20	0.51±0.08	0.53±0.21
	(264.50±38.11)	(1.98±0.04)	(0.58±0.09)	(0.54±0.20)
150mg kg^{-1} b.w *A. indica* plus 3.33 mg kg^{-1} b.w oLH	185.13±14.19	1.09±0.20	0.50±0.01	0.51±0.12
	(243.10±27.20)	(1.13±0.19)	(0.53±0.05)	(0.56±0.09)

**Significantly different from respective control values (P<0.05). Values in brackets are measurements obtained from recovery experiments.*

Table 2: Effect of A. indica on the weight (g) of the liver, pituitary (Pit), thyroid and adrenal glands

Treatment Group (n=5)	Final Body weight (g)	Pit. Gland x 10^{-3}	Thyroid gland	Adrenal gland	Liver
Control (Peanut oil)	203.30±23.60	4.70±0.43	0.041±0.002	0.018±0.002	8.10±0.40
	(230.11±25.20)	(5.00±0.09)	(0.045±0.019)	(0.018±0.004)	(7.40±1.70)
50 $mgkg^{-1}$ b.w A. indica	185.00±15.17	4.40±0.49*	0.050±0.009*	0.021±0.002*	6.64±0.96
	(185.00±18.00)	(4.80±0.40)	(0.046±0.019)	(0.016±0.005)	(5.13±0.31)
100 $mgkg^{-1}$ b.w A.indica	87.10±5.10	4.60±0.49*	.037±0.002*	0.024±0.003*	8.86±0.57
	(196.10±25.30)	(4.70±0.43)	(0.044±0.010)	(0.020±0.006)	(6.95±1.22)
150 $mgkg^{-1}$ b.w A. indica	179.00±8.00	5.00±0.01	0.040±0.010*	0.028±0.006*	7.29±0.66
	(196.10±26.70)	(5.00±0.01)	(0.051±0.013)	(0.022±0.004)	(6.13±1.00)
3.33 $mgkg^{-1}$ b.w oLH	205.40±2.70	5.00±0.41	0.042±0.004	0.018±0.001	8.60±0.51
	(265.50±38.00)	(5.12±0.19)	(0.047±0.008)	(0.018±0.003)	(8.90±0.71)
150 $mgkg^{-1}$ b.w A.indica plus 3.33$mgkg^{-1}$b.w oLH	185.13±14.19	4.90±0.03	0.041±0.008	0.029±0.005	8.00±0.71
	(243.10±27.20)	(5.10±0.16)	(0.049±0.014)	(0.027±0.004)	(8.32±0.90)

*Values different from control(P<0.05). Values for recovery experiments are shown in brackets.

during the treatment period, became significant P<0.01), especially in the 150 $mgkg^{-1}$ extract treated rats. There was also no change in liver weight in the extract treated rats when compared with the control.

Azadirachta indica treated rats did not show any unusual behaviour, different from their control counterparts throughout the period of the study. Mortality rate was nil. Water and feed intake (data not shown) remained almost the same as in the control.

Effects of A. indica on sperm functions

As shown in Table 3, the sperm motility appeared to decrease as the concentration of the extract administered was increased. There was a significant reduction in sperm motility when the 150 $mgkg^{-1}$ b.w test group was compared with the control group (P<0.01). Although this trend was also observed in the recovery group, the decrease in sperm motility was not as pronounced as in those rats that were not allowed a recovery period.

With regards to sperm viability (Table 3), the treatment group showed a dose-dependent decrease in the percentage live spermatozoa as the concentration of the extract administered was increased. There was also a significant reduction in percentage viability when the 100 mgkg^{-1} test group was compared with the control group ($P < 0.01$); 150 mgkg^{-1} test group showed a much more lower value than other test groups when compared with the control ($P < 0.001$).

In almost all test groups, the most common abnormality encountered for the epididymal sperm was "detached heads" which accounted for over 60% of the abnormalities. During the treatment period (Table 3), as the concentration of the extract administered was increased the total observable abnormalities were also increased. The difference between the 50 mgkg^{-1} test group and the control group was significant ($P < 0.05$); a more significant increase was observed for the 100 mgkg^{-1} test group when compared with control group ($P<0.01$), and the highest abnormalities were observed in the 150 mgkg^{-1} test group ($P<0.001$).

The female rats mated with the male rats from the control group gave birth to 8 ± 1 litters. The 50 mgkg^{-1} *A. indica* did not induce any observable deleterious effect on the fertility of the male rats as the female rats mated with them delivered 6 ± 1 offspring. However, the female rats mated with the 100 mgkg^{-1} *A. indica* male rats sired 4 ± 1 litters while those from the 150 mgkg^{-1} group did not conceive throughout the duration of the study. However, the female rats mated with male rats that received 150 mgkg^{-1} b.w *Azadirachta* extract conceived and sired physically normal litters (8 ± 2 litters) about four weeks after re-cohabitation with untreated male rats. Injection of oLH to the 150 mgkg^{-1} male rats did not produce any measurable effect on sperm motility, viability, morphology and the litter size of female rats cohabited with them (Table 3).

Effects of A. indica on Serum levels of LH, FSH and Testosterone *Azadirachta indica* produced dose-dependent reduction in serum LH and testosterone levels in male albino rats. Each of the dose regimens of *A. indica* extracts induced a significant reduction ($P < 0.01$) in serum levels of LH and testosterone in rats. Injection of oLH to 150 mgkg^{-1} *A. indica* treated rats did not produce any significant effect on the inhibitory action of *A indica* on LH and testosterone secretion. However, this extract seems to have no effect on the secretion of FSH; none of the doses of the extract produced any significant change in serum levels of FSH when compared with the normal control values (Table 4).

Table 3: Effect of A. indica on sperm functions.

Treatment Group (n=5)	%Motility	%Viability	Morphology (%)		Litter size
			% Normal	% Abnormal*	
Control (Peanut oil)	76.67±4.71	98.33±1.70	94.33±0.47	5.67±0.47	8±1
	(80.33±0.47)	(92.5±4.50)	(90.67±0.47)	(9.33±0.47)	(8±1)
50 mgkg^{-1} b.w A. indica	74.00±4.90	95.20±3.43	91.40±1.74	8.60±1.74	6±1
	(76.00±4.90)	(82.60±4.03)	(87.80±2.48)	(12.2±2.48)	(8±1)
100 mgkg^{-1} b.w A.indica	68.00±7.48**	85.40±3.26**	82.6±1.74**	17.40±1.74**	4±1*
	(72.50±4.33)	(77.25±3.11)	(89.25±2.59)	(10.75±2.59)	(8±1)
150 mgkg^{-1} b.w A. indica	50.00±6.32**	69.00±3.03**	63.80±4.02**	36.20±4.02**	0
	(66.00±4.90)	(77.80±6.62)	(82.60±5.16)	(17.40±5.16)	(4±2)
3.33mgkg^{-1}b.w oLH	80.10±4.55	94.21±2.70	94.12±1.36	5.88±1.36	8±1
	(79.14±3.12)	(93.41±3.61)	(92.18±1.17)	(7.82±1.17)	(8±1)
150 mgkg^{-1} b.w A. indica plus3.33 mgkg^{-1} b.w oLH	56.31±3.41**	69.12±2.04**	74.49±3.01**	25.51±3.01	0
	(69.14±2.90)	(81.61±3.41)	(89.34±2.06)	(10.66±2.06)	(4±1)

*In this study, a spermatozoon was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, detached acrosome, no tail, looped tail, tail coiled below head, tail coiled, round head, and detached head

** Significantly different from control values ($P<0.05$).

Testicular Histology: Figures 1 and 2 show the sections through the testes from control and *Azadirachta indica*

Table 4: Effects of *A. indica* on Serum LH, FSH and Testosterone levels

Treatment Group (n=5)	LH (ng/ml)	FSH (ng/ml)	Testosterone (ng/ml)
Control (Peanut oil)	168±18	237±14	10.4±1.2
	(165±15)	(244±16)	(10.1±1.0)
50 mgkg^{-1} b.w *A. indica*	89±11*	231±13	6.7±0.9*
	(110±12)	(240±14)	(7.7±1.3)
100 mgkg^{-1} b.w *A.indica*	62±13*	229±15	4.5±0.3*
	(90±14)	(243±17)	(6.9±0.8)
150 mgkg^{-1} b.w *A. indica*	57±8*	230±15	3.9±0.6*
	(69±16)	(236±15)	(4.9±0.8)
3.33 µgkg^{-1} b.w oLH	171±19	235±14	15.3±1.4**
	(169±16)	(239±17)	(14.9±1.6)
150 mgkg^{-1} b.w *A. indica* plus 3.33 µgkg^{-1} b.w oLH	60±10*	230±17	4.1±0.8*
	(72±8)	(240±19)	(7.3±0.8)

(*) Significantly lower (P< 0.05) and (**) significantly higher (P< 0.05) than control values.

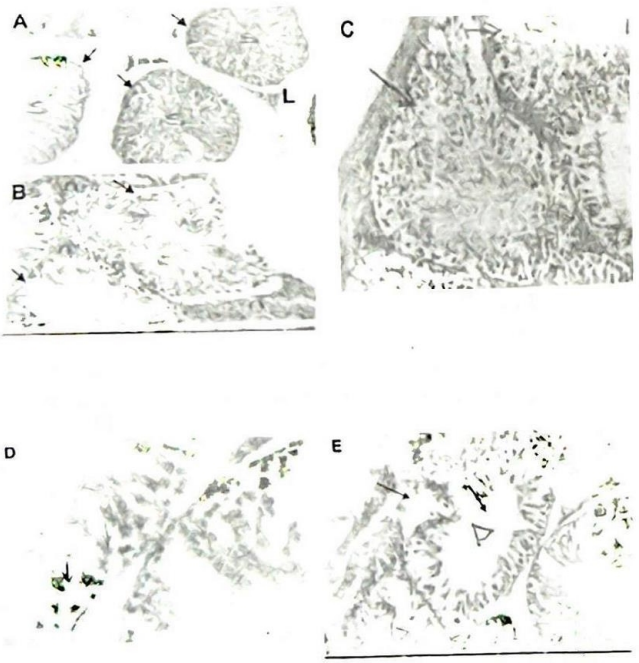

Fig.1: Cross sections through the testes from control (A) and *Azadirachta indica* extract treated rats (**B**, 50mg/kg b.w; **C**, 100mg/kg b.w; **D**, 150- mg/kg b.w of the extract and **E**, 150 mg/kg b.w extract plus oLH). Arrows point to the seminiferous tubules while **L** indicates location of Leydig cells. Light arrows in **D** show Sertoli cells while light alphabets in the micrographs indicate congestion of blood vessels(C), seminiferous tubules (S) and tubular degeneration (D). Mag.X 400.

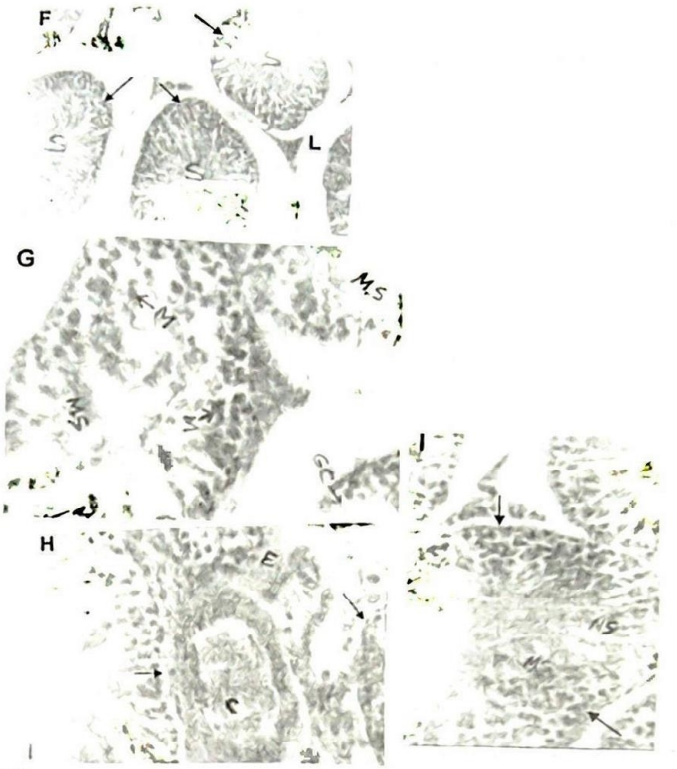

Fig.2: Cross sections through the testes from control (**F**) and *Azadirachta indica* extract treated rats in recovery study (**G**, 50mg/kg b.w; **H**, 100mg/kg b.w and **I**, 150mg/kg b.w, of the extract). Light alphabets show the seminiferous tubules (S), regenerative changes in the seminiferous tubules including mitotic figures (M), the mature spermatozoa (MS), intertubular edema (E) and germinal cells (GC). Mag.X 400

treated rats. Compared with the control (A), there were histological alterations ranging from slight congestion of blood vessels and mild degeneration of seminiferous tubules (B) to increasing congestion of blood vessels and degree of degeneration of the seminiferous tubules(C, D, E) in Fig.1. These changes were gradually being restored following withdrawal from the extract (F, G, H, I, in Fig.2).

Discussion

The results suggest that *Azadirachta indica* extract used in this study impaired reproductive performance in male albino rats. Decreases in testicular weight and reduction in serum LH and testosterone levels occurred in these rats within ten weeks of treatment with the *indica* extract. Sperm counts, motility, viability and morphology were appreciably reduced in a dose-dependent manner. These findings demonstrate the sensitivity of rat testes to *A. indica*, even at low doses. However, because the normal untreated female rats cohabited with the male rats that were treated with 50 and 100 mgkg^{-1} *A. indica*, conceived, it appears the antifertiltiy activity of the *indica* is more

pronounced at higher concentrations. None of the female rats cohabited with the male rats on 150 $mgkg^{-1}$ extract conceived. These findings corroborate the report of Upadhyay *et al.* (7) that a single intra vas application of *Azadirachta indica* oil resulted in infertility with a block of spermatogenesis.

The reduction in both the serum levels of LH and testosterone may suggest that the extract either has an inhibitory action on LH secretion from the pituitary gland or testosterone secretion from the Leydig cells or both. That the extract is capable of inhibiting testosterone secretion is consistent with the report that oral administration of *A. indica* extract decreased serum testosterone levels [8]. The inability of exogenously administered LH to enhance secretion of testosterone in *A. indica* treated rats may also suggest that the action of the plant is directly related to testicular cells other than Leydig cells. Another way *A. indica* might induce sterility could be through its action on the Sertoli cells. Since Sertoli cells provide nourishment for the germinal sperms as they mature, it can be deduced that the fewer the Sertoli cells, the lesser the nourishment provided for the maturation of the germinal sperm. This is consistent with the reduced LH level in *A. indica* treated rats and corroborated by the report that as the number of Sertoli cells decreases the infertility rate increases proportionately [13].

The sterility induced in the male rats by *A. indica* could be a result of decrease in seminiferous tubular spermatozoa, decrease in adenosine triphosphatase (ATPase) activity of the spermatozoa, cellular changes and degeneration of spermatids, spermatocytes and germinal cells similar to those in gossypol induced sterility [10]. The degeneration of the seminiferous epithelium and tubules could lead to inhibition of spermatogenesis. At higher concentrations of the extract, the seminiferous tubular degeneration appeared more severe (Figs.1 and 2) accounting for the sterility induced at these concentrations. As the concentration of *A. indica* was increased the number of abnormal sperm cells increased with a high percentage of detached heads suggesting that one way the extract induced infertility is probably by detaching or deforming the heads of the sperm cells. Moreover, LH is known to enhance fructolysis and sperm adenyl cyclase activity, which are important means by which spermatozoa derive energy for their motility [11]. Since live spermatozoa (though, low percentage) were still found in the epididymis, it appears that *A.indica* did not completely inhibit spermatogenesis. In addition, the presence of deformed and non-motile spermatozoa and change in LH and testosterone levels might imply *A. indica* induced infertility through an array of factors. A general reduction in the diameters of seminiferous tubule and nuclei of the germinal elements and mass atrophy of the spermatogenic elements and Leydig cells in *A. indica* treated rats [12] are consistent with the present findings. Consequently, these activities probably summate leading to a decrease in percentage live spermatozoa as the concentration of the extract increased. However this assumption could be confirmed in a more detailed *in vitro* experiment in which the extract is topically administered to semen and its effects on sperm characteristics observed in suitable media and environment. The actions of *A. indica* on male reproduction appear reversible. The active principle in this extract with these activities is not yet known. Future studies will attempt to identify this and also further mechanism by which the stem bark extract of *A. indica* induces infertility in male rats.

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