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Neem leaf extracts induced biochemical and histological changes in male albino mice

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Abstract
Oral administration of alcoholic extract of neem leaves at the rate of 132, 200 and 300 mg/kg body weight /day for 24 days caused reduction in the weights of accessory reproductive organs like epididymides and seminal vesicles and change in testicular biochemical parameters in albino mice. The distinct changes in the histology of epididymides and seminal vesicles were observed. The higher dose groups showed more prominent change.

Keywords: Neem, Testicular, Biohemical parameters, Histological , Accessory reproductive organs, Albino mice.

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INTRODUCTION

Our old system of treatment consisted of products/extracts prepared from medicinal plants. Our ancient literature is enriched with the information indicating the benefits of herbal products (Sharma et al 2013). These plant products have the capacity to fight against bacterial, fungal and parasitic infections and also hold anti-pyretic, anti-inflammatory and anti-fertility properties (Kumar et al 2018, Zihadi et al 2019, Khan et al 2020, Nandi et al 2022, Njoga et al 2022) and may be used against various other types of diseases. WHO has suggested that these locally available herbal products can be used as substitute of drugs and a task force has been setup on plant research to find the compounds which are non steroidal and can be used as an oral contraceptive (WHO, 2000). In India also, different medicinal plants/extracts have been screened for their antifertility effects both in male and female animal species.

There is clear cut evidence on the regulation of fertility with herbal products in ancient literature of indigenous system of medicine (Joshi et al 2011). These plant products may induce abortifacient, anti-implantation, emengogue like effects in females and anti- spermatogenic, spermicidal and anti-androgenic effects in males (Shaikh et al 2017). Various synthetic compounds are known to induce contraceptive effects in males (Franca et al 2000, Grima et al 2001). But obviously these chemical compounds are toxic for animal as well as human use. Hence old traditions of using plants as medicine without side effects should be revised.

The effect on reproductive organs has been studied after the oral administration of different plant extracts (Njar et al 1995, Raji and Bolarinwa, 1997). Neem extracts have also been tested in males for their anti-fertility effects (Parshad et al 1997, Pandey et al 1999, Shaikh et al 2009). This study deals with an attempt to check the effect of neem extracts on different testicular metabolites and to study the histological aspects of accessory reproductive organs in male mice to explore its potential as an antifertility agent in male animal species.

MATERIALS AND METHODS:

Adult male albino mice of 8 weeks old and 30 grams (g) body weight (bw) approximately were procured from breeding house of Small Animal Colony, Department of Zoology, PAU, Ludhiana. The mice were given time to acclimatize to new conditions. They were kept under standard conditions and fed with standard rat diet and fed ad libitum. Leaves were collected from neem plant in botanical garden. Leaves were dried in shade for several days. The dried leaves were then powdered. The powder was extracted by percolation at room temperature with 70 per cent ethanol. The extract was then concentrated under reduced pressure and dried in vacuum desiccator. The final residue was dissolved in propylene glycol (vehicle) at the rate of 100mg/ml and was used for present experimental study. The different groups of mice were maintained having 8 mice in each group. Dose 1 in which mice received leaf extract at the rate of 132 mg/kg bw/day for 24 days and dose 2 and dose 3 received leaf extract at the rate of 200 and 300 mg/kg bw/day for 24 days respectively. The mice of all groups were sacrificed 24 hours after the administration of last dose. The testes, epididymides and seminal vesicles were

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removed from the dissected mice. The mucus was cleared and weight of every organ is measured separately. The testes were processed for the study of biochemical parameters.
Various testicular metabolites such as Total proteins, Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA), Acid phosphatase (ACP), Alkaline phosphatase (AKP), Total lipids, Total cholesterol, Total phospholipids were estimated from :
-testes of control mice.
-testes of mice treated with different doses of neem extracts.
The total proteins were estimated by the method of Lowry et al (1951).DNA and RNA were estimated by the method of Work and Burdon(1980). ACP and AKP activity was estimated by the method of Linhardt and Walter (1974). The total lipids from testes were extracted by the method of Folch et al (1957). Total cholesterol was estimated by the method of Chaimory and Henry (1959). Total phospholipids were estimated by the method of Ames (1966).

In another experiment a single control group was maintained for all the three doses of leaf extracts. Epididymis and seminal vesicles were removed after the completion of treatment and processed for histological studies. Epithelial height of caput and cauda epididymis and luminal diameter of cauda epididymis were determined by Stage-occular meter. About 15 different tubules of caput as well as cauda epididymis were studied from each group to record epithelial height.

RESULTS AND DISCUSSIONS:

Effect on weight of epididymis and seminal vesicles: Oral administration of leaf extract at the dose level of 132, 200 and 300 mg/kg bw/day for 24 days resulted in significant reduction in mean epididymal weight in all the treated groups as compared to their control groups (Table 1). The weight of caput epididymis reduced significantly in all the groups who were given dose1, 2 and 3 as compared to their control groups while the weight of cauda epididymis reduced significantly in dose 3 treated group as compared to its control (Table 1). Androgens play an important role in the functioning of these reproductive organs so deficiency of androgens is the main cause of reduction in the weight of accessory reproductive organs in different animals treated with different plant extracts such as Malva viscus conzattii in gerbils (Dixit, 1977), Aristolochia indica in mice (Pakrashi and Pakrasi, 1977), Calotropis procera in gerbils (Garg,1979), Andrographis paniculata in rats (Akbarsha *et al* 1990).

Out of all the treated groups , the dose 3 treated group showed significant reduction in the weight of seminal vesicles in comparision to its own control group (Table 1). Dixit (1977) and Akbarsha et al *(*1990) suggested that the plant extract has anti-androgenic effect which is responsible for reduction in the weight of seminal vesicles. Administration of neem leaf extracts may have caused anti-androgenic effect due to which there is reduction in the weight of seminal vesicles.

Effect on testicular metabolites:
A drastic decrease in the testicular proteins was observed in the treated mice after the administration of different doses of leaf extracts for 24 days. The total proteins reduced significantly in all the three dose groups in comparison to their controls respectively (Table 2). Reduction in the total protein content may be due to impairment in spermatogenesis (Bir Hans *et al* 1999). Different plant extracts such as Malvaviscus conzattii Greenm in rats and gerbils (Dixit 1977), in mice (Verma *et al* 1980) affected testicular protein content after the administration of plant extracts.
The mean content of Deoxyribonucleic acid also reduced significantly in all the treated groups i.e. in 132, 200 and 300 mg/kg bw treated groups as compared to their control groups respectively while there was dose dependent reduction in the RNA content of leaf extract treated groups (Table 2). A decrease in testicular DNA was also observed due to abnormal spermatogenesis(Bhiwgade et al 1990). When nucleic acid synthesis is decreased, it results in apoptosis in mouse treated with mitomycin (Nakagawa *et al* 1997). Dixit, (1977) suggested that when androgen production is decreased , it actually suppresses spermatogenesis and result in decrease in the RNA levels

Table 1: Effect of neem leaf extracts on the weights of Epididymis and seminal vesicles-

Organ	Control	Dose 1	Control	Dose 2	Control	Dose 3
Epididymis (g/100 g bw)	0.15 ± 0.003	0.137 ± 0.004(91)*	0.125 ± 0.001	0.095 ± 0.004(76)**	0.122 ± 0.001	0.079 ± 0.004(65)**
Caput	0.081 ± 0.004	0.0683 ± 0.003(84)*	0.073 ± 0.004	0.055 ± 0.005(75)**	0.068 ± 0.003	0.039 ± 0.002(57)**
Corpus	0.024 ± 0.001	0.023 ± 0.003	0.0151 ± 0.002	0.014 ± 0.003	0.015 ± 0.001	0.014 ± 0.002

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Cauda	0.044 ± 0.001	0.041 ± 0.001	0.036 ± 0.005	0.025 ± 0.001	0.038 ± 0.004	0.026 ± 0.002(68)*
Seminal vesicles(g/100 g bw)	0.869 ± 0.025	0.812± 0.043	0.884 ± 0.034	0.768 ± 0.065	0.864 ± 0.026	0.473 ± 0.050(55)**

Values are mean ± S.E., values in parenthesis are % of control. P ≤ 0.01: ** indicates significant change as compared to control. P ≤ 0.05: * indicates significant change as compared to control.

A dose dependent reduction in the activity of ACP was seen in the treated groups. The activity of ACP decreased significantly in dose 2 and dose 3 treated groups as compared to their control group while the activity of AKP increased significantly in all three treated groups(Table 2). Mann (1964) suggested that acid phosphatase is an indicator of androgen activity and is dependent on the circulating levels of testosterone. The decrease in acid phosphatase activity after the administration of neem extracts exerts anti-androgenic effect and it interferes with the process of spermatogenesis. Similar observations have been made by different workers after the administration of different plant extracts in rats, like Solanum xanthocarpum (Rao, 1988) and Azadirachta indica seeds (Choudhary et al 1990; Kasturi *et al* 1995; Joshi et al 1996).

Increase in alkaline phosphatase activity indicates suppression of spermatogenesis and extreme lytic activity (Verma et al 1980; Kaur et al 1997) and similar activity has also been reported after the administration of Calotropis procera(Ait.)R. Br. (Garg 1979) and Andrographis paniculata (Akbarsha et al 1990) in rodents.

A dose dependent increase in total lipids was seen in treated groups (Table 2). Mean lipid content of dose 2 and dose 3 treated groups increased significantly in comparison to their control.

There was also significant increase in testicular total cholesterol content in mice treated with leaf extract at the dose level of 200 and 300 mg/kg bw/day for 24 days(Table 2). When lipids remain unutilized by tubular elements , it leads to accumulation of lipids (Guraya 1995). Various anti-fertility and anti-spermatogenic extracts have been known to cause less utilization of lipids and consequently accumulation in various vertebrate testes (Khanna 1994; Kaur 1998; Bir Hans et al 1999). Increase in testicular cholesterol levels was also reported in rats administered with leaf powder of Azadirachta indica (Joshi et al 1996). Verma et al (1980) suggested that the complete arrest in spermatogenesis was the main reason for increase in cholesterol level. Unavailalability of hormones required for steroidogenesis may also be the reason. (Reddy et al 1997).

A non significant reduction in the total phospholipids of testes was observed in groups treated with 132 and 200 mg/kg bw/day while a significant reduction in total phospholipid content occurred in group treated with 300 mg/kg bw/day for 24 days in comparison to its control (Table 2).Administration of nimbecidine also caused decreased phospholipid content (Kaur,1998).

Table 2: Effect of oral administraton of neem leaf extracts (132, 200 and 300 mg/kg bw/day for 24 days on testicular metabolites in male albino mice:

Testicular metabolites	Control	Dose 1	Control	Dose 2	Control	Dose 3
Proteins (mg/g wet tissue)	6.867 ± 0.160	5.697 ± 0.126(83)**	7.267 ± 0.225	5.407 ± 0.127(74)**	7.203 ± 0.092	4.900 ± 0.170(68)**
Deoxyribonucleic acid (mg/g wet tissue)	0.580 ± 0.052	0.298 ± 0.009(51)**	0.664 ± 0.047	0.286 ± 0.006(73)**	0.577 ± 0.028	0.258 ± 0.010(45)**
Ribonucleic acid(mg/g wet tissue)	0.998 ± 0.003	0.947± 0.027	1.003 ± 0.007	0.94 ± 0.021(94)*	1.047 ± 0.030	0.704 ± 0.046(67)**
Acid phosphatase (µmoles /min/mg protein)	1.830 ± 0.095	0.803 ± 0.813	1.847 ± 0.068	0.813± 0.052(44)**	1.827 ± 0.030	0.723 ± 0.072(40)**
Alkaline phosphatase (µmoles/min/mg protein	0.797± 0.017	2.197± 0.452(276)*	0.795 ± 0.013	2.615 ± 0.292(329)**	0.903 ± 0.052	3.070 ± 0.057(340)**
Total lipids (mg/g wet tissue)	55.983± 3.215	67.560 ± 8.170	60.863± 0.486	68.267 ± 2.077(112)*	60.307± 0.704	118.227 ± 0.995(196)*
Total Cholesterol (mg/g wet tissue)	1.084 ± 0.054	1.607 ± 0.201	1.213 ± 0.174	1.733 ± 0.047(143)*	1.108 ± 0.052	2.087 ± 0.109(188)**

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Total phoshpholipids (mg/g wet tissue)	7.163 ± 0.121	6.933 ± 0.401	7.554 ± 0.552	6.850± 0.201	7.403 ± 0.508	5.897 ± 0.119(79)*

Values are mean ± S.E., values in parenthesis are % of control. P ≤ 0.01: **indicates significant change as compared to control. P ≤ 0.05: *indicates significant change as compared to

Histological changes in accessory reproductive organs:

In the control group ,the caput epididymis consisted of highly contorted tubules with some tissue in between the tubules. The tubules showed uniform diameter. There was tall and ciliated epithelium in all the tubules. Sperms were clearly visible in the lumen. (Figure. 1, mpg 1). The oral administration of leaf extracts at the dose level of 132, 200 and 300 mg/kg bw/day for 24 days caused changes in the epithelial height. There was significant reduction in the epithelial height in the dose 3 treated group as compared to control (Table 3).

The tubules of control mice had more number of sperms while the number of sperms were less in the caput epididymal tubules of treated mice (Figure I)

Table 3: Effect of neem leaf extracts on Epithelial height and luminal diameter of epididymis:

Epithelial height of: Caput epididymis(µm)	31.040 ± 2.367	29.860 ± 2.346	28.180 ± 1.542	22.880 ± 1.054**
Cauda epididymis (µm)	21.120 ± 1.349	21.040 ± 1.362	17.149± 0.618**	15.400 ±0.955**
Luminal diameter of Cauda epididymis(µm)	204.750±11.140	199.620 ± 9.400	163.313± 8.632*	116.188± 6.544**

Values are mean S.E. ;
P ≤ 0.01: ** indicates significant change as compared to control. P ≤ 0.05: * indicates significant change as compared to control

In the cauda epididymis of control mice, the tubules were compactly arranged and there was very less inter-tubular tissue. The epithelium was made up of low cuboidal and ciliated cells. Sperms were seen in the tubules of cauda epididymides (Figure II). There was dose dependent reduction in the mean epithelial height of cauda epididymis. The mean epithelial height of cauda epididymis reduced significantly in dose 2 and dose 3 treated groups in comparison to control group (Table 3). The luminal diameter of tubules of cauda epididymides also showed significant reduction in 200 and 300 mg/kg bw/day treated mice. The epithelial height of caput epididymis was also reduced in rats after the administration of Azadirachta indica leaf powder (Kasturi et al 1995). Androgen deficiency leads to reduced sperm concentration as androgens are required for normal spermatogenesis. Reduced androgen synthesis leads to degenerative changes in epididymis.

Histomorphological changes such as reduction in the epithelial height and reduced number of sperms in cauda epididymis were also reported by Kasturi et al (1995) in rats fed orally with dry leaves of Azadirahchta indica. Verma et al (1980) reported that androgen deficiency is the main cause of inhibition of spermatogenesis which is further responsible for decreased number of sperms in the lumen of cauda epididymis.

Histological examination of seminal vesicles of control mice showed a large cavity with several mucosal crypts. The epithelial lining of mucosa is formed of single layer of tall columnar cells (Figure III). Lu A viscous seminal fluid is present in the lumen which contains fructose for giving energy to sperms and also prostaglandins which are responsible for the onset of contractions in the female reproductive tract so that the two gametes can meet in the oviduct. In the seminal vesicles of treated mice, the secretion in the lumen was limited because of inward growth of mucosa towards lumen in all the groups administered with neem leaf extracts. Inward growth of mucosal crypts in the lumen of seminal vesicles was also observed by other workers in male rodents fed with different plant extracts (Verma et al 1980; Akbarsha et al 1990).

CONCLUSION:

Plant extracts/products can be exploited to induce antifertility effect in male mammals. Neem leaf extracts induced anti-androgenic

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effect which in turn is responsible for affecting testicular biochemistry and caused histological changes in accessory reproductive organs. Further research is required to see whether the changes are reversible or not, only then these products can be used in human males as contraceptive

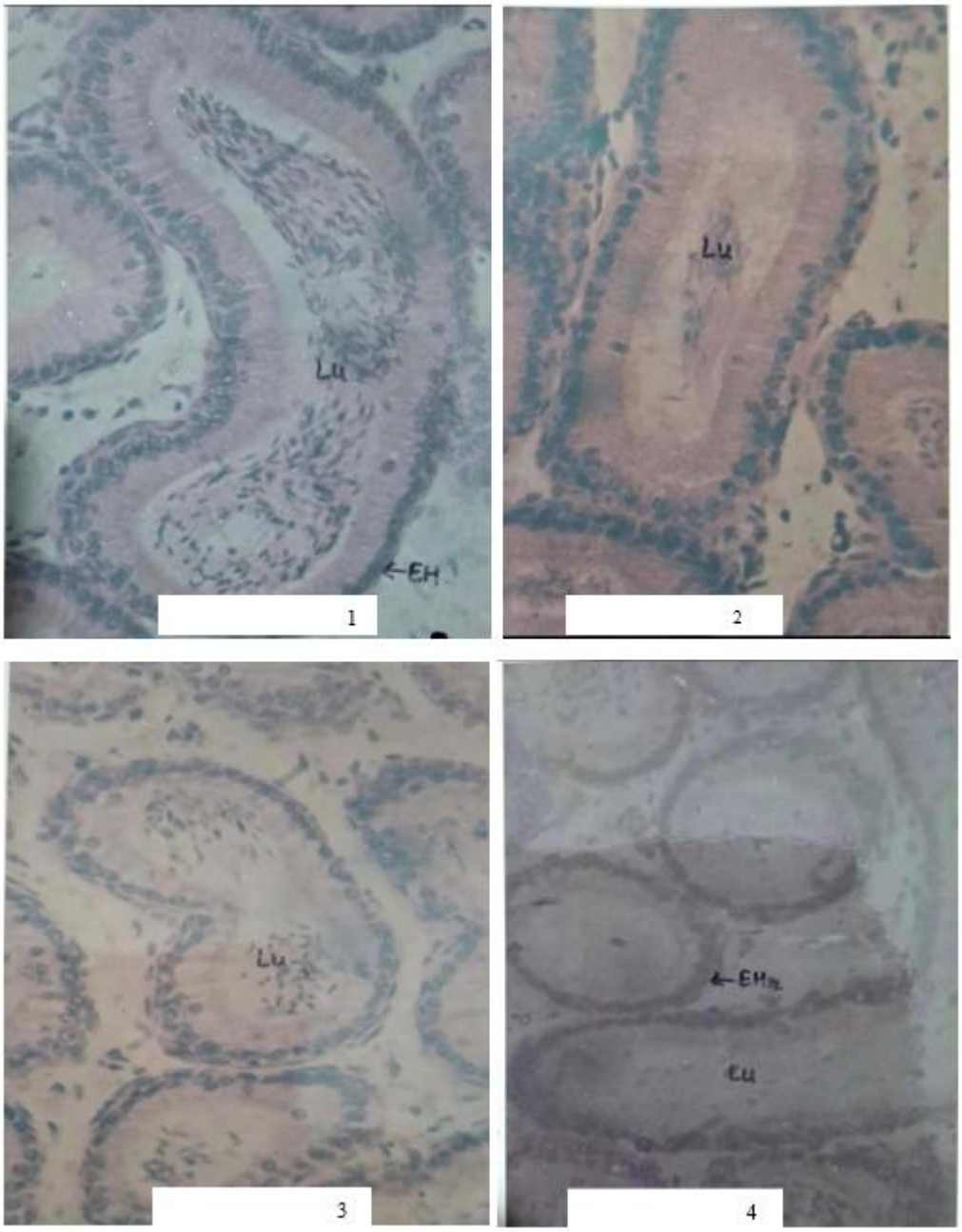

Figure I: Haematoxylin /eosin stained sections of Caput epididymis of;
mpg 1: control mice showing lumen full of sperms(10 X 40); mpg 2,3 & 4: mice treated with leaf extracts at the dose level of 132,200 and 300 mg/kg bw/day for 24 days respectively showing less number of sperms in lumen(10X40).
Lu: Lumen; EH: Epithelial height; EHr: Reduced epithelial height

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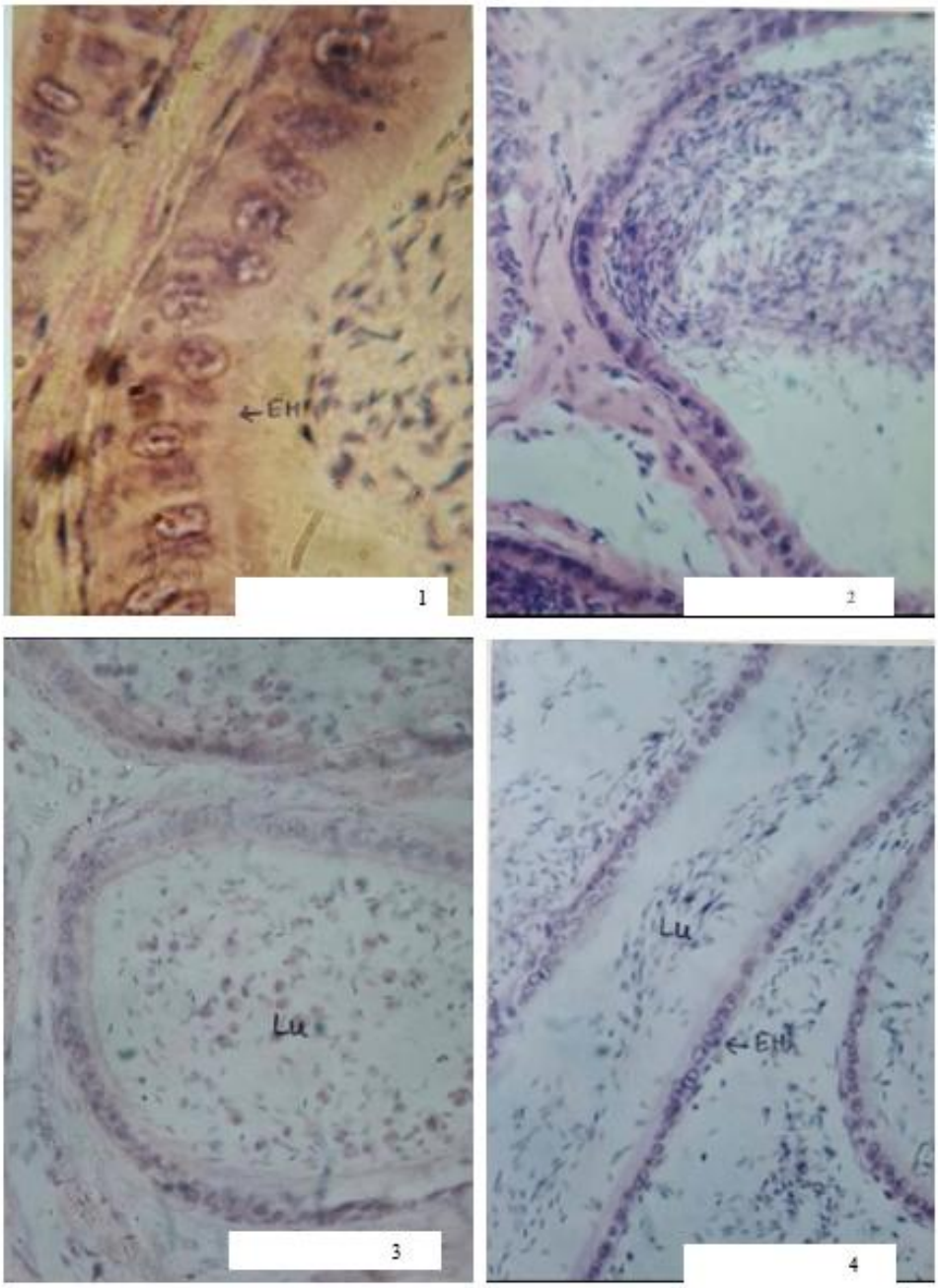

Figure II: Haematoxylin /eosin stained sections of Cauda epididymis of; mpg 1: control mice showing epididymal tubule with full epithelial height and part of lumen filled with sperms(10X40). mpg: 2,3 & 4: mice treated with leaf extract at the dose level of 132, 200 and 300 mg/kg bw/day for 24 days respectively showing reduced epithelial height and lumen with less number of sperms(10 X 40). Lu: Lumen; EHr: Reduced epithelial height.

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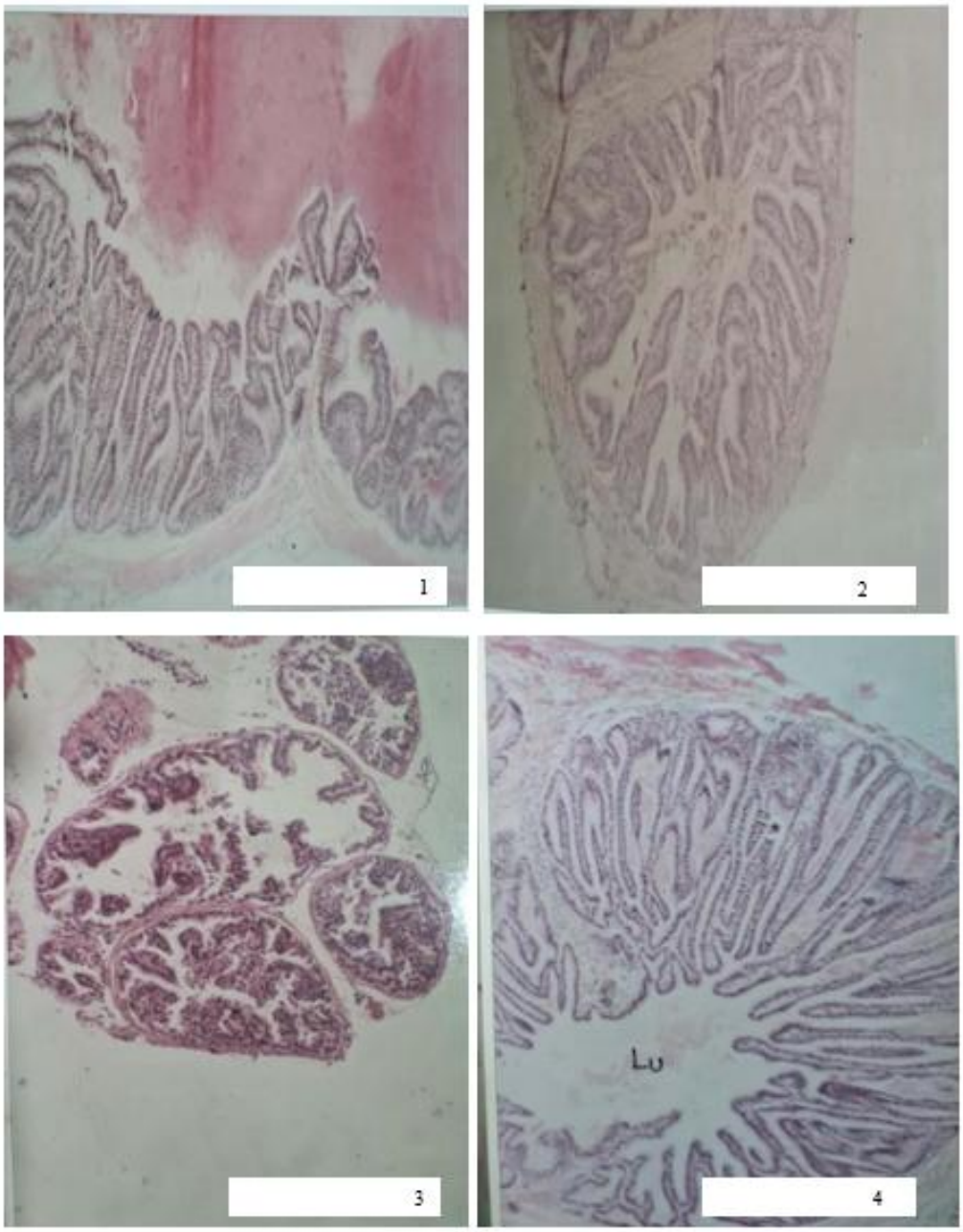

Figure III : Haematoxylin / eosin stained section of Seminal Vesicles of; mpg:1 :control mice with elongated cavity with viscous seminal fluid(10X 40). mpg 2,3 &4: mice treated with 132,200 and 300 mg/kg bw/day for 24 days respectively showing inward growth of mucosal crypts leading to reduced lumen with very less seminal fluid(10- X 40) .Lu: Lumen

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