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Evaluation of the Anabolic and Reproductive Activity of *Eulophia Herbacea* Lindl. In male rats

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ABSTRACT

Orchid such as *Eulophia herbacea*, commonly called as Kukud-kand (Family *Orchidaceae*). According to the folklore, 'Salep' of *Eulophia* tuber is used as an aphrodisiac drug and tonic. Decoction of tuber is used on spermatorrhoea, and menses. Methanolic extract of the tuber was studied for its effect on anabolism, spermatogenesis, and sperm count. Serum profile alteration in blood of albino rat was also recorded. Lead acetate was used to induce reproductive dysfunction in rats. Two doses i.e. 35 and 70 mg/kg of methanolic extract on concomittent administration of it in albino rat showed pronounced anabolic and spermatogenic effect in animals of respective groups. The sperm count in testicular tissues, serum glucose and serum protein level was markedly increased. The extract had dose dependent influence on sperm count and glucose concentration which increased significantly. Restoration of sperm density, sperm viability after plant extract fed treatment in reproductive dysfunctioned animals is noted on the basis of histomorphological studies of testes in rat.

Keywords: • *Eulophia herbacea* Salep Aphrodisiac Anabolic Spermatogenesis, Reproductive dysfunction

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INTRODUCTION

Infertility is one of the major problems in life and approximately 30% of contribution in infertilies is due to male and female factor each[1,2]. A number of plants like *Asparagus racemosus, Chlorophytum borivilianum, Curculigo orchiodies, Dactylorhiza hatagirea, Orchis latifolia, Anacyclus pyrethrum* have been traditionally employed among different cultures in order to improve sexual performances[3-6]. Orchid such as *E. herbacea* (Kukud-kand) from the family *Orchidaceae* is used to prepare a drink called Salep. According to the folklore, 'Salep' of *Eulophia* tuber is used as an aphrodisiac drug. Tubers of *E. herbacea* used to make a nutritious beverage by treating the powdered preparation with hot water as tonic. Decoction of tuber is used on spermatorrhoea, and menses[7]. Its seed powder is consumed as an ailment in weakness,[8] rhizome paste as an aliment on pimples[7] and on rheumatism[9,10]. It is traditionally used in the treatment of tumors of scrofulous glands of neck[11] and urinary complaints also.

Few Indian *Eulophia* species are reported for their phytoconstituents. The *Eulophia* species are reported to contain eullophiol, nudol[12,13]. β-sitostorol[14] ephemeranthol, fimbriol[12] etc. as active phyto-constituents. Phenanthenes in various *Eulophia* species which have often been used in traditional medicines. Tatiya[15]et al., (2012) has reported presence of acidic compounds, carbohydrates, amino acids, mucilage, tannins, steroids and terpenoids in *E. herbacea*.

The various biological activities of *Eulophia* tubers are reported such as, antitumor[16, 17], hypolipidomic[18], antimicrobial and anthalmintic[19], antidiarrheal[20], anti-inflammatory[21], antioxidant[22] and aphrodisiac activities[23]. They confirmed earlier folkloric claim. They also reported spermatogenic parameters in herbal composition containing *Macuna pruriens* (Linn), *Chlorophytum borivilianum* (Sant and Fernand) and *E. campstris* (Wall). This encourages us to evaluate reproductive functions of *Eulophia* species.

Although, *Eulophia* is a constituent of number of herbal formulations that are known for aphrodisiac nature and improving fertility, there is no scientific report on its usage as sexual tonic or stimulant. Keeping in view the growing popularity and market interest for the drug, present studies were undertaken to provide scientific support for its purported folkloric usage.

MATERIALS AND METHOD

Animal Stock

The protocol for experimentation was approved by Institutional Animal Ethics Committee in Moolji Jaitha College, Jalgaon (Reg. No. **1062/c/07/CPCSEA dated 24th May 2007)**. The animals were allowed to acclimatize for at least 10 days before the start of the experiments. Sixteen Wistar strain male albino rats weighing 200–250 g were fed on standard diet and water *ad*

libitum. The animals were maintained under standard conditions of light-dark cycles (12 hours each) and temperature (22 ± 2 ^{0}C). Animal handling was conducted between 8.00 am and 10.00 am to minimize the effects of environmental changes. The guidelines of CPCSEA, India the governing body for animal experimentations in India, were strictly adhered to during the whole animal experimentation protocol. The number for approval of ethical committee is IAEC/ 23/CPCSEA/MJ/2014-15 and the proposal was approved.

The tubers of *E. herbacea* and were collected and also purchased from local market from Vani (District Nasik) and Manudevi (Taluka Yawal, District Jalgaon) respectively. The tubers were identified and authenticated by Dr. V. V. Bhadane. Voucher specimens (PCA/ BOT H.S.1641 and PCA/ BOT H.S. 1642) were deposited at the herbarium of Department of Botany, Pratap College, Amalner.

Preparation of extracts

The shade dried tubers of *E. herbacea* were pulverized to form coarse powder and extracted with 5 solvents of increasing polarity viz. petroleum ether (60-80^{o}C) benzene (80^{o}C), chloroform (61^{o}C), acetone (56^{o}C) and methanol (65^{o}C) in a Soxhlet extractor for 8h each. Each extract was concentrated by distilling off the solvent and then evaporating to dryness on the water bath. After determining the yields, sediment extracts were stored at 4^{o}C for further study in a dessicator. All the extracts were analyzed phytochemically and the methanolic extract of *E. herbacea* (MEEH) were screened for their biological activities.

Experimental design

After one week of acclimatization, 16 male rats weighing about 200-250 g each, were randomized into 4 groups comprising of 4 animals each (n = 4). Following treatments were administered to respective groups from the study day 1 to day 28. Here lead acetate was used as reproductive disfunctioning agent[24].

1. Group I – Normal – Saline solution (1.0 mL)

2. Group II – Rd (Reproductive disfunctioned)-3% Lead acetate solution was administered orally once a day at the dose of 0.5 mL/100 g body weight

3. Group III (low dose) **–** MEEH 35 mg/kg b.w.p.o along with 3% Lead acetate solution at 0.5 mL/100 g b. w.

4. Group IV (high dose) **–** MEEH 70 mg/kg b.w.p.o along with 3% Lead acetate solution at 0.5 mL/100 g b. w.

Studies Performed

1) Effect on sexual organ weight After 28 days of treatment the body weight of animals was recorded. The animals were then sacrificed by cervical decapacitation and testis, epididymis were carefully removed and weight of each organ was determined[25].

B Sperm parameters – sperm density, motility and viability

Sperm density per mL of the diluted sperm suspension was evaluated by Dorostghoal[26] method. The left cauda epididymis from all males was used for sperm motility analysis. Motion parameters included total percentage of motile sperm, percentage of progressively motile sperm and sperms showing circular motion. The sperm viability was determined using Eosin stain as described by Raji[27] et al., (2003). Sperm viability was expressed as % live sperms.

C Serum biochemical profile

Approximately 1 mL of blood volume were taken and dispensed into labelled plain tubes. The blood samples were then centrifuged at 3000 rpm for 10 min to separate the serum. The serum was stored at 4°C until assays were carried out. Estimations of serum testosterone, serum cholesterol, blood glucose and serum total proteins were done using commercial kits (Erba Pvt. Ltd.).

D Histological studies

After 28 days of treatments to animals of all respective groups, testis of animals from each group was dissected out and one of the excised testes was fixed in 10% Formalin solution. During the preparation, slides of each testis were fixed in 70% ethanol and embedded in paraffin wax and longitudinal sections of 5 µm thickness were cut on a microtome. Each section was taken on a microscopic slide, deparaffinized and stained with hematoxyllin - Eosin stain. Microscopic evaluation of the thin section was undertaken and variations in histoarchitecture were recorded[28].

Statistical analysis

The data was expressed as mean ± SEM and the difference in the central tendencies of treatment groups was tested for statistical significance using ANOVA followed by Bonferroni's multiple comparison test for parametric data. For non-parametric data Kruskal-Wallis test followed by Dunnette's multiple comparison tests was applied; $p < 0.05$ was considered statistically significant for both these tests.

RESULTS AND DISCUSSION

Anabolic effect

No statistical difference of mean body weight in first 5days of experiment in all groups. While from second to fifth interval (5-25days) the mean body weight was increased in all the groups. On 25th day the % weight gain was very much lowered (35%) in G-II in comparison to MEEH treatment at low (28.33%) and high dose (20%) respectively. In control group the body weight rise continuously throughout all the intervals. The treatments with MEEH led to enhance the body weight as well as gonadal organs (testis and epididymis) weight **(Figure 1a**

and 1b) which was lowered in reproductive disfunctioned group. The Rd led to decrease in the average weights of testes and epididymis by 39.58% and 35.45% respectively. The protective effect of MEEH was dose dependent**.**

Anabolic effect of MEEH is noted as compared to the control group. Such effect is noted in *Anacyclus pyrethrum*[25] and *Fumaria parviflora*[26].

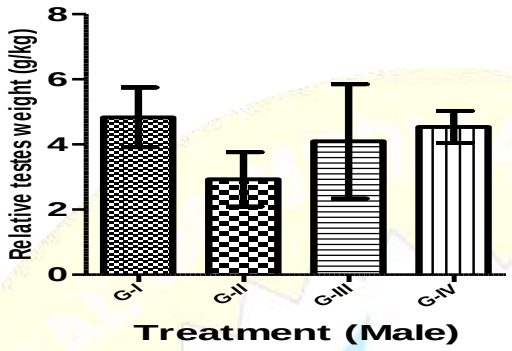


Figure 1 a. Effect of oral treatment of MEEH on testicular weight in different groups of adult rat.

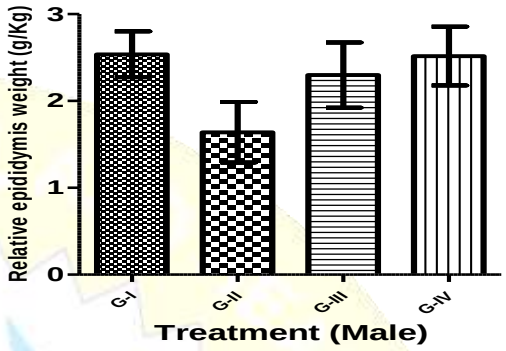


Figure 1 b. Effect of oral treatment of MEEH on epididymal weight in different groups of adult rat.

Data was analysed by One way ANOVA followed by Dunnette's post hoc test. $p<0.05$

The androgens possess anabolic activity[29]. Androgens are necessary for the development, growth and normal functioning of the testes and male accessory reproductive glands and studies have shown that the level of testosterone is positively correlated with the weights of testis and epididymis[26]. In the present study the testes and epididymal weights are also increased. Since androgenic effect is attributable to testosterone levels in the blood, it is likely that the plant extracts may have a role in testosterone secretion allowing better availability of hormone to gonads[3] as in *Anacyclus pyrethrum*[25], *Zingiber officinale*[30] and *Orchis anatolica*[31]. Therefore, the increase in body weight in MEEH treated groups could be due to the androgenic properties of MEEH.

B. Sperm parameters – sperm density, motility and viability

As expected, Rd group showed significant decrease in the number of testicular and epididymal sperm count by 77.68% and 71.77% respectively as compared to the control ($p<0.01$). In **Figure 2a**, MEEH administrated concomitant groups with highest dose almost recovered the loss of sperm density by 92.9 %.

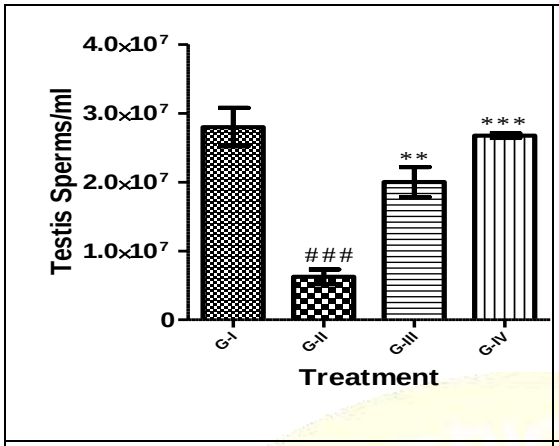


Figure 2a. Influence of oral treatment of MEEH on testicular sperm density in adult rats.

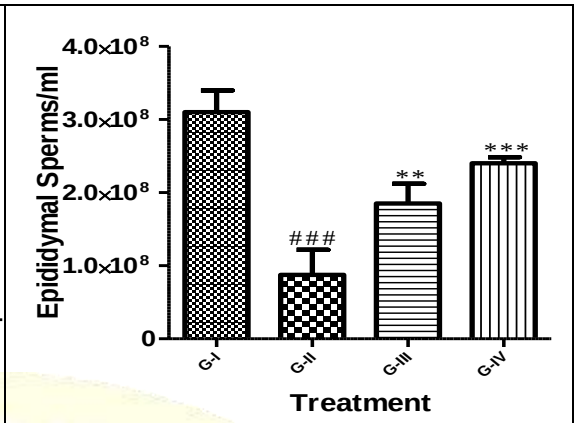


Figure 2b. Influence of oral treatment of MEEH on epididymal sperm density in adult rats.

Data was analysed by One way ANOVA followed by Dunnette's post hoc test; **p<0.01, ***p<0.001 as compared to LA and ### p<0.001 as compared to control

The entire treatment groups also showed higher epididymal sperm count (**Figure. 2b**). The effect of MEEH showed increased sperm density (71.4%) at higher dose as compare to Rd group. Rd group revealed diminish in motility by 91.23%. In this group the percentage of motile sperms dwindled down as compared to the control group (p<0.001).

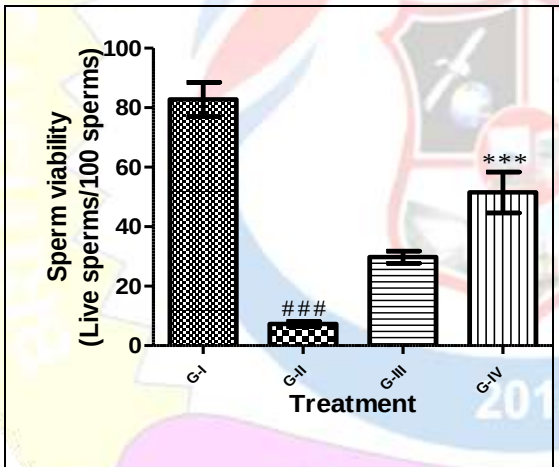


Figure 3a. Effect of oral treatment of MEEH on sperm motility in different groups of adult male rats.

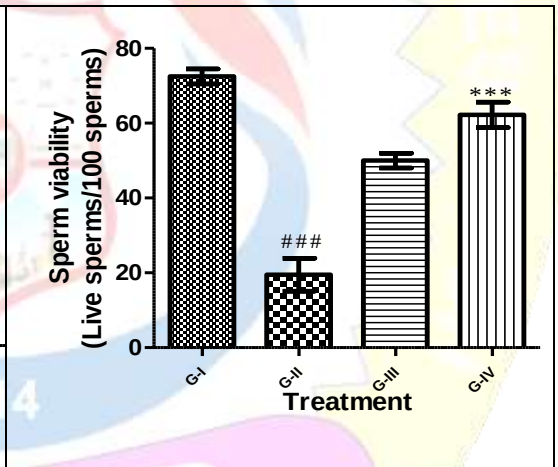


Figure 3b. Effect of oral treatment of MEEH on sperm viability in different groups of adult male rats.

Data was analysed by One way ANOVA followed by Dunnette's post hoc test; ***p<0.001 as compared to LA and ### p<0.001 as compared to control.

Sperm count is often used as a measure of sperm production, testicular function and/or male fertility. The significant enhancement of testis weight is mostly related to number of spermatozoa present in the tissue. The significant increase in the weight of reproductive organs indirectly supports the increased availability of androgen. Androgen surge not only accelerates spermatogenesis, leading to update sperm concentration, but also alters the epididymal milieu which supports the maturation and survival of spermatozoa[32]. One of the most sensitive tests for evaluating spermatogenesis is sperm count in the epididymis[33].

However, number of stored sperm determines the weight of epididymis. The MEEH treatment causes highly significant ($p<0.001$) increase in sperm counts and motility of spermatozoa may be due to higher testosterone level.

Lead acetate inhibits spermiogenesis and reduces young spermatids, spermatocytes and mature spermatids. Such lead toxicity effect on male reproduction was ameliorated by vitamin E and pumpkin seeds oil[24].

In **Figure 3a,** MEEH has increased the motility by 73.94% and 85.92% respectively at low and high dose and thus the concurrent administration of *E. herbacea* ameliorated the sperm motility 3 folds and 7 folds more than G-II. MEEH achieved sperm motility almost same as that of control group, dose dependently. The % viability found in test plant extract treated groups was higher. Rd treatment for 28 days resulted into significant fall in the number of live sperms by 73.6% ($p<0.001$) as compared to control group. The concomitant groups of MEEH treatment (**Figure. 3b**) showed increased viability of sperms by 156.4%, 219.23% respectively. The higher doses of MEEH significantly increased the live sperm count ($p< 0.001$).

The energy for sperm motility is derived from their energy supplying metabolic processes like oxidation of glucose and fructose and ultimately related with the enzyme (protein) lactate dehydrogenase that catalyses the reversible reaction between lactate and pyruvate. The increased sperm motility in MEEH treated rats may be due to a significant increase of total testicular proteins. This suggests that the extracts are not disturbing the blood-testis barrier. The increase in sperm motility caused by chemical agents had earlier been reported to be due to their non-disturbing ability to the blood-testis barrier and thus, preventing a different microenvironment in the inner part of the wall of the seminiferous tubules from that in its outer part[27]. It is known that in order to accomplish fertilization, sperms must be motile to migrate through female genital tract to the site of fertilization. Thus, sperm motility is considered one of the most important predictors of sperm ability in fertilization[34].

C. Serum biochemical profile

Serum testosterone

From the calibration of standard curve, the testosterone level of serum sample of each group was estimated in ng/ml.

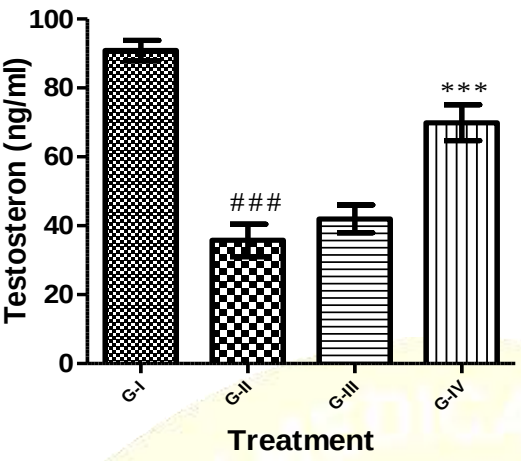


Figure 4. Effect of oral treatment of MEEH on serum testosterone in different groups of adult male rats.

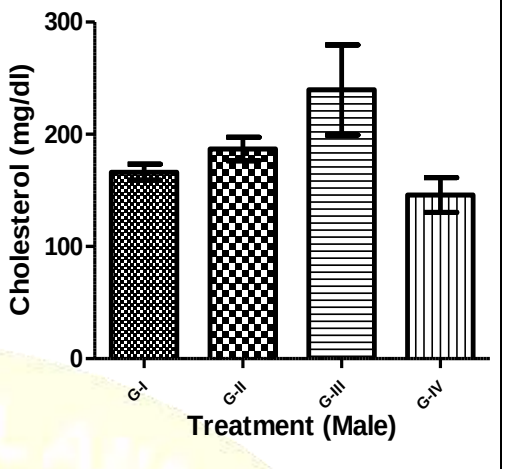


Figure 5. Effect of oral treatment of MEEH on serum cholesterol level in different groups of male rats.

Data was analysed by One way ANOVA followed by Dunnette's post hoc test; ***p<0.001 as compared to LA and ### p<0.001 as compared to control.

Testosterone in association with follicle stimulating hormone, acts on the seminiferous tubules to initiate and maintain spermatogenesis[27]. At any stage the induction of spermatogenic elements is completely androgenic dependent. The testosterone levels in MEEH group at high dose were about double than that of Rd group and nearby to that of control group. Rd group had significantly lowered the levels of serum testosterone as compared to that of the control group (**Figure 4).** It was observed that the treatment with MEEH group rise in the serum testosterone levels even when the rats received concomitant effect of Rd was highly significant (p<0.001).

A significant increase in serum testosterone levels in the extract-treated rats for 28 days suggests that the extracts have improving effect on testicular androgen secretory function and the direct effect on the Leydig cells, steroidogenesis *in vivo*[35]. The 90% restoration (compared to G-II) of sperm performance in the extract treated rats is an indication that the rats would require at least 4 weeks treatment to show their restorative effects.

Serum cholesterol

In Rd group (G-II), rats had less rise in serum cholesterol. In G-III (Low dose of MEEH), serum cholesterol was increased (**Figure 5)** as compared to the control group. High dose of MEEH (G-IV) successfully lowered the cholesterol as compared to normal and also other groups of rats. The significant increase in the cholesterol level of the group receiving extract (at low dose) indicates that cholesterol was not fully used for steroidogenesis hence accumulated in the reproductive organs. However, higher dose treatment results lowering of the cholesterol level. This result is in accordance with *Ruta graviolens*[36].

Serum glucose

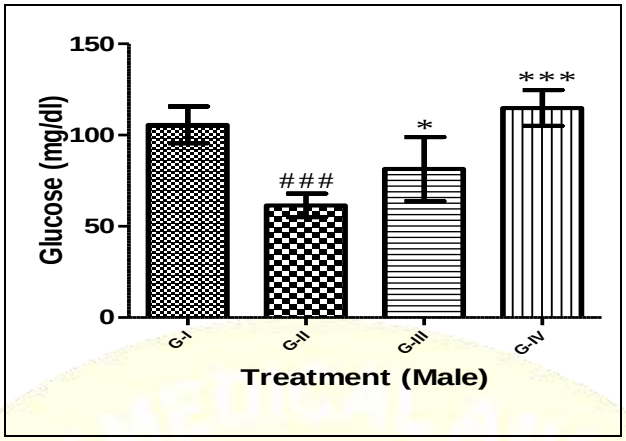


Figure 6. Effect of oral treatment of MEEH and MEEO on serum glucose in male rats.

Data was analysed by One way ANOVA followed by Dunnette's post hoc test. Figures in the parenthesis indicated dose in mg/kg per oral; ***p<0.001 as compared to LA and ### p<0.001 as compared to control (B and C- MEEO, D and E- MEEH)

The serum glucose level was significantly (p<0.001) lowered in Rd group as compared to control group. However, G-III and G-IV (lower and higher dose of MEEH respectively) had serum glucose levels reaching close to that in the control G-I. **Figure 6** indicates that MEEH has significantly updated serum glucose levels up to the control group especially in their high doses. The increased serum glucose level could be justified by the increase of testosterone in the serum since accessory sex organs are controlled by androgen[29]. Indirectly the significant increase in carbohydrate metabolism in the MEEH treated groups as compared to control, confirmed androgenic nature of extracts. The mobility of sperms utilise glucose/fructose as an energy source. The increased glucose level indicates more energy source available for the restoration, motility and viability of sperms[29].

Total proteins

As the body weight increased the protein contents were also increased in normal rats. In Rd group protein contents were reduced (25%). MEEH had successfully enhanced the protein content more (7%) than control group, especially at their high doses.

D. Histological studies

Histological examination of testes of rats in control group I (**Figure 7a**) observed normal histological structure of the mature active seminiferous tubules with complete spermatogenic series were recorded. Testes of rats of Rd group (G-II) showed focal degeneration of seminiferous tubules with loss of spermatogenic series in some seminiferous tubules. While testes of concomitant treatment with MEEH of group III and IV recovered all the deformities in spermatogenic series and showed focal regeneration of seminiferous tubules thereby to attain its normal structure as in control group as seen in **Figure 7b.**

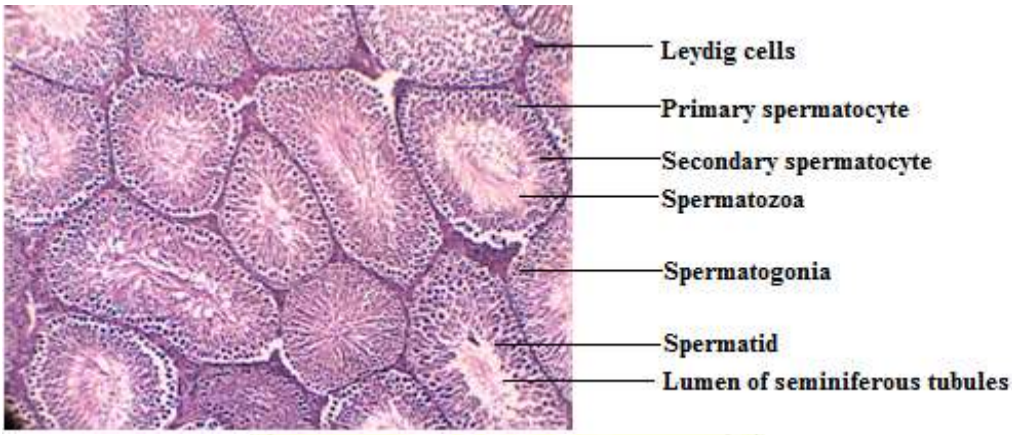


Figure 7a. Histoarchitecture of testis of control group.

MEEH treated groups showed increase in the diameter of seminiferous tubules however, in Rd group (G-II) it was reduced to about 60%. Basement membrane was tightly bound with germinal epithelium. The number of leydig's cells also increased. The lumen of seminiferous tubule was filled with bundles of spermatozoa. Under normal condition the sertoli cells are pyramidal in shape and present near the basement membrane and are equally spaced apart at regular intervals to support the developing spermatogenic cells. Their nucleus is at right angles to the wall. In MEEH treated groups, the primary spermatogenic cells, the spermatogonia are in the first state of repetitive cell division.

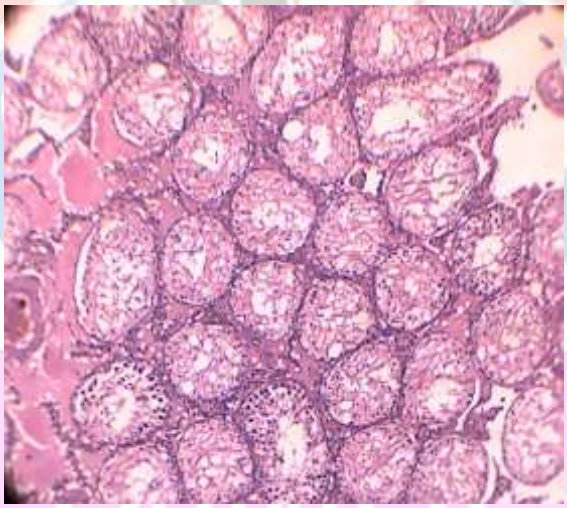
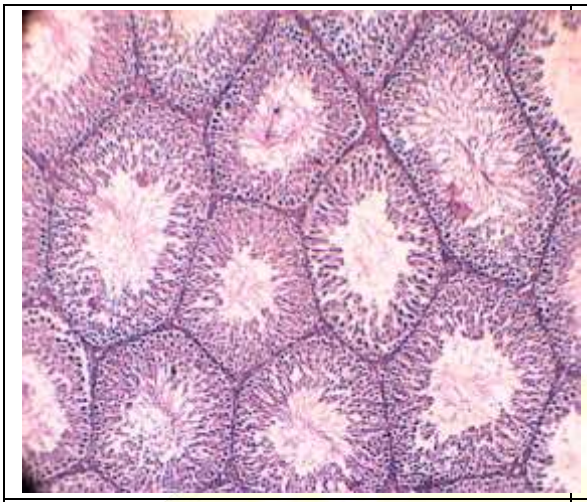
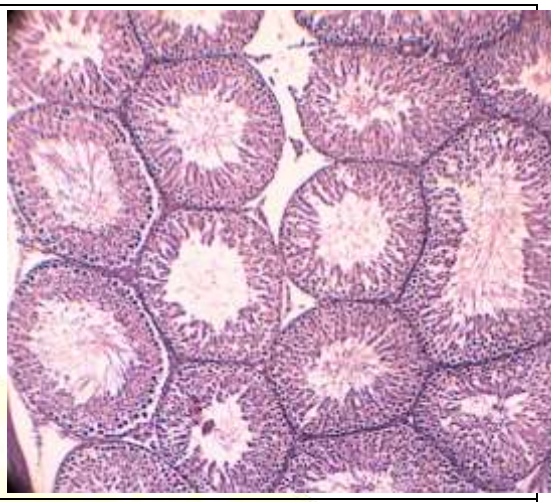


Figure 7b. Histoarchitecture of testis of Reproductive disfunctioned (Rd) group II.



Group III(MEEH-35mg/kg)



Group IV(MEEH-70mg/kg)

Figure 7c. Histoarchitecture of testis of MEEH treated groups (III and IV)

Leydig cells and sertoli cells were also following the same pattern significantly ($p<0.01$). The result states that the plant extract treated groups **(Figure 7c)** ameliorate the spermatic series and the structure of seminiferous tubules that was disturbed in reproductive disfunctioned[37] treatment. Darkness in stained control slide and the vascularized mounts of the test group testis suggests a proper differentiation and vascularization of the spermatids and spermatogonia as compared to pale slide in Rd group. Histomorphological studies revealed that the spermatid separation was enhanced by the administration of extracts. The tubules are widened by seminiferous epithelium resting on a basement membrane which in turn, is bordered by thin layer of fibrous connective tissues. The interstitial stroma is full of blood and lymph vessels and contains small group of interstitial cells of leydig. The results were analogous to that of *A. pyrethrum*[25] in spermatogenic activity on rat.

Histomorphological analysis of extract treated groups also show a profile similar to control group. High number of spermatozoa in seminiferous tubules confirmed the increased spermatogenesis **(Figure 8)** which is also evident by an increase in spermatogenic elements as compared to control.

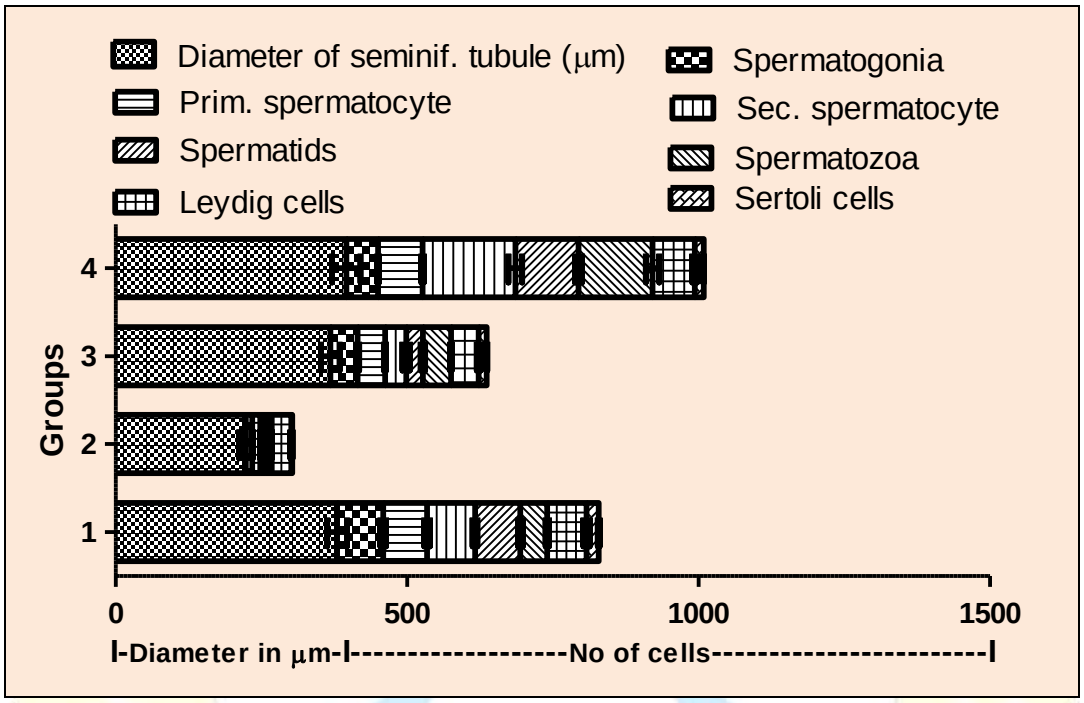


Figure 8. Histomorphological analysis of Testicular tissues.

In case of Rd group, a distinct degeneration compared to control group animals was observed in case of leydig cells and interstitial cells. The solid packing in extract also suggests a supposed role of testosterone in increasing the vascularization of testicular tissue. Methanolic extract of *E. herbacea* treated groups showed increase in the size of seminiferous tubules. Basement membrane was tightly bound with germinal epithelium. The lumen of seminiferous tubule was filled with bundles of spermatozoa. There was also increase in number of leydig's cells as cytoplasm was highly stained with eosin[38]. Under normal condition the sertoli cells lie down near the basement membrane and are spaced at quite regular intervals whereby they perform their functions of supporting the developing spermatogenic cells, in general the nucleus is at right angles to the wall and the cell is pyramidal in shape. The primary spermatogenic cells, the spermatogonia are seen at state of repetitive cell division in test plant extract treated groups. The lumen size is decreased and vascularization is increased in extract treated groups. Similarly, a very clear view of leydig cells can be seen in microphotograph for different extract treated groups, further confirming the efficacy of extracts of *E. herbacea* in spermatogenic activity.

CONCLUSION

High sperm count, increased viability and low percentage of abnormal spermatozoa each has been associated with improved fertility. Higher dose of *E. herbacea* is comparatively more effective in restoration of sperm density and viability than lower dose. The folk claimed efficacy of *E. herbacea* as male fertility agent is proved experimentally in rat and thus provide an alternative for management of infertility due to reduced spermatogenesis.

ACKNOWLEDGEMENT

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ANIMAL RIGHTS

The institutional and (inter) national guide for the care and use of laboratory animals was followed. See the experimental part for details.

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