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APHRODISIAC AND SPERMATOGENIC POTENTIAL OF UNSAPONIFIABLE FRACTION FROM SEEDS OF *HYGROPHILA SPINOSA* T. ANDER IN RATS

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ABSTRACT: Seeds of *Hygrophila spinosa* T. Ander (Acanthaceae) are traditionally used as an aphrodisiac and spermatogenic in the Indian System of Medicine. Studies were planned to evaluate the effect of unsaponifiable (sterol-enriched) fraction from chloroform extract, of the seeds of *Hygrophila spinosa* as spermatogenic and as aphrodisiac *in-vivo*, using rats as experimental animals. An unsaponifiable fraction was evaluated for its ability to stimulate testosterone production using isolated rat leydig cells *in-vitro*. The fraction was administered for 28 days to animals. At the end of the study period, biochemical evaluation of selected parameters, determining the increase in weight of reproductive organs, histological studies of testes, and sexual behavioral studies were performed as evaluation parameters. Increased serum cholesterol and testosterone level, increased weights of reproductive organs, and marked improvement in testes histo-architecture of rats suggested the spermatogenic potential of the fraction. Mounting behavior of test animals improved while the latency period was decreased, as observed in behavioral studies. Stimulation to leydig cells and increased serum testosterone level *in-vivo* might be responsible for the higher number of spermatozoa in the testicular lumen as seen in testicular histology as well as increased libido as observed in behavioral studies.

INTRODUCTION: Infertility affects around 10 to 15 percent of the couple and considered a major reproductive health problem today [1]. Male infertility problems contribute to about 30 to 40 percent of infertility [2-3]. One retrospective study based on an article published spanned over a period of fifty years showed that the average sperm count has reduced to 66 million sperms per ml 1990 from 113 million sperms per ml in 1940 [4].

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The efficacy of allopathic drugs in improving the sperm count or inducing the libido (as an aphrodisiac) has not been fully established. The therapies adopted to improve sperm count or to improve libido are accompanied by serious side effects. *Hygrophila spinosa* (Acanthaceae) (HS) is known as Kokilaksha in the Indian system of medicine [5]. Seeds of the plant are traditionally used as an aphrodisiac and spermatogenic [6]. The seeds of HS found to contain essential oils, fatty acids, waxy substances, gums, mucilage, alkaloids, sterols [7-14]. Studies have been carried out on crude alcoholic extract of seeds for aphrodisiac potential [15]. The studies also have been carried out on the unsaponifiable fraction of the plant to assess its ability to stimulate leydig cells isolated from rat testes *in-vitro* [18].

The results of that study indicated that the fraction was able to stimulate leydig cells for their ability to produce more amount of testosterone locally in testes [17-18]. The set of studies are aimed to assess unsaponifiable (sterol-enriched) fraction prepared from seeds of plant as spermatogenic and aphrodisiac using rats as an experimental animal *in-vivo*.

MATERIALS AND METHODS:

Reagents and Chemicals: All the solvents, reagents, and chemicals used in the study were of analytical grade (AR) and procured from Lobachemie, Mumbai, India. Materials and media used for *in-vitro* and *in-vivo* studies were purchased from Sigma Aldrich, USA, and Hi-media, India.

Plant Materials: Seeds of plant were collected in August from nearby tribal area. The identification of the plant sample was done by a taxonomist at J & J Science College, Nadiad, Gujarat, India. The plant specimen was submitted to the Pharmacognosy Department of the institute for future reference with specimen no 2011/NV/HS. Seeds were separated from the plants, dried under shade, and powdered using laboratory grinder to 70# powder. This powder was used for further extraction process.

Preparation of Unsaponifiable Fraction: Unsaponifiable fraction was prepared from seeds of plant as per the method described elsewhere [16-19]. 5 kg of seeds were extracted using 4000 ml n-hexane at in Soxhlet extractor at 60 °C for 48 h. The hexane extract was concentrated to one-fourth of the original volume using a rotary vacuum evaporator at 30 °C. The concentrated hexane extract was mixed with an equal volume of 10% w/w aqueous potassium hydroxide solution and refluxed for 8 h for saponification at 60 °C. Equal volume of water was added to refluxed solution after cooling, and partitioned with equal quantity of diethyl ether (solvent ether). The procedure was repeated three times. This process could separate sterols and triterpenoid compounds in the organic solvent phase. The saponified matter remained in the aqueous phase only. Pooled ether fractions were passed through sodium sulphate bed and evaporated to dryness at 30 °C in the rotary vacuum evaporator. The yield of the crude unsaponifiable fraction was determined to be 5.2% w/w. The fraction was then subjected to TLC studies for the detection of sterols.

Development of TLC Fingerprint of Unsaponifiable Fraction: The fraction was evaluated for the presence of sterols using TLC. The experiments were planned to optimize the chromatographic procedure, which could resolve Phytoconstituent of the fraction on the plate. Optimized mobile phase employed to separate sterol on silica gel coated TLC plates (silica gel G 60 $F_{254,}$ Merck) was Petroleum ether: Ethyl acetate: Methanol: Glacial acetic acid (6:4.5:0.5:0.2 v/v/v/v) with 10 min saturation time. The plate was subjected to post chromatographic derivatization using Anisaldehyde - Sulphuric acid reagent[16-20] and dried at 110 °C. The plate was observed in daylight, as seen in **Fig. 1**.

Animals: Protocols for acute toxicity studies, *in-vitro* studies, and *in-vivo* studies were approved by Institutional Animal Ethics Committee (IAEC) constituted as per the norms of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The protocol numbers assigned were RPCP/IAEC/2013-14/R29, RPCP/ IAEC/2011-12/R7, RPCP/IAEC/2011-12/R24, respectively. Healthy male wistar rats of weight 250 to 350 g were used in the experiments. Rats were received from Anand Agricultural University (AAU), Anand, Gujarat, India. All animals were housed at 25 °C ± 2 °C, with a relative humidity of 75% ± 5%, under 12 h light/dark cycle. A basal diet and water were provided *ad libitum*.

Acute Toxicity Studies: Acute toxicity studies were conducted as per OECD guidelines [21]. Briefly, 6 were divided into two groups of three animals each. After depriving the animals for food overnight, the animals in the control group received 1 ml of 0.3% gum acacia suspension orally, while animals in the test group received a single dose of 2000 mg/kg of body weight of animal of sterol-enriched fraction suspended in 0.3 % acacia gum suspension (volume of suspension 1 ml) p.o. The animals were observed continuously for the first 4 h and then for the next 24 h, 48 h & then after once in a day for 7 days. They were observed for any death or alterations in general behavior as well as physiological activities.

At the end of the studies, blood samples were withdrawn from animals through retro-orbital plexus, under light ether anesthesia. Samples were analyzed for hemoglobin content, RBC count, WBC count, and Platelet count. Serum was separated and analyzed for the amount of GOT, GPT, Creatinine & total protein. Different organs like heart, kidney, liver, and testes were dissected out and subjected to histology. Slides were stained with hematoxylin-eosin (H and E) stain following fixation with 10% formalin and embedding using paraffin wax. Any alteration in architecture of the tissue structure of these vital organs as compared to the control group was observed under microscope.

***In-vivo* studies:**

Dosing and Treatment: *In-vivo* studies were performed by methods described by Vyas *et al*., using rats as an experimental animals [17-19]. A total of thirty male rats were divided into 5 groups; each contained six rats. Group 1 served as a control group and received 0.3% gum acacia suspension orally for 28 days. Group 2 served as a positive standard, and each animal was administered 0.5 mg/kg b.w. dose of testosterone propionate in arachis oil intramuscularly, twice a week for 28 days. Group 3 received 100 mg/kg, 200 mg/kg and 300 mg/kg of body weight of unsaponifiable fraction suspended in 0.3% gum acacia suspension p.o. Female rats were made sexually receptive by administering a single subcutaneous (s.c.) dose of 500 µg/rat of progesterone prior to 48 h and singles. c. a dose of 5 µg/rat of estradiol benzoate prior to 8 h of mating studies, including sexual behavior experiments. Male to female animal ratio was maintained 1:1 in the experiment.

Biochemical Studies: Alteration in serum cholesterol level and serum testosterone level of test animals were determined. Blood was collected from retro-orbital plexus from each animal. Serum was separated by centrifugation at 10000 rpm at 4°C and stored at -80 °C until further analysis. Serum testosterone was estimated using commercially available ELISA kits (CE 1875, Cal Biotech), while cholesterol was estimated using kit procured from SPAN Diagnostics.

Total Sperm Count: Epididymis was dissected out from all the animals, and the caudal part was cut, and tubules were dispersed using medium 199 (contained Hank's salts supplemented with 0.5% w/v BSA, pH 7.4) in a 35 mm plastic petri dish. It was incubated at 37 °C for 10 min to allow the sperm to disperse. The supernatant containing sperm was collected, and it was diluted 20 times with PBS; total sperm count was measured manually using a hemocytometer.

Histological Studies: Testes of all experimental animals were dissected and fixed in 10% formaline solution. Testicular tissues were dehydrated with varying concentration of ethanol. Thin sections were cut of 5 µ size and stained with eosin. The sections were fixed on slides and observed under microscopes with different magnification.

Histo-architecture of testes of treated animals was compared with control. Various parameters like sperm density in seminiferous tubule, shape of seminiferous tubule, density of matured sperma-tozoa, epithelial lining of seminiferous tubules were analyzed for variations, especially in HSU treated animals to animals from blank group as well to testosterone treated animals.

Sexual Behavior Studies: On 29th day of the experiment, each male rat was placed in a glass chamber (20 cm × 40 cm × 60 cm) having top lid closed. Three sides of chamber were covered with black sheet. The rat was kept inside for 10 min to acclimatize with the cage environment. A receptive female was introduced from one side of the glass chamber. The sexual behavior of male rat toward female was observed. Observations for various parameters were made as follows: Mount frequency (MF): the number of mounts without intromission from the time of introduction of the female until ejaculation, Intromission frequency (IF): the number of intromissions from the time of introduction of the female until ejaculation, Mount latency (ML): the time interval between the introduction of the female and the first mount by the male, Intromission latency (IL): the time interval from the time of introduction of the female to the first intromission by the male and Ejaculatory latency (EL): the time interval between the first intromission and ejaculation.

Statistical Analysis: Results are expressed as Mean ± SEM. The statistical significance for alteration between the means was determined by

one-way Analysis of Variance (ANOVA) followed by Dunnett's test. In all statistical tests, a value of $p<0.05$ was considered significant. Statistical analysis was performed using Microsoft Excel 2007.

RESULTS AND DISCUSSION:

Preliminary Phytochemical Studies: Phyto-chemical evaluation of unsaponificable fraction using TLC, showed the presence of sterols in the fraction. The plate was subjected to post chromato-graphic derivatization using Anisaldehyde-sulphuric acid reagent. Sterols and triterpenoids reacted with anisaldehyde in acidic media and formed violet to blue color.

The Plate was subjected to post chromatographic derivatization yielded three major violet spots confirmed presence of at least three triterpenoidal steroidal moieties. TLC fingerprint of fraction is shown in **Fig. 1**.

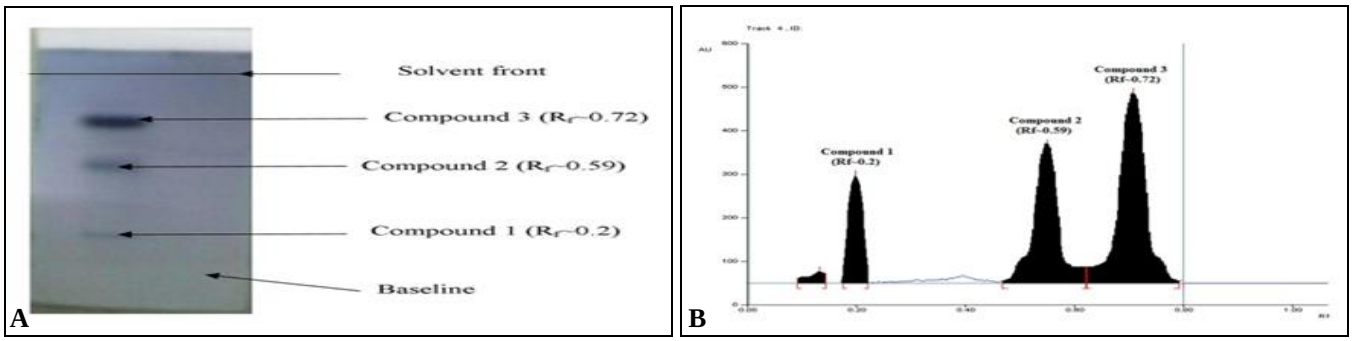

FIG. 1: (A) TLC PROFILE OF FRACTION AFTER DERIVATIZATION WITH ANISALDEHYDE SULPHURIC ACID REAGENT (B) HPTLC CHROMATOGRAM OF UNSAPONIFIABLE FRACTION

Acute Toxicity Studies: Acute toxicity studies were performed to evaluate, preliminarily, the lethality of the fraction. It was also of value in selecting dose level to assess the efficacy of the fraction. There was no mortality observed in test group animals (2000 mg/kg) throughout the test period following a single oral dose of test fraction of the plant. The animals were observed for 24 h, immediately after dosing, for any visible alterations in locomotor activity or behavior. The studies confirmed unaltered locomotor activity with no behavioral alterations during the observation period as well as at the end of studies. The results of the blood analysis showed that there was no significant alteration in hematological parameters as compared to control animals. There were no structural alterations seen in microarchitecture in histology studies of different vital organs of animals as compared to control. Further, SGOT, SGPT, serum creatinine, and serum total protein levels were not altered significantly as compared to those of control animals. The results of biochemical and histological studies thus confirmed that the fraction did not alter liver, heart, and kidney function.

***In-vivo* studies:**

Biochemical Studies: Results of *in-vivo* studies showed an increased level of serum cholesterol, and serum testosterone in HSU treated groups **Fig. 2**. Serum testosterone was found increased nearly three times, as compared to that of untreated animals when HSU was administered at 300 mg kg^{-1} dose.

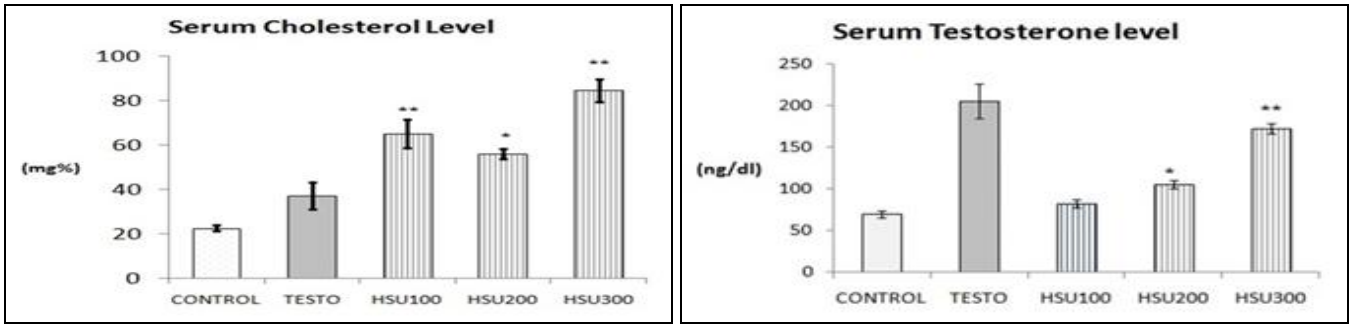

FIG. 2: EFFECT OF UNSAPONIFIABLE FRACTION ON SERUM TESTOSTERONE AND CHOLESTEROL LEVEL

n=6, Values are mean ± SEM, * P<0.05, **P<0.01 as compared to control

As cholesterol is a precursor in testosterone synthesis, increased levels of cholesterol suggested the ability of fractions to stimulate the metabolism of steroidal hormones. Inflated serum level of testosterone was in line with results of *in-vitro* experiments [18], performed using isolated rat Leydig cells. The results of *in-vitro* studies and *in-vivo* studies, when considered together, confirmed the ability of HSU to stimulate testosterone synthesis through direct stimulation to Leydig Cells in testis.

Sexual Organ Weight: At the end of experiments, animals were sacrificed, and weights of secondary sexual organs were determined. The results **Table 1** showed that the weight of organs was not increased significantly except for the weight of testes in the HSU treatment group at all dose levels. Secondary sexual organs like testes, prostate, seminal vesicle, and epididymis are sensitive to hormones.

Their weights were found to be increased as a function of steroidal stimulus [17]. Weight gain in testes was thought to be attributed to the increase in the spermatogenesis process in the lumen of seminiferous tubules in testes.

TABLE 1: EFFECT OF UNSAPONIFIABLE FRACTION ON REPRODUCTIVE ORGANS WEIGHT

Group	Weight in gm			
	Testes	Prostate	Seminal vesicle	Epididymis
Group I	1.44±0.03	0.12±0.001	0.54±0.001	0.48±0.04
Group II	1.46±0.004	0.13±0.005	0.64±0.005*	0.51±0.01*
Group III	1.54±0.01*	0.12±0.002	0.62±0.003*	0.52±0.04*
Group IV	1.62±0.03*	0.14±0.001	0.64±0.003*	0.58±0.03*
Group V	1.56±0.03	0.13±0.001	0.67±0.003	0.51±0.03

Compared to control group, * p<0.05, **p<0.01, Values are expressed as Mean ± SEM, (n=6) **.**Group I- Control (vehicle only)**.** Group II- Unsaponifiable fraction (100 mg/kg b.w.) p.o. daily for 28 days. Group III- Unsaponifiable fraction (200 mg/kg b.w.) p.o. daily for 28 days. Group IV- Unsaponifiable fraction (300 mg/kg b.w.) p.o. daily for 28 days Group V- testosterone (500 µg/kg b.w.) twice a week for 28 days by intramuscular route

Total Sperm Counts: It was observed that total sperm counts in HSU treated animals was 29.2 million/ml as compared to 26.1 million/ml in control animals, which were administered with vehicle only. The testosterone-treated group did not show any improvement in sperm count compared to control. The result of the studies is shown in **Table 2**. It must be noted that these sperms were counted from the fluid collected from epididymis after dissecting the animals.

TABLE 2: EFFECT ON TOTAL SPERM COUNTS

Group	Total sperm counts (millions/ml)
Group I	26.1±0.87
Group II	20.6±0.5
Group III	22.8±0.66
Group IV	29.2±1.06*
Group V	24.2±0.89

Compared to control group, * p<0.05, **p<0.01, Values are expressed as Mean ± SEM, (n=6). Group I- Control (vehicle only). Group II- Unsaponifiable fraction (100 mg/kg b.w.) p.o. daily for 28 days. Group III- Unsaponifiable fraction (200 mg/kg b.w.) p.o. daily for 28 days. Group IV- Unsaponifiable fraction (300 mg/kg b.w.) p.o. daily for 28 days. Group V- testosterone (500 µg/kg b.w.) twice a week for 28 days by intramuscular route

Histological Studies: Testes section of control group animals showed that seminiferous tubules having maximum diameter were not abundant. Germinal epithelium showed a normal shape and size. Spermatozoa were embedded in Sertoli cells, showed normal cytoplasmic granulation. Leydig cells had a normal appearance. Luminal part of the tubule was not excessively filled with spermatozoa **Fig. 3a**.

The testes section of treated group animal showed increased spermatogenesis evident from high numbers of spermatozoa in seminiferous tubules. Almost all seminiferous tubules showed greater diameter. The lumen of every tubule was filled with enormous numbers of spermatozoa. Germinal epithelial cells were hyperactive. Large numbers of different cells at different stages of spermato-genesis were seen. The cytoplasm of sertoli cells was found highly granulated. The nucleus of the Leydig cells was enlarged, and cytoplasm appeared to be stained darkly **Fig. 3b, c, d**.

Histo architecture of testosterone-treated group also exhibited similar profile to that of HSU treated group **Fig. 3e**. Histological studies, along with *in-vitro* and biochemical studies, further suggested that HSU might stimulate Leydig cells and

upregulated testosterone synthesis. The inflated testosterone concentration might be attributed to the higher number of spermatids and spermatozoa in the lumen of somniferous tubules.

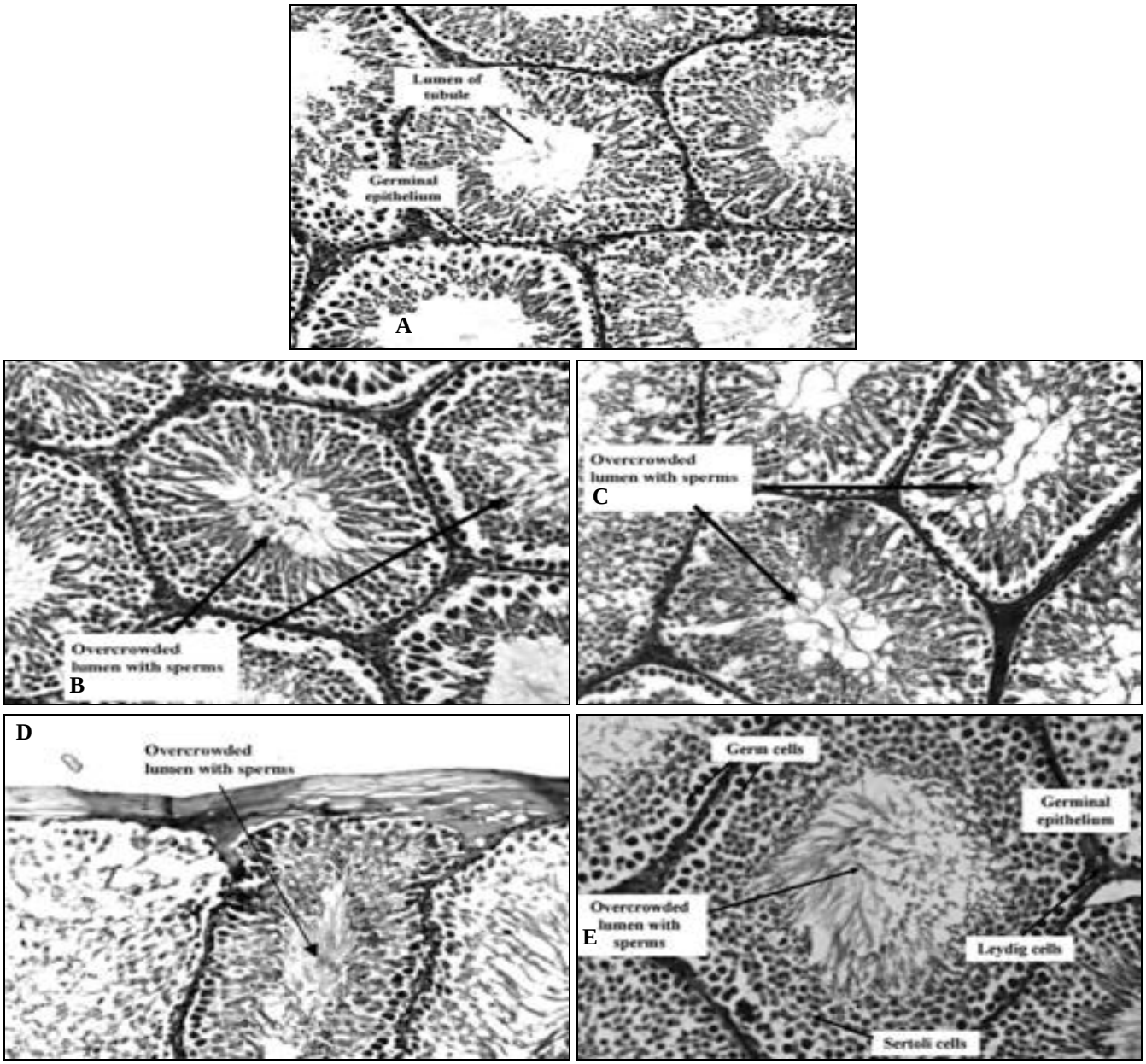

FIG. 3: PHOTOMICROGRAPHS OF HISTOLOGY OF TESTIS. (A) CONTROL, (B) UNSAPONIFIABLE FRACTION 100 mg/kg, (C) UNSAPONIFIABLE FRACTION 200 mg/kg, (D) UNSAPONIFIABLE FRACTION 300 mg/kg, (E) TESTOSTERONE 500 mg/kg, MAGNIFICATION: 200X, PHOTOMICROGRAPHS CAPTURED BY CARL ZEISS AXIO LAB A1 MICROSCOPE

Sexual Behavior Studies: Sexual desire in humans and in rats, too, is under control of the central nervous system. The centers for sexual arousal in male are believed to be present in the hypothalamus. They were shown to be stimulated through dopaminergic agonist. Correlation between testosterone level and libido in males has been established. Basal testosterone level is essential for sexual arousal. Though rats might not simulate human sexual behavior, they were used to assess, preliminarily, the efficacy of the fraction on libido.

Treatment with HSU increased the mount frequency, intromission frequency and ejaculatory latency while a significant decrease was observed in mount latency and intromission latency as compared to control. The results, as shown in **Fig. 4**, showed dose-dependent improvement in all selected behavioral parameters. The results thus confirmed that the fraction might act as an aphrodisiac due to its ability to stimulate circulating testosterone concentration.

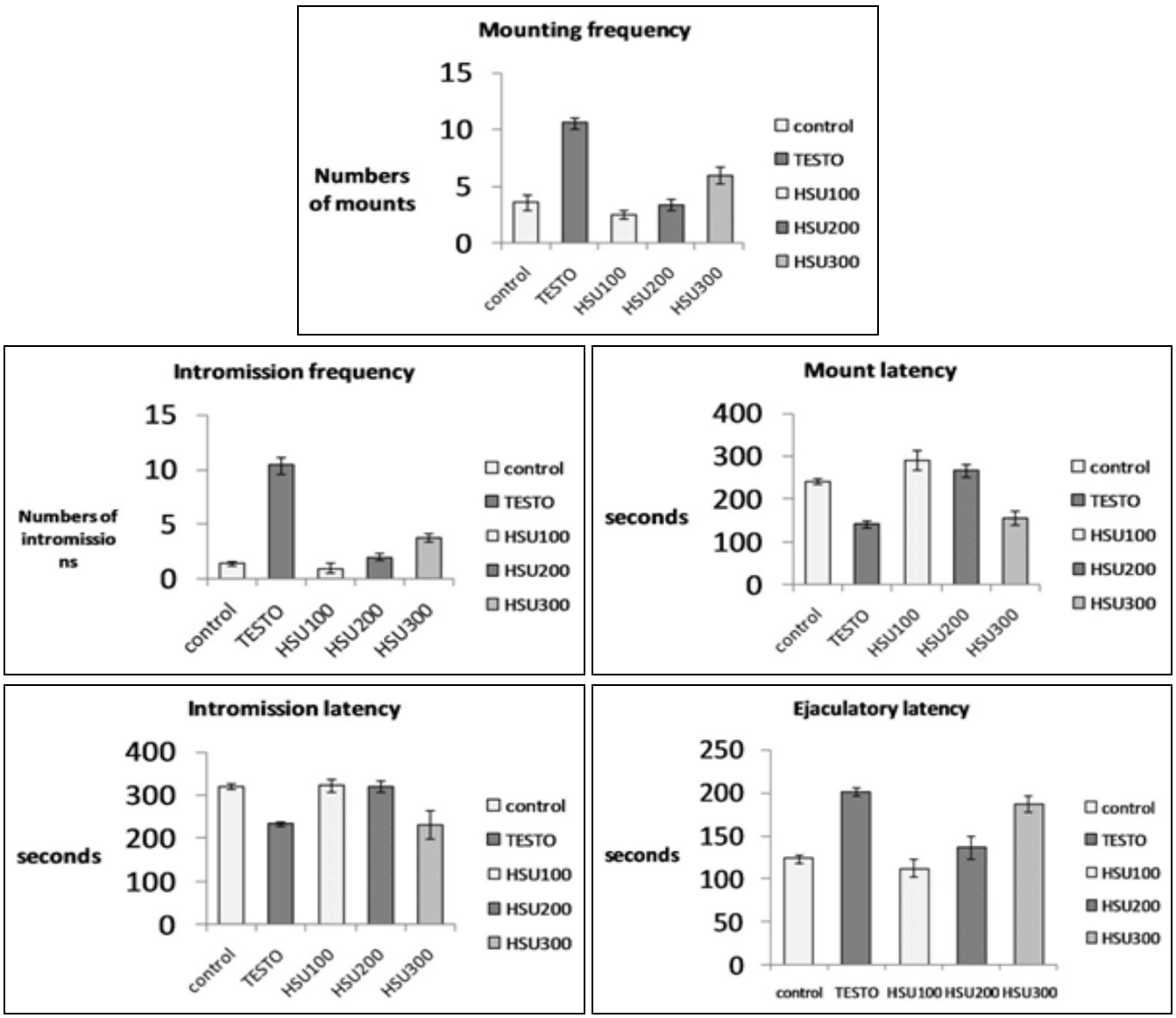

FIG. 4: EFFECT OF UNSAPONIFIABLE FRACTION OF HS ON SEXUAL BEHAVIOR OF MALE RATS

n=6, values are mean±SEM, * P<0.05, ** P<0.01, as compared to respective control.

CONCLUSION: HSU is a sterol-enriched (unsaponifiable) fraction prepared from seeds of HS. The maximum tolerable dose of the fraction was 2000 mg kg^{-1} body weight. This might be considered as GRAS dose for a fraction. *In-vitro* studies confirmed that the fraction could act upon Leydig cells and stimulated testosterone biosynthesis [18]. Fraction, thus, could stimulate serum testosterone concentration in treated animals through action on Leydig cells. An increased concentration of serum testosterone was believed to be responsible for the spermatogenic and aphrodisiac potential of the fraction. The studies supported bioactivity guided chemical isolation approach while identifying probable bioactive constituents. As a result of these studies, detailed investigations were carried out, and few potential bioactive steroidal moieties were isolated from fractions using column chromatography.

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CONFLICTS OF INTEREST: Authors declare no conflict of interest.

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