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# Dose-related Reversible Suppression of Spermatogenesis and Fertility in Male Laboratory Mice after Treatment with Hibiscus rosa-sinensis (Linn.)

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**Abstract**

The development of an orally active, safe, reversible, and effective male contraceptive of plant origin has long been a major research focus, owing to its ready availability, cost-effectiveness, and, most importantly, the preservation of privacy. To evaluate the effects of oral administration of Hibiscus rosa-sinensis (H. rosa-sinensis; family: Malvaceae) on male reproductive endpoints and fertility, 30 adult male albino mice of proven fertility were allocated to six groups. Mice in the treated groups received the benzene leaf extract of H. rosa-sinensis (100 and 200 mg/kg b. w. daily), whereas those in the control group received olive oil for 35 days. H. rosa-sinensis treatment induced dose-related histological alterations in reproductive organs, including reduced organ weights, decreased height of the seminiferous epithelium, reduced diameter of stage VII tubules, and increased percentage of affected seminiferous tubules in the testes. Treatments adversely affected sialic acid and fructose levels and sperm parameters; libido was not affected, but fertility was significantly suppressed in extract-treated males compared with controls. Forty-two days after treatment withdrawal, reproductive endpoints and fertility returned to levels comparable with controls. Furthermore, the histoarchitecture of the liver and kidney, serum levels of ALT, AST, and creatinine, and haematological parameters remained unaltered compared with controls. The results suggest that treatment with H. rosa-sinensis causes dose-dependent, reversible suppression of spermatogenesis and fertility in albino mice, without any visible toxicity. Therefore, it might be a potential candidate for herbal male contraception.

**Keywords:** Malvaceae; Testis; Fertility; Creatinine; Libido

## 1. Introduction

The design of a safe, effective, reversible, and orally active male contraceptive has consistently intrigued scientists. Regarding the control of male fertility, none of the existing methods meets the criteria for an ideal male contraceptive. In India and globally, plants and their preparations have often been used to regulate human fertility; this propensity can be attributed to their accessibility, affordability, and, crucially, the preservation of privacy [1] . Further, a plant-based male contraceptive is generally considered to be free from unwanted side effects. Hibiscus rosa-sinensis (H. rosa-sinensis; Family: Malvaceae), with several local names such as China rose (English), Jasum (Hindi), Japa (Sanskrit), etc., has been studied extensively for a variety of biological and pharmological properties [2-5] including antifertility effects in both sexes of rats and mice [6-10] . The results of our previous study in albino mice [11] clearly indicate that treatment with a benzene extract (100 mg/kg body weight for 35 days) from H. rosa-sinensis suppressed spermatogenesis. This study examines the dose-dependent effects of Hibiscus on reproductive endpoints and fertility; recovery experiments were conducted to evaluate the reversibility of H. rosa-sinensis&39;s influence on spermatogenesis and fertility in albino mice. Different indicators of male reproductive function, such as sperm characteristics, histological, biochemical and fertility measures, were evaluated. Additionally, toxicological studies related to the treatment were also conducted.

## 2. Materials and Methods

### 2.1 Selection, identification, and preparation of plant extract

Fresh and infection-free leaves of H. rosa-sinensis were selected for the trial study since it has not been investigated earlier for their antifertility potential in males, as revealed after a thorough literature survey. The sample was collected from the campus of Banaras Hindu University (B.H.U.), Varanasi, after proper taxonomic identification (Voucher specimen no. Malva. 2023/02) of the plant by Professor N. K. Dubey at the Department of Botany, B.H.U., Varanasi (India). Benzene extract of leaves was prepared strictly according to the WHO practice [12] with minor modifications in a soxhlet apparatus [11] . The yield of extract per 100 g of Hibiscus leaf powder was approximately 20.0 g (20 %). The lyophilized extract was stored at 40°C, and the doses were expressed as the dry weight of the extract.

### 2.2 Ethical approval and animal treatments

This was a randomized controlled trial conducted in the Department of Zoology at K. N. Government P. G. College, Gyanpur, Bhadohi (India). A total of 36 adult (age 12-14 wk; weight 28-38 g) male laboratory mice of proven fertility belonging to the Parkes (P) strain were used in the investigation after ethical approval of the Institutional Animal Ethics Committee of Mahatma Gandhi Kashi Vidyapith, Varanasi (India). Animals were obtained from a randomly bred colony maintained in our animal room under standard conditions (temperature 23 ± 2 0 C, photoperiod 12 h, and relative humidity 50±20% with proper ventilation) in polypropylene cages (450 mm X 270 mm X 150 mm) having dry rice husk as the bedding material, following the guidelines of CPCSEA, New Delhi. [13] Animals were given standard pellet feed (Mona Laboratory Animal Feeds, Varanasi) and fresh drinking tap water *ad libitum*. Animals were randomly allocated into six (I–VI) groups, each comprising five individuals (Table 1).

**Table 1.** The experimental design.

| Groups | Treatments | Duration (Day |
|---|---|---|
| I | Untreated controls (UN) | 35 |
| II | Olive oil-treated controls (OL) | 35 |
| III | *H. rosa-sinensis* at 100 mg/kg b.w. | 35 |
| IV | *H. rosa-sinensis* at 200 mg/kg b.w. | 35 |
| V | *H. rosa-sinensis* at 200 mg/kg b.w. (TR) | 35 |
| VI | Olive oil-treated controls (CR) | 35 |

The benzene extract from *H. rosa-sinensis* leaves was dissolved in olive oil and given orally each day at 10:00 AM with separate, properly sterilized feeding needles; mice in groups II and VI (vehicle control) were given an equivalent volume of only olive oil (5.0 ml/kg b.w. daily) orally. The dosages for the therapy using *H. rosa-sinensis* were established following several initial experiments performed on albino mice in our laboratory. The animals' overall health status and body weight were consistently observed during the treatment duration.

### 2.3 Animal autopsy and sample collection

On day 36, animals in groups I–IV were euthanized collectively 24 h post-treatment cessation by decapitation after recording their final body weights; meanwhile, those in groups V (treatment recovery; TR) and VI (control recovery; CR) were also euthanized together through decapitation 42 days following treatment withdrawal after their final body weights were documented (Table 1). Trunk blood was collected, and serum was separated and stored at $-20^{\circ}$C for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine levels. Haematological tests were performed in fresh blood. The testis, epi-

didymis, vas deferens, seminal vesicle, ventral prostate, brain, liver, kidney, adrenal gland, and spleen were excised, cleared of fat and connective tissue, and weighed. The epididymes and seminal vesicles from five animals in each group were stored at −20°C for assays of sialic acid and fructose, respectively.

### 2.4 Analyses of sperm parameters

At autopsy, the cauda epididymidis was taken out randomly from the left or right sides of each of the five animals in each group, and placed in a watch glass containing 0.5 ml of 0.9% normal saline maintained at 37°C on a hot plate [14]. The tissue was minced properly, and the sperm suspension was used for analyses of percentage motility, viability, abnormality, and number of spermatozoa according to the WHO protocol [15]. The criteria of Wyrobek and Bruce, as well as Zaneveld and Polakoski were followed to evaluate morphological abnormalities in spermatozoa [16].

### 2.5 Assays of sialic acid and fructose levels

The concentration of sialic acid in the epididymis was assessed using the thiobarbituric acid method and computed based on Warren's equation 2 with slight adjustments [17]. In summary, the entire epididymis was homogenized in chilled 0.1 N sulfuric acid and maintained in a water bath at 80°C for 1 h to release bound sialic acid. The homogenate was cooled for 2 h, then oxidized with a 25 mM periodate solution for precisely 30 min, followed by reduction using a 2% sodium arsenite solution. Next, the mixture was incubated in a 0.1 M thiobarbituric acid solution, followed by boiling in a water bath for precisely 7.5 min. The chromophore, obtained in this way, was extracted in acidic butanol, and optical density (O.D.) was measured at 549 nm and 532 nm, respectively, using a spectrophotometer with distilled water as a blank. Fructose levels in the seminal vesicle were evaluated with slight adjustments [18]. In summary, the entire seminal vesicle was mixed in 80% ethanol and spun at 5,000 rpm for 10 min. The supernatant was treated with 0.3 N barium hydroxide and 5% zinc sulfate (1:1) for deproteinization and then cooled for 2 h. The homogenate was subsequently centrifuged at 5,000 rpm for 10 min, and the supernatant was utilized for the fructose assay. The sample solution was mixed well with 0.1% ethanolic resorcinol, then 30% hydrochloric acid was added. The samples were maintained at 80°C in a water bath for precisely 10 min, then quickly cooled in an ice bath. The optical density of the sample solution was measured at 410 nm in a spectrophotometer within 30 min, using distilled water as the blank.

### 2.6 Toxicological investigations

**Haematology and serum biochemistry:** Trunk blood was analyzed for mean counts of blood cells (RBC and WBC), haemoglobin (Hb), and haematocrit (Hct) according to standard laboratory procedures [19]. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were also calculated. Serum levels of ALT and AST were estimated to assess liver function [20]. Serum creatinine was measured using a diagnostic kit (Span Diagnostics Limited, India) to assess kidney function.

### 2.7 Fertility tests

A total of 10 adult males (TR and CR) and 40 females with verified fertility were used in the fertility assessments. The fertility of male mice from groups V (CR: treated with olive oil) and VI (TR: treated with *Hibiscus* at 200 mg/kg b.w. for 35 days) was assessed at 24 h, 2 wk, 4 wk, and 6 wk (42 days) after treatment ended by letting each male mate overnight with an age-matched, virgin female in proestrus, showing regular cycles. The features such as libido and fertility rates in treated males, along with the number of live implants and the losses before and after implantation in pregnant females, were assessed [21] . The males were considered fertile if impregnated females showed live implants.

### 2.8 Histological techniques

To examine histologic changes, sections of the testis, epididymis, vas deferens, seminal vesicle, and ventral prostate gland, parts of the liver, kidney, and spleen were fixed in aqueous Bouin's solution overnight, dehydrated using a graded series of ethanol, cleared in benzene, and then embedded in paraffin wax (60–62°C). Tissues were cut into 6 $\mu$m sections, which were then stained with periodic acid-Schiff (PAS) and subsequently counter-stained using Harris haematoxylin. The stained tissue samples were analyzed using a Leitz/Leica (Germany) light microscope. Quantitative changes in the testes were assessed by evaluating the height of the seminiferous epithelium, the diameter of round or slightly oblique stage VII seminiferous tubules, the percentage frequency of spermatogenesis stages, and the prevalence of affected seminiferous tubules from randomly chosen testis samples. Histological changes in the mouse testis were examined based on the criteria outlined by Russell, Ettlin, Sinha Hikim, and Clegg [22]; for histological study, the epididymis was divided into five (I–V) segments [23].

**Table 2.** Effect of treatment with benzene leaf extract (mg/kg b.w for 35 days) of *H. rosa-sinensis*, and 42 days after removal of the treatment on body weight, and weights of reproductive organs.

| Groups/Treatments | Body weight (g) | | Reproductive organ weight (mg/100 g b.w.) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | Final | Testis | | Epididymis | | Vas deferens | | Seminal vesicle | | Ventral prostate | |
| | | | Absolute | Relative | Absolute | Relative | Absolute | Relative | Absolute | Relative | Absolute | Relative |
| I, Untreated (UN) | 30.40 ±0.75 | 31.60 ±1.16 | 99.62 ±3.70 | 306.14 ±3.28 | 40.00 ±2.55 | 116.07 ±5.01 | 14.96 ±0.84 | 41.22 ±1.75 | 93.72 ±2.80 | 260.16 ±25.23 | 36.38 ±1.34 | 106.17 ±5.86 |
| II, Olive-oil Control (OL) | 30.00 ±0.63 | 31.80 ±0.91 | 97.56 ±3.59 | 301.40 ±5.41 | 40.68 ±2.24 | 126.21 ±7.11 | 15.02 ±1.02 | 44.55 ±2.54 | 91.74 ±7.92 | 228.23 ±20.42 | 32.24 ±2.23 | 111.91 ±7.00 |
| III, 100 mg dose | 29.20 ±0.49 | 30.20 ±0.49 | 77.68* ±5.23 | 241.13* ±19.53 | 37.06 ±0.97 | 109.17 ±4.93 | 13.48 ±1.22 | 45.73 ±4.21 | 52.18* ±5.09 | 150.52* ±13.74 | 34.90 ±1.91 | 103.45 ±4.71 |
| IV, 200 mg dose | 32.20 ±0.66 | 33.40 ±0.68 | 67.88* ±3.80 | 217.80* ±16.82 | 27.14[a] ±0.86 | 91.01[a] ±5.64 | 12.96 ±1.53 | 36.12 ±1.98 | 40.48* ±5.01 | 130.20* ±17.26 | 20.98* ±3.27 | 65.11* ±1.12 |
| V, Treatment recovery (TR)[#] | 33.00 ±1.58 | 33.40 ±1.51 | 95.90 ±3.66 | 314.39 ±19.93 | 39.86 ±2.65 | 119.89 ±9.13 | 15.10 ±0.67 | 46.29 ±2.27 | 71.62 ±6.06 | 192.96 ±11.72 | 28.34 ±2.69 | 119.92 ±16.41 |
| VI, Olive oil recovery (CR)[#] | 30.6 ±2.79 | 31.20 ±2.58 | 97.60 ±3.74 | 308.52 ±22.26 | 43.58 ±2.58 | 136.41 ±6.10 | 12.66 ±1.29 | 41.49 ±3.45 | 88.82 ±10.87 | 271.90 ±6.37 | 32.02 ±2.75 | 109.37 ±6.29 |

*Values are mean ± S.E.M. for five animals; * significantly ($p < 0.05$) different from controls; [a] significantly ($p < 0.05$) different from controls and those in groups III by ANOVA followed by Newman-Keuls' multiple range test; [#] treatments were discontinued aker 35 days, and animals were sacrificed 42 days aker removal of the treatment; organ weight refers to the weight of the unpaired organ; absolute refers to the real weight, while relative refers to the weight of the organ per 100 g b.w. of the animal.*

**Table 3.** Effect of treatment with benzene leaf extract (mg/kg b.w. for 35 days) of *H. rosa-sinensis*, and 42 days after removal of the treatment on weights of vital body organs.

| Groups/Treatments | Body organ weight (mg/100 g b.w.) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Brain | | Liver | | Kidney | | Adrenal | | Spleen | |
| | Absolute | Relative | Absolute | Relative | Absolute | Relative | Absolute | Relative | Absolute | Relative |
| I, Untreated (UN) | 330.40 ±9.56 | 1017.76 ±35.33 | 1236.98 ±72.16 | 3835.31 ±114.61 | 199.36 ±7.75 | 630.07 ±27.57 | 3.98 ±0.22 | 12.06 ±1.06 | 89.14 ±2.98 | 263.54 ±19.45 |
| II, Olive-oil Control (OL) | 315.24 ±8.90 | 1117.02 ±95.68 | 1204.12 ±43.21 | 3859.68 ±196.60 | 223.46 ±14.70 | 619.52 ±38.47 | 3.66 ±0.30 | 11.80 ±0.51 | 83.02 ±4.03 | 294.70 ±16.71 |
| III, 100 mg dose | 342.42 ±10.64 | 1048.86 ±42.29 | 1202.46 ±41.27 | 3933.04 ±150.75 | 229.72 ±16.13 | 612.48 ±38.55 | 4.16 ±0.35 | 12.82 ±0.94 | 79.72 ±4.55 | 247.65 ±20.02 |
| IV, 200 mg dose | 359.12 ±16.73 | 1112.88 ±47.43 | 1215.04 ±48.85 | 3903.86 ±245.87 | 199.04 ±8.43 | 668.46 ±24.33 | 3.88 ±0.19 | 10.89 ±0.48 | 85.4 ±2.72 | 250.42 ±13.86 |
| V, Treatment recovery (TR)[#] | 331.10 ±15.11 | 1003.97 ±63.70 | 1244.06 ±61.89 | 3826.67 ±161.51 | 192.38 ±9.17 | 634.02 ±27.07 | 4.00 ±0.20 | 11.70 ±0.37 | 81.78 ±5.02 | 256.73 ±11.69 |
| VI, Olive oil recovery (CR)[#] | 313.86 ±15.21 | 1010.83 ±29.98 | 1224.86 ±60.67 | 3825.08 ±169.65 | 201.62 ±7.24 | 660.34 ±22.63 | 4.16 ±0.23 | 10.54 ±0.56 | 92.52 ±6.02 | 250.83 ±14.50 |

*Values are mean ± S.E.M. for five animals; [#] treatments were discontinued aker 35 days, and animals were sacrificed 42 days aker removal of the treatment; organ weight refers to the weight of the unpaired organ; absolute refers to the real weight, while relative refers to the weight of the organ per 100 g b.w. of the animal.*

**Table 4.** Effect of treatment with benzene leaf extract (mg/kg b.w. for 35 days) of *H. rosa-sinensis*, and 42 days after removal of the treatment on haematology and serum biochemistry.

| Groups/Treatments | Hb (g/100 ml) | RBC ($\times 10^6$/mm$^3$) | WBC ($\times 10^3$/mm$^3$) | Hct (%) | MCV (fl) | MCH (pg) | MCHC (%) | ALT (U/L) | AST (U/L) | Creatinine (mg/100 ml) |
|---|---|---|---|---|---|---|---|---|---|---|
| I, Untreated (UN) | 12.44 ±0.23 | 6.42 ±0.13 | 1.54 ±0.17 | 50.91 ±3.76 | 67.52 ±3.97 | 20.09 ±0.50 | 27.21 ±2.88 | 23.94 ±0.71 | 24.32 ±1.61 | 1.39 ±0.11 |
| II, Olive-oil Control (OL) | 12.84 ±0.78 | 6.66 ±0.11 | 1.80 ±0.25 | 45.59 ±2.37 | 74.31 ±5.71 | 19.51 ±1.25 | 25.07 ±1.48 | 22.96 ±2.07 | 25.05 ±1.58 | 1.32 ±0.13 |
| III, 100 mg dose | 12.52 ±0.18 | 6.90 ±0.26 | 1.86 ±0.22 | 47.80 ±1.72 | 66.55 ±2.40 | 18.75 ±0.50 | 27.05 ±1.67 | 23.12 ±1.94 | 26.60 ±2.27 | 1.59 ±0.09 |
| IV, 200 mg dose | 12.48 ±0.33 | 6.72 ±0.17 | 1.86 ±0.27 | 47.14 ±1.50 | 69.10 ±2.44 | 18.93 ±1.19 | 27.73 ±2.16 | 20.84 ±1.08 | 24.73 ±2.34 | 1.67 ±0.20 |
| V, Treatment recovery (TR)[a] | 12.20 ±0.11 | 6.51 ±0.19 | 1.47 ±0.10 | 48.55 ±3.25 | 72.32 ±4.98 | 19.01 ±0.62 | 25.38 ±1.69 | 21.98 ±1.32 | 28.63 ±1.07 | 1.49 ±0.08 |
| VI, Olive oil recovery (CR)[a] | 12.92 ±0.15 | 6.41 ±0.11 | 1.75 ±0.16 | 50.37 ±4.43 | 70.83 ±4.10 | 19.43 ±0.65 | 26.20 ±1.20 | 21.49 ±1.70 | 27.61 ±1.74 | 1.47 ±0.16 |

*Values are mean ± S.E.M. for five animals; [a] treatments were discontinued aker 35 days, and animals were sacrificed 42 days aker removal of the treatment.*

### 2.9 Statistical analyses

All data, except for body weight, were subjected to one-way analysis of variance (ANOVA), followed by Neuman-Keuls' multiple range test. Body weight data were assessed using Student's *t*-test. Values were reported as mean ± S.E.M. Results were deemed significant at $p < 0.05$ level.

**Table 5.** Effect of treatment with benzene leaf extract (200 mg/kg b.w. for 35 days) of *H. rosa-sinensis*, on fertility of male and pregnancy outcome in impregnated female mice at intervals (24 h, 2 wk, 4 wk and 6 wk) after removal of the treatment.

| Time after withdrawal of treatment/Parameters | | Number of males | | | Number of females | | | Index of libido (%) | Pre-implantation loss (%) | Post-implantation loss (%) | Number of live implants | Index of fertility (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | T | M | F | T | M | P | | | | | |
| 24 hr | Control | 5 | 5 | 5 | 5 | 5 | 5 | 100 | 0.0 | 0.0 | 8.60±0.51 | 100 |
| | Treated | 5 | 5 | 0 | 5 | 5 | 0 | 100 | 100.00±0.0* | 0.0 | Nil* | Nil* |
| 2 wk | Control | 5 | 5 | 5 | 5 | 5 | 5 | 100 | 2.22±2.22 | 2.00±2.00 | 9.00±0.45 | 100 |
| | Treated | 5 | 5 | 0 | 5 | 5 | 0 | 100 | 100.00±0.0* | 0.0 | Nil* | Nil* |
| 4 wk | Control | 5 | 5 | 5 | 5 | 5 | 5 | 100 | 5.33±3.44 | 4.22±2.60 | 9.00±0.45 | 100 |
| | Treated | 5 | 5 | 5 | 5 | 5 | 5 | 100 | 44.93±13.38* | 10.66±6.88 | 3.60±1.16* | 100 |
| 6 wk | Control | 5 | 5 | 5 | 5 | 5 | 5 | 100 | 3.81±2.35 | 0.0 | 9.20±0.58 | 100 |
| | Treated | 5 | 5 | 5 | 5 | 5 | 5 | 100 | 9.50±4.65 | 6.89±2.94 | 7.20±0.49 | 100 |

*Values are mean ± SEM for five animals; * significantly different from controls ($p < 0.05$) by ANOVA followed by Newman-Keuls' multiple range test.*
*T- Tested; M- Mated; F- Fertile; and P- Pregnant*
*Index of libido= (number mated/number paired) x 100; Index of fertility = (number of males siring live implants/ number mated) x 100;*
*Pre-implantation loss = (total number of corpora lutea - total number of implantations/ total number of corpora lutea) X 100;*
*Post-implantation loss = (total number of implantations - total number of viable implantation/ total number of implantations) X 100*

**Table 6.** Effect of treatment with benzene leaf extract (mg/kg b.w. for 35 days) of *H. rosa-sinensis*, and 42 days after removal of the treatment on the height of seminiferous epithelium, diameter of stage VII seminiferous tubules, percentage frequency of affected tubules, and on the frequency of stages of spermatogenesis in the testis.

| Groups/Treatments | Height of seminiferous epithelium ($\mu$m) | Diameter of stage VII tubules ($\mu$m) | Affected seminiferous tubules (%) | Frequency of stages of spermatogenic cycle | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Stages I-IV | Stages V-VI | Stages VII-VIII | Stages IX-X | Stages XI-XII | Unidentifiable stages |
| I, Untreated (UN) | 67.74 ±2.15 | 200.25 ±4.54 | 12.6 ±0.76 | 17.63 ±0.97 | 10.53 ±0.53 | 35.54 ±1.83 | 10.60 ±0.94 | 25.66 ±1.40 | 6.54 ±1.35 |
| II, Olive-oil Control (OL) | 64.49 ±3.71 | 199.20 ±3.44 | 12.50 ±1.46 | 18.72 ±1.36 | 11.58 ±0.76 | 36.98 ±2.61 | 10.14 ±0.66 | 25.87 ±1.03 | 6.20 ±1.41 |
| III, 100 mg dose | 56.74 ±2.68 | 148.45* ±12.85 | 51.55* ±3.35 | 14.38 ±0.83 | 9.62 ±0.68 | 28.45* ±2.36 | 11.14 ±0.83 | 26.83 ±4.44 | 15.31* ±6.32 |
| IV, 200 mg dose | 44.99[a] ±3.57 | 133.67[a] ±13.13 | 92.80[a] ±2.05 | 15.06 ±1.13 | 9.65 ±0.50 | 18.84[a] ±2.55 | 9.69 ±0.32 | 19.28 ±1.55 | 25.44[a] ±4.88 |
| V, Treatment recovery (TR)[#] | 64.55 ±3.33 | 193.16 ±3.45 | 22.39 ±4.09 | 16.29 ±1.18 | 9.05 ±0.41 | 34.07 ±1.92 | 10.17 ±0.79 | 28.02 ±0.68 | 4.76 ±0.77 |
| VI, Olive oil recovery (CR)[#] | 66.74 ±2.12 | 197.19 ±2.50 | 15.06 ±0.43 | 18.31 ±1.01 | 10.53 ±0.47 | 38.30 ±0.59 | 9.14 ±0.41 | 23.85 ±1.23 | 5.54 ±1.78 |

*Values are mean ± S.E.M. for five animals; * significantly ($p < 0.05$) different from controls; [a] significantly ($p < 0.05$) different from controls and those in group III by ANOVA followed by Newman-Keuls' multiple range test; [#] treatments were discontinued aker 35 days, and animals were sacrificed 42 days aker removal of the treatment.*

## Results

### Body and organs weight

Hibiscus treatment had no significant impact on the body weight (Table 2) as well as the weights of the vital body organs (brain, liver, kidney, adrenal gland, and spleen) when compared to controls (Table 3); the general health and behaviour of the animals remained normal throughout the period of investigation. Marked decreases were observed in the weights of testis, epididymis, seminal vesicle, and prostate gland, barring the vas deferens, in mice administered *H. rosa-sinensis* extract at a dosage of 200 mg/kg b.w. for 35 days relative to controls (Table 2); administration of the lower dosage (100 mg/kg b.w.) of Hibiscus, however, did not significantly impact the weights of the epididymis, vas deferens, and prostate gland; furthermore, the reduction in epididymis weight was notably greater in mice treated with the 200 mg dosage compared to those on the 100 mg dosage of Hibiscus (Table 2). After 42 days of removal of the treatment, the weights of the aforementioned organs returned to control levels (refer to Table 2).

### Motility, viability, number, and morphology of spermatozoa

A notable reduction in motility, viability, and count, alongside a rise in the percentage of morphologically

abnormal spermatozoa, was observed in mice administered the benzene leaf extract of Hibiscus (100 and 200 mg/kg b.w. for 35 days) compared to controls (Figure 1 A); however, the changes in these sperm parameters were more pronounced in mice receiving the 200 mg dose of Hibiscus than in those given the 100 mg dose of the plant (see Figure 1). The caudal spermatozoa in the control group of mice displayed normal structure (Figure 2 A); however, the spermatozoa from the Hibiscus-treated group presented morphological defects categorized as follows: i) headless or missing head, ii) abnormal head shapes (oval, amorphous, microcephalic, macrocephalic, and lacking a hook), iii) neck anomalies (bent and coiled), iv) tail irregularities (bent, weak, and coiled), and v) other issues (sperm precursors and immature sperm with cytoplasmic droplets) (Figure 2 B–D). Forty-two days post-treatment removal, the changes in sperm parameters were similar to control levels (Figure 1 A).

**Sialic acid and fructose levels**

Considerable decreases were observed in sialic acid levels in the epididymis and fructose levels in the seminal vesicle in mice administered Hibiscus extract (100 and 200 mg/kg b.w. for 35 days) compared to control groups; however, the lower dosage (100 mg/kg BW) of the extract did not significantly affect the sialic acid levels in the epididymis (refer to Figure 1 B). Forty-two days post-treatment removal, the sialic acid levels in the epididymis and fructose levels in the seminal vesicle returned to control values (see Figure 1 B).

**Haematology and serum biochemistry**

The average counts of RBC and WBC, along with the indices Hb, Hct, MCV, MCH, and MCHC, stayed unchanged in Hibiscus-treated mice when compared to the control group (Table 4). No notable variations were observed in serum levels of ALT, AST, and creatinine in Hibiscus-treated mice when compared to control groups (Table 4).

**Fertility test and pregnancy outcome**

Libido remained unchanged in mice treated with Hibiscus leaf extract; however, a notable decrease in the count of viable implants was observed in females mated with Hibiscus-treated (200 mg/kg b.w. for 35 days) males at 24 h, 2 wk, and 4 wk post-treatment removal compared to olive-oil controls (refer to Table 5). The fertility index was significantly impacted in the treated mice at 24 h and 2 wk following treatment removal, as none of the females mated with treated males displayed live implants in contrast to the controls (Table 5). The pre-implantation loss was absolute in these impregnated females, which stayed markedly elevated even after 4 wk of treatment removal compared to controls (Table 5); however, the post-implantation loss was not significant in females mated with Hibiscus-treated males at any time point post-withdrawal compared to controls (Table 5). Forty-two days (6 wk) after stopping the treatment, the aforementioned parameters returned to control levels, and all five females mated with Hibiscus-treated males exhibited live implants similar to those of the controls (Table 5).

**Histologic observations**

**Testis** Histologic observations of testes in olive oil-treated (Figure 3 A) controls showed normal spermatogenesis in nearly all the seminiferous tubules (see Table 6). By contrast, noticeable histologic alterations were observed in testes of *H. rosa-sinensis*-treated mice (Figures 3 B–G), as supported by significant increase in the frequency of affected tubules (Table 6), compared to controls. Out of 10 Hibiscus-treated mice examined for histologic alterations in testes, 8 (3 in group III and all 5 in group IV) showed moderate to severe degenerative histologic alterations in the seminiferous tubules, while the remaining 2 (Group III) showed almost normal histologic features in nearly all tubules. Testes in 2 (1 in group III and 1 in group IV) out of the above 10 Hibiscus-treated mice showed non-uniform histologic alterations; on the other hand, testes in the remaining 6 out 10 Hibiscus-treated mice (2 in group III and 4 in group IV) showed uniform histologic alterations with almost all the tubules in degenerating and atrophic conditions (Figures 3 B–G). All 5 mice treated with benzene leaf extract of Hibiscus (*H. rosa-sinensis*) at 200 mg/kg b.w. for 35 days (Group IV) showed complete suppression of spermatogenesis in their testes, with almost all the seminiferous tubules severely affected (Table 6; Figures 3 F–G), suggesting the dose-related anti-spermatogenic effect of Hibiscus. The frequency of affected tubules was significantly higher (92.80%) in the testes of the above treated mice compared to those treated with a 100 mg dose of Hibiscus (see Table 6). Testes of Hibiscus-treated mice showed some common histologic alterations, which are described together. In general, the affected tubules in testes of extracts-treated mice showed loosening of seminiferous epithelium, intraepithelial vacuolation, exfoliation of germ cells, mixing of spermatids of different stages of spermatogenic cycle (particularly in stages IX, X, and XI) in the same tubule, failure of spermiation, phagocytosis of elongated spermatids, occurrence of apoptotic germ cells, pycnotic round spermatids as well as multinucleate giant cells, and thinning and disorganization of seminiferous epithelium in severe conditions due to marked depletion of germ cells (Figures 3 B–G). The seminiferous tubules in the testes of mice treated

with a 200 mg dose of Hibiscus frequently showed the presence of multinucleate giant cells containing two or more pycnotic round spermatids, and giant cells containing two to four pachytene spermatocytes (Figure 3 E–F). There was a complete absence of elongated spermatids in most of the seminiferous tubules in the testes of the above treated mice (see Figures 3 E–F).

Quantitative assessment of histologic alterations in testes showed a significant increase in the percent frequency of affected seminiferous tubules in Hibiscus-treated mice in both treated groups (Groups III–IV) compared to controls; however, in mice treated with 200 mg dose of Hibiscus extract, the percent frequency of affected tubules in the testes was significantly higher than in mice treated with 100 mg dose of the plant (Table 6). Testes in mice treated with Hibiscus extract at 200 mg/kg b.w., showed significant reductions in the height of the seminiferous epithelium as well as the diameter of stage VII tubules compared to controls and those in group III; the lower dose (100 mg), however, have no significant impact on the height of the seminiferous epithelium, though, the diameter of stage VII tubules reduced significantly compared to controls (Table 6). The frequency of seminiferous tubules in stages VII–VIII of the spermatogenic cycle, and of those with unidentifiable stages, decreased significantly in testes in Hibiscus-treated mice in a dose-related manner compared to controls (Table 6); the extract treatment, however, had no significant effect on the frequency of tubules in stages I–IV, V–VI, IX–X and XI–XII of spermatogenesis in mice in all treated groups (Table 6). Forty-two days after removal of the Hibiscus treatments (100 and 200 mg/kg b.w. for 35 days), however, the alterations caused in the testes of Hibiscus-treated mice recovered to control levels (Table 6, Figure 3 H).

**Epididymis and vas deferens** The epididymis in olive oil-treated controls exhibited normal histologic features in segments I–V (Figures 4 A–C, G–H). Treatment with *H. rosa-sinensis* extract at different doses (100 and 200 mg/kg b.w. for 35 days) brought detectable but non-uniform histologic alterations in the epididymis (Figures 4 D–F, I–L), as the alterations varied among the treated groups and also among individuals in a treated group. However, in mice treated with a 200 mg dose of Hibiscus (Group IV), the epididymis in all 5 mice showed severe degenerative alterations. The epididymes in 8 (3 in group III and all 5 in group IV) out of 10 extract-treated mice examined (5 mice per group) showed detectable histologic alterations in their epididymes (Figures 4 D–F, I–L), while the remaining 2 (2 in group III and none in group IV) showed normal histologic features in all the epididymal segments (I–V). As the alterations in the epididymis were more or less similar in the treatment groups, these were described together. In Hibiscus-treated mice, segments I, II and III (Figures 4 D–F) of the epididymis presented almost a normal appearance, except that the lumen in segments II–III of the epididymis was either empty or contained relatively less sperm, sperm fragments or exfoliated germ cells with PAS-positive materials (Figures 4 D–F). Segments IV–V of the epididymis in extract-treated mice frequently showed relatively less sperm, sperm fragments, or no sperm and/or exfoliated germ cells with PAS-positive materials (Figures 4 I–L) in the tubular lumen; epithelial cells in segment IV frequently showed distinct PAS-positive inclusions (Figure 4 K); the lumen was found to be relatively reduced in diameter, and an increase in stroma was noticed in segments IV–V of the epididymis (Figures 4 I–L).

No distinct histologic alterations were observed in the vas deferens in Hibiscus-treated mice compared to controls, except that the tubular lumen in mice treated with Hibiscus extract at 200 mg/kg b.w. showed the presence of a few or no sperm, sperm fragments and/or exfoliated germ cells with accumulation of PAS-positive material (Figure 5 A–B). Forty-two days after removal of the treatment, however, the epididymis (Figure 4 M–N) and vas deferens (Figure not presented) in treated mice presented normal histologic features similar to controls.

**Seminal vesicle and prostate gland** Histologically, the seminal vesicle (Figure 5 C) and prostate gland (Figure 5 D) in olive oil-treated controls showed normal features; in contrast, mice treated with Hibiscus extract (100 and 200 mg/kg b.w. for 35 days) exhibited non-uniform but detectable histologic alterations in the above glands compared to controls. The seminal vesicle and prostate gland in mice treated with a 200 mg dose of Hibiscus frequently showed reduced height of the secretory epithelium, fewer epithelial mucosal folds, and increased calcareous secretion in the lumen (Figures 5 E–F, respectively) compared to controls (Figures 5 C–D, respectively). Forty-two days after the treatment removal, however, the above glands recovered to histologic features similar to controls (Figures 5 G–H, respectively).

**Liver, kidney and spleen** No histopathological alterations were noticed in the liver, kidney, and spleen in mice in all treated groups compared to controls (Figures not given).

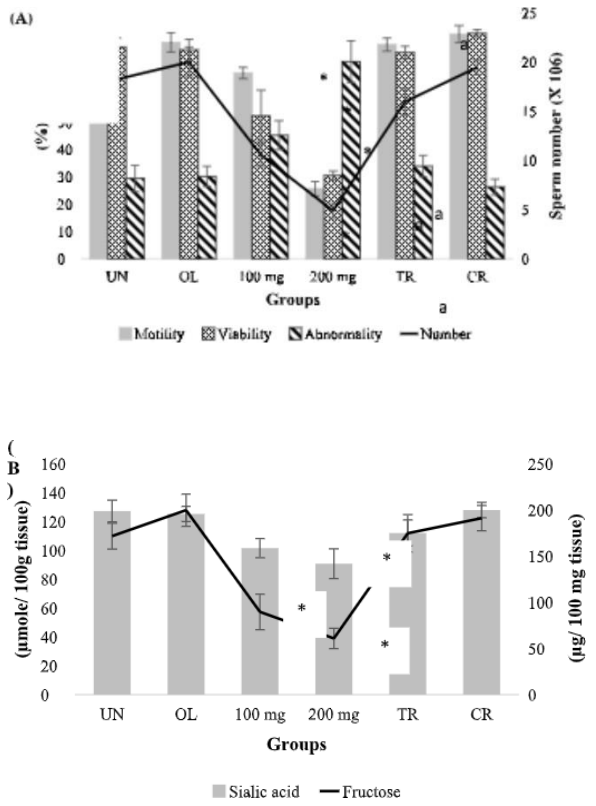



**Figure 1.** Effect of treatment with benzene leaf extract (mg/kg b.w. for 35 days) of H. rosa-sinensis, and 42 days after removal of the treatment on (A) Sperm motility, viability and abnormality, and number of spermatozoa in cauda epididymis; (B) Level of sialic acid in the epididymis and that of fructose in the seminal vesicle. Values are mean ± S.E.M. for five animals; * significantly (plt;0.05) different from controls; a significantly (plt;0.05) different from controls and those in group III by ANOVA followed by Newman-Keuls' multiple range test; treatments were discontinued after 35 d, and animals were sacrificed 42 d after withdrawal of the treatment.

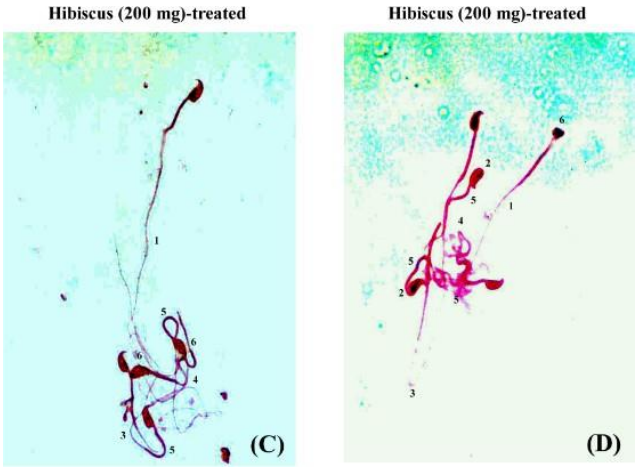
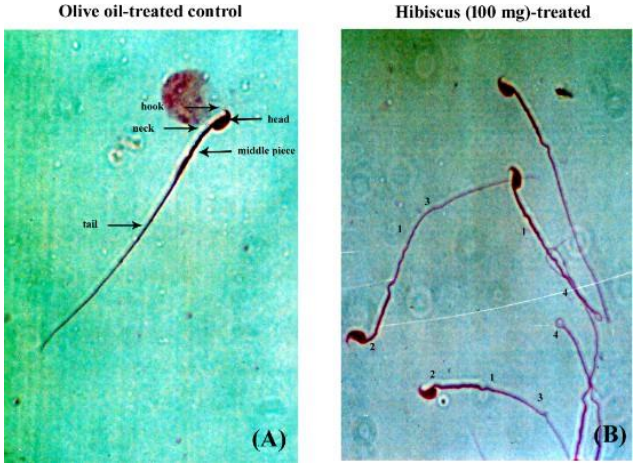



**Figure 2.** Photomicrographs of eosine-nigrosine stained spermatozoa of cauda epididymidis of mouse. (Original magnification: A-D: X 400). (A) Olive oil-treated control to show normal features of spermatozoon; (B) After treatment with benzene extract of H. rosa-sinensis (100 mg/kg b.w. for 35 days) to show spermatozoa with fragile tail (1), bent neck (2) and bent tail (3) and coiled tail (4); (C-D) After H. rosa-sinensis treatment at 200 mg/kg b.w. for 35 days to show spermatozoa with fragile tail (1), bent neck (2), bent tail (3), coiled tail (4), bent and coild middle piece (5), and amorphous head (6).



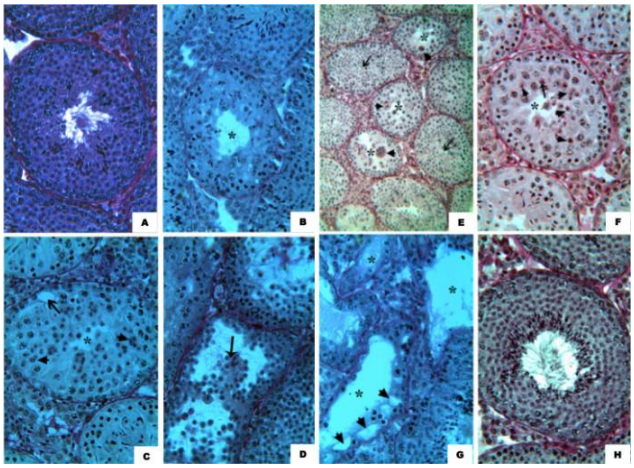


**Figure 3.** (A-H): Photomicrographs of PAS-H-stained sections of mouse testis. (Original magnification: A-D, F, H: X 252; E: X 160; G: X 200). (A) Oilve oil-treated control to show the normal spermatogenesis in the seminiferous tubule; (B) After treatment with benzene leaf extract of H. rosa-sinensis (100 mg/kg b.w. for 35 d) to show the presence of relatively less number of germ cells in the middle tubule (asterisk); (C) After the same treatment as in (B) to show the vacuolation (arrow) and formation of multinucleate giant cells in the seminiferous epithelium (aroow heads) in middle tubule (asterisk); (D) After treatment with benzene leaf extract of H. rosa-sinensis (200 mg/kg b.w. for 35 d) to show the mass exfoliation of germ cells (arrow) in the tubule; (E) After the same treatment as in (D) to show the presence of degenerating seminiferous tubules (asterisk) with remarkably reduced diameter and the absence of lumen (arrows). Note the formation of multinucleate giant cells (arrow heads) and complete absence of elongated spermatids in tubules (asterisk); (F) After the same treatment as in (D) to show the pycnotic round spermatids (arrow heads) and multinucleate giant cells in the seminiferous epithelium (aroow) in middle tubule (asterisk); (G) After the same treatment as in (D) to show the atrophic tubules (asterisk) with disorganised seminiferous epithelium containing vacuolations (arrow heads), Sertoli cells, a few spermatogonia and spermatocytes; (H) Testis of mouse, 42 days after treatment removal to show normal spermatogenesis in the tubules.

## Discussion

In the current study with Parkes strain albino mice, administration of benzene leaf extract from H. rosa-sinensis Linn. (100, 200 mg/kg b. wt.) for 35 days decreased testicular weights and induced histopathological aberrations in seminiferous tubules with occurrence of both histopathologically altered and typical tubules in the same testis sections. Altered tubules showed loss of cohesion among seminiferous epithe-

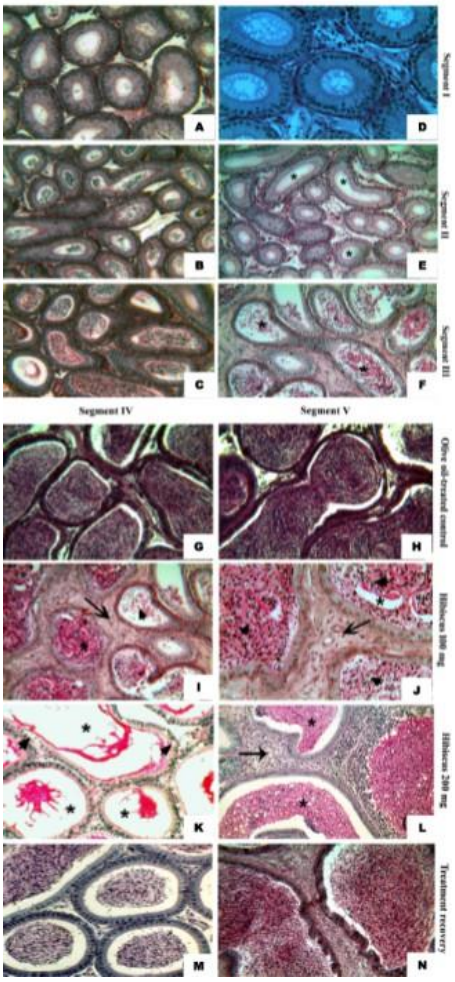



**Figure 4.** (A-N): Photomicrographs of PAS-H-stained sections of mouse epididymis (segments I-V). (Original magnification: A-C and E-F: X 160; D, G-I, K-N: X 200; J: X 252). Oilve oil-treated control (A-C; G-H) to show the normal histologic features of epithelium. Note the presence of spermatozoa in segments II (B) and III (C), and lumen in segments IV (G) and V (H) distended with spermatozoa; (D-F) After treatment with benzene extract of H. rosa-sinensis (200 mg/kg b.w. for 35 days) to show the normal appearance of the segments, except that the lumen is devoid of sperm, and contains exfoliated germ cells with accumulation of PAS-positive materials (asterisk); (I-J) After treatment with benzene extract of H. rosa-sinensis at 100 mg/kg b.w. for 35 days to show relatively less number of sperm (asterisk), exfoliated germ cells (arrow heads), and accumulation of PAS-positive materials in the tubular lumen; (K-L) After treatment with H. rosa-sinensis at 200 mg/kg b.w. for 35 days to show complete the absence of sperm in segment IV (K), and presence of sperm fragments in segment V (L) with accumulation of PAS-positive materials (asterisk) in the tubular lumen. The lumen was relatively reduced in diameter, and an increase in stroma (arrow) was noticed in segments IV-V (K-L); (M-N) Epididymis of mouse 42 days after treatment removal to show the normal histologic features of epithelium, and lumen distended with sperm in segments IV (M) and V (N).

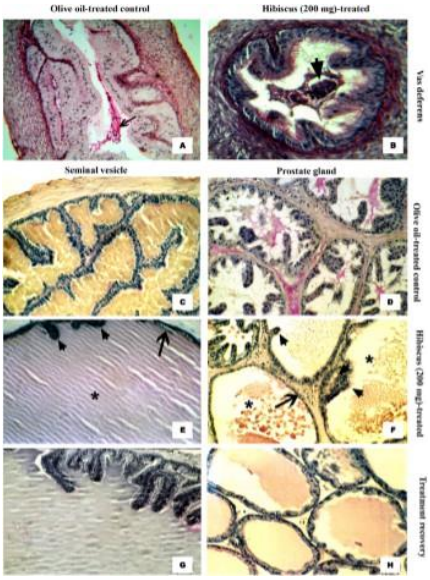



**Figure 5.** (A-H): Photomicrographs of PAS-H stained tissue sections of vas deferens, seminal vesicle and prostate gland of mouse. (Original magnification: A-B: X 160 and C-H: X 200). Vas deferens of Olive oil-treated control (A) and that (B) of mice treated with benzene extract of H. rosa-sinensis (200 mg/kg b.w. for 35 days) to show the normal histologic features of epithelium. Note the presence of spermatozoa in lumen (arrow) of olive oil-treated control (A), while presence of sperm fragments and exfoliated germ cells in lumen (asterisk) of Hibiscus-treated mice (B); Seminal vesicle (C) and prostate gland (D) of an olive oil-treated control to show the normal histologic features of epithelium; those (E & F respectively) of mice treated with H. rosa-sinensis (same dose and duration) to show the reduced height of the secretory epithelium (arrow), fewer mucosal folds (arrow heads), and increased calcareous secretion in the lumen (asterisk); and those (G & H respectively) of mice, 42 days after the treatment removal.

lium cells, intraepithelial vacuolation, exfoliation of germ cells, presence of spermatids at different stages of development in the same tubule, failure of spermiation, presence of pyknotic rounded spermatids with only marginal chromatin condensation, and the appearance of multinucleated giant cells. It is crucial to note that non-uniform histologic changes in the testis have also been documented in mice in our previous study with this plant [11] , and also in other similar studies following treatment with extracts from Albizia lebbeck (L) Benth bark [24] , Curcuma longa L. rhizome [25] , Bacopa monnieri [26] , Citrus limon leaf [27] , Coccinia indica leaf [28] , Leonotis nepetifolia [29] , and Terminalia chebula Retz. Bark [30] . It has been proposed that uneven focal injuries in the testis arise because tubules at specific phases of spermatogenesis are more susceptible to harm from different treatments than others [22] . It is known that stages VII-VIII of spermatogenesis are highly androgen-dependent [31-32] , and therefore, a noticeable decrease in the frequency of tubules in stages VII-VIII of spermatogenesis, as observed in testes of Hibiscus-treated mice, might be the result of androgen-dependent adverse effects on the kinetics of spermatogenesis [33-35] . Hibiscus extract treatment (100 and 200 mg/kg b.w.) resulted in a dose-dependent fall in spermatogenesis, with mice receiving 200 mg/kg b.wexhibiting the most significant degenerative histological changes, alongside the highest percentage of affected tubules (92.80%) and those in unidentifiable stages (25.44%), as well as notable reductions in the height of seminiferous epithelium and the diameter of stage VII tubules. The round spermatids appeared to be the most affected germ cells in the testes of P mice after treatment with benzene leaf extract of H. rosa-sinensis, since these cells often showed marginal condensation of chromatin, premature exfoliation and giant cell formation. Similar observations have been reported in mouse testis after treatment with aqueous leaf extract of Allamanda cathartica [36] and fruits of Piper nigrum [37] , and in rat testis after cadmium toxicity [38] , and treatment with Tamoxifen [39] . The testes in mice treated with Hibiscus at 200 mg/kg b.w. frequently showed atrophied seminiferous tubules consisting of Sertoli cells and a few spermatogonia with rare spermatocytes, and occurrence of giant cells in the seminiferous epithelium, as observed in other studies [14, 27] [40-42] , the frequency of tubules in unidentifiable stages (25.44%) was significantly high in testes of above treated mice as stated above; these findings suggest that Hibiscus treatment at 200 mg/kg b.w. for 35 days causes complete suppression of spermatogenesis in testis in albino mice. Treatment with H. rosa-sinensis (100 and 200 mg/kg b.w. for 35 days) had a dose-related effect on the weight of the epididymis, with detectable histologic alterations in extract-treated mice compared to controls. Similar to the testis, histologic alterations in the epididymis were comparatively more severe in mice treated with the 200 mg dose of Hibiscus than in those treated with the 100 mg dose of the plant. Epididymis in the above Hibiscus-treated mice frequently showed vacuolization and accumulation of PAS-positive inclusions in the epithelial cells of segment IV (corpus) of the epididymis as reported in mice following treatment with rhizome extract of Curcuma longa [25] , leaf extract of Ficus bengalensis [43] , Citrus limon [27] ,and Coccinia indica [28] , Terminalia chebula [30] . Principal cells in segment II (caput) are said to secrete PAS-positive substances into the lumen that sperm use for maturation in later segments, and

when sperm are absent, this material is reabsorbed by the principal cells of segment IV (corpus) of the epididymis as intracellular inclusions [44] [23] . The occurrence of PAS-positive inclusions in epithelial cells of segment IV is therefore linked to a small number of sperm in the epididymal duct, as in photomicrographs of various segments (I-V) of the epididymis from the treated mice. This argument is further backed by the fact that such inclusions were absent in segment IV of the epididymis in treated mice with an adequate number of spermatozoa in the tubular lumen. Treatment with Hibiscus extract had dose-related adverse effects on motility, viability, morphology and number of spermatozoa in the cauda epididymidis. The significant decrease in the number of spermatozoa in Hibiscus-treated mice is likely to result from the suppressive effects of the treatment on spermatogenesis [45] , and also because the sperm number returned to control level after withdrawal of the treatment; on the other hand, alterations in sperm motility, viability, and morphology could have resulted from testosterone mediated disturbance in the secretory function of epididymis [46-48] , as indicated by a marked reduction in the level of sialic acid in epididymis in mice after treatment with benzene extract of Hibiscus in the present study. Significant reductions were noticed in the weight as well as the level of fructose in the highly testosterone-sensitive seminal vesicle, accompanied by histological alterations in Hibiscus-treated mice; the prostate gland, however, also showed a significant reduction in weight with detectable histological alterations in mice treated with a 200 mg dose of Hibiscus compared to the control. The observation of marked reduction in vesicular fructose level, accompanied by histological alterations in the seminal vesicle and prostate gland in Hibiscus-treated mice, is likely to be the result of the decreased level of testosterone [49] [50] , supporting the above hypothesis. Libido remained unchanged in males treated with Hibiscus, but there was a total inhibition of fertility in males at 24 h and 2 wk after stopping treatment due to total pre- implantation loss in the impregnated females. This may be due to a significant decrease in both the quantity and quality of the cauda spermatozoa. At 4 weeks post-treatment cessation, the fertility of mice treated with Hibiscus returned to 100count of live implants in pregnant females was still notably lower than in the control group. Forty-two days (6 wk) after stopping treatment, the fertility of the Hibiscus- treated mice returned to control levels; this indicates that the spermatogonial proliferation was unaffected by the treatment. The minimal post-implantation loss in impregnated females seen in this study suggests that Hibiscus treatment does not relate to any teratogenic or genotoxic effects [51] . The absence of any alterations in mean body weight and in weights of the brain, liver, kidney, adrenal gland and spleen, in histological features of liver, kidney, and spleen accompanied by unaltered serum levels of ALT, AST and creatinine respectively, as well as in haematological indices suggest that treatment with H. rosa-sinensis (100 and 200 mg/kg b.w. for 35 days) does not produce toxic effects. The findings of the present investigation show that the oral administration of benzene leaf extract of Hibiscus at the dose of 200 mg/kg b.w. for 35 days caused complete suppression of spermatogenesis in the testis in albino mice, but the mechanism by which such effect is caused remains poorly understood. It is well documented that testosterone is essential for the maintenance of spermatogenesis in testis and accessory sex organs. Thus, it is probable that Hibiscus-induced suppression of spermatogenesis in

24 albino mice is caused by a deficiency of testosterone. The epididymis and seminal vesicle are two important reproductive organs largely dependent on androgen for the maintenance of structure and function, and therefore, histologic alterations in the above organs accompanied by significant reductions in the levels of sialic acid and fructose, respectively, strongly indicate disturbance in the intratesticular androgen level [52] . However, whether the antispermatogenic action of H. rosa-sinensis results from a reduction in serum testosterone levels in Hibiscus-treated mice due to interference with Leydig cell function, and/or from interference with Sertoli cell function, remains unanswered.

## Conclusion

The results of the present study in albino mice suggest that treatment with the benzene leaf extract of Hibiscus (H. rosa-sinensis) at the dose of 200 mg/kg b.w. for 35 days causes marked histological alterations in the male reproductive organs with complete suppression of spermatogenesis and fertility in albino mice, which are, however, reversible by 42 days of treatment removal. Besides, no detectable signs of toxicity were found in Hibiscus-treated mice. Therefore, H. rosa-sinensis might be a potential candidate for herbal male contraception.

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clared Ethical approval: The study was approved by the Institutional Ethical Committee.

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