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Effect of Hibiscus-Rosa-Sinensis (Linn.) on the Reproductive Endpoints and Fertility in Male Albino Mice

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KEYWORDS

Testis; Fertility; Creatinine; Libido;

Liver

ABSTRACT:

Introduction: Plants and plant products have been regularly used for fertility regulation in humans. This could be because plant-based remedies are easily available, cheap, reliable, and most importantly free from unwanted side effects. The different parts viz. leaves, stems, roots, and flowers of Hibiscus rosa-sinensis Linn. (family: Malavaceae) are known to exhibit many medicinal and pharmacological properties. Though Hibiscus has been known for its antifertility properties both in males and females, we do not have much information about the use of this plant in the control of fertility in males.

Objectives: The present study deals with the effect of Hibiscus-rosa-sinensis on the reproductive endpoints and fertility in male albino mice after oral administration of different extracts prepared from the leaves of Hibiscus.

Methods: The aqueous, ethanol, and benzene leaf extracts of H. rosa-sinensis were administered orally (100 mg/kg BW/d for 35 d) to albino mice, and the effect of the treatments on the male reproductive organs, sperm parameters, biochemical and fertility indices in albino mice was examined. Toxicological as well as hematological studies were also carried out.

Results: Treatment with Hibiscus extracts brought non-uniform but detectable histologic alterations in the testis, epididymis, and seminal vesicle; the alterations caused in the reproductive organs as well as the number, motility, viability, and morphology of caudal spermatozoa were, however, severe in mice treated with the benzene extract of Hibiscus compared to controls and those treated with other extracts of the plant. The level of sialic acid in the epididymis and that of fructose in the seminal vesicle were adversely affected in Hibiscus-treated mice with no significant differences among treated groups. The fertility was reduced significantly in benzene extract-treated males, though, libido remained unaffected after Hibiscus treatment compared to controls. Further, treatment with Hibiscus did not alter histological features of the liver and kidney, levels of ALT, AST, and creatinine in serum, and hematological indices compared to controls.

Conclusions: The results of the present study suggest that the treatment with benzene leaf extract of H. rosa-sinensis results in suppression of spermatogenesis and fertility in albino mice, and therefore, Hibiscus might be a source of a male contraceptive of plant origin.

1. Introduction

There has been a growing interest among researchers to develop an orally active, safe, reversible, and effective male contraceptive since none of the currently available approaches fulfill the requirements of an ideal male contraceptive. Plants and plant preparations have been used regularly for fertility regulation of both males and females; this may be because of their easy availability, cost-effectiveness, absence of drug-associated side effects, and most importantly protection of the privacy of the user [1,2] Thus, plants have a viable future in the search for a plant-based orally active male contraceptive.

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Hibiscus (Genus) belongs to the family Malavaceae and has as many as 275 species widely distributed in tropical and subtropical regions of the world. Out of 275 species of Hibiscus, about 40 species have been reported to be present in India only with most common species viz. H. rosa-sinensis, H. syriacus, H. cannabinus, H. radiatus, H. vitifolius, H. sabdariffa, H. schizopetalus, etc [3]. Hibiscus rosa-sinensis (H. rosa-sinensis) Linn. is a common ornamental perennial shrub in Indian gardens. The large beautiful flowers of the plant are usually dark red in color and carry several common names such as China rose (English), Jasum (Hindi), Japa (Sanskrit) etc. The leaves, stems, roots, and flowers of H. rosa-sinensis have been known to contain numerous medicinal properties like antihypolipidemic, antiproliferative, antioxidant, antimicrobial, antiinflammatory and other pharmacological properties [4,3,5]. Hibiscus has been known for its antifertility properties both in males and females [6-8], but we do not have much information about the use of this plant in the control of fertility in males. There are a few reports on the effect of various extracts of the flowers of H. rosa-sinensis on spermatogenesis and fertility in rats [9-12]. However, leaves of the plant have not been studied for antispermatogenic and antifertility effects in male mice. Since plants may play an important role in the regulation of fertility, the present investigation was undertaken to evaluate the possible effects of crude extracts from the leaves of H. rosa-sinensis on reproductive organs and fertility in male mice.

2. Objectives

The present study deals with the effect of Hibiscus-rosa-sinensis on the reproductive endpoints and fertility in male albino mice after oral administration of different extracts prepared from the leaves of Hibiscus. Various male reproductive endpoints such as histopathology, sperm parameters, and biochemical and fertility indices were assessed. Further, toxicological studies were also performed.

3. Methods

Preparation of plant extracts: Fresh leaves of H. rosa-sinensis were collected from the campus of Banaras Hindu University (BHU), Varanasi after proper taxonomic identification (Voucher specimen no. Malva. 2023/02) of the plant by Professor N. K. Dubey of the Department of Botany, B.H.U., Varanasi (India). Plant extracts were prepared strictly according to WHO protocol [13]. In brief, leaves were washed properly with sterile DW, shade dried for 1 w, and then crushed into fine powder using an electric grinder. The leaf powder (500 g) was extracted successively with benzene, 95% ethanol, and DW (2000 ml, w/v 1:20) in a soxhlet apparatus each for 3 h. The filtrates were evaporated to dryness and lyophilized to get brownish-blackish extracts. All the extracts were stored at 4^0C.

Experimental design and animal autopsy: A total of 30 adult (age 12-14 w; weight 28-38 g) male laboratory mice of proven fertility belonging to the Parkes (P) strain were used in the present investigation after approval of the Institutional Animal Ethics Committee. Animals were 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, following the guidelines of CPCSEA, New Delhi [14]. Animals were given standard pellet feed (Mona Laboratory Animal Feeds, Varanasi, India) at regular intervals, and fresh drinking tap water ad libitum. Animals were randomly allocated into six (I-VI) groups, each comprising five animals. The experimental design and autopsy schedule (Chart 1) were as follows:

Chart 1: Experimental design and autopsy schedule**.**

Groups	Treatments	Duration	Autopsy (after the last treatment)
I	Untreated controls	35 d	24 h
II	DW-treated controls		
III	OL-treated controls		
IV	Aqueous leaf extract		
V	95% Ethanol leaf extract		
VI	Benzene leaf extract		

Control groups included untreated (UN) controls and vehicle (DW: distilled water and OL: olive oil)-treated

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controls. Mice in experimental groups (IV-VI) were treated (100 mg/kg BW/d) with extracts of H. rosa-sinensis for 35 d. The dose and duration for the treatment with H. rosa-sinensis were determined based on a series of preliminary studies conducted in albino mice in our laboratory. The aqueous leaf extract of H. rosa-sinensis was suspended in sterile DW, while the ethanol and benzene leaf extracts were suspended in OL. The doses were expressed as the dry weight of the extract. The drugs were administered orally, using separate and properly sterilized oral feeding needles. Mice in Groups II-III (vehicle controls) received either only sterile DW or OL (5.0 ml/kg BW/d). The animals in groups I-VI were sacrificed together 24 h after the last treatment after recording their final body weights. At autopsy, trunk blood was collected by decapitation for hematological tests and separation of sera which were stored at -20^{0}C for the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine levels. The testis, epididymis, vas deferens, seminal vesicle, brain, liver, kidney, adrenal gland, and spleen were excised and weighed using an electric balance. The epididymes and seminal vesicles from five animals in each group were stored at -20^{0}C for sialic acid and fructose assays, respectively.

Histological techniques: To study histologic alterations, testis, epididymis, and seminal vesicle, a portion of the liver and kidney were fixed in aqueous Bouin's fluid overnight at RT, dehydrated in graded ethanol series, cleared in benzene, and embedded in paraffin wax (60-62^{0}C). Tissue sections (6 µm) were stained with periodic acid-Schiff (PAS) and counter-stained with Harris hematoxylin (H) for examination under a light microscope (Leica, Germany). Histologic alterations in the testes were determined by quantifying the height of the germinal epithelium, the diameter of stage VII seminiferous tubules, the percent frequency of tubules in different stages (I-IV, V-VI, VII-VIII, IX-X, and Xi-XII) of spermatogenesis, and that of affected tubules from random testis sections. Qualitative alterations in mouse testis were described according to criteria given by Russell et al. [15]; the histologic alterations in the epididymal segments (I-V) were studied following Abe et al. [16].

Analyses of sperm parameters: 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 370C on a hot plate [17]. The tissue was minced properly and the sperm suspension was used for analyses of percent frequency of motility, viability, abnormality, and number of spermatozoa according to WHO protocol [18]. The morphological abnormalities in spermatozoa were studied as per the criteria of Wyrobek and Bruce [19], and Zaneveld and Polakoski [20].

Biochemical assays in reproductive tissues: The level of sialic acid in the epididymis [21] and that of fructose in the seminal vesicle [22] were estimated by standard procedures.

Toxicological investigations: Trunk blood was analyzed for mean counts of blood cells (RBC and WBC), hemoglobin (Hb) and hematocrit (Hct) according to standard laboratory procedures [23]. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were also calculated. The levels of ALT as well as AST [24], and creatinine (Span Diagnostics Limited, India) in sera were determined biochemically.

Fertility tests: A total of 25 adult males (five animals per group), and 25 females of proven fertility were employed in the fertility tests. The fertility of males in groups II-VI was tested after the last treatment (on 35th d) by allowing each male to mate with one coeval, virgin female in proestrus. The presence of a vaginal plug in a mated female confirmed successful mating. After 12/13 d of pregnancy, females were autopsied to record the total number of implants in both the uteri and the total number of corpora lutea in both ovaries. The resorption sites were detected by treating the uteri with 10% ammonium sulphide solution [25]. The males were considered fertile if impregnated females showed live implants.

Statistical analyses: All data, except for body weight, were analyzed by one-way analysis of variance (ANOVA), and employing Neuman-Keuls' multiple range test. Student's t-test was used for data on body weight. Values were presented as mean ± S.E.M. and considered significant at $p< 0.05$ level.

4. Results

Body and organ weight: Hibiscus treatment did not affect the body weight (Table 1), and weights of the brain, liver, kidney, adrenal gland, and spleen (Table 2)

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of the treated mice. Significant reductions were noticed in the weights of the testis in extracts-treated mice; the weights of the epididymis, vas deferens, and seminal vesicle remained unaltered in mice in groups IV-V (Table 1), but the weight of the above organs reduced significantly in those treated with benzene extract of Hibiscus; further, the reduction in absolute weight of the testis was significantly high in the above treated mice compared to those in other treated groups and controls (Table 1).

Table 1. Effect of Hibiscus leaf extracts (100 mg/kg BW/d for 35 d) on body weight and weights of reproductive organs.

Groups/ Treatments	Body weight		Reproductive organs weight (mg/100 g BW)							
	Initial	**Final**	**Testis**		**Epididymis**		**Vas deferens**		**Seminal vesicle**	
			Absolut	Relativ	Absol	Relativ	Absolut	Relativ	Absolu	Relati
I, Untreated (UN)	31.16 ±0.40	31.83 ±0.40	93.48 ±4.79	293.93 ±14.21	36.96 ±0.63	116.37 ±3.08	11.48 ±1.08	36.13 ± 3.44	72.80 ±2.51	223.04 ±8.14
II, Control (DW)	34.33 ±0.66	35.00 ±0.68	100.50 ±2.87	292.30 ±7.11	36.42 ±1.92	106.03 ±5.75	11.38 ±0.65	33.11 ±1.87	64.14 ±2.56	184.43 ±6.99
III, Control (OL)	35.33 ±1.22	36.00 ±1.23	108.30 ±5.15	302.55 ±11.03	37.72 ±2.24	102.37 ±4.38	12.46 ±0.67	34.00 ±1.87	75.68 ±4.32	202.80 ±12.49
IV, Aqueous extract	31.33 ±0.42	32.83 ±1.83	68.62* ±5.39	225.84* ±18.13	34.24 ±1.63	112.58 ±4.97	12.66 ±0.86	41.60 ±2.67	65.20 ±5.29	214.05 ±15.78
V, Ethanol extract	37.83 ±0.54	38.33 ±0.80	71.48* ±4.91	190.64* ±13.78	39.54 ±3.08	108.72 ±10.17	12.34 ±0.57	33.71 ±1.31	79.16 ±3.60	216.72 ±10.46
VI, Benzene extract	37.00 ±1.12	37.33 ±1.51	51.64[a] ±8.46	167.52* ±26.51	28.06* ±1.15	85.19* ±2.79	7.30* ±1.01	24.23* ±3.03	36.42* ±9.71	125.5* ±24.76

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Table 2. Effect of Hibiscus leaf extracts (100 mg/kg BW/d for 35 d) on weights of vital body organs.

Groups/ Treatments	Body organs weight (mg/100 g BW)									
	Brain		Liver		Kidney		Adrenal		Spleen	
	Absolut	Relativ	Absolut	Relativ	Absolut	Relativ	Absolut	Relativ	Absolut	Relativ
I, Untreated (UN)	250.96 ±10.63	791.95 ±46.63	1056.14 ±45.21	3320.79 ±129.36	229.60 ±7.72	721.55 ±17.92	2.90 ±0.18	9.11 ±0.56	73.60 ±5.19	230.74 ±13.21
II, Control (DW)	275.44 ±17.74	800.31 ±47.33	1113.74 ±42.03	3238.00 ±102.42	221.59 ±11.98	643.44 ±28.17	3.02 ±0.23	8.75 ±0.57	83.08 ±5.68	240.96 ±13.15
III, Control (OL)	291.15 ±24.34	789.02 ±54.52	1192.20 ±21.77	3250.63 ±102.73	237.68 ±10.08	646.68 ±25.16	3.18 ±0.33	8.60 ±0.83	83.08 ±6.19	218.61 ±13.96
IV, Aqueous extract	267.82 ±11.24	882.59 ±43.89	1016.58 ±61.31	3342.59 ±192.93	192.24 ±16.10	632.11 ±52.19	2.60 ±0.15	8.54 ±0.46	80.14 ±13.62	301.64 ±46.54
V, Ethanol extract	274.02 ±9.23	750.79 ±30.66	1253.08 ±41.39	3428.44 ±110.57	217.88 ±14.23	594.34 ±29.87	3.10 ±0.19	8.51 ±0.67	91.40 ±7.00	258.72 ±22.99
VI, Benzene extract	256.82 ±9.77	864.41 ±74.02	1012.14 ±68.28	3362.55 ±218.40	196.72 ±21.64	648.69 ±63.14	2.74 ±0.19	8.44 ±0.49	94.14 ±18.62	298.45 ±43.25

Values are mean ± S.E.M. for five animals; Organs weight refers to the weight of the unpaired organ; * significantly different from controls; [a]significantly different from controls and those in groups IV and V by

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ANOVA followed by Newman-Keuls' multiple range test.

Histopathological observations: **Testis**: Histologically, testes in controls (Figure 1 A) showed normal features in nearly all the seminiferous tubules except in a few (see Table 3). In contrast, noticeable histologic alterations were observed in testes of Hibiscus-treated mice (Figures 1 B-F). Out of 15 extracts-treated mice examined for histologic alterations, 10 (2 in group IV, 3 in group V, and all 5 in group VI) showed noticeable histologic alterations in the seminiferous tubules; in contrast, the remaining 5 (3 in group IV, 2 in group V and none in group VI) showed almost normal histologic features in nearly all tubules of their testes. Testes in 13 out of the above 15 extracts-treated mice showed non-uniform histologic alterations as both affected and normal tubules were observed in the same testis section (Figure 1 F). On the other hand, testes in 2 (benzene extract-treated, group VI) out of 15 extracts-treated mice (groups IV-VI) showed uniform histologic alterations with many tubules in degenerating and atrophic conditions (Figure 1 E-F). The histologic alterations in testes, therefore, were severe in mice treated with benzene extract compared to those in other treated groups suggesting that the effect was dependent on the type of plant extract administered. The frequency of affected tubules was significantly high (70.66%) in the testes of above treated mice compared to mice in other treated groups (groups IV-V; see Table 3). Testes in Hibiscus-treated mice, in general, showed loosening of germinal epithelium, intraepithelial vacuolation, exfoliation of germ cells, mixing of spermatids of different stages in the same tubule, failure of spermiation, phagocytosis of elongated spermatids, occurrence of apoptotic germ cells, multinucleate giant cells, and thinning and disorganization of germinal epithelium due to marked depletion of germ cells (Figure 1 B-F). In benzene extract-treated mice, testes frequently contained atrophic and severely degenerating tubules with a very thin and disorganized germinal epithelium consisting of Sertoli cells and a few spermatogonia with rare spermatocytes and/or round spermatids (Figure 1 E-F); quantitative assessment of histologic alterations in testes showed a significant reduction in the height of germinal epithelium and diameter of stage VII tubules in the above treated mice compared to those in other treated and controls (Table 3). The height of germinal epithelium and diameter of stage VII tubules, however, were not affected in testes of mice treated with aqueous extract; while, mice treated with ethanol extract showed significant reduction in the height of germinal epithelium (Table 3). The frequency of stages (I-IV, V-VI, IX-X and XI-XII) of the spermatogenic cycle (Table 3) remained unaltered, though, a significant decrease in the frequency of tubules in stages VII-VIII was noticed in testes of mice treated with ethanol and benzene extracts of Hibiscus; further, the number of tubules with unidentifiable stages (27.20%) was significantly high in testes of benzene extract-treated mice compared to those in other treated groups and controls (Table 3). **Epididymis, seminal vesicle, liver, and kidney**: Epididymis in OL-treated (Figures 2 A-C; 4 A-B) controls exhibited normal histologic features. In contrast, treatment with Hibiscus brought histologic alterations in the epididymis (Figure 3 C-H), though similar to testes, the alterations were more severe in mice treated with benzene extract compared to those treated with other extracts of the plant. In benzene extract-treated mice, segments I-III of the epididymes showed nearly normal histologic features, except that the lumen of the tubules in these segments was empty, and contained PAS-positive materials (Figure 2 D-F); the tubular lumen in segments IV (Figure 3 G) and V (Figure 3 H) was relatively reduced in diameter, and frequently filled with relatively less sperm or sperm fragments and PAS-positive materials; stroma was also increased (Figure 3 G). Histologically, seminal vesicles in controls presented normal features; in contrast, in benzene extract-treated mice these showed reduced height of the secretory epithelium, fewer epithelial mucosal folds, and increased calcareous secretion in the lumen (Figures not presented); no such alterations were, however, observed in mice in other treated groups (Figures not presented). Further, the liver and kidney in mice in all treated groups showed normal histologic features compared to controls (Figures not presented).

Motility, viability, number, and morphology of spermatozoa: Significant decrease in motility, viability, and number, while an increase in the percent frequency of morphologically abnormal spermatozoa was noticed in cauda epididymides of Hibiscus-treated mice compared to controls (Figure 4); further, alterations in the above sperm parameters were dependent on the type of extract administered since mice treated with benzene extract showed severe alterations in the sperm

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parameters compared to those treated with other extracts of H. rosa-sinensis and controls (Figure 4).

Table 3. Effect of Hibiscus leaf extracts (100 mg/kg BW/d for 35 d) on the height of germinal epithelium, diameter of stage VII tubules, percent frequency of affected tubules, and on the frequency of stages of spermatogenic cycle in the testis.

Groups/ Treatments	Height of germinal epithelium(µm)	Diameter of stage VII tubules (µm)	Affected seminiferous tubules (%)	Stages I-IV	Stages V-VI	Stages VII-VIII	Stages IX-X	Stages XI-XII	Unidentifiable stages
I, Untreated (UN)	57.97 ±1.47	187.59 ±4.39	9.23 ±0.43	15.40 ±0.91	11.83 ±1.03	35.21 ±0.50	10.75 ±0.71	22.57 ±1.98	3.70 ±0.46
II, Control (DW)	59.09 ±1.17	200.75 ±5.58	10.24 ±0.20	15.66 ±0.62	11.60 ±0.83	36.26 ±0.38	10.53 ±0.68	22.19 ±1.51	3.87 ±0.99
III, Control (OL)	59.80 ±2.00	188.92 ±7.10	9.23 ±0.40	15.95 ±1.62	9.18 ±0.87	35.73 ±0.79	11.34 ±0.67	23.65 ±1.15	4.11 ±0.83
IV, Aqueous extract	57.73 ±1.04	180.24 ±7.75	30.54* ±2.55	12.20 ±1.35	9.15 ±0.41	33.33 ±1.76	10.43 ±0.86	23.50 ±1.54	8.85 ±3.37
V, Ethanol extract	53.17* ±1.49	167.75 ±1.94	26.69* ±2.62	11.65 ±1.50	9.53 ±0.84	27.49* ±2.98	9.93 ±0.28	21.83 ±1.89	9.27 ±1.76
VI, Benzene extract	42.37[a] ±1.12	146.30[a] ±7.72	70.66[a] ±12.29	12.53 ±0.97	10.25 ±0.80	17.02[a] ±1.96	10.04 ±0.70	18.20 ±1.50	27.20[a] ±4.27

Table 4. Effect of Hibiscus leaf extracts (100 mg/kg BW/d for 35 d) on toxicological parameters.

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Groups/ Treatments	Hb (g/ 100 ml)	RBC (X 10^6/ mm^3)	WBC (X 10^3/ mm^3)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (%)	ALT (U/L)	AST (U/L)	Creatinine (mg/ 100ml)
I, Control (UN)	11.88 ±0.25	6.53 ±0.11	2.49 ±0.24	48.86 ±2.90	74.96 ±4.84	19.10 ±0.68	24.99 ±1.99	36.47 ±4.23	26.76 ±3.14	1.69 ±0.20
II, Control (DW)	12.04 ±0.30	6.51 ±0.16	2.63 ±0.25	52.17 ±2.63	80.28 ±4.22	18.56 ±0.84	23.31 ±1.31	39.73 ±6.22	22.88 ±0.82	1.84 ±0.16
III, Control (OL)	11.84 ±0.27	6.40 ±0.15	3.09 ±0.25	48.43 ±1.43	75.84 ±2.94	18.55 ±0.72	24.47 ±0.44	43.80 ±5.82	24.32 ±3.75	1.50 ±0.19
IV, Aqueous extract	12.20 ±0.27	6.47 ±0.15	2.35 ±0.30	47.76 ±2.54	74.39 ±5.55	18.90 ±0.66	25.73 ±1.08	42.99 ±9.36	20.43 ±3.34	2.08 ±0.25
V, Ethanol extract	12.24 ±0.22	6.68 ±0.05	3.20 ±0.17	47.65 ±1.34	71.30 ±1.69	18.32 ±0.40	25.75 ±0.77	32.24 ±7.34	30.40 ±3.58	2.15 ±0.33
VI, Benzene extract	12.04 ±0.22	6.53 ±0.15	3.30 ±0.17	46.61 ±1.79	71.6 ±3.38	18.47 ±0.57	26.10 ±1.10	42.17 ± 5.97	28.28 ±3.04	2.07 ±0.58

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 IV and V by ANOVA followed by Newman-Keuls' multiple range test.

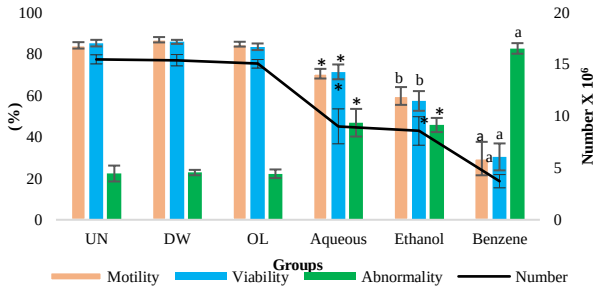

Figure 4. Effect of Hibiscus leaf extracts (100 mg/kg BW/d for 35 d) on sperm parameters.

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Values are mean ± S.E.M. for five animals; *significantly ($p<0.05$) different from controls;); [a] significantly different from controls and those in groups IV and V; [b] significantly different from controls and those in groups IV by ANOVA followed by Newman-Keuls' multiple range test.

Sialic acid and fructose levels: The level of sialic acid in the epididymis and that of fructose in seminal vesicle reduced significantly in Hibiscus-treated mice compared to controls, though, no significant differences were observed concerning the above parameters among treated groups (Figure 5).

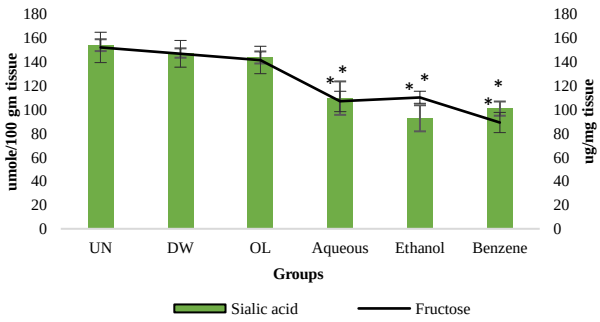

Figure 5. Effect of Hibiscus leaf extracts (100 mg/kg BW/d for 35 d) on the level of sialic acid in the epididymis and that of fructose in the seminal vesicle.

Values are mean ± S.E.M. for five animals; *significantly different from controls ($p<0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

Hematology and Serum biochemistry: The mean counts of RBC and WBC, and indices Hb, Hct, MCV, MCH, and MCHC remained unaltered in Hibiscus-treated mice compared to controls (Table 4). No significant differences were found in serum levels of ALT, AST, and creatinine in Hibiscus-treated mice compared to controls (Table 4).

Fertility test and pregnancy outcome: Libido was not affected in Hibiscus-treated males that mated with females after the last treatment. A significant reduction was noticed in the number of live implants in females impregnated by Hibiscus-treated males compared to controls (see Table 5). However, the fertility reduced significantly for benzene extract-treated males; the reduction in the number of live implants in females impregnated by the above treated males was significantly high compared to those impregnated by males in other treated groups and controls (Table 5). Treatment with Hibiscus caused a significant increase in pre-implantation loss in females impregnated by extracts-treated males; though, the pre-implantation loss was severe in females impregnated by benzene extract-treated males compared to those impregnated by males in other treated groups (Table 5). The treatments, however, did not affect post-implantation loss in impregnated females compared to controls (Table 5).

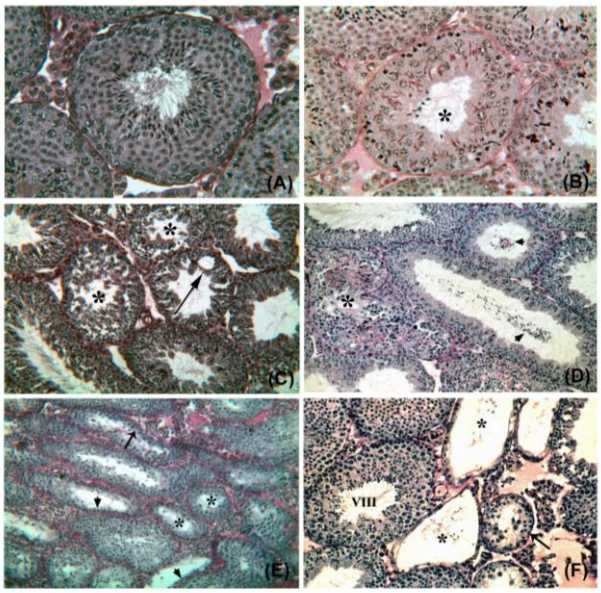

Figure 1 (A-D): Photomicrographs of PAS-H stained sections of mouse testis. (Original magnification: A-B: X 252; C-F: X 200). (A) OL-treated control showing normal spermatogenesis in the seminiferous tubules; (B) After treatment with aqueous leaf extract of H. rosa-sinensis (100 mg/kg BW/d for 35 d) showing the presence of spermatids of different stages in the same tubule (asterisk); (C) After treatment with ethanol extract (same dose and duration). Note the intraepithelial vacuolation (arrow) and loosening of the germinal epithelium (asterisk) in the seminiferous tubules; (D) After the same treatment as in (B) to show exfoliation of germ cells in tubules (arrowheads). Note a tubule (asterisk) filled with apoptotic germ cells; (E) After treatment with benzene extract (same dose and duration) showing the presence of degenerating tubules with

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reduced diameter (asterisk), disorganization (arrow) and marked thinning (arrowheads) of the germinal epithelium; (F) After the same treatment as in (E) to show atrophic (asterisk) and degenerating (arrow) tubules near a normal tubule (VIII).

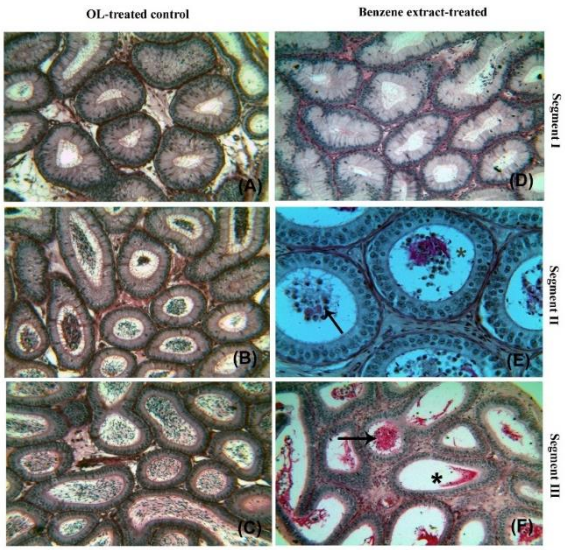

Figure 2 (A-F): Photomicrographs of PAS-H stained sections of mouse epididymis (segments I-III). (Original magnification: A-D and F: X 160; E: X 200). OL-treated control (A-C) showing normal histologic features of epithelium. Note the presence of sperm in segments II (B) and III (C); (D-F) After treatment with benzene extract of H. rosa-sinensis (100 mg/kg BW/d for 35 d) showing normal appearance of the segments, except that the lumen is devoid of sperm, and contains exfoliated germ cells (arrows) with accumulation of PAS-positive materials (asterisk).

5. Discussion

The findings of the present study in albino mice show that oral administration of the leaf extracts (aqueous, 95% ethanol, and benzene) of H. rosa-sinensis (100 mg/kg BW daily) for 35 d results in a significant reduction in the weight of testis accompanied with non-uniform histologic alterations in the seminiferous tubules. The affected tubules, in general, showed exfoliation of germ cells, intraepithelial vacuolation, loosening of germinal epithelium, presence of spermatids of different stages of spermatogenic cycle in the same tubule, failure of spermiation, and frequent occurrence of apoptotic and giant cells. Similar treatment-associated non-uniform alterations in mouse testis have also been described by different authors after treatment with Albizia lebbeck (L) Benth [26], Curcuma longa L. [27], Bacopa monnieri [28], Citrus limon [29], and Coccinia indica [30]. It has been anticipated that such non-uniform degeneration in the testis occurs because tubules in certain stages of spermatogenic cycle are sensitive to damage by several treatments [15]. This is established that stages VII-VIII of spermatogenesis are highly testosterone-dependent [31-32].

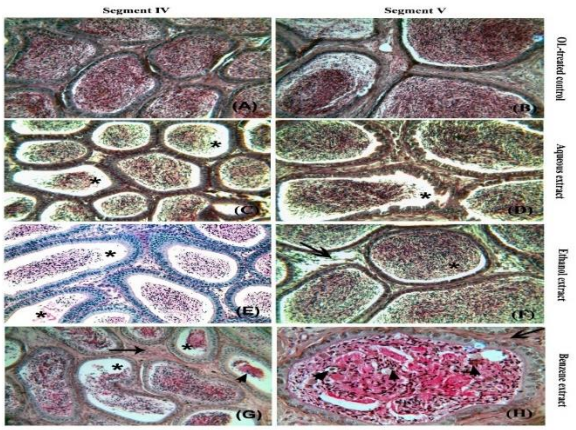

Figure 3 (A-H): Photomicrographs of PAS-H stained sections of mouse epididymis (segments IV-V). Original magnification: A-F: X 200; G: X 160; H: X 252). OL-treated control (A-B) showing normal histologic features of epithelium, and lumen distended with sperm; (C-H) After treatment with an aqueous extract (C-D), ethanol extract (E-F) and benzene extract (G-H) of H. rosa-sinensis (100 mg/kg BW/d for 35 d) showing relatively less number of sperm (asterisk) in tubular lumen; Segments IV (G) and V (H) of mice treated with benzene extract of H. rosa-sinensis (same dose and duration) showing the presence of sperm fragments and exfoliated germ cells (arrowheads) with the accumulation of PAS-positive materials in the tubular lumen. The lumen was relatively reduced in diameter, and an increase in stroma (arrow) was noticed in segments IV-V (G-H) of the epididymis.

Thus, a decrease in the frequency of stages VII-VIII of the spermatogenic cycle as observed in the present study in albino mice after treatment with benzene extract of Hibiscus, suggests that such non-uniform histologic alterations might be the result of testosterone deficiency in the testis. The present study also indicates that mice treated with benzene extract of Hibiscus showed severe alterations in their testes compared to those treated with

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other extracts of the plant; the percent frequency of affected tubules was significantly high (70.66 %) in the testes of the above treated mice compared to testes of mice in other treated groups.

Table 5: Effect of Hibiscus leaf extracts (100 mg/kg BW/d for 35 d) on libido and fertility of males, and pregnancy outcome in impregnated females.

Groups/ Treatments	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					
II, Distilled water	5	5	5	5	5	5	100 ±0.0	1.81 ±4.06	2.00 ±4.47	9.60 ±0.24	100 ±0.0
III, Olive oil	5	5	5	5	5	5	100 ±0.0	2.00 ±4.47	2.22 ±4.96	9.00 ±0.44	100 ±0.0
IV, Aqueous extract	5	5	5	5	5	5	100 ±0.0	41.08* ±16.68	4.00 ±8.94	6.00* ±0.63	100 ±0.0
V, Ethanol extract	5	5	4	5	5	4	100 ±0.0	55.93* ±26.86	2.85 ±6.38	4.40* ±1.16	80 ±20.0
VI, Benzene extract	5	5	1	5	5	1	100 ±0.0	84.00[a] ±35.77	2.50 ±5.59	1.40[a] ±1.39	20[a] ± 20.0

Values are mean ± S.E.M. 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.

Further, the testes in above treated mice frequently contained atrophied and degenerating seminiferous tubules consisting of Sertoli cells and a few germ cells. The presence of apoptotic germ cells and the formation of multinucleate giant cells in the germinal epithelium of tubules were very common, as observed in similar studies [33,34,29]; the percent frequency of tubules with unidentifiable stages (27.20 %) was also maximum in these treated mice. There were significant reductions in the height of germinal epithelium and the diameter of stage VII tubules in the testes of mice treated with benzene extract suggesting that Hibiscus interferes with spermatogenesis [29]. Treatment with benzene leaf extract of Hibiscus caused significant reductions in weights of epididymis and vas deferens, though, obvious histologic alterations were observed in epididymes and not in vas deferens of above treated mice. Similar to the testis, histologic changes in epididymis were severe in mice treated with benzene extract of Hibiscus. The lumen of segments IV-V of the epididymis in above treated mice frequently showed sperm fragments, exfoliated germ cells, and the accumulation of PAS-positive materials. Several authors have reported similar results after treatment with Curcuma longa [27], Citrus limon

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[29], and Coccinia indica [30], etc. Treatment with Hibiscus brought a significant reduction in the number of caudal spermatozoa, which further indicates the inhibitory action of Hibiscus on spermatogenesis. The epididymis is an important site of sperm maturation [35], and the changes in motility, viability, and morphology of spermatozoa might be the result of disturbance in the

secretory function of epididymis [36-38], since the level of sialic acid in epididymis reduced significantly in Hibiscus-treated mice. The treatment with benzene extract of Hibiscus caused a significant reduction in weight of the seminal vesicle accompanied by detectable histologic alterations; though, the level of fructose in the seminal vesicle decreased significantly in mice in all treated groups compared to controls. It is known that the structure and function of epididymis and seminal vesicle are principally dependent on testosterone, and therefore, histologic alterations in the above organs accompanied by a reduction in their weights, and a significant decrease in the levels of sialic acid and fructose indicate disturbance in the serum level of testosterone [39]. However, further studies are required to understand the mechanism of the antispermatogenic effect of Hibiscus.

Libido was not affected in Hibiscus-treated males; the fertility after the last treatment, however, was adversely affected in males in all treated groups as revealed by the reduced number of live implants in impregnated females; further, mice treated with benzene extract of Hibiscus showed remarkable reduction in fertility because of significantly increased pre-implantation loss in impregnated females. The antifertility effect of Hibiscus especially concerning benzene extract in males might be due to the adverse effect of Hibiscus on sperm parameters. The absence of any alterations in mean body weight and weights of the brain, liver, kidney, adrenal gland, and spleen, in histological features of liver and kidney accompanied by normal serum ALT, AST, and creatinine respectively, and unchanged hematological indices suggest that treatment with H. rosa-sinensis is not associated with toxic effects.

6. Conclusion

The results of the present investigation suggest that treatment with the benzene leaf extract of Hibiscus (H. rosa-sinensis) results in the suppression of spermatogenesis and fertility in albino mice without any toxic effects and, therefore, Hibiscus might be a source of a male contraceptive of plant origin.

Conflict of interest

The authors have no conflicts of interest regarding this investigation.

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