Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 16/10/2023;
Date Revised: 27/12/2023;
Date Published Online: 25/02/2024;

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How to Cite:
Okasha HS, Helal EGE, Mansoury MMS, Alsrehy GMA, Khattab HAH(2024). The Effects of *Hibiscus sabdariffa* and *Salvia officinalis* Aqueous Extracts on Reproductive Function, Antioxidant Status, and Epididymal Sperm Quality in Male Rats. Adv. Life Sci. 11(1): 104-111.

Keywords:
Hibiscus sabdariffa (*H. sabdariffa*); Salvia officinalis; Male fertility; Testosterone; FSH; LH; Prolactin; Testicular histology Humid subtropical climate; ITS; nrDNA; Taxonomy

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The Effects of *Hibiscus sabdariffa* and *Salvia officinalis* Aqueous Extracts on Reproductive Function, Antioxidant Status, and Epididymal Sperm Quality in Male Rats

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Abstract

Background: Phytoestrogens are non-estradiol molecules categorized as endocrine-disrupting chemicals, which means they can alter the hormonal equilibrium and cause reproductive problems. Studies suggested *Hibiscus sabdariffa* (*H. sabdariffa*) possible use as alternative to hormone replacement therapy in ovarian hypofunction patients. *Salvia officinalis* (*S. officinalis*) contains phytoestrogen steroids and isoflavonoids. The purpose of this research was to examine the estrogenic effects of *H. sabdariffa* and *S. officinalis* aqueous extracts on male reproductive hormones and testicular histology and function.

Methods: Three groups of six male rats each were utilized as follows: control (distilled water), *H. sabdariffa* (500 mg/kg aqueous extract), and *S. officinalis* (500 mg/kg aqueous extract). Blood, epididymis. Testis and serum samples were gathered after 30 days for biochemical analysis, sperm characteristics, and histological evaluations.

Results: Compared to the control and *S. officinalis* groups, *H. sabdariffa* caused a significant reduction in body weight (BW) and percent BW gain (%BWG). Relative to the control rats, the two extracts dramatically reduced testicular weight, sperm motility, concentration, and viability while raising abnormalities. Furthermore, the herbal extract groups demonstrated a significant decrease in blood testosterone levels while increasing serum prolactin, FSH, and LH levels. Consumption of both herbs also caused testicular histological changes such as degenerated seminiferous tubules, with a diminished number of mature spermatozoa in the tubular lumen, reduced diameter of seminiferous tubules, and the presence of exfoliated cells in the tubular lumen.

Conclusion: The results of this study revealed that the aqueous extracts of *H. sabdariffa* and *S. officinalis* may negatively impact male fertility. However, more research is needed to validate these findings and investigate the mechanistic components of these impacts.

Introduction

Phytoestrogens are secondary plants' metabolism. They have biological actions analog to estrogen in animals. Regular ingestion of non-steroidal estrogenic compounds can impact the reproductive functions in males [1]. Estrogenic-rich compounds are widespread in several herbs, seasonings, vegetables, fruits, and grains [2,3].

Roselle (*Hibiscus sabdariffa,* L, family Malvaceae), red sorrel, or Karkade, is a common garden plant native to warm-temperate, tropical, and subtropical regions as Egypt, Sudan, Saudi Arabia, India, and Nigeria [4]. *H. sabdariffa* extract is a prevalent medicinal plant; it acts as hypotensive, diuretic, antioxidant, and choleretic, stimulating the intestinal peristalsis, anti-inflammatory, hepatoprotective, antifungal, and anti cholesterol activities [5]. The therapeutic effects of *H. sabdariffa* extract are due to its content of numerous phytochemical constituents such as citric acid, anthocyanins, organic acids, protocatechuic acid, and other flavonoids, alkaloids, triterpenoids, polysaccharides, and steroids [6,7]. Besides *H. sabdariffa* being used as a beverage, it is also used to prepare various food products such as tea, puddings, jellies, jams, cakes, and syrup [8].

Several studies assessed the phytoestrogen effect of *H. sabdariffa* extract, which is rich in anthocyanin, and revealed that oral ingestion of its extract resulted in deterring the ovarian hypofunction adverse effects on memory performance *via* the modulation of estrogen receptor expression in ovariectomized female rats, thus suggested its possible use as alternative to hormone replacement therapy in ovarian hypofunction patients [9,10]. Previous studies revealed that *H. sabdariffa* calyces aqueous extract (rich in phytoestrogen) have endocrine-disrupting actions and causes a significant reduction in reproductive hormones in male rats [5,11].

Salvia officinalis L. (sage) Lamiaceae family is native to the Middle East and the Mediterranean, so it is named "Maramia" in Saudi Arabia [12]. Maramia is usually consumed as tea and in various cooking and medicinal preparations [13]. Various studies revealed that *S. officinalis* extract is used for several therapeutic and pharmaceutical purposes as hypoglycemics, anti-inflammatory, anticancer, antioxidant, hypolipidemic, and antimicrobial [14,15]. The biological-related activities of salvia are attributed to its active components such as phenolic compound, polyacetylene, glycosidic products (saponins, flavonoid, glycosides), terpenoids (mono-, di-, and triterpenoids), alkaloids, terpenes, and steroids [16]. Salvia contains phytoestrogen steroids and isoflavonoids, which can improve and recover infertility in females [16, 17].

Although the numerous therapeutic and pharmaceutical actions of *H. sabdariffa* and *S. officinalis* aqueous extracts, the estrogenic effect on male reproductive features should have been investigated. Therefore, this research was undertaken to compare the effects of *H. sabdariffa* and *S. officinalis* aqueous extracts on male rats' reproductive hormones and antioxidant status, as well as on testicular histopathological changes and epididymal motility and morphology of spermatozoa.

Methods

Plant materials

Dried calyces of *Hibiscus sabdariffa* (*H. sabdariffa)* and *Salvia officinalis* (*S. officinalis)* leaves were obtained from Harraz Medicinal and Herbal Company, Bab Al Khalq, Cairo, Egypt.

Extraction procedures of *H. sabdariffa*

H. sabdariffa aqueous extract was prepared according to the method of Nwabufo and Olusanya [5] with some modifications. Briefly, 100g of dried *H. sabdariffa* was soaked in 200 ml of boiled distilled water for 30 minutes and left to cool, then filtered and saved in dark bottles at 4 ºC until use.

Extraction procedures of *S. officinalis*

Aqueous extract of *S. officinalis* was prepared according to the procedure of Abd El-Motelp *et al.* [18] with some changes. Briefly, 50g of dried *S. officinalis* was soaked in boiled distilled water (100 ml) for 30 minutes and then left to cool. The cooled extract was filtered and stored in dark bottles at 4 ºC until use.

Experimental care and protocol

Male albino rats (*Rattus rattus*) (18 rats) weighing (120 - 150g) were used in the experiment. They were purchased from the animal unit of the El-Nile Comp for Pharmaceutical Products, Cairo, Egypt. Throughout the experiment, the animals were kept in metal cages under the standard humidity, temperature, and light/dark cycle conditions. Throughout the experiment, food and water were available. The experimental procedures were performed in the Faculty of Science, Al-Azhar University, Cairo, Egypt, Biology lab. Rats were given a week to acclimate pre-beginning the experiment. After acclimation time, rats were at random distributed to three groups, each with 6 rats, according to the following protocol:

- **A- Control group,** in which rats received distilled water by gavage.
- **B- *H. sabdariffa* group,** in which rats received *H. sabdariffa* aqueous extract by gavage (500 mg/kg) [19].

C- ***S. officinalis* group,** in which rats received *S. officinalis* aqueous extract by gavage (500 mg/kg) [20].

Initial body weight (BW) was measured at the beginning of the study; the final BW was measured at the end of the study. The percentage of BW gain (%BWG) was also computed. At the end of the experimental treatment, all rats were sacrificed. Samples of sperm were isolated from the left epididymis, and sperm characteristics were assessed. Blood samples were withdrawn from the retro-orbital sinus of each rat, then the separated plasma and serum were frozen till use.

Gonadosomatic index

The testicle weight of each rat was recorded, and the ratio of the testicle's weight to the BW was calculated. After that, the testes were preserved in formalin (10 %), while the epididymis was gathered for semen analysis.

Estimation of reproductive hormones

Serum follicle-stimulating hormone (FSH), testosterone, prolactin hormone, and luteinizing hormone (LH) were measured by ELISA procedure (LDN Labor Diagnostika Nord Gmbh & CO, KG kits, Germany; Kamiya Biomedical Company kits; USA. Genie kits, Ireland; and Abnova kit, Taiwan, respectively).

Estimation of lipid peroxidation and antioxidant indices

Superoxide dismutase (SOD), catalase (CAT), and lipid peroxide (MDA) were estimated using colorimetric kits (Biodiagnostic Co. Dokki, Giza, Egypt).

Estimation of epididymal sperm motility

Left caudal epididymis was excised using scissors in 10 ml saline (0.9% NaCl, at 37°C); the epididymal procedures were done as described by Sakhaee *et al.* [21]. Sperm quality was judged by sperm concentration, viability, motility, and abnormality. Sperm concentration was measured by a Neubauer hemocytometer (deep1/10mm, LABART, Darmstadt, Germany) and expressed as x 10^6 ml. Sperm motility was determined by counting the non-motile and motile spermatozoa and expressed as motility percent. Sperm viability was determined by the Eosin-Nigrosine staining. Sperm suspensions (one drop) were mixed with Eosin Y 1% (one drop). Three drops of Nigrosine 10 % were added after 30 seconds and mixed well. One drop of the mixture was placed on a clean glass slide to make a smear, then allowed to air dry. The slide was studied and counted under a light microscope (dead spermatozoa were pink-stained, and live spermatozoa were unstained). The spermatozoa viability and abnormalities were expressed as percentages.

Examination of the morphology of spermatozoa

The sperm solution samples were placed on clean glass slides. Smears were created and allowed to air dry, placed in methanol for 15 min, then stained with Eosin-Nigrosine for 15 min. It was examined under a microscope with 400 x magnification. The sperm morphology includes normal live and dead sperm and other abnormal types using a standard procedure [22].

Histopathological examination

Testis samples were excised and fixed in formalin (10 %); after applying the procedures of preparing the samples, the sections were stained with hematoxylin and eosin. The examination studies were conducted through a Zeiss light microscope. The seminiferous tubules and various cells in the seminiferous epithelium were examined, and photomicrographs were made.

Statistical analysis

Statistical significances were tested by ANOVA, Tukey's post hoc test using SPSS 27. Results were expressed as mean ± SD and were considered statistically significant at $p \leqslant 0.05$.

Results

Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on BW and % BWG. The initial BW, final BW, and %BWG were presented in Figure 1. There were no significant differences among all the experimental groups concerning the initial BW. However, the obtained data indicated a significant decrease in the final BW and %BWG in *H. sabdariffa* group relative to the control group. Besides, significant decreases in the final BW and %BWG of *H. sabdariffa* group relative to the *S. officinalis* ingested group. No significant difference in biological evaluation was found between *S. officinalis* group and the control group.

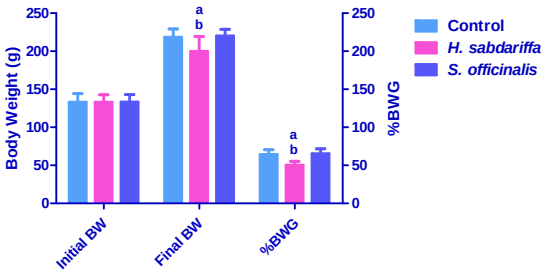

Figure 1: Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on biological evaluation (the initial body weight (BW), final BW, and the percentage BW gain (%BWG)) determined in male rats. Significance was considered at $p \leq 0.05$. Data were represented as mean ± SD (n = 6). [a]Significant *versus* the control group; [b]Significant *versus* the *H. sabdariffa* group.

Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on the testicular weights and the gonadosomatic indices

The testicular weights and the gonadosomatic indices were presented in Table 1. Administration of *H. sabdariffa* and *S. officinalis* aqueous extracts (30 days) resulted in significant a decrease in the testicular weights relative to the control group. However, the gonadosomatic indices showed non-significant changes in *H. sabdariffa* and *S. officinalis* groups relative to the control group.

Groups	Testicular weight (g)	Gonadosomatic index
Control	1.37 ± 0.04	0.63± 0.07
H. sabdariffa	1.28 ± 0.06[a]	0.64 ± 0.06
S. officinalis	1.26 ± 0.04[a]	0.57 ± 0.04

Significance was considered at $p \leq 0.05$. Data were represented as mean ± SD (n = 6). [a]Significant *versus* the control group.

Table 1: Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on testicular weight and gonadosomatic index determined in male rats.

Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on sperm characteristics

The sperm motility, count, viability, and abnormalities were presented in Table 2. Administration of *H. sabdariffa* and *S. officinalis* aqueous extracts for 30 days resulted in significant decreases in sperm motility, concentration, and viability relative to the control group. However, the sperms abnormalities percentages were significantly higher in H. sabdariffa and S. officinalis groups than in the control group. There were significant reductions in the sperm concentration and viability concurrent with a significant elevation in the sperm abnormalities percentage in the *S. officinalis* group relative to the *H. sabdariffa* group.

Groups	Sperm characteristics			
	Motility (%)	Concentration (10^6/ml)	Viability (%)	Abnormalities (%)
Control	53.17 ± 4.88	46.17 ± 4.83	66.83 ± 6.88	9.95 ± 1.46
H. sabdariffa	48.17 ± 3.31[a]	41.83 ± 4.62[a]	57.33 ± 5.28 [a]	13.00 ± 1.79[a]
S. officinalis	47.50 ± 2.88[a]	29.83 ± 2.14[a,b]	46.40 ± 4.72[a,b]	31.00 ± 3.35[a,b]

Significance was considered at $p \leq 0.05$. Data were represented as mean ± SD (n = 6). [a]Significant *versus* the control; [b]Significant *versus* the *H. sabdariffa* group.

Table 2: Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on sperm characteristics in male rats.

Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on the morphology of semen samples

The morphology of semen samples was presented in Figure 2. Normal live sperm of the control group was presented in Figure 2 A. Sperms of *H. sabdariffa,* and *S. officinalis* groups showed many abnormalities concerning their morphological appearance including increased sperms with abnormal head (hookless) (Figure 2 B&C); abnormal tail (Figure 2 D); amorphous and banana shapes (Figure 2 E&F, respectively).

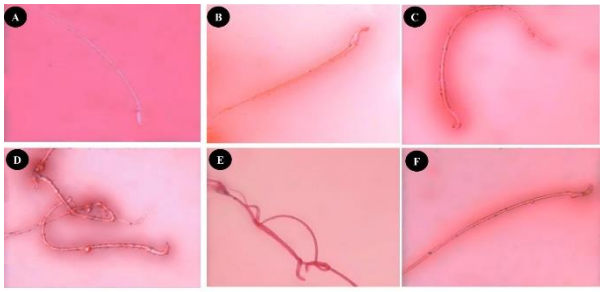

Figure 2: Photomicrographs illustrating sperm morphology normal and various sperm abnormalities (stained by Eosin-Nigrosin stain and original magnification x 400). A- Normal live; B&C- Hookless sperm; D- Abnormal tail; E- Amorphous shape; F- Banana shape.

Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on serum levels of reproductive hormones

The serum levels of testosterone, prolactin, FSH, and LH were presented in Figure 3. Administration of *H. sabdariffa* and *S. officinalis* extracts for 30 days resulted in significant disturbances in the male rats' serum levels of reproductive hormones. There was a significant decline in serum level of testosterone (Figure 3 A) concurrent with significant elevations in the serum prolactin (Figure 3 B), FSH (Figure 3 C), and LH (Figure 3 D) levels in the groups ingested *H. sabdariffa* and *S. officinalis* relative to the control group.

Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on serum levels of reproductive hormones

The serum levels of testosterone, prolactin, FSH, and LH were presented in Figure 3. Administration of *H. sabdariffa* and *S. officinalis* extracts for 30 days resulted in significant disturbances in the male rats' serum levels of reproductive hormones. There was a significant decline in serum level of testosterone (Figure 3 A) concurrent with significant elevations in the serum prolactin (Figure 3 B), FSH (Figure 3 C), and LH (Figure 3 D) levels in the groups ingested *H. sabdariffa* and *S. officinalis* relative to the control group.

Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on plasma antioxidant indices

The plasma levels of MDA, CAT, and SOD were presented in Table 3. Administration of *H. sabdariffa* and *S. officinalis* extracts for 30 days resulted in non-significant disturbances in the antioxidant indices of the male rats. There were no significant elevations in plasma level of MDA concurrent with non-significant

reductions in plasma levels of CAT and SOD in *H. sabdariffa* and *S. officinalis* groups relative to the control group.

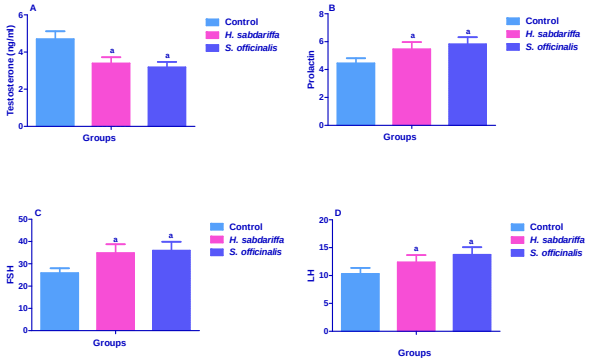

Figure 3: Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on serum levels of reproductive hormones (A: testosterone, B: prolactin, C: follicle-stimulating hormone (FSH), and D: luteinizing hormone (LH)) in male rats. Significance was considered at $p \leq 0.05$. Data were represented as mean ± SD (n = 6). [a]Significant *versus* control.

Groups	MDA (nmol/ml)	CAT (U/L)	SOD (U/ml)
Control	7.97 ± 0.77	146.10 ± 13.81	5.19 ± 0.52
H. sabdariffa	8.55 ± 0.94	138.12 ± 10.59	4.87 ± 0.49
S. officinalis	8.77 ± 0.99	136.93 ± 11.95	4.65 ± 0.49

Significance was considered at $p \leq 0.05$. Data were represented as mean ± SD (n = 6).

Table 3: Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on plasma levels of antioxidant indices (malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) in male rats.

Histopathological examination of testicular tissues

The histopathological examinations of testicular tissues from all the study groups were presented in Figure 4. Examination of testicular tissues of the control rats showed normal spermatogenesis and structure of the seminiferous tubules Figure 4 A&B. Administration of *H. sabdariffa* aqueous extract resulted in degenerated, and irregularity of seminiferous tubules with a diminished number of mature spermatozoa in some the tubular lumen together with the presence of exfoliated cells and widening of the inter-tubular spaces Figure 4 C&D. Administration of *S. officinalis* aqueous extract resulted in noticeable irregularity and degenerated of the seminiferous tubules, diminished number of mature spermatozoa in the tubular lumen, reduced diameter of seminiferous tubules, intact basement membrane, and the presence of exfoliated cells in the tubular lumen Figure 4 E&F.

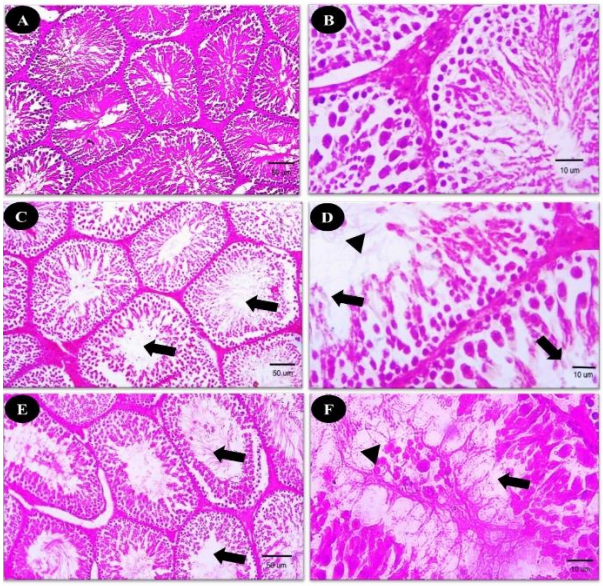

Figure 4: Effect of *H. sabdariffa* and *S. officinalis* aqueous extracts on histopathology changes in testis (H&E, bar 50 & bar=10 mμ). Photos A&B represented the testis of control rats and showed normal histological arrangements of spermatogonia in the seminiferous tubules with Sertoli cells resting on the intact basement membrane. Photos C&D represented testis of *H. sabdariffa* aqueous extract rats showed degenerated seminiferous tubules, with a diminished number of mature spermatozoa in some tubular lumen (arrow) and the presence of exfoliated cells in the tubular lumen (arrowhead). Photos E&F represented testis of *S. officinalis* aqueous extract rats showed degenerated seminiferous tubules, with a diminished number of mature spermatozoa in the tubular lumen (arrow), the reduced diameter of seminiferous tubules, intact basement membrane, and the presence of exfoliated cells in the tubular lumen (arrowheads).

Discussion

Endocrine-disrupting chemicals (EDCs) have a major influence on the body and have been proven to interfere with the functioning of cells, particularly through affecting hormone homeostasis [23]. Phytoestrogens are non-estradiol molecules categorized as EDCs, which means they can alter the hormonal equilibrium and cause developmental and reproductive problems [24,25]. This study aimed to examine the estrogenic effects of *H. sabdariffa* and *S. officinalis* aqueous extracts on male reproductive hormones and testicular histology and function.

The findings of this investigation demonstrated that *H. sabdariffa* aqueous extract significantly reduced the male rats' final BW and %BWG. It also reduced testicular weight considerably. The aqueous extract of *H. sabdariffa* dramatically reduced sperm motility, concentration, and viability while increasing their abnormalities. Concurrently, *H. sabdariffa* aqueous extract lowered serum testosterone levels while increasing serum prolactin, FSH, and LH levels.

Consumption of *H. sabdariffa* extract also caused testicular histological changes such as deteriorated seminiferous tubules, a decline in the quantity of mature spermatozoa, and the presence of exfoliated cells.

A previous study found that administration of *H. sabdariffa* calyx ethanolic extract (250 mg/kg) for 28 days to male rats significantly decreased serum testosterone (agree with our results), while no change was detected in prolactin or FSH levels (disagree with our results), besides no histological changes on the testes (agree with our results) [11]. Orisakwe *et al*. [26] findings showed that the aqueous *H. sabdariffa* calyx extract caused testicular damage in rats. According to their findings, sub-chronic administration of *H. sabdariffa* calyx aqueous extract (1.15, 2.30, and 4.60 g/kg) did not significantly alter absolute and relative testis weights (disagree with our results), significantly decreased final BW, significantly decreased epididymal sperm counts in the 4.60 g/kg group, deformed the seminiferous tubules and destroyed normal epithelial in the 1.15 g/kg group, caused testicular hyperplasia with basement membrane thickening the in the 2.30 g/kg group and degraded the sperm cells in the 4.60 g/kg group. By using transmission electron microscopy, it is clear that an aqueous extract from the dried calyx of *H. sabdariffa* alters normal sperm morphology and testis ultrastructure, adversely affecting reproductive function in male albino mice [27]. Wahabi *et al*. [28] linked the discrepancies in findings to a lack of standardization in the quantity and conditions of Hibiscus preparation, the apparent differences in the quantities of water and temperature utilized in preparation, and thus the yield and biological efficacy of anthocyanins administered to the animals.

H. sabdariffa extracts have been shown to be high in phytoestrogens like quercetin and daidzein [29]. In addition, *H. sabdariffa* contains large amounts of the polyphenolic compounds, anthocyanin, with potent estrogenic activity [30]. The findings presented by the present study suggested that the lowering of serum testosterone levels in male rats caused by *H. sabdariffa* extract might be explained by its anthocyanin content [31], and the plant's estrogenic action [26]. *H. sabdariffa* extract induces sperm head anomalies due to abnormal spermatogenesis that might result from hormonal changes, testicular DNA mutations, alerted chromatin condensation patterns, and acrosome development [27]. Furthermore, the present whole-testicular changes caused by *H. sabdariffa* extract are believed to be due to its hypotensive effect. The blood flow is particularly important to the testes as the tubules are avascular, and the impaired blood flow to the testicles can have serious impacts [32].

The findings of this investigation demonstrated that *S. officinalis* aqueous extract significantly reduced sperm motility, concentration, and viability while increasing their abnormalities. Concurrently, *S. officinalis* aqueous extract lowered serum testosterone levels while increasing serum prolactin, FSH, and LH levels. Consumption of *S. officinalis* extract also caused testicular histological changes such as degenerated seminiferous tubules, with diminished number of mature spermatozoa in the tubular lumen, reduced diameter of seminiferous tubules, and the presence of exfoliated cells in the tubular lumen.

A previous study Abdrabou *et al*. [17] found that ingestion of *S. officinalis* aqueous extract to female rats at doses of 60 mg/kg and 100 mg/kg significantly increased FSH and LH levels (agree with our results). This raise may be related to the occurrence of biologically active components identified in *S. officinalis* that can stimulate GnRH release and increase FSH and LH hormone synthesis in the pituitary [33]. In agreement with our results, Alrezaki *et al*. [34] also showed that *S. officinalis* extract (60 mg/kg) can boost the synthesis of FSH and LH hormones in mice. Moreover, it found that *S. officinalis* extract can potentiates the production of FSH and LH hormones in female rabbits [35]. Consistent with our results, administration of *S. officinalis* (3g/100g diet) to male mice significantly decreased seminiferous tubule diameter [36]. In contrast to our findings, the *in vitro* effects of *S. officinalis* ethanolic extract on the TM3 Leydig cell revealed that *S. officinalis* extract (200, 250, and 300 µg/ml) significantly increased testosterone production [37]. *S. officinalis* leaf hydroalcoholic extract has a stimulating effect on the male reproductive system, increasing blood testosterone levels and spermatogenesis (disagree with our results). *S. officinalis* extracts (150 and 200 mg/kg) increased seminiferous tubule width, and sperm count within the tubular lumen (disagree with our results). It did not significantly alter serum LH and FSH concentrations (disagree with our results) [38]. Concerning the effects of *S. officinalis* aqueous extract on the male rat reproductive system, we might attribute the disparities between our results and previously published data to various extraction procedures and kinds, apparent changes in dosages employed, and animal species used. Hence, more research is needed to resolve this issue.

The administration of *H. sabdariffa* and *S. officinalis* extracts to male rats for 30 days resulted in non-significant changes in antioxidant indicators. These are expected findings given that several published research have shown the antioxidant effects of these extracts [39,40].

The results of this investigation revealed that aqueous extracts of *H. sabdariffa* and *S. officinalis* may

affect male fertility by decreasing testosterone production, sperm counts, motility and viability, and increasing sperm abnormalities. The extracts also disrupted the normal histology of the testicular and seminiferous tubules. More research is needed to validate these findings and investigate the mechanistic components of these impacts.

Author Contributions

MSc. Hager S. Okasha, performing experiments and writing the article. Prof. Dr. Eman G.E. Helal, is responsible for supervision and reviewing the original article. Assoc. Prof. Manal M.S. Mansoury, writing and reviewing the original article. Assis. Prof. Ghadeer M. Alsrehy, writing and reviewing the original article. Prof. Dr. Hala A.H. Khattab, corresponding author, is responsible for supervision and writing and reviewing the original article.

Conflict of Interest

The authors declare that there is no conflict of interest.

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