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Effects of sub-chronic oral intake of Hibiscus sabdariffa calyx extract on some reproductive indices in adult male rats

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ABSTRACT

The effects of *Hibiscus sabdariffa* Linn (Malvaceae) calyx methanol extract (HSME) on some indices of reproduction in male rats were evaluated. Twelve weeks old male Sprague Dawley rats were randomly grouped (n=6) and orally administered HSME at doses of 200, 400 and 800 mg/kg daily for 13 weeks. Body weights of the rats were determined on days 0, 28, 56 and 91 of HSME administration. At the end of 13 weeks, serum testosterone levels and cauda epididymal sperm reserve were evaluated, and sections of the testes were subjected to histological examination. The extract was subjected to phytochemical analysis and acute toxicity (LD_{50}) test. Results showed a significant (P<0.05) increase in the testosterone level of animals administered 200 and 400 mg/kg and a significant (P<0.05) decrease in those administered 800 mg/kg of HSME. The HSME (200-800 mg/kg) elicited a significant (P<0.05) and dose-related reduction in the epididymal sperm count, but did not cause any visible pathological lesions in the testes. There was a significant (P<0.05) reduction in weight gain of the HSME-treated animals compared to control. The HSME tested positive for anthocyanins, alkaloids, coumarins, flavonoids, proteins and steroids, while the oral LD_{50} was estimated to be greater than 5000 mg/kg. The study demonstrated that sub-chronic daily oral intake of *H. sabdariffa* calyx extract at 400 mg/kg or less increased testosterone level, while higher doses produced remarkable reduction; also the extract (200-800 mg/kg) reduced cauda epididymal sperm count, but did not cause any obvious pathological lesions in the rat testes.

KEYWORDS: Cauda epididymal sperm reserve, serum testosterone, testes, zobo

INTRODUCTION

Hibiscus sabdariffa L. (Malvaceae), commonly known as roselle or red sorrel (English), 'Karkadeh' (Arabic) and 'Zobo' or 'Zoborodo' (Nigeria), is an annual, erect, bushy, herbaceous sub-shrub that grows up to 2.4 m tall, with smooth or nearly smooth, cylindrical and typically red stems. The morphological features are well described [1-3]. It is commonly grown in West and Central Africa, South East Asia, Mexico and some other parts of the world.

Beverages produced from the thick, fleshy, red, cup-shaped calyces of the flower are freely consumed in many tropical and sub-tropical countries. Extracts are also used in folk medicine as an aphrodisiac, and in the treatment of a wide array of diseases including high blood pressure, liver diseases and fever [2, 4-6]. The red anthocyanin pigment in the calyces is used as food colouring [7]. In a bid to unravel and characterize the pharmacological properties and potentials of this widely and freely consumed substance, *H. sabdariffa* extract has been the subject of a myriad of *in vitro* and *in vivo* studies, as well as some clinical trials [3].

Earlier studies demonstrated that sub-chronic administration of *H. sabdariffa* aqueous extract (1150 – 4600 mg/kg) reduced sperm count and spermatogenesis with marked degenerative histological changes on the rat testes [6]. Deleterious effects on the testes and spermatozoa and an adverse influence on the male reproductive

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fertility of albino mice were reported on daily administration of *H. sabdariffa* calyx aqueous extract (200 mg/kg) for 4 weeks [8]. In contrast, administration of *H. sabdariffa* aqueous extract for 10 weeks and hibiscus anthocyanins (50–200 mg/kg) for 5 days did not affect the male reproductive system in rats [9]. Previous related studies were done using 50-200 mg/kg and 1150 – 4600 mg/kg.

This study sought to evaluate the effects of sub chronic oral intake of *H. sabdariffa* calyx methanol extract (200 – 800 mg/kg) on serum testosterone, cauda epididymal sperm count and histo-architecture of the rat testes.

MATERIALS AND METHODS

Animals

Ten weeks old male Sprague-Dawley rats (115-140 g) and conventional grade UN-FERH:NS outbred strain of male albino mice (*Mus musculus*) (19–29 g) bred in the laboratory animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used for the study. The animals were individually housed in metal cages, and allowed to acclimatize for two weeks before the commencement of the study. They were maintained on standard pellets and clean drinking water. Animal experiments were done in compliance with National Institute of Health Guide for Care and Use of Laboratory Animals (Pub No. 85 – 23, revised 1985) and with prior permission from the National Health Research Ethics Committee (NHREC) of the University of Nigeria, with protocol ethical clearance number NHREC/05/01/2010C.

Preparation of extract

Hibiscus sabdariffa calyces were collected in September at Wukari, Taraba State, Nigeria. The plant material was identified and authenticated at the International Centre for Ethnomedicine & Drug Development (InterCEDD) Nsukka, Enugu State where a voucher specimen (InterCEDD/967), has been deposited. The calyces of *H. sabdariffa* were air dried under shade for 2 days and ground with a mechanical grinder into coarse powder. The powered material (1 kg) was extracted by maceration in methanol at room temperature (28 ±1 ˚C) for 24 h, and the mixture filtered using muslin cloth, and subsequently Whatman paper No. 2. The plant material was repeatedly washed with fresh solvent until the filtrate became clear. The filtrate was concentrated using a rotary vacuum evaporator under reduced pressure at 40°C to obtain 227.1 g of *H. sabdariffa* methanol extract (HSME; 22.71% w/w). The HSME was subjected to phytochemical analysis using standard procedures [10,11].

Acute toxicity

The acute toxicity and lethality of the HSME was determined using the method described by Lorke (1983) [12]. Briefly, nine mice randomly divided into three groups (n=3) were orally administered 10, 100, and 1,000 mg/kg of HSME respectively and observed for 24 h for death. Since no death was recorded, 1,600, 2,900 and 5,000 mg/kg of HSME were administered to a fresh batch of animals at one animal per dose; the number of deaths in 24 h was recorded. Also, animals were monitored for signs of toxicity.

Treatment of animals

Animals were randomly grouped (n=6) to receive oral administrations of HSME 200, 400 or 800 mg/kg for 13 weeks (91 days), control rats received distilled water (5 ml/kg). The animals were weighed every week and appropriate doses administered based on the body weight.

Testosterone assay

Prior to sacrificing each rat, 2 ml of blood was collected from the medial cantus of the eye into a test tube using non-heparinized micropipette capillary tube. The blood was left for 30 min to clot, subsequently the serum was harvested and used for the testosterone assay. The assay was carried out using ELISA technique [13]. The Testosterone AccuBind® Microplate Enzyme Immunoassay Test System (Monobind Inc., USA) was used for the quantitative determination of testosterone concentration.

Determination of cauda epididymal sperm count

At the end of 13 weeks treatment, the rats were sacrificed, the cauda epididymes dissected out, and extraneous tissues trimmed. The epididymes were put into a porcelain mortar and then ground thoroughly with a pestle to release the sperm cells contained in the highly convoluted tubules of the epididymes. Normal saline (10 ml) was poured into the porcelain mortar to homogenize the ground epididymis. The normal saline containing the epididymal sperm cells was filtered, and the filtrate transferred into a clean test tube. The filtrate of epididymal cell fluid (0.1 ml) was added to another test tube followed by 0.9 ml of formol saline. The

cauda epididymal sperm count was determined using the standard hemocytometric method [14].

Histomorpholophy of the testes

The testes were dissected and fixed by immersion in Bouin's fluid for 48 h. They were subsequently dehydrated in graded concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. The 5 µm thick sections were cut, mounted on glass slides and stained with hematoxylin and eosin (H&E) for light microscopy. Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic China group Ltd. 1999-2004).

Statistical analysis

Data obtained were analyzed using One-Way ANOVA in Graph Pad Prism 5 and subjected to Dunnett's Multiple Comparison Test. Results were presented as Mean±SEM, and differences between means of treated and control groups accepted significant at $P<0.05$.

RESULTS

Acute toxicity (LD_{50})

There was no mortality on administration of HSME up to 5,000 mg/kg, hence the oral LD_{50} was greater than 5,000 mg/kg. Also, no obvious signs of toxicity were observed in the animals at the administered doses.

Phytochemical analysis

The HSME tested positive for anthocyanins, alkaloids, coumarins, flavonoids, proteins and steroids (Table 1).

Table 1: Phytoconstituents of methanol extract of *H. sabdariffa* calyx

Phytoconstituent	Relative Presence
Alkaloids	+++
Anthocyanins	+++
Carbohydrates	—
Coumarins	+++
Fats and oil	—
Flavonoids	+++
Glycosides	++
Protein	++
Reducing sugars	+++
Resins	—
Saponins	—
Steroids	++
Tannins	+
Terpenoids	—

Key: - = absent; + = present; ++ = moderately present; +++ = abundantly present; HSME = methanol extract of H. sabdariffa calyx.

Table 2: Body weight of male rats administered varied doses of methanol extract of *H. sabdariffa* calyx

Treatment	Dose (mg/kg)	Body weight (g)			
		Pre-treatment	Week 4	Week 8	Week 13
Control	—	176.6±3.2	205.9±9.8 (14.23)	240.0±9.4 (26.42)	265.0±9.3 (33.36)
HSME	200	169.00±6.1	197.0±4.0 (14.21)	218.0±4.4 (22.48)	242.0±6.0 (34.34)
	400	162.5±0.9	191.3±5.2 (15.0)	206.3±5.5 (21.23)	247.5±9.7 (34.34)
	800	174.2±9.9	187.5±7.2 (7.09)	208.3±10.0 (16.37)	225.8±11.1* (22.85)

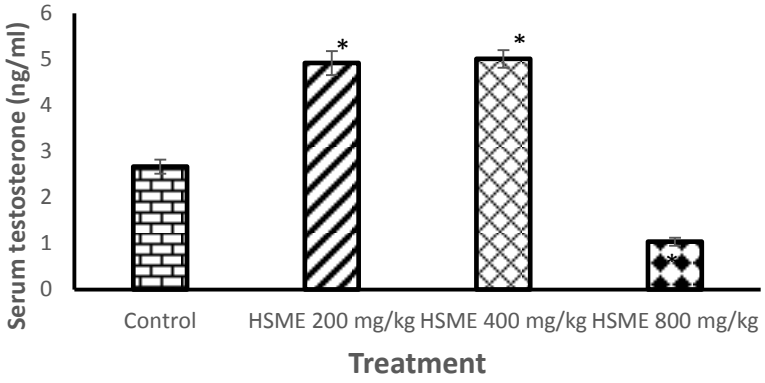
n=6; * reduced weight gain significant ($P<0.05$) compared to control (LSD post hoc test); value in parenthesis represent increase in weight (%) relative to pre-treatment value; HSME = methanol extract of *H. sabdariffa* calyx.

Effect of HSME on serum testosterone level

The HSME at 200 and 400 mg/kg evoked significant (P<0.05) and dose- related increase in serum testosterone level of 84.27 and 87.64% respectively, however 800 mg/kg elicited a significant (P<0.05) 61.05% decrease in testosterone level compared to control (Figure 1).

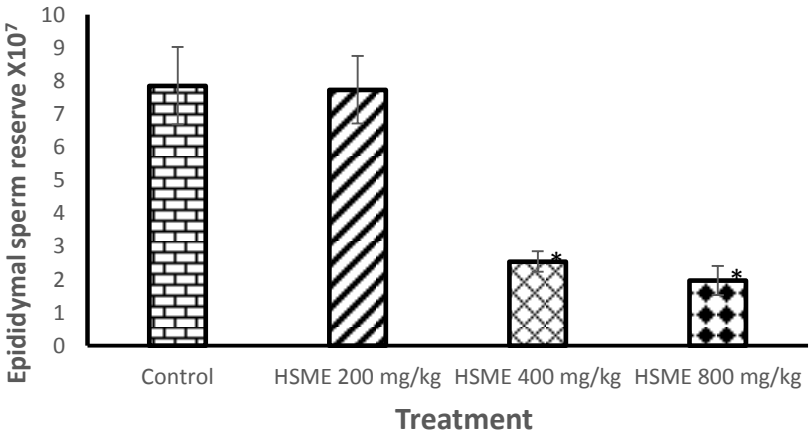
Effect of HSME on cauda epididymal sperm count

The HSME elicited dose-related and significant (P<0.05) inhibition of cauda epididymal sperm count, with 200, 400 and 800 mg/g causing 1.5, 67.6 and 74.9% inhibition respectively (Figure 2).



n=6; *P<0.05 compared to control (LSD post hoc test) HSME = methanol extract of *H. sabdariffa* calyx

Figure 1: Effect of methanol extract of *H. sabdariffa* calyx on serum testosterone level



n=6; *P<0.05 compared to control (LSD post hoc test); HSME = methanol extract of *H. sabdariffa* calyx

Figure 2: Effect of methanol extract of *H. sabdariffa* calyx on cauda epididymal sperm count

Effect of HSME on body weight

Rats treated with HSME 200 and 400 mg/kg gained weight, similar to that of control. However, the weight gain of the animals administered 800 mg/kg was significantly (P<0.05) lower than that of control (Table 2).

Histomorphology of the testes

Sections of the testes of control rats showed normal seminiferous tubules and interstices (Figure 3). There were no overt pathological changes in the seminiferous epithelia or the interstitial spaces of the testes of HSME- treated rats. The morphology of the testes of HSME- treated rats did not appear different from those of control rats (Figures 4 and 5).

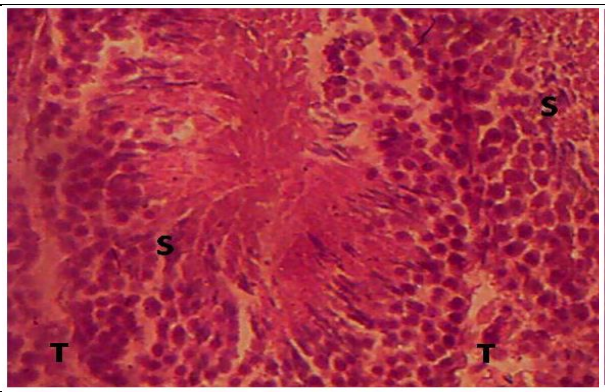


Figure 3: A representative histological section of testes of control rats administered distilled water for 13 weeks. Note active seminiferous tubules (S) and interstitial spaces (T) [H&E stain; x400].

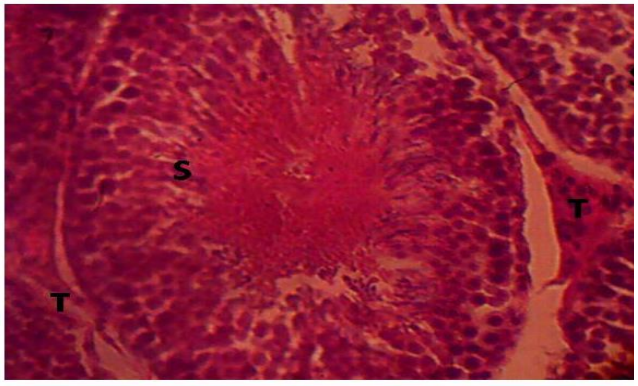


Figure 4: A representative histological section of testes of rats administered methanol extract of *H. sabdariffa* calyx (800 mg/kg) for 13 weeks showing no obvious lesions. Note active seminiferous tubules (S) and interstitial spaces (T) [H&E stain; x400].

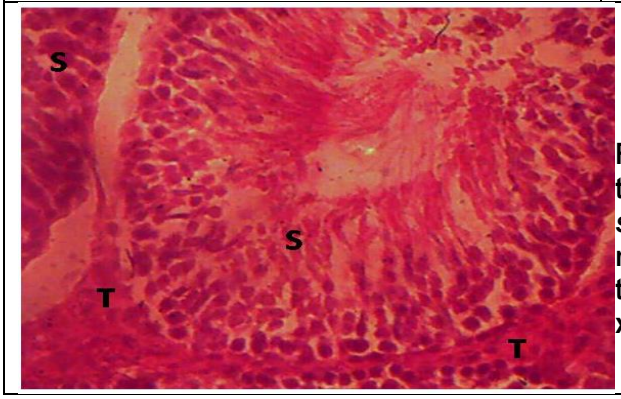


Figure 5: A representative histological section of testes of rats administered methanol extract of *H. sabdariffa* calyx (200 mg/kg) for 13 weeks showing no obvious lesions. Note active seminiferous tubules (S) and interstitial spaces (T) [H&E stain; x400].

DISCUSSION

H. sabdariffa calyx extract, a beverage freely consumed in Nigeria and many parts of the world, is used as an aphrodisiac in Northern Nigeria. Assessment of the effects of *H. sabdariffa* calyx methanol extract on some reproductive indices in male rats demonstrated that low doses increased testosterone level, while higher amounts reduced testosterone level. Furthermore, the extract reduced sperm count, though there were no visible pathological lesions in the testes of treated rats.

A number of exogenous substances may modulate the synthesis, secretion or actions of hormones. Results obtained from this study suggest that daily intake of about 400 mg/kg of the extract or less may increase the level of testosterone, while intake of higher amounts may reduce testosterone level. The principal source of testosterone in male is the testes. The regulation of serum testosterone is via a dynamic feedback interaction among the hypothalamus, anterior pituitary and testis. The hypothalamus synthesizes and releases gonadotropin releasing hormone (GnRH) into the hypothalamohypophyseal portal system. The GnRH stimulates release of pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which traverses to the testes to regulate testosterone synthesis and spermatogenesis respectively. While the major effects of LH are thought to be on testosterone synthesis by Leydig cells and that of FSH on spermatogenesis in the seminiferous tubules, there is a complex interaction/interplay of their roles [15]. The increase in testosterone levels elicited by low doses of HSME may have triggered a negative feedback response at both the hypothalamus and the pituitary level, resulting in the reduction of GnRH concentration, inhibition of pituitary gonadotropin, suppression of LH and FSH, and consequent reduction in the production of testosterone. This may account for the marked reduction in testosterone produced by 800 mg/kg.

Testosterone is one of the hormones essential for spermatogenesis; hence any substance that modulates testosterone level may affect spermatogenesis. The HSME elicited dose-related and significant reduction in epididymal sperm count,

with 800 mg/kg eliciting the highest reduction in sperm count. This indicates an adverse effect on spermatogenesis, possibly due to depletion of testosterone in the spermatogenic tissues, ultimately affecting the development and maturation of sperm. The result is consistent with that of other studies that reported reduced sperm count with intake of higher doses of *H. sabdariffa* calyx extract [6]. Spermatogenesis occurs in the walls of seminiferous tubules, and involves a series of mitotic and meiotic cell divisions, differentiation and maturation to produce the sperm [15]. Hormones that stimulate spermatogenesis include testosterone, LH, FSH, estrogens and growth hormone. The LH, FSH and growth hormone are anterior pituitary hormones, while testosterone and estrogen are produced in the gonads on stimulation by FSH and LH. Thus, the hypothalamus-anterior pituitary axis is essential for initiation and maintenance of spermatogenesis. Androgens suppress gonadotropin secretion when taken in high doses; this results in suppression of endogenous testicular function, with consequent decrease in endogenous testosterone and sperm production [16]. The marked increase in testosterone level (84-88%) evoked by HSME (200 and 400 mg/kg) may have triggered a negative feedback mechanism resulting in the reduction of GnRH concentration, inhibition of pituitary gonadotropin, and suppression of LH and FSH; ultimately, resulting in the marked decrease in testicular production of testosterone and spermatozoa at 800 mg/kg.

Animals treated with 800 mg/kg of HSME had reduced weight gain compared to those given lower doses, and the control rats. The antiobesity [17] and hyperlipidemic [3, 18-20] activities of *H. sabdariffa* calyx have been reported. Also, previous studies have reported the ability of *H. sabdariffa* calyx extract to cause weight loss/reduce weight gain [6,21]. These aforementioned effects have been attributed to decrease in food and fluid intake, inhibition of α-amylase, modulation of fat absorption-excretion, inhibition of lipid droplet accumulation and expression of preadipocytes [6, 22-27].

Though HSME reduced testosterone level and cauda epididymal sperm reserve, histological sections of the testes of the rats showed normal seminiferous tubules and interstitial spaces, and normal histomorphology, suggesting no overt pathological lesions on the tissue architecture of the testes. This observation suggests that consumption of moderate amount of HSME (≤ 800 mg/kg), may not have any direct toxic effect on the testes. This is particularly important considering earlier report of reduced sperm count and spermatogenesis with marked degenerative histological changes in rats administered high doses of *H. sabdariffa* aqueous extract for 12 weeks [6]. The HSME may have impaired spermatogenesis through disruption of the hypothalamus-pituitary-testis regulatory axis, and not through direct toxic effect on the testes. Testosterone, the principal androgen secreted by the testes, is formed by the interstitial cells of Leydig, which lie in the interstices between the seminiferous tubules [15]. Any damage or pathological lesion on the seminiferous tubules will adversely affect formation of sperm, consequently resulting in reduced sperm count. The observation that HSME did not have any direct toxic action on tissue architecture of the testes indicates that its effect on testosterone level and sperm count may derive from modulation of the hypothalamus-anterior pituitary axis. The reduced cauda epididymal sperm reserve observed in HSME-treated rats may be due to a decline in the influence of testosterone on spermatogenesis, possibly secondary to effect on the hypothalamus-anterior pituitary axis.

The oral LD_{50} of the LHE was greater than 5,000 mg/kg, suggesting that *H. sabdariffa* calyx may be regarded as safe, with remote risk of acute intoxication on consumption.

CONCLUSION

Sub-chronic daily oral intake of low to moderate amounts of *H. sabdariffa* calyx extract may increase and subsequently reduce serum testosterone, through a negative feedback mechanism involving the hypothalamus-anterior pituitary axis; this may ultimately reduce spermatogenesis. However, these doses elicited no overt pathological lesion on the testes.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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