CONFIDENTIAL

STUDIES &
CERTIFICATES

TESTOSURGE™

TESTOSURGE® is a natural, highly standardized ingredient proven in clinical studies to significantly increase total, biologically active & free testosterone. Designed for products to boost testosterone & increase performance.

Increases Bioavailable, Free & Total Testosterone Levels by increasing competitive binding to SHBG, steroidogenesis, and Aromatase & 5 -reductase Inhibition

10H
A cross-over clinical study assessed the effects of a single 600 mg dose of TESTOSURGE® on serum testosterone levels of 16 healthy sedentary male subjects. There was a significant increase in free, total and bioavailable testosterone levels after 10 hours.

PDE5
PDE5 enzyme regulates blood flow causing vasoconstriction. This hinders post-exercise recovery. TESTOSURGE® inhibits PDE5 activity and promotes vasodilation, which increases blood flow to the muscles, aiding in recovery. Testosurge exhibits 90% inhibition rate at 1000 µg/mL.

35mg
TESTOSURGE® was tested on male rats to see its effect on testosterone production and spermatogenesis. The results showed that Testosurge significantly increased testosterone levels and the number of normal sperm. This suggests that Testosurge may improve male fertility and reproductive health.

8W
A study was conducted to evaluate the effects of 500mg TESTOSURGE® supplementation on 30 resistance-trained males during an 8-week resistance training program. The study was randomized, double-blind, and placebo-controlled. The results showed a significant impact on body fat percentage, total and bioavailable testosterone levels. Importantly, the testosterone levels remained within the physiological limits.

Pharmacogn J. 2024; 16(1): 9-19
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Androgenic Efficacy and Mechanism of Glycosides-Based Standardized Fenugreek Seeds Extract Through Aromatase And 5-Alpha Reductase Inhibition

Urmila M Aswar[1], Savita R. Nimse[2], Prasad A. Thakurdesai[2,*]

Urmila M Aswar[1], Savita R. Nimse[2], Prasad A. Thakurdesai[2,*]

[1]Department of Pharmacology, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune, INDIA.

[2]Department of Scientific affairs, Indus Biotech Limited, 1, Rahul Residency, Off Salunke Vihar Road, Kondhwa, Pune 411048, INDIA.

Correspondence

Dr. Prasad Thakurdesai

Department of Scientific Affairs, Indus Biotech Limited, 1 Rahul Residency, Off Salunke Vihar Road, Kondhwa, Pune - 411048, Maharashtra, INDIA.

Tel. +91-20-26851239

E-mail: prasad@indusbiotech.com

History

- Submission Date: 05-10-2023;
- Review completed: 13-12-2023;
- Accepted Date: 18-12-2023.

DOI : 10.5530/pj.2024.16.2

Article Available online

http://www.phcogj.com/v16/i1

ABSTRACT

Introduction: Fenugreek seeds glycosides content have many health benefits. **Objective**: To evaluate the androgenic efficacy and probable mechanism of glycosides-based standardized fenugreek seed extract (SFSE-G) in laboratory rats. **Methods**: Male Wistar rats were administrated with 28-days of once-daily oral administration of SFSE-G (10 or 35 mg/kg) on sexual and orientational behavior with female rats, serum testosterone concentrations, weights of reproductive system-related organs (seminal vesicles, prostate, levator ani), nitric oxide level in penis homogenate, sperm count in the cauda epididymis, and testis histology were evaluated. Separate groups of rats with a positive control (testosterone propionate (10 mg/kg, s.c. bi-weekly) and vehicle control (distilled water) were maintained. In addition, the safety of acute intravenous administration of SFSE-G (1 mg/kg) on cardiovascular function parameters was evaluated. Moreover, the inhibitory potential of SFSE-G against aromatase and 5-alpha-reductase enzymes was evaluated *in vitro*. **Results**: Subacute administration of SFSE-G (35 mg/kg, oral) to male rats showed androgenic efficacy in sexual behavior (increased mounting and intromission latency and rearing), with increased weights of seminal vehicles, prostate and levator ani muscles, serum testosterone levels, sperm count, and penile NO concentration, while preserving the normal architecture of the testes. Acute intravenous administration of SFSE-G to rats increased intracavernous pressure but retained normal cardiovascular parameters, such as blood pressure, heart rate, and corrected QT interval (QTc). SFSE-G showed significant inhibition of aromatase and 5-alpha-reductase *in vitro*. **Conclusion**: SFFE-G exhibited significant androgenic and spermatogenic efficacy, mediated through testosterone metabolism inhibition, without affecting the cardiovascular system in laboratory rats.

Keywords: Androgenic, Fenugreek extract, Glycosides, Spermatogenic, Sexual Behavior, Testosterone.

INTRODUCTION

Testosterone, a potent male sex hormone, plays a vital role in antagonizing catabolic stress caused by daily physical challenges. After the age of 30 years in males, serum testosterone levels start dropping at about 1% per year and gonadal function slows down.[1] This can lead to multiple clinical manifestations such as decreased bone mass, erectile function, hematopoiesis, muscle mass, and strength.[2] Owing to its hydrophobic nature, most circulating testosterone is bound to plasma proteins, including sex hormone-binding globulin (SHBG) and albumin. The SHBG-bound fraction is irreversible and biologically inactive. Albumin-bound testosterone is readily dissociable and thus bioavailable as free testosterone (active) and circulating in the blood.[3] In middle-aged men, the rise in SHBG levels results in an age-dependent decline in free testosterone despite normal total testosterone levels. [4]

Both medical and surgical treatment modalities are available for testosterone deficiency and male sexual dysfunction. Testosterone replacement is useful for restoring health status. [5,6] In recent years, plant-based natural plant extracts have become a popular choice to overcome testosterone deficiency, maintain hormonal balance[7], and improve physical performance[8, 9] and sex life.[10] However, only a few contain standardized phytoconstituents with scientifically validated evidence of their efficacy and safety.

Recent scientific evidence has confirmed the benefits of a standardized extract of fenugreek (*Trigonella foenum-graecum* L. Family Fabaceae) seed as a dietary supplement for healthy volunteers for many exercise physiology applications[11], including anabolic[12] and androgenic activities[13]. Fenugreek seeds are known as spices and have a history of traditional medicinal use.[14, 15] In ethnobotanical literature, fenugreek seeds have been reported to have beneficial effects on the male reproductive system[16], including aphrodisiac potential[17] and endurance enhancement.[18] Fenugreek seeds are certified as GRAS (generally recognized as safe) items under clause §182.20 (Essential oils, oleoresins, and natural extractives including distillates) by the US Food and Drug Administration. Moreover, many standardized extracts of fenugreek seeds have demonstrated their safety for long-term use in humans.[16, 19]

Recently, glycoside-based standardized fenugreek seed extract (SFSE-G) was reported to increase bioavailable and free testosterone within physiological limits.[20] Furthermore, androgenic potential in sedentary and resistance-trained male subjects[13] with excellent preclinical safety profiles[21] has also been reported. In addition, anabolic action without the involvement of testosterone was reported by oral administration of galactomannan-based fenugreek seed extract (10 and 35 mg/kg).[22] As testosterone plays a vital role in the functional efficacy of SFSE-G in the maintenance of the erectile process, the enhancement of sexual desire

Cite this article: Aswar UM, Nimse SR, Thakurdesai PA. Androgenic Efficacy and Mechanism of Glycosides-Based Standardized Fenugreek Seeds Extract Through Aromatase And 5-Alpha Reductase Inhibition. Pharmacogn J. 2024;16(1): 9-19.

and anabolic activity is expected. However, role of testosterone in mechanism of anabolic and androgenic efficacy of SFSE-G is not yet investigated. Furthermore, long-term use of testosterone-boosting anabolic steroids[23] and testosterone replacement therapy[24] in men is reported with cardiovascular events. Therefore, the present study aimed to evaluate the functional efficacy and cardiovascular safety of subacute SFSE-G treatment in male rats.

MATERIALS AND METHODS

Animals

Male and female Wistar rats (150-200 g) were purchased from the National Toxicology Center, Pune, India. Male rats capable of mounting over female rats were selected to evaluate sexual behavior, following a previously reported procedure.[25] They were maintained at 25 ± 1 °C and relative humidity of 45–55% under a 12-h light and 12-h dark cycle. The animals had free access to food pellets (Chakan Oil Mills, Pune, India) and water. The experimental protocol was approved by the Institutional Animal Ethics Committee of Poona College of Pharmacy, Pune, India and was conducted according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA).

The chemicals

Sildenafil citrate (SIL) was a gift sample from Varma Pharmaceuticals (Pune, India). Testosterone enanthate solution (5 mg/ml in olive oil) was prepared from Testoviron ® (Zydus Healthcare Limited, Mumbai, India). All chemicals were of analytical grade and the solvents were of the highest purity.

The test compound, SFSE-G

The test compound, SFSE-G, is a standardized fenugreek seed extract with not less than 80% glycosides. It is available as a bioactive ingredient, Testosurge™. SFSE-G was prepared in a GMP-compliant manufacturing facility by Indus Biotech Limited (Pune, India) and used for the study. The raw material for SFSE-G, fenugreek seeds, was authenticated at the Agharkar Research Institute, Pune, India. The SFSE-G solution was freshly prepared daily for oral administration to rats in distilled water to obtain a dose volume of 10 ml/kg. A fresh solution of SFSE-G in sterile water for injection was used for the experiment involving intravenous administration. Testosterone solution was injected by subcutaneous (s.c.) route to rats at dose of 10 mg/kg.

Androgenic effects by observing sexual behavior in male rats

Male rats capable of mounting over female rats were selected to evaluate sexual behavior, as previously reported.[25] The male Wistar rats were weighed and divided into four groups of 6 rats each and treated orally once a day for the next 28 days as follows: (1) Vehicle, distilled water (10 ml/kg, p.o.) (VC group); (2) testosterone (10 mg/kg in sesame oil suspension, s.c.) bi-weekly (3) SFSE-G (10 mg/kg p.o.) alone, and (4) SFSE-G (35 mg/kg p.o.) alone; female rats were primed by sequential administration of estradiol benzoate (10 µg/kg body weight) and hydroxyprogesterone (1.5 mg/kg body weight), through subcutaneous injections, at 48 h and 4 h, respectively, to induce the estrous phase before the sexual behavior studies. Sexual behavioral studies were conducted in a separate room under dim red illumination. Male rats were placed in a rectangular plexiglass chamber 10 min before the introduction of a primed female for acclimatization to the chamber conditions. Primed females were then introduced into the chamber. The sexual (copulatory) behavior parameters[25, 26], such as mount frequency (MF), intromission frequency (IF), mount latency (ML), and orientational activities parameters, such as rearing and anogenital grooming, were observed and recorded by the observer blind to the treatments. The copulatory behavioral parameters included MF (the number of mounts without intromission from the time of introduction of the female until ejaculation), IF (the number of intromissions from the time of introduction of the female until ejaculation), ML (the time interval between the introduction of the female and the first mount by the male), orientational activity parameters, including rearing (standing on rear limbs), and anogenital grooming (brushing and cleaning) related to both the anal and genital regions.

Measurement of serum testosterone levels

At the end of the treatment period, the rats were anesthetized with urethane, blood was withdrawn by retro-orbital puncture, and biochemical parameters were analyzed. On day 28, blood was withdrawn from each rat by using the retro-orbital plexus. Blood samples were centrifuged and analyzed for serum testosterone using a radioimmunoassay (RIA) kit (Enzo Life Sciences Kit, Biogenuix, New Delhi, India).

Measurement of NO level in rat penis homogenate

Rat penile homogenate was prepared using a previously reported method.[27] Briefly, the rats were sacrificed, and the penile bulb and shaft (excluding the skin and glans) were excised. The removed penis was treated with cold saline and homogenized with 1:10 w/v of cold 0.1M Phosphate Buffer Saline (pH 7.4), and homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was used to measure NO synthase (NOS) activity using the Griess reaction.[28] In the penis homogenate sample, nitrate was reduced to nitrite with the help of copper-cadmium alloy fillings.[29] In brief, 0.4 ml of homogenates/ standard nitrate was treated with 150 mg copper-cadmium fillings in a clean Eppendorf tube and was intermittently shaken for one h, followed by centrifugation for 10 min at 4000 rpm. Then, 10.0 µl of the sample (supernatant) was injected into the acid-iodide bath, and the corresponding change in current was recorded using a NO-measuring system (Innovative Instruments Inc, Mumbai, India).

Recording of reproductive organ weights

At the end of the treatment period, the rats were sacrificed using an overdose of urethane. Seminal vesicles, ventral prostate, skeletal muscle, and levator ani were carefully dissected and weighed. The reported dissection procedure for the isolation of the levator ani muscle was followed.[30] The body weight of the rats was recorded at the beginning and end of the experiment. An increase in the weight of the seminal vesicles and ventral prostate indicates an androgenic response, whereas a gain in weight of the musculus levator ani is considered an anabolic response.[30] The cauda epididymis was carefully removed to collect sperm and sperm (motile) counts were recorded.

Histology of testes

Testes from each group were removed and placed in a 10% formalin solution for 24 h. The organ specimens were subjected to dehydration by placing them three times in xylene (for one h each) and then in 70%, 90%, and 100% alcohol for 2 h. Infiltration and impregnation were performed by treatment with paraffin wax twice, each time for one hour. Paraffin wax was used to prepare paraffin molds. Specimens were cut into sections of 3-5 µm thickness. The sections were mounted on a glass slide using Distrene Phthalate Xylene and stained with hematoxylin and eosin (H and E).

Study of acute intravenous (i.v.) administration on cardiovascular parameters in male rats

In a separate set of experiments, the effects of intravenous administration of SFSE-G (1 mg/kg, i.v.) and SIL (1 mg/kg, i.v.) in the

male rats on blood pressure (BP), heart rate (HR), electrocardiogram (ECG), and intracavernous pressure (ICP) were investigated. ICP measurements were performed after cavernous nerve stimulation, as per a previously reported procedure.[31] The ICP is considered a measure of penile erection.[32] The rats were anesthetized using urethane, and their body temperature was maintained at 36–37 °C using heating pads. An intravenous line was established through the right external jugular vein for saline infusion and intravenous supplements of the anesthetic agent if needed. The trachea was cannulated to avoid respiratory disturbances and maintain the stable physiology of the rats throughout the procedure. The left internal carotid artery was cannulated, and blood pressure was recorded through perineal dissection. A surgical needle (27 G) filled with heparinized saline (250 units/ml) and PE10 tubing was inserted into the right crus to record ICP. Through abdominal dissection, the cavernous nerve was traced towards the penis.[33] The cavernous nerve was gently torn from the prostatic capsule and hooked to a stainless steel bipolar electrode for nerve stimulation. A distance of 1 mm was used to separate the two arms of the electrode; each arm was 0.2 mm in diameter. The stimulation parameters were 2 volts and frequency of 20 Hz, which produced consistent pressure recordings. The contact time was 45 sec per stimulation. At the end of the study, the animals were sacrificed and the wet weight of the prostate was measured in all groups.

Effect on aromatase using human breast epithelial cells

The quantitative inhibitory potential of SFSE-G against increased aromatase gene expression induced by Vitamin D3 (VD3) was evaluated in human breast epithelial cells (MCF7 cell line) at Abich S. r. l. (Verbania, Italy) using real-time polymerase chain reaction (RT-PCR).[34, 35] Briefly, a human epithelial cell line isolated from the breast tissue of a patient with metastatic adenocarcinoma (MCF7: ATCC code HTB-22, batch 70011012) was cultured, seeded in 24-wells plates and allowed to grow for 24 h at 37 °C in a 5% CO_2 incubator (Model CCL-170B-8, Esco Lifesciences, Rome, Italy). At the end of the incubation period, cell viability was assessed by incubating the cells for 2 h with MTT solution at sample concentrations of 0.1 ng/ml and 0.01 ng/ml. Cell viability was expressed in percentage terms: % of cell viability = [OD cells treated with sample/mean OD negative control] × 100. The average cell viability of the three replicates was calculated. Fresh medium containing 1-alpha,25-Dihydroxyvitamin D3 (VD3) at 100 nM concentration and supplemented with two viable dilutions of the tested sample concentrations in the culture medium was then added to the cells. Untreated cells in culture medium (Euroclone S.p.A., Pero, Italy) were used as a negative control (NC), and cells treated with medium containing only VD3 (100nM) were used as positive controls.

After 48 h of exposure, total Ribonucleic acid (RNA) was extracted and eluted in 50 µl of nuclease-free water using an AS1390 kit (Promega Corporation, Madison, WI, USA). The RNA concentration was quantified using a spectrophotometer (MySpec®, VWR International, USA). Total RNA (300 ng) was reverse-transcribed into cDNA using random primers with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA) according to the following protocol:25 °C for 10 min, 37 °C for 2 h, and 85 °C for 5 min.

The gene expression profile was analyzed by RT-PCR (QuantStudio 3, Thermo Fisher Scientific, MA, USA) using TaqManTM Fast Advance Master Mix (Life Technologies, CA, USA), ad-hoc specific commercially available primers, and a TaqMan probe for aromatase (CYP19A1). GAPDH: Hs99999905_m1 and CYP19A1: Hs00903411_m1 (Applied Biosystems, Waltham, MA, USA). Changes in gene expression profiles from triplicate readings were analyzed using the $2^{-\Delta\Delta Ct}$ method as a fold change[36], with GAPDH as a housekeeping gene. A fold change ≥ 1.5, together with $P < 0.05$ (vs. untreated cells) is an index of gene upregulation in VD3-only treated cells.[37]

Effect on 5-alpha-reductase (type 2) gene expression using human prostate epithelial cells

The inhibitory potential of SFSE-G for 5-alpha-reductase was evaluated using human prostatic epithelial cells immortalized with SV-40 (PNT2 cell line) at Abich S. r. l. (Verbania, Italy) by RT-PCR.[38] Briefly, PNT2 cells (ECAC, 95012613) were seeded in 6-well plates and incubated for 24 h at 37 °C in a 5% CO_2 incubator (Model CCL-170B-8, Esco Lifesciences, Rome, Italy). At the end of the incubation, a cell viability assay was performed by incubating the cells for two h with (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) solution at 6 different sample concentrations. Cell viability was expressed in percentage terms: % of cell viability = [OD cells treated with sample/mean OD negative control] × 100. The average cell viability of the three replicates was calculated.

Thereafter, the cultures were treated for 24 h with 10 ng/ml testosterone and fresh medium was added. The medium contained directly dissolved supplementation of SFSE-G at selected concentrations of 10 and 20 µg/ml, which were found to be sub-toxic in the cell viability assay. Untreated cells in RPMI culture medium (Euroclone S.p.A., Pero, Italy) were used as negative controls (NC). In contrast, cells treated with Saw Palmetto/*Serenoa repens* extract (Seppic, Courbevoie, France) at 10 µg/ml (SRE-10) which was used as a positive control.[39] Every sample was tested in duplicate.

After 48 h of exposure, total RNA was purified from cells using the RNAlater protocol (Thermo Fisher Scientific, MA, USA). After precipitation and centrifugation, RNA was collected and dissolved in 20 µL of sterile purified water, and its concentration was quantified using a spectrophotometer (MySpec®, VWR International, USA). Total RNA (300 ng) was reverse-transcribed into cDNA at 37 °C for 2 h in a thermal cycler using random primers following the manufacturer's instructions (Applied Biosystems, Waltham, MA, USA).

Changes in gene expression profiles were analyzed by quantitative polymerase chain reaction (qPCR) using SYBR Green real-time PCR master mix (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Changes in gene expression profiles from triplicate readings were analyzed using the $2^{-\Delta\Delta Ct}$ method as fold change and % fold change[36], with β-actin as a housekeeping gene. A fold change ≤ 0.5 or ≥ 2 compared to untreated cells, was taken as an index of gene expression inhibition. In addition, the data are transformed into a normal scale according to the following formula: Fluorescence intensity arbitrary unit normalized = 2^{-DCT} and expressed as a percentage as compared with NC.

Statistical analysis

The data of animal experiment are presented as the mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism version 4.03 for Windows (GraphPad Software, La Jolla, California, USA). Data for each parameter of body weight, organ weight, sperm count, serum testosterone levels, and penile NO concentration were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. The data for each of the parameters of sexual behavior were analyzed using the Kruskal-Wallis test, followed by Dunn's multiple comparisons test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of SFSE-G on body weight and reproductive organ weights in male rats

The body weight and reproductive organ weight data are presented in Table 1. Treatment of rats with testosterone (10 mg/kg, subcutaneous,

Table 1: Effects of SFSE-G (10 and 35 mg/kg, p. o. daily) on body and reproductive organ weights of rats.

	VC	Testosterone (10)	SFSE-G (10)	SFSE-G (35)
Body weight (g)	99.83 ± 1.08	108.00 ± 2.44**	106.00 ± 1.73*	113.00 ± 1.24***
Weight of Seminal vesicle (mg)	40.53 ± 3.87	254.17 ± 18.13***	63.30 ± 4.26^{ns}	145.33 ± 11.15***
Weight of Prostate (mg)	38.81 ± 3.74	143.10 ± 20.52***	47.75 ± 4.50^{ns}	65.55 ± 3.03^{ns}
Weight of Levator ani muscle (mg)	139.11 ± 11.63	297.74 ± 26.00***	178.68 ± 3.79^{ns}	215.32 ± 11.50**

n = 6, Data represented as mean weight (g.) ± SEM. Each parameter was analyzed using a separate one-way ANOVA followed by Dunnett's test. ns, not significant; * P < 0.05, ** P < 0.01, *** P < 0.001, compared to the VC group. VC – Vehicle control, SFSE-G - Glycosides based standardized fenugreek seed extract, Numbers in bracket indicate dose (mg/kg).

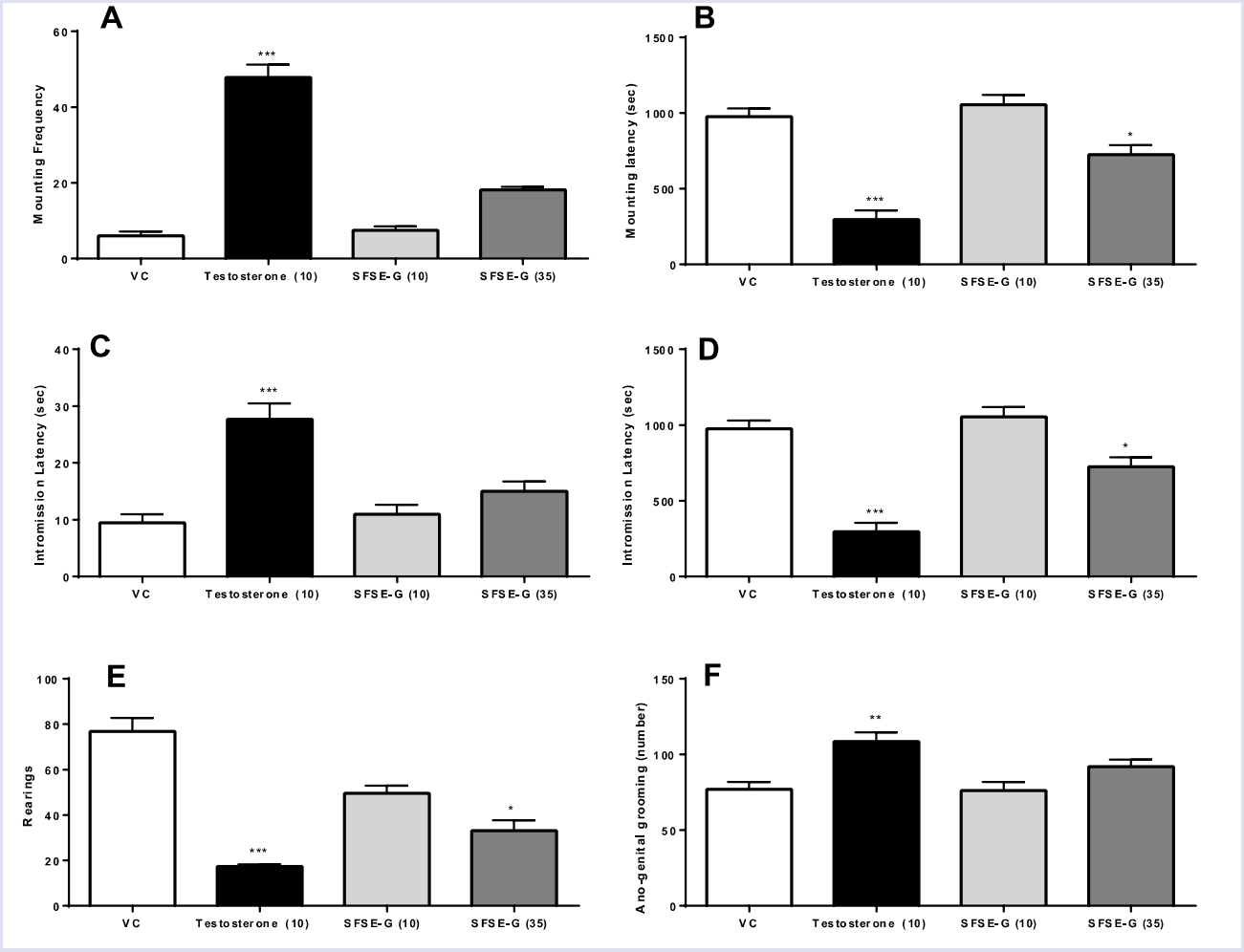


Figure 1: Effect of SFSE-G (10 and 35 mg/kg, p. o. daily) on sexual behavior-related parameters in male rats. n= 6, Data represented are as the mean ± SEM. Each parameter was analyzed using a separate one-way ANOVA followed by Dunnett's test. * P < 0.05, ** P < 0.01, *** P < 0.001 as compared to VC group. VC – Vehicle control, SFSE-G - Glycosides based standardized fenugreek seed extract, Numbers in bracket indicate dose (mg/kg).

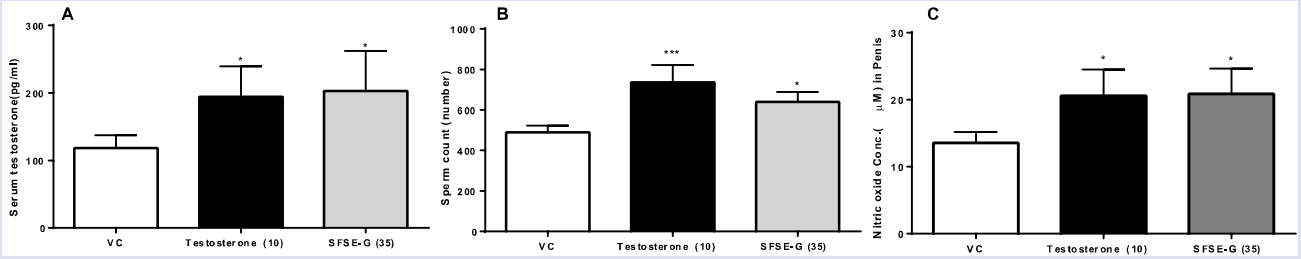


Figure 2: Effect of subacute oral administration SFSE-G (10 and 35 mg/kg) on (A) serum testosterone, (B) sperm count, (C) Penile nitric oxide (NO) concentration in male rats. n= 5, Data represented are mean ± SEM. Each parameter was analyzed using a separate one-way ANOVA followed by Dunnett's test. * P < 0.05, *** P < 0.001 as compared to VC group. VC, vehicle control; SFSE-G, glycosides-based standardized fenugreek seed extract; numbers in brackets indicate dose (mg/kg).

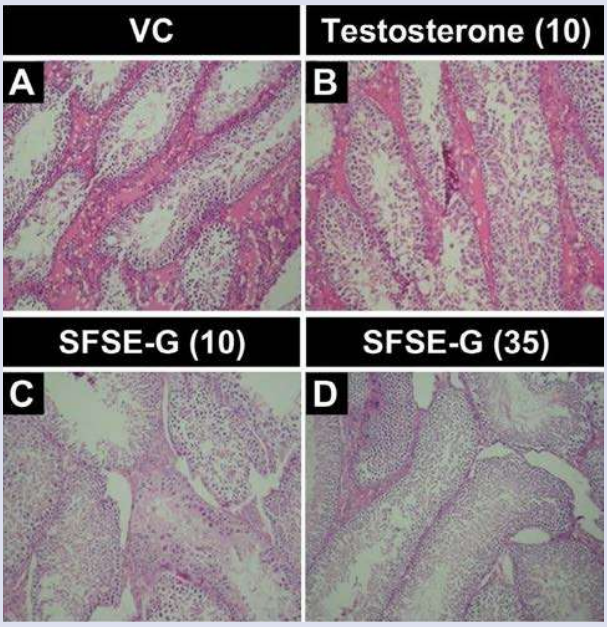


Figure 3: Photomicrographs of sections of representative testes of male rats showing the effects of subacute administration of (A) VC (vehicle control)) (B) testosterone (10 mg/kg, biweekly, subcutaneous), (C) SFSE-G (10 mg/kg, oral), and (D) SFSE-G (25 mg/kg, oral). H and E stain, ×100 magnifications. SFSE-G - Glycosides-based standardized fenugreek seed extract. Numbers in brackets indicate the dose (mg/kg).

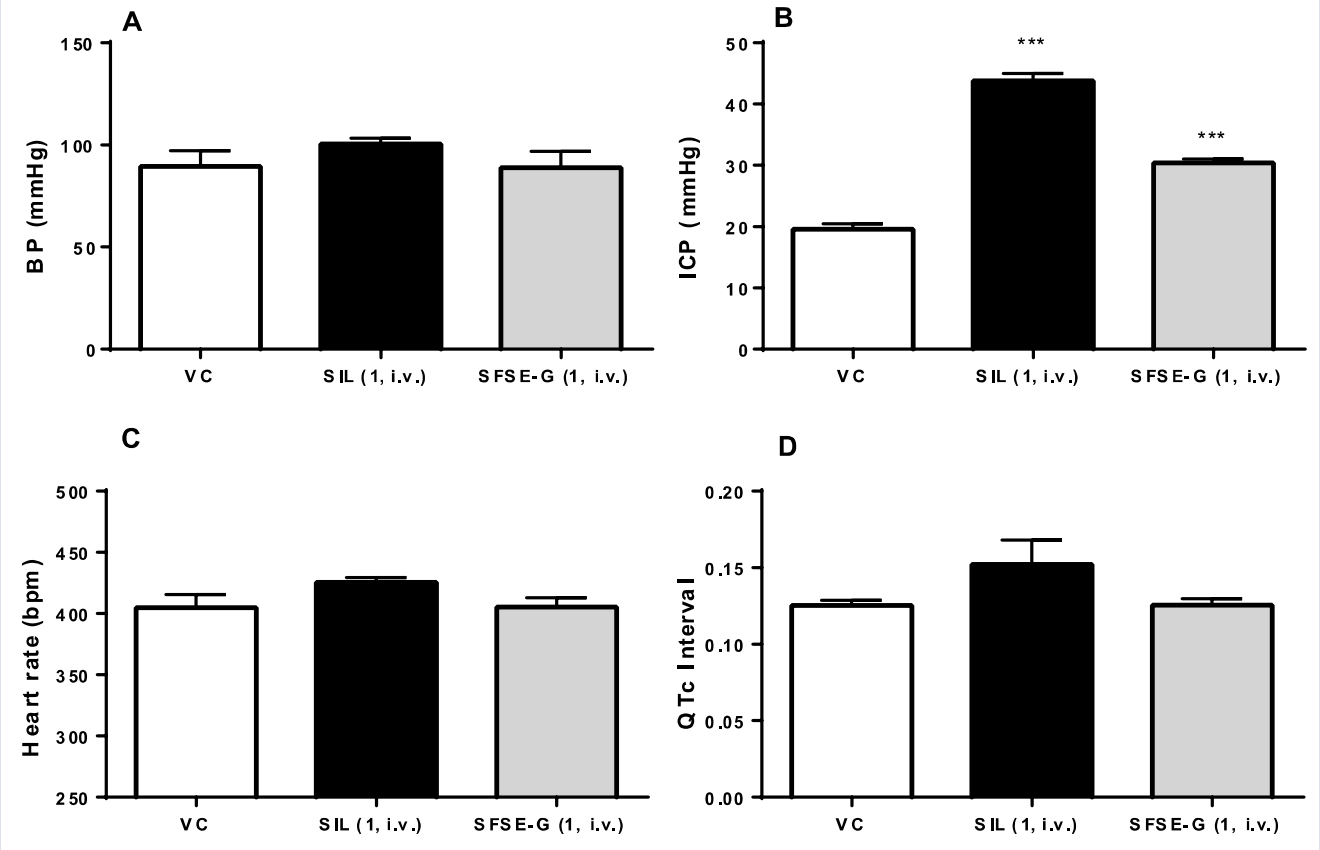


Figure 4: Effect of intravenous administration of SIL and SFSE-G at dose of 1 mg/kg on (A) blood pressure, (B) intracavernous pressure, (C) heart rate, (D) and QTc (corrected QT interval from ECG in male rats. n= 5, Data represented are mean ± SEM. Each parameter was analyzed using a separate one-way ANOVA followed by Dunnett's test. *** $P < 0.001$ as compared to VC group. VC, vehicle control; SIL, sildenafil; SFSE, glycoside-based standardized fenugreek seed extract. Numbers in brackets indicate the dose (mg/kg).

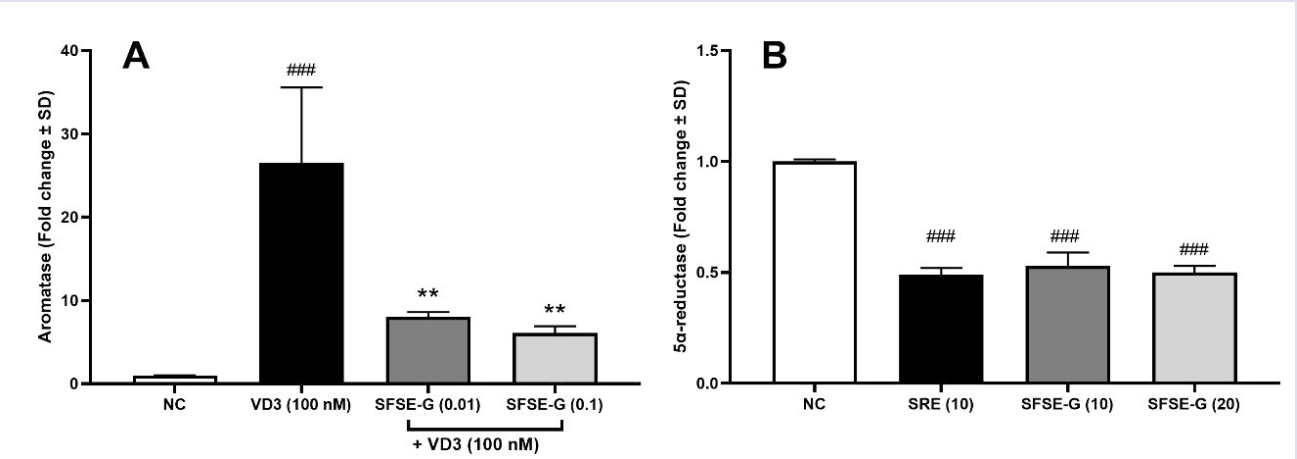


Figure 5. Effect of SFSE-G on gene expression in Fold change of enzymes (A) aromatase in human breast epithelial cells (MCF7 cell line) and (b) 5-alpha-reductase in human prostate epithelial cells (PNT2 cell line). The figures in parentheses indicate the dose in (A) ng/ml and (B) µg/ml. VD3, Vitamin D3 (1-alpha,25-Dihydroxyvitamin D3), NC: Negative control, SRE: *Serenoa repens* extract, SFSE-G: Glycosides based standardized fenugreek seed extract, ### P < 0.001 (v/s NC), ** P < 0.01 (v/s VD3).

bi-weekly for four weeks) showed a significant (P <0 .01) increase in body weight as compared to the VC group (from 99.83 g to 108 g). The body weight of SFSE-G (10 and 35 mg/kg)-treated rats showed significant increases of 6.18% and 13.19%, respectively, compared to the VC group. The weights of the seminal vesicle, prostate gland, and levator ani muscle of the testosterone group showed a significant (P < 0.001) increase as compared those with of the VC group. The SFSE-G (35) group showed a significant increase in the weights of the seminal vesicle and Levator ani muscle, but no changes in the prostate gland compared to the VC group. The seminal vesicles, prostate gland, or levator ani muscle of SFSE-G (10)-treated rats did not show a significant increase compared to the VC group.

Effects of SFSE-G on sexual behavior in male rats

The data obtained from the sexual behavior recordings are shown in Figure 1. The testosterone (10) group of rats showed a significant increase in MF and IF, whereas significant decrease in ML, IL and rearing was found compared to the respective VC group scores. The anogenital grooming frequency in the testosterone (10) group was significantly higher than that in the VC group. The SFSE-G (10 mg/kg)-treated rats did not show a significant change in any of the parameters compared with the VC group. The SFSE-G (35 mg/kg) treated rats showed a significant decrease in ML, IL, and rearing (but not in MF, IF and anogenital grooming scores) as compared to the VC group.

Effects of SFSE-G on serum testosterone levels, sperm count, penile NO levels in male rats

The data on serum testosterone level, sperm count, and penile NO concentration are presented in Figure 2. The testosterone (10) and SFSE-G (35) groups showed a significant increase in serum testosterone levels, sperm count, and penile NO concentration as compared to VC group.

Histopathology of testes

Photomicrographs representing the sections of the tastes of male rats are shown in Figure 3. The testes of rats from the VC, testosterone (10), SFSE-G (10), and SFSE-G (35) groups showed normal histological features with successive stages of transformation of the seminiferous epithelium into spermatozoa. No signs of toxicity to pachytene spermatocytes, germ cells, Leydig cells, or Sertoli cells were observed in any of the sections.

Effects of acute intravenous treatment of SFSE-G on cardiovascular parameters in male rats

The data of cardiovascular safety parameters, namely BP, ICP, HR, and corrected QT (QTc) interval on ECG, are presented in Figure 4. Acute treatment with SIL or SFSE-G-treated group (1 mg/kg, intravenous) did not show a significant change in BP, HR, or ECG parameters (including QTc interval) as compared to the VC group. However, both the SIL and SFSE-G groups showed a significant (P < 0.001) increase in ICP at a single dose of 1 mg/kg compared to the VC group.

Effects on aromatase inhibition

The cell viability assay showed cytotoxicity in MCF7 cells when the SFSE-G at (as low as 0.0001 mg/ml). The treatment of SFSE-G (0.1 and 0.01 ng/mL) in addition to VD3 (100 nM) to MCF-7 cell line showed more than 50% cell viability (98.48% and 95.60% respectively) at 48 h. The positive control, VD3 (100 nM), resulted in 76.09% viability, whereas the NC had 100% viability.

The 48-h treatment of VD3 (100 nM) to MCF7 cell showed 26.48-fold increase (P < 0.01) in aromatase gene expression (vs. NC). The addition of SFSE-G (0.01 and 0.1 ng/ml) to VD3 (100 nM) in MCF7 cell line for 48 h resulted in 8.03-fold and 6.08-fold (P < 0.05) gene expression (v/s NC), which are 70 % and 77 % inhibition as compared to VD3-induced aromatase gene expression in MCF7 cell line (Figure 5A)

Effect on 5-alpha-reductase (type 2) gene expression using human prostatic epithelial cells

The cell viability at various concentrations of SFSE-G (0.5, 0.25, 0.125, 0.06, 0.03, and 0.016 mg/ml) was 3.95%, 17.71%, 56.62%, 79.44%, 89.37%, and 96.05%, respectively. Concentrations that were lower than 1/5th of the IC_{50}, namely 10 µg/ml and 20 µg/ml, were selected for the 5-alpha-reductase inhibition assay. The positive control, SRE (10 µg/ml) treatment for 48 h, showed a 0.49-fold change (2.0-fold reduction, met acceptance criteria) in 5-alpha reductase expression. The 48-h treatment with SFSE-G (10 and 20 µg/ml) showed 0.53-fold and 0.50-fold changes (1.9- and 2.0-fold reduction, acceptance criteria met). The fold reductions in 5-alpha-reductase gene expressions showed by SRE and of SFSE-G treatments showed statistical significance as compared to NC (Figure 5B).

DISCUSSION

The physiologically beneficial properties of a wide range of plant-derived glycosides have been extensively reported and reviewed [40]. Natural glycosides are among the most potent natural androgenic compounds.[41, 42] Fenugreek seeds contain numerous furostenol[43, 44] and flavanol[45, 46] glycosides. The results of the present study indicate the role of testosterone in the androgenic potential of SFSE-G (improved sexual behavior of male rats) at 10 mg/kg and 35 mg/kg, whereas galactomannan-based standardized fenugreek seed extract did not show testosterone involvement.[22]

Existing data suggest a cause-and-effect relationship between serum testosterone levels and sexual function.[47] Decreased testosterone production is also associated with an age-related decline in libido (male menopause or andropause).[2] In the present study, the improvement in sexual behavior was noted by an increase in MF, IF, and anogenital grooming, and a decrease in ML IL and rearing by testosterone (10 mg/kg, twice a week) and SIL (5 mg/kg) treatment implied the desired component of sexuality. These results correlate well with the effects of testosterone replacement therapy[31] and SIL.[48, 49] Subacute oral treatment with SFSE-G (35 mg/kg) showed pro-erectile effects, like those of testosterone and SIL. In our study, SFSE-G was found to decrease rearing in male rats, which is an indication of increased sexual stimulation of male rats towards females. In the present study, subacute oral treatment with SFSE-G in male rats increased serum testosterone levels (like external testosterone treatment). These results are in line with reported testosterone-enhancing effects.[13, 20] This improved serum testosterone level probably contributed to enhanced sexual performance in male rats.

Various neurotransmitters and inter/intracellular signaling molecules, such as NO, are responsible for relaxation of the smooth muscle of the corpus cavernosum. Androgens that increase NO levels are known to benefit penile erection.[50] Testosterone and its metabolites play a direct role in erection by affecting NO synthase within the corpus cavernosum.[51] Androgen-dependent NO release in rat penises correlates with the levels of constitutive NO synthase isoenzymes.[52] In the present study, subacute administration of SFSE-G resulted in a significant increase in NO concentration in the penis, suggesting a role of NO in the action of SFSE-G. The levator ani is the largest muscle on the pelvic floor and plays a crucial role in male sexual function.[53] In the present study, the increase in levator ani weight after SFSE-G treatment may have resulted in improved sexual behavior in male rats. This notion was also supported by the increased ICP with acute intravenous SFSE-G treatment in the present study.

A direct correlation between serum testosterone levels and sperm count has been reported.[54, 55] The results of the present study are in line with these reports and show a significant increase in both serum testosterone levels and sperm count by external testosterone and SFSE-G treatment. These results are also supported by earlier reports of improved sperm counts by fenugreek seed extract in healthy volunteers.[56]

Testosterone plays a significant role in the central and peripheral neural pathways for the maintenance and restoration of erectile capacity.[57] Testosterone levels are affected by testicular conditions because the testes produce most testosterone in men. Therefore, histology of the testes was carried out during the present study. SFSE-G showed elevated sperm count through enhanced spermatogenesis via testosterone increase. The standard architecture of the testes with spermatogenesis stages was maintained in testosterone-treated and SFSE-G-treated rats and showed no toxicity. Furthermore, the enhanced sperm count in SFSE-G-treated rats in the present study and an earlier published toxicology study[21] support the morphological and functional safety of SFSE-G treatment in male rats.

Exogenous testosterone therapy is known to increase the risk of cardiovascular-related events.[58, 59] The increase in testosterone levels in males is known to shorten the action potential and QT interval in ECG.[60] Recently, the absence of structural damage to heart tissues with an oral subchronic (90-day) repeated dose of SFSE-G in male and female rats was reported.[21] The results of the present study indicate the absence of changes in BP, HR, and QTc interval with intravenous administration of SFSE-G and strongly support the functional safety of the cardiovascular system.

Agents that enhance testosterone levels may show unintended estrogenic effects such as hirsutism.[61] Testosterone is metabolized by the body by aromatase (alias CYP19A1) through aromatization, which plays a key role in maintaining the delicate balance between testosterone and estrogen.[62] Moreover, aromatase inhibitors has been reported to enhance sperm count.[63] However, an increase in aromatase can lead to excess estrogen production and a reduction in muscle tone and strength[64] and libido[65], increased abdominal body fat.[66]

In the present study, 48 h exposure of the MCF-7 cell line to SFSE-G potent inhibition of VD3-induced upregulation of aromatase gene expression. The aromatase inhibition properties correlated well with testosterone and sperm count enhancement efficacy demonstrated in the present study. Therefore, SFSE-G can be used safe supplementation for testosterone levels enhancement with aromatase inhibition to balance the hormonal profile in males in normal, aging or low-testosterone conditions.[67, 68]

Although testosterone itself does not cause Benign prostatic hyperplasia (BPH), its development requires the presence of testicular androgens during prostate development, whereas prostatic testosterone levels decline with age.[69] In addition, enhanced endogenous testosterone levels, especially in aging males, have been linked to the risk of BPH development.[70] This risk is attributed to the metabolism of testosterone to dihydrotestosterone (DHT), a more potent form of the hormone, by the 5-alpha reductase enzyme.[71]

DHT is predominantly generated by prostatic 5-alpha-reductase[72]. Higher DHT activity in prostate tissues is a permissive mediator of the development of BPH[69] and prostate cancer.[72] The enzyme 5-alpha-reductase exists in two forms, type 1 and type 2. Type 1 is produced primarily in liver and skin and is carried to the prostate, whereas Type 2 is the major form in the prostate.[73, 74] Therefore, 5-alpha-reductase type 2 inhibitors are typical therapeutic agents that are used against BPH to reduce DHT production and prostate size.[75] In the present study, the addition of SFSE-G to human prostatic epithelial cells (PNT2 cell line) inhibited 5-alpha-reductase (type 2) gene expression (1.9 to 2.0-fold), which is equivalent to the positive control, Saw Palmetto (*Serenoa repens*) extract. These results indicate the potential of SFSE-G as a safer agent for BPH prevention and management. However, specific studies on BPH in animals and/or clinical settings are required.

CONCLUSIONS

In conclusion, the glycoside-based standardized fenugreek seed extract showed significant androgenic and spermatogenic potential probably through aromatase and 5-alpha reductase inhibition, without affecting cardiovascular function in male rats.

CONFLICTS OF INTEREST

None.

ABBREVIATIONS

ANOVA : Analysis of variance; BP: Blood pressure; CPCSEA: Committee for Control and Supervision of Experiment on Animals; ECG: Electrocardiogram; GMP: Good Manufacturing Practices;

GRAS: Generally recognized as safe; H and E: Hematoxylin and eosin; HR: Heart rate; HR: Heart rate; ICP: Intracavernous pressure; IF: Intromission frequency; MF: Mount frequency; ML: Mount latency; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NC: Negative control; NO: Nitric oxide; NOS: Nitric oxide synthase; qPCR: Quantitative polymerase chain reaction; QTc: Corrected QT interval; RIA: Radioimmunoassay; RT-PCR: Real-Time polymerase chain reaction; S.C.: Subcutaneous; SEM: Standard error of the mean; SFSE-G: Glycosides based standardized fenugreek seed extract; SGBG: Sex hormone-binding globulin; SIL: Sildenafil citrate; SRE : Saw Palmetto/Serenoa repens extract; VC: Vehicle control; VD3: 1α,25-Dihydroxyvitamin D3

REFERENCES

1. Morales A, Heaton JP, Carson CC. Andropause: a misnomer for a true clinical entity. *J Urol.* 2000;163:705-712 https://doi.org/10.1016/s0022-5347(05)67788-9; PMid:10687961;
2. Utiger RD. Testosterone "fix": youth or consequences? Should we treat "male menopause"? *Health News.* 2003;9:3; PMid:12710389;
3. Bhasin S, Matsumoto AM. Patient information page from The Hormone Foundation. Patient guide to testosterone therapy in adult men with androgen deficiency syndromes. *J Clin Endocrinol Metab.* 2010;95:2536-2559 https://doi.org/10.1210/jc.2009-2354; PMid:20525905;
4. Yeap BB, Almeida OP, Hyde Z, et al. In men older than 70 years, total testosterone remains stable while free testosterone declines with age. The Health in Men Study. *Eur J Endocrinol.* 2007;156:585-594 https://doi.org/10.1530/EJE-06-0714; PMid:17468195;
5. Haider KS, Haider A, Doros G, Traish A. Long-Term Testosterone Therapy Improves Urinary and Sexual Function, and Quality of Life in Men with Hypogonadism: Results from a Propensity Matched Subgroup of a Controlled Registry Study. *J Urol.* 2018;199:257-265 https://doi.org/10.1016/j.juro.2017.07.039; PMid:28728990;
6. Corona G, Rastrelli G, Morgentaler A, Sforza A, Mannucci E, Maggi M. Meta-analysis of Results of Testosterone Therapy on Sexual Function Based on International Index of Erectile Function Scores. *Eur Urol.* 2017;72:1000-1011 https://doi.org/10.1016/j.eururo.2017.03.032; PMid:28434676;
7. Tiwari P, Sahu P. Plants altering hormonal milieu: A review. *Asian Pac J Reprod.* 2017;6:49-53 https://doi.org/10.12980/apjr.6.20170201;
8. Qureshi A, Naughton DP, Petroczi A. A systematic review on the herbal extract *Tribulus terrestris* and the roots of its putative aphrodisiac and performance enhancing effect. *J Diet Suppl.* 2014;11:64-79 https://doi.org/10.3109/19390211.2014.887602; PMid:24559105;
9. Kumar BS, Kumar JV, Selvaraj R. Aphrodisiac activity of *Cycas circinalis* L and *Ionidium suffruticosum* Ging on male wister albino rats. *Asian J Pharm Clin Res.* 2013;6:215-217;
10. Stasiak M, Zarlok K, Tomaszewski W. Erectile dysfunction - treatment with substances of natural origin. *Wiad Lek.* 2016;69:576-581; PMid:27717948;
11. Nagulapalli Venkata KC, Swaroop A, Bagchi D, Bishayee A. A small plant with big benefits: Fenugreek (*Trigonella foenum-graecum* Linn.) for disease prevention and health promotion. *Mol Nutr Food Res.* 2017;61:1600950 https://doi.org/10.1002/mnfr.201600950; PMid:28266134;
12. Poole C, Bushey B, Foster C, et al. The effects of a commercially available botanical supplement on strength, body composition, power output, and hormonal profiles in resistance-trained males. *J Int Soc Sports Nutr.* 2010;7:34 https://doi.org/10.1186/1550-2783-7-34; PMid:20979623; PMCid:PMC2978122.
13. Wilborn C, Taylor L, Poole C, Foster C, Willoughby D, Kreider R. Effects of a purported aromatase and 5alpha-reductase inhibitor on hormone profiles in college-age men. *Int J Sport Nutr Exerc Metab.* 2010;20:457-465 https://doi.org/10.1123/ijsnem.20.6.457; PMid:21116018;
14. Mohana L, Sandhya R, Kiran U. A review on diabetes milletus and the herbal plants used for its treatment. *Asian J Pharm Clin Res.* 2012;5:15-21;
15. World Health Organization. *WHO monographs on selected medicinal plants.* Geneva: World Health Organization; 2007.
16. Basch E, Ulbricht C, Kuo G, Szapary P, Smith M. Therapeutic applications of fenugreek. *Altern Med Rev.* 2003;8:20-27; PMid:12611558;
17. Santillo H, Dharmananda S. *Natural healing with herbs.* Prescott Valley, AZ: Hohm Press; 1984.
18. Ikeuchi M, Yamaguchi K, Koyama T, Sono Y, Yazawa K. Effects of fenugreek seeds (*Trigonella foenum greaecum)* extract on endurance capacity in mice. *J Nutr Sci Vitaminol.* 2006;52:287-292 https://doi.org/10.3177/jnsv.52.287; PMid:17087056;
19. Kandhare AD, Thakurdesai PA, Wangikar P, Bodhankar SL. A systematic literature review of fenugreek seed toxicity by using ToxRTool: Evidence from preclinical and clinical studies. *HELIYON.* 2019;5:e01536 https://doi.org/10.1016/j.heliyon.2019. e01536;
20. Mokashi M, Singh-Mokashi R, Mohan V, Thakurdesai PA. Effects of glycosides based fenugreek seed extract on serum testosterone levels of healthy sedentary male subjects: A exploratory double blind, placebo controlled, crossover study. *Asian J Pharm Clin Res.* 2014;7:177-181;
21. Deshpande P, Mohan V, Thakurdesai P. Preclinical safety assessment of glycosides based standardized fenugreek seeds extract: Acute, subchronic toxicity and mutagenicity studies. *J Appl Pharm Sci.* 2016;6:179-188 https://doi.org/10.7324/JAPS.2016.60927;
22. Aswar U, Mohan V, Bhaskaran S, Bodhankar SL. Study of galactomannan on androgenic and anabolic activity in male rats. *Pharmacologyonline.* 2008;2:56-65;
23. Baggish AL, Weiner RB, Kanayama G, et al. Cardiovascular Toxicity of Illicit Anabolic-Androgenic Steroid Use. *Circulation.* 2017;135:1991-2002 https://doi.org/10.1161/CIRCULATIONAHA.116.026945; PMid:28533317; PMCid:PMC5614517.
24. Elsherbiny A, Tricomi M, Bhatt D, Dandapantula HK. State-of-the-Art: a Review of Cardiovascular Effects of Testosterone Replacement Therapy in Adult Males. *Curr Cardiol Rep.* 2017;19:35 https://doi.org/10.1007/s11886-017-0838-x; PMid:28361372;
25. Park SW, Lee CH, Shin DH, Bang NS, Lee SM. Effect of SA1, a herbal formulation, on sexual behavior and penile erection. *Biol Pharm Bull.* 2006;29:1383-1386 https://doi.org/10.1248/bpb.29.1383; PMid:16819173;
26. Ageel AM, Islam MW, Ginawi OT, Al-Yahya MA. Evaluation of the aphrodisiac activity of *Litsea chinesis* (Lauraceae) and *orchis maculate* (Orchidaceae) extracts in rats. *Phytother.* 1994;8:103-105;
27. Seo SI, Kim SW, Paick JS. The effects of androgen on penile reflex, erectile response to electrical stimulation and penile NOS activity in the rat. *Asian J Androl.* 1999;1:169-174; PMid:11225889;
28. Kiechle FL, Malinski T. Nitric oxide. Biochemistry, pathophysiology, and detection. *Am J Clin Pathol.* 1993;100:567-575 https://doi.org/10.1093/ajcp/100.5.567; PMid:7504395;
29. Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors.* 2003;3:276 https://doi.org/10.3390/s30800276;
30. Eisenberg E, Gordan GS. The levator ani muscle of the rat as an index of myotrophic activity of steroidal hormones. *J Pharmacol Exp Ther.* 1950;99:38-44; PMid:15429015;
31. Gauthaman K, Adaikan PG, Prasad RN. Aphrodisiac properties of *Tribulus terrestris* extract (Protodioscin) in normal and castrated rats. *Life Sci.* 2002;71:1385-1396 https://doi.org/10.1016/s0024-3205(02)01858-1; PMid:12127159;

32. Chen KK, Chan JY, Chang LS, Chen MT, Chan SH. Intracavernous pressure as an experimental index in a rat model for the evaluation of penile erection. *J Urol.* 1992;147:1124-1128 https://doi.org/10.1016/s0022-5347(17)37500-6; PMid:1552608;

33. Martinez-Pineiro L, Brock G, Trigo-Rocha F, Hsu GL, Lue TF, Tanagho EA. Rat model for the study of penile erection: pharmacologic and electrical-stimulation parameters. *Eur Urol.* 1994;25:62-70 https://doi.org/10.1159/000475249; PMid:8307078;

34. Danza K, Porcelli L, De Summa S, et al. The ERRα–VDR axis promotes calcitriol degradation and estrogen signaling in breast cancer cells, while VDR-CYP24A1-ERR-alpha overexpression correlates with poor prognosis in patients with basal-like breast cancer. *Mol Oncol.* 2021;16:904-920 https://doi.org/10.1002/1878-0261.13013; PMid:34003583; PMCid:PMC8847991.

35. Chan HJ, Petrossian K, Chen S. Structural and functional characterization of aromatase, estrogen receptor, and their genes in endocrine-responsive and –resistant breast cancer cells. *J Steroid Biochem Mol Biol.* 2016;161:73-83 https://doi.org/10.1016/j.jsbmb.2015.07.018; PMid:26277097; PMCid:PMC4752924.

36. Jensen EC. Real-Time Reverse Transcription Polymerase Chain Reaction to Measure mRNA: Use, Limitations, and Presentation of Results. *Anat Rec.* 2011;295:1-3 https://doi.org/10.1002/ar.21487; PMid:22095866;

37. McCarthy DJ, Smyth GK. Testing significance relative to a fold-change threshold is a TREAT. *Bioinformatics.* 2009;25:765-771 https://doi.org/10.1093/bioinformatics/btp053; PMid:19176553; PMCid:PMC2654802.

38. Kang J-I, Kim E-JI, Kim M-K, et al. The Promoting Effect of *Ishige sinicola* on Hair Growth. *Marine Drugs.* 2013;11:1783-1799 https://doi.org/10.3390/md11061783; PMid:23708185; PMCid:PMC3721205.

39. Blair HA. Hexanic Extract of *Serenoa repens* (Permixon®): A Review in Symptomatic Benign Prostatic Hyperplasia. *Drugs Aging.* 2022;39:235-243 https://doi.org/10.1007/s40266-022-00924-3; PMid:35237936; PMCid:PMC9192452.

40. Patel S. Plant-derived cardiac glycosides: Role in heart ailments and cancer management. *Biomed Pharmacother.* 2016;84:1036-1041 https://doi.org/10.1016/j.biopha.2016.10.030; PMid:27780131;

41. Aswar U, Bodhankar SL, Mohan V, Thakurdesai PA. Effect of furostanol glycosides from *Trigonella foenum-graecum* on the reproductive system of male albino rats. *Phytother Res.* 2010;24:1482-1488 https://doi.org/10.1002/ptr.3129; PMid:20878698;

42. da Cruz AC, Guerra NG, de Souza KEBP, et al. The action of herbal medicine on the libido: aspects of nutritional intervention in increasing sexual desire. *Nutrire.* 2017;42:29 https://doi.org/10.1186/s41110-017-0051-0;

43. Kang LP, Zhao Y, Pang X, et al. Characterization and identification of steroidal saponins from the seeds of *Trigonella foenum-graecum* by ultra high-performance liquid chromatography and hybrid time-of-flight mass spectrometry. *J Pharm Biomed Anal.* 2013;74:257-267 https://doi.org/10.1016/j.jpba.2012.11.005; PMid:23245259;

44. Murakami T, Kishi A, Matsuda H, Yoshikawa M. Medicinal foodstuffs. XVII. Fenugreek seed. (3): structures of new furostanol-type steroid saponins, trigoneosides Xa, Xb, XIb, XIIa, XIIb, and XIIIa, from the seeds of Egyptian *Trigonella foenum-graecum* L. *Chem Pharm Bull (Tokyo).* 2000;48:994-1000 https://doi.org/10.1248/cpb.48.994; PMid:10923829;

45. Pang X, Kang LP, Yu HS, et al. New kaurene diterpenoid glycosides from fenugreek seeds. *Nat Prod Res.* 2013;27:1202-1207 https://doi.org/10.1080/14786419.2012.722087; PMid:22950814;

46. Rayyan S, Fossen T, Andersen OM. Flavone C-glycosides from seeds of fenugreek, *Trigonella foenum-graecum* L. *J Agric Food Chem.* 2010;58:7211-7217 https://doi.org/10.1021/jf100848c; PMid:20486688;

47. Marberger M, Roehrborn CG, Marks LS, Wilson T, Rittmaster RS. Relationship among serum testosterone, sexual function, and response to treatment in men receiving dutasteride for benign prostatic hyperplasia. *J Clin Endocrinol Metab.* 2006;91:1323-1328 https://doi.org/10.1210/jc.2005-1947; PMid:16434455;

48. Hadidi KA, Aburjai T, Battah AK. A comparative study of F*erula hermonis* root extracts and sildenafil on copulatory behaviour of male rats. *Fitoterapia.* 2003;74:242-246 https://doi.org/10.1016/s0367-326x(03)00032-7; PMid:12727488;

49. Behr-Roussel D, Gorny D, Mevel K, et al. Chronic sildenafil improves erectile function and endothelium-dependent cavernosal relaxations in rats: lack of tachyphylaxis. *Eur Urol.* 2005;47:87-91 https://doi.org/10.1016/j.eururo.2004.09.005; PMid:15582254;

50. Chamness SL, Ricker DD, Crone JK, et al. The effect of androgen on nitric oxide synthase in the male reproductive tract of the rat. *Fertil Steril.* 1995;63:1101-1107; PMid:7536692;

51. Zvara P, Sioufi R, Schipper H, Begin L, Brock G. Nitric oxide mediated erectile activity is a testosterone dependent event: a rat erection model. *Int J Impot Res.* 1995;7:209-219; PMid:8770664;

52. Marin R, Escrig A, Abreu P, Mas M. Androgen-dependent nitric oxide release in rat penis correlates with levels of constitutive nitric oxide synthase isoenzymes. *Biol Reprod.* 1999;61:1012-1016 https://doi.org/10.1095/biolreprod61.4.1012; PMid:10491638;

53. Cohen D, Gonzalez J, Goldstein I. The Role of Pelvic Floor Muscles in Male Sexual Dysfunction and Pelvic Pain. *Sex Med Rev.* 2016;4:53-62 https://doi.org/10.1016/j.sxmr.2015.10.001; PMid:27872005;

54. Meeker JD, Godfrey-Bailey L, Hauser R. Relationships between serum hormone levels and semen quality among men from an infertility clinic. *J Androl.* 2007;28:397-406 https://doi.org/10.2164/jandrol.106.001545; PMid:17135633;

55. Lenau H, Gorewoda I, Niermann H. Relationship between sperm count, serum gonadotropins and testosterone levels in normo-, oligo- and azoospermia. *Reproduccion.* 1980;4:147-156; PMid:6772497;

56. Maheshwari A, Verma N, Swaroop A, et al. Efficacy of Furosap™, a novel *Trigonella foenum-graecum* seed extract, in Enhancing Testosterone Level and Improving Sperm Profile in Male Volunteers. *Int J Med Sci.* 2017;14:58-66 https://doi.org/10.7150/ijms.17256; PMid:28138310; PMCid:PMC5278660.

57. Suzuki N, Sato Y, Hisasue S, Kato R, Suzuki K, Tsukamoto T. Effect of testosterone on intracavernous pressure elicited with electrical stimulation of the medial preoptic area and cavernous nerve in male rats. *J Androl.* 2007;28:218-222 https://doi.org/10.2164/jandrol.106.000695; PMid:16957136;

58. Xu L, Freeman G, Cowling BJ, Schooling CM. Testosterone therapy and cardiovascular events among men: a systematic review and meta-analysis of placebo-controlled randomized trials. *BMC med.* 2013;11:108 https://doi.org/10.1186/1741-7015-11-108; PMid:23597181; PMCid:PMC3648456.

59. Oskui PM, French WJ, Herring MJ, Mayeda GS, Burstein S, Kloner RA. Testosterone and the cardiovascular system: a comprehensive review of the clinical literature. *J Am Heart Assoc.* 2013;2:e000272 https://doi.org/10.1161/JAHA.113.000272; PMid:24242682; PMCid:PMC3886770.

60. Sedlak T, Shufelt C, Iribarren C, Merz CN. Sex hormones and the QT interval: a review. *J Womens Health.* 2012;21:933-941 https://doi.org/10.1089/jwh.2011.3444; PMid:22663191; PMCid:PMC3430484.

61. Jędrejko K, Lazur J, Muszyńska B. Risk Associated with the Use of Selected Ingredients in Food Supplements. *Chem Biodivers.* 2021;18:e2000686 https://doi.org/10.1002/cbdv.202000686; PMid:33410585;

62. Stocco C. Tissue physiology and pathology of aromatase. *Steroids.* 2012;77:27-35 https://doi.org/10.1016/j.steroids.2011.10.013; PMid:22108547; PMCid:PMC3286233.

63. Shuling L, Sie Kuei ML, Saffari SE, et al. Do men with normal testosterone–oestradiol ratios benefit from letrozole for the treatment of male infertility? *Reproductive BioMedicine Online.* 2019;38:39-45 https://doi.org/10.1016/j.rbmo.2018.09.016; PMid:30449700;

64. Oza S. Aromatase Inhibitor-induced Musculoskeletal Syndrome: a Review of Rehabilitation Interventions. *Curr Phys Med Rehabil Rep.* 2021;9:119-126 https://doi.org/10.1007/s40141-021-00318-3; PMid:26556210;

65. Shay DA, Vieira-Potter VJ, Rosenfeld CS. Sexually dimorphic effects of aromatase on neurobehavioral responses. *Front Mol Neurosci.* 2018;11:374 https://doi.org/10.3389/fnmol.2018.00374; PMid:30374289; PMCid:PMC6196265.

66. Jones H. Testosterone for the aging male; current evidence and recommended practice. *Clin Interv Aging.* 2008;Volume 3:25-44 https://doi.org/10.2147/cia.s190; PMid:18488876; PMCid:PMC2544367.

67. Yang C, Li P, Li Z. Clinical application of aromatase inhibitors to treat male infertility. *Hum Reprod Update.* 2021;28:30-50 https://doi.org/10.1093/humupd/dmab036; PMid:34871401;

68. Korani M. Aromatase inhibitors in male: A literature review. *Medicina Clínica Práctica.* 2023;6:100356 https://doi.org/10.1016/j.mcpsp.2022.100356; PMid:21693046; PMCid:PMC3143915.

69. Roberts RO, Jacobson DJ, Rhodes T, Klee GG, Leiber MM, Jacobsen SJ. Serum sex hormones and measures of benign prostatic hyperplasia. *Prostate.* 2004;61:124-131 https://doi.org/10.1002/pros.20080; PMid:15305335;

70. Kusuma Duarsa GW, Sari YA, Gde Oka AA, et al. Serum testosterone and prostate-specific antigen levels are major risk factors for prostatic volume increase among benign prostatic hyperplasia patients. *Asian J Urol.* 2021;8:289-297 https://doi.org/10.1016/j.ajur.2020.06.001; PMid:34401336; PMCid:PMC8356038.

71. Andriole G, Bruchovsky N, Chung LWK, et al. Dihydrotestosterone and the prostate: The scientific rationale for 5-alpha reductase inhibitors in the treatment of benign prostatic hyperplasia. *J Urol.* 2004;172:1399-1403 https://doi.org/10.1097/01.ju.0000139539.94828.29; PMid:15371854;

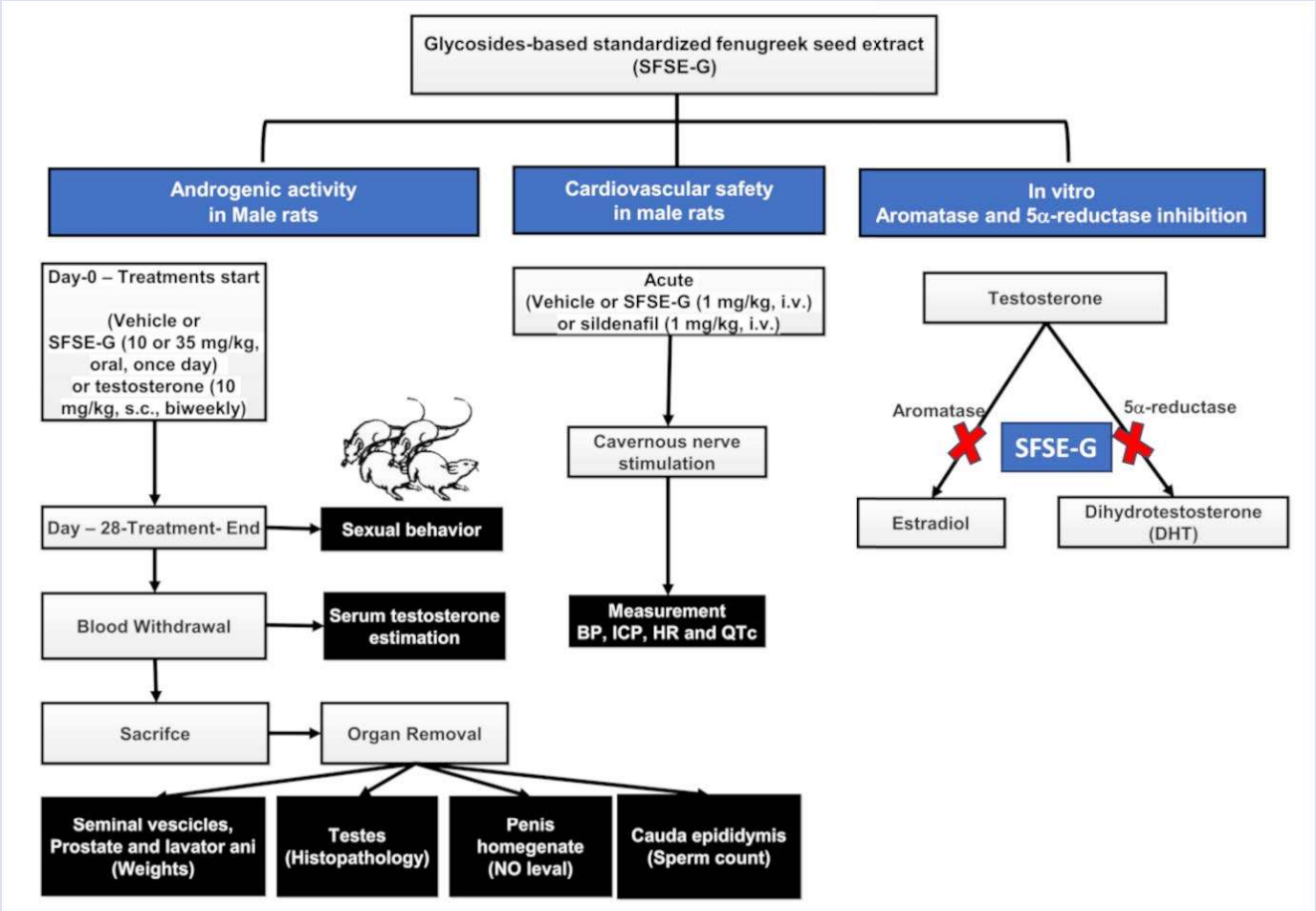
72. Björnebo L, Nordström T, Discacciati A, et al. Association of 5-alpha Reductase Inhibitors With Prostate Cancer Mortality. *JAMA oncology.* 2022;8:1019 https://doi.org/10.1001/jamaoncol.2022.1501; PMid:35587340; PMCid:PMC9121300.

73. Marks LS. 5-alpha reductase: history and clinical importance. *Rev Urol.* 2004;6 Suppl 9:S11-21; PMid:16985920; PMCid:PMC1472916.

74. Marcelli M, Mediwala SN. Male Hypogonadism: A Review. *J Investig Med.* 2020;68:335-356 https://doi.org/10.1136/jim-2019-001233; PMid:31988219;

75. Devlin CM, Simms MS, Maitland NJ. Benign prostatic hyperplasia – what do we know? *BJU International.* 2020;127:389-399 https://doi.org/10.1111/bju.15229;

GRAPHICAL ABSTRACT



SUMMARY

- Androgenic efficacy and safety of glycosides-based standardized fenugreek seeds extract (SFSE-G) was evaluated in laboratory rats.
- Subacute oral administration of SFSE-G to rats showed androgenic efficacy to increase sexual behavior and organ parameters with preservation of testes architecture.
- Subacute oral administration of SFSE-G to rats showed an increase in androgenic markers (serum testosterone levels, sperm count and penile nitric oxide concentration)
- Acute intravenous administration of SFSE-G resulted in increased intracavernous pressure and normal cardiovascular function.
- SFSE-G showed enzyme aromatase and 5-alpha reductase inhibition efficacy *in vitro*.

ABOUT AUTHORS

Urmila M. Aswar, MPharm, PhD. is a Professor, Pharmacology, Poona College of Pharmacy, Bharati Vidyapeeth Deemed to be University, Erandwane, Pune. She has a total of 20 years of academic and research experience. She has more than 40 publications in peer-reviewed journals and 15 awards in her name. She has published 2 patents, completed around 50 consultancy research projects.

Savita R. Nimse, BPharm, is Assistant General Manager, Regulatory and Quality, Indus Biotech Limited, Pune, India. She has 21 years of professional experience in the pharmaceutical and nutraceutical Industry in quality and regulatory functions.

Dr. Prasad A. Thakurdesai, MPharm, PhD, is a Chief Scientific Officer, Indus Biotech Limited, Pune, India, with 34 years of professional experience in academia and industry. He has published more than 100 journal publications, 60 conference presentations, two books, six book chapters, and eleven popular science magazine articles, and has delivered several lectures at conferences, workshops, and exhibitions. He has guided four PhDs and more than 40 M. Pharm students for their dissertations.

Cite this article: Aswar UM, Nimse SR, Thakurdesai PA. Androgenic Efficacy and Mechanism of Glycosides-Based Standardized Fenugreek Seeds Extract Through Aromatase And 5-Alpha Reductase Inhibition. Pharmacogn J. 2024;16(1): 9-19.

International Journal of Sport Nutrition and Exercise Metabolism, 20, 2010, 457-465

Effects of a Purported Aromatase and 5 α-Reductase Inhibitor on Hormone Profiles in College-Age Men

Colin Wilborn, Lem Taylor, Chris Poole, Cliffa Foster, Darryn Willoughby, and Richard Kreider

The purpose of this study was to determine the effects of an alleged aromatase and 5-α reductase inhibitor (AI) on strength, body composition, and hormonal profiles in resistance-trained men. Thirty resistance-trained men were randomly assigned in a double-blind manner to ingest 500 mg of either a placebo (PL) or AI once per day for 8 wk. Participants participated in a 4-d/wk resistance-training program for 8 wk. At Weeks 0, 4, and 8, body composition, 1-repetition-maximum (1RM) bench press and leg press, muscle endurance, anaerobic power, and hormonal profiles were assessed. Statistical analyses used a 2-way ANOVA with repeated measures for all criterion variables ($p \leq .05$). Significant Group × Time interaction effects occurred over the 8-wk period for percent body fat (AI: –1.77% ± 1.52%, PL: –0.55% ± 1.72%; $p = .048$), total testosterone (AI: 0.97 ± 2.67 ng/ml, PL: –2.10 ± 3.75 ng/ml; $p = .018$), and bioavailable testosterone (AI: 1.32 ± 3.45 ng/ml, PL: –1.69 ± 3.94 ng/ml; $p = .049$). Significant main effects for time ($p \leq .05$) were noted for bench- and leg-press 1RM, lean body mass, and estradiol. No significant changes were detected among groups for Wingate peak or mean power, total body weight, dihydrotestosterone, hemodynamic variables, or clinical safety data ($p > .05$). The authors concluded that 500 mg of daily AI supplementation significantly affected percent body fat, total testosterone, and bioavailable testosterone compared with a placebo in a double-blind fashion.

Keywords: fenugreek, anabolic, resistance training

Athletes are continuously searching for ways to enhance performance, which has directed many to the use of anabolic steroids. Anabolic steroids are testosterone derivatives capable of inducing a positive nitrogen balance and increasing fat-free mass by stimulating protein synthesis and/or minimizing protein breakdown. Several studies have shown that administration of testosterone derivatives to younger (Bhasin et al., 1996) and older men (Ferrando et al., 2002; Schroeder, Terk, & Sattler, 2003; Snyder et al., 1999), as well as those classified as hypogonadal (Bhasin et al., 1997; Bhasin et al., 2000), increases muscle size and strength. This is in contrast to exercise-induced changes in testosterone that do not appear to have such a profound effect on muscle protein synthesis (West et al., 2009).

Testosterone is produced from its cholesterol substrate and almost exclusively binds to the blood proteins albumin (40%) and sex-hormone-binding globulin (40%). The remaining portion of testosterone that is not bound to blood proteins is the active constituent and labeled free testosterone. Exogenous testosterone can bind to an androgen receptor and promote intracellular transcriptional and translational events that ultimately increase fat-free mass (muscle hypertrophy). However, once bound to its receptor, testosterone can convert to dihydrotestosterone (DHT) and estradiol through enzymatic action of 5-α reductase and aromatase, respectively.

Because of the legal and ethical repercussions surrounding anabolic steroid use, nutritional supplement companies have designed prohormone compounds, or testosterone precursors, that are marketed to increase testosterone production similarly to anabolic steroids. Even though acute sublingual ingestion of androstenediol was effective in elevating free and total testosterone concentrations up to 180 min after intake (Brown, Martini, Roberts, Vukovich, & King, 2002), this protocol does not resemble the manner in which the supplement is purported to work. Other inquiries have established that prolonged supplementation with prohormone compounds over the course of weeks to months does not increase endogenous testosterone levels in conjunction with resistance training (Broeder et al., 2000; Brown et al., 2000; Brown et al., 1999).

In spite of this, nutritional supplement companies are continuing to try to develop products that have ergogenic potential comparable to that of anabolic steroids. The latest line of nutritional supplements fitting this category is aromatase inhibitors (AIs), which are proposed to suppress estrogen levels and thereby increase endogenous free testosterone levels (increased free testosterone:estrogen [Test:Est] ratio), resulting in increased fat-free mass and strength. It is assumed that

Wilborn, Taylor, Poole, and Foster are with the Dept. of Exercise and Sport Science, University of Mary Hardin-Baylor, Belton, TX. Willoughby is with the Dept. of Health, Human Performance & Recreation, Baylor University, Waco, TX. Kreider is with the Dept. of Health and Kinesiology, Texas A&M University, College Station, TX.

these supplements will increase testosterone within normal physiological levels, but that is not clear at this point. AIs are not a new classification of drugs; they have been used as a medicinal preventive and treatment for breast cancer, and the effects of pharmacologic AIs such as anastrozole and exemestane on the Test:Est are well substantiated in both young and old men (Hayes, Seminara, Decruz, Boepple, & Crowley, 2000; Leder, Rohrer, Rubin, Gallo, & Longcope, 2004; Mauras et al., 2003; Taxel et al., 2001). Nevertheless, there are limited data to support the claims that nutritional companies make regarding AI supplementation.

Testosterone deficiency in males is related to a considerable decrease in protein synthesis, decreased strength, decreased fat oxidation, and increased adiposity (Mauras et al., 1998), which are all regarded as negative physiological conditions. Elderly men exhibiting a state of hypogonadism were orally supplemented with the AI anastrozole for 12 weeks and effectively elevated bioavailable and total testosterone levels to a normal range, while estradiol was mildly suppressed (Leder et al., 2004). Similar results were seen in young, eugonadal men over the course of 10 days (Mauras et al., 2003), indicating that AIs have the potential to blunt estrogen concentrations while concomitantly increasing serum testosterone levels beyond normal levels.

Nutritional supplements designed with the purpose of inhibiting aromatase activity are alleged to work in the same mechanistic manner as AI drugs such as exemestane. One particular AI supplement (Novadex XT) increased total and free testosterone by 283% and 625%, respectively, while only slight increases in estrogen levels were observed in young, eugonadal men over 8 weeks (Willoughby, Wilborn, Taylor, & Campbell, 2007). Another investigation using an AI product concluded that aromatase activity was not completely blocked, even though increases were detected for free testosterone and Test:Est (Rohle et al., 2007). These findings demonstrate that AI nutritional supplements appear to provide some possible benefits to those interested in increasing their anabolic status. As noted previously, research is clear that supraphysiological levels of testosterone are capable of inducing myofibrillar hypertrophy and are vital in the regulation of muscle mass. It is possible that an herbal AI could increase testosterone significantly without increasing values over normal physiological levels and, according to recent research (West et al., 2009), may not be sufficient to induce appreciable change. However, neither of the previous studies examined the effects of the AI on performance measures, which are important variables of interest in athletic and physically active populations. In addition, no studies to our knowledge have investigated the performance benefits of an over-the-counter 5-α reductase inhibitor such as saw palmetto or willow bark. Therefore, it was our purpose to determine the effects of a commercially available product (Trigonella foenum-graecum [standardized for Grecunin]) purported to inhibit aromatase and 5-α reductase activity on strength, body composition, and hormonal profiles in resistance-trained men during an 8-week resistance-training program.

Methods

Participants

Thirty resistance-trained (>1 year of total-body resistance training) male participants (placebo [PL] $n = 13$, 21 ± 3 years, 180 ± 6.4 cm, 84 ± 15 kg, 18.3% ± 6.8% body fat; AI $n = 17$, 21 ± 2.8 years, 178 ± 5.8 cm, 85 ± 9.6 kg, 18.8% ± 4.8% body fat) participated in this study. Participants were not allowed to join this study if they had any metabolic disorder including known electrolyte abnormalities or heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism or a history of hypertension, hepatorenal, musculoskeletal, autoimmune, or neurologic disease; were taking thyroid, hyperlipidemic, hypoglycemic, antihypertensive, or androgenic medications; or had taken ergogenic levels of nutritional supplements that may affect muscle mass (e.g., creatine, HMB) or anabolic/catabolic hormone levels (androstenedione, DHEA, etc.) within 6 months before the start of the study. Participants were asked to maintain their normal dietary intake for the duration of the study and refrain from ingesting any dietary supplement with potential ergogenic benefits. Those meeting eligibility criteria were informed of the requirements of the study and signed informed-consent statements in compliance with the human participant guidelines of the University of Mary Hardin-Baylor and the American College of Sports Medicine.

Experimental Design

The study was conducted as a double-blind, placebo-controlled clinical trial using parallel groups matched according to total body weight. The independent variable was the nutritional supplements. Dependent variables included estimated dietary energy intake; body composition; upper and lower body one-repetition-maximum (1RM) strength, upper and lower body muscle endurance (80% of 1RM), anaerobic sprint power, and fasting clinical blood profiles (substrates, electrolytes, muscle and liver enzymes, red cells, white cells) and anabolic hormones (total testosterone, bioavailable testosterone, dihydrotestosterone, estradiol).

Entry and Familiarization Session

Participants believed to meet eligibility criteria were then invited to attend an entry/familiarization session. During this session, they signed informed-consent statements and completed personal and medical histories. Participants meeting entry criteria were familiarized with the study protocol via a verbal and written explanation outlining the study design. This included describing the training program, familiarizing participants with the tests to be performed, and having them practice the bench-press and leg-press strength tests.

Testing Sessions

After the familiarization/practice session, participants recorded all food and fluid intake on dietary record forms

on 4 consecutive days before each experimental testing session to evaluate nutritional intake. Dietary intake was assessed using Food Processor nutrition software (ESHA, Salem, OR). Participants were instructed to refrain from exercise for 48 hr and fast for 12 hr before baseline testing (T1). They then reported to the human performance laboratory for body-composition and clinical assessments. Height was measured using standard anthropometry, and total body weight was measured using a calibrated electronic scale (Health o Meter, Electromed Corp., Flint, MI) with a precision of ±0.02 kg. Heart rate was determined by Polar (Finland) heart-rate monitor. Blood pressure was assessed in the supine position after participants had rested for 5 min, using a mercurial sphygmomanometer via standard procedures (Adams, 2002).

We then drew ~20 ml of fasting blood using venipuncture techniques of an antecubital vein in the forearm according to standard procedures. Blood samples were shipped to Quest Diagnostics (Dallas, TX) to run clinical chemistry profiles (glucose, total protein, blood urea nitrogen, creatinine, BUN:creatinine ratio, uric acid, AST, ALT, CK, LDH, GGT, albumin, globulin, sodium, chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, HDL, LDL) and whole blood cell counts (including hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, RDW, white blood cell counts, neutrophils, lymphocytes, monocytes, eosinophils, basophils). Blood samples were collected, allowed to sit for 5 min, and then centrifuged at room temperature. Serum was extracted, aliquotted into microcentrifuge tubes, and stored at –20 °C for future analysis. Serum samples were then assayed in duplicate for free testosterone, total testosterone (Diagnostics Systems Laboratories, Webster, TX), DHT, and estradiol (Alpco Diagnostics, Windham, NH), using enzyme-linked immunoabsorbent assays (ELISA) and enzyme-immunoabsorbent assays using a Wallac Victor-1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays were performed at wavelengths of 450 and 405 nm, respectively, in the exercise and biochemical nutrition laboratory at Baylor University.

Participants then had body composition determined using hydrodensitometry. They reported to the underwater weighing tank in swimsuits, and body weight was determined out of water by an electronic scale. Body composition was analyzed using an Exertech (La Cresent, MN) body-density-measuring system. The Exertech consists of a shallow tank (4′ wide × 6′ long × 3′ deep) with a weighing platform with electronic (load cell) weighing system connected to a PC. Calibration is conducted daily by establishing linear interpolation from two known weights. Data points were recorded with data-acquisition software from the force transducer. Residual volume was estimated using standard procedures (Quanjer, 1983). Participants were submerged in warm water and asked to exhale a maximal amount of air, after which a signal from the force transducer produced a readable analog wave. The most stable waveform was selected, and the mean value was recorded. Participants performed this procedure until at least two trials were within a 0.10% difference or a total of seven trials had been completed. Body density was calculated after weight was recorded in and out of water, and the Siri equation was used to calculate percent body fat (Siri, 1993). Fat-free mass was also calculated from percent body fat (Siri, 1961).

Participants then performed 1RM lifts on the isotonic bench press and leg press to assess strength and then muscle endurance. All strength/exercise tests were supervised by laboratory assistants experienced in conducting strength/anaerobic exercise tests using standard procedures. Participants warmed up (two sets of 8–10 repetitions at approximately 50% of anticipated maximum) on the bench press. They then performed successive 1RM lifts starting at about 70% of anticipated 1RM and increased by 5–10 lb until reaching 1RM. They then rested for 10 min and performed a muscle-endurance test at 80% of their 1RM. Participants then rested for 10 min and warmed up on the 45° leg press (two sets of 8–10 repetitions at approximately 50% of anticipated maximum). They then performed successive 1RM lifts on the leg press starting at about 70% of anticipated 1RM and increased by 10–25 lb until reaching 1RM. Participants then rested for 10 min and performed a muscle-endurance test at 80% of their 1RM. Both 1RM protocols were followed as outlined by the National Strength and Conditioning Association (Baechle & Earle, 2008).

After the strength assessments and 15 min of rest, participants performed a 30-s Wingate anaerobic capacity test using a Lode computerized cycle ergometer (Groningen, The Netherlands). Cycle-ergometer measurements (seat height, seat position, handlebar height, and handlebar position) were recorded and kept identical for each participant across testing sessions to ensure test-to-test reliability. Before leaving the laboratory, participants were randomly assigned to a supplement group based on their body weight and given a training regimen. Participants repeated all testing after 4 (T2) and 8 (T3) weeks of training and supplementation.

Supplementation Protocol

Participants were matched into one of two groups according to total body weight. They were then randomly assigned in a double-blind manner to ingest capsules containing 500 mg of placebo (maltodextrin; PL) or 500 mg of T. foenum-graecum (standardized for Grecunin; AI; Indus Biotech, India). The doses investigated represent the current recommended doses sold in nutritional supplements. Participants ingested the assigned capsules once per day in the morning on nontraining days and before their workout on training days for 8 weeks. The supplements were prepared in capsule form and packaged in generic bottles for double-blind administration by Indus Biotech. Supplementation compliance was monitored by having research assistants watch participants take the supplements before supervised workouts and by having the participants return empty bottles of the supplement at the end of 4 and 8 weeks of supplementation. Participants

reported to a research assistant on a weekly basis throughout the study to answer a questionnaire regarding side effects and health status.

Training Protocol

Participants underwent a periodized 4-day/week resistance-training program, split into two upper and two lower extremity workouts per week, for a total of 8 weeks. This training regimen has been shown to increase strength and lean body mass without additive dietary or supplementary interventions (Kerksick et al., 2009). The participants performed an upper body resistance-training program consisting of nine exercises (bench press, lat pull, shoulder press, seated rows, shoulder shrugs, chest flies, biceps curl, triceps press-down, and abdominal curls) twice a week and a seven-exercise lower extremity program (leg press, back extension, step-ups, leg curls, leg extension, heel raises, and abdominal crunches) twice a week. They performed three sets of 10 repetitions with as much weight as they could lift per set during Weeks 1–4 and three sets of eight repetitions during Weeks 5–8, also with as much weight as could be lifted per set (typically 60–80% of 1RM). Rest periods between exercises lasted no longer than 3 min, and rest between sets, no longer than 2 min. Training was conducted at the Mayborn Campus Center at the University of Mary Hardin-Baylor under the supervision of trained research assistants, documented in training logs, and signed off to verify compliance and monitor progress.

Statistical Analysis

Analysis of variance (ANOVA) for repeated-measures univariate tests was used to analyze data. Data were considered statistically significant when the probability of Type I error equaled .05 or less. All statistical procedures were analyzed using SPSS (Statistical Package for Social Science) version 16.0. All data are reported as $M \pm SD$.

Results

Medical Monitoring, Dietary Analysis, and Training Volume

Although a few cases of gastrointestinal discomfort were reported, no participants experienced any major clinical side effects related or unrelated to the study. All participants completed the training protocol without any complications. No significant differences ($p > .05$) between groups were detected for total daily caloric intake, macronutrient intake, or training volume.

Hematological Variables

There were no significant Group × Time interactions ($p > .05$) or main effects for time ($p > .05$) for red blood cell count, white blood cell count, triglycerides, cholesterol variables, liver enzymes or proteins, or markers of kidney function or muscle damage.

Body Composition

Baseline total body weight was not significantly different ($p = .809$) between AI and PL groups. A significant main effect for time ($p = .034$) was observed for total body weight for AI (T1= 85.13 ± 9.69 kg, T2 = 85.74 ± 10.59 kg, T3 = 85.31 ± 10.68 kg) and PL (T1 = 84.02 ± 15.21 kg, T2 = 85.04 ± 15.73 kg, T3 = 85.63 ± 16.07 kg) groups, although no between-groups differences ($p = .083$) were noticed over the 8-week study period. Significant main effect for time ($p = .001$) and interaction ($p = .048$) effects for mean body-fat percentage occurred between AI (T1 = 18.87% ± 4.87%, T2 = 17.91% ± 4.98%, T3 = 17.09% ± 5.04%) and PL (T1 = 18.37% ± 6.85%, T2 = 17.59% ± 7.04%, T3 = 17.82% ± 7.19%) groups (Figure 1). In addition, a significant main effect for time ($p < .001$) was noticed for fat-free mass (AI: T1 = 68.81 ± 6.30 kg, T2 = 70.06 ± 6.57 kg, T3 = 70.40 ± 6.45 kg; PL: T1 = 67.91 ± 8.28 kg, T2 = 69.33 ± 8.27 kg, T3 = 69.55 ± 8.06 kg).

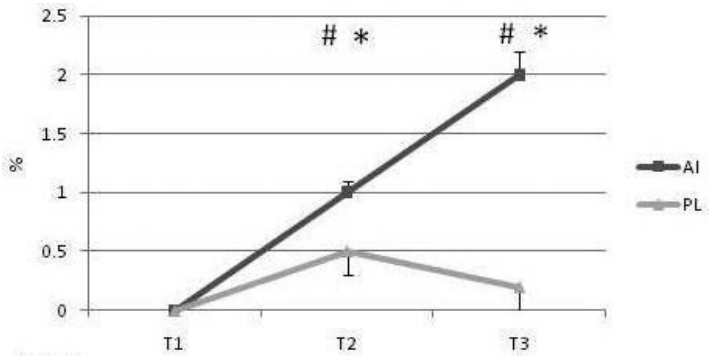


Figure 1 — Body-fat changes from baseline testing (T1) through Week 8 (T3), mean Delta ± SD. #Significant Group × Time interaction ($p < .05$). *Significant main effect for time ($p < .05$) over baseline at T2 (after 4 weeks) and T3.

Training Adaptations

A significant main effect for time was detected for AI and PL groups for bench-press 1RM ($p < .001$; AI: T1 = 108.55 ± 24.98 kg, T2 = 112.97 ± 24.84 kg, T3 = 114.04 ± 23.39 kg; PL: T1 = 95.10 ± 26.89 kg, T2 = 100.17 ± 28.89 kg, T3 = 102.10 ± 29.29 kg) and leg-press 1RM ($p < .001$; AI: T1 = 329.15 ± 61.20 kg, T2 = 371.12 ± 68.20 kg, T3 = 398.53 ± 74.33 kg; PL: T1 = 295.80 ± 71.25 kg, T2 = 332.17 ± 80.72 kg, T3 = 355.77 ± 81.53 kg) over the 8-week resistance-training program, despite no between-groups differences for 1RM tests (Table 1). No significant interactions were noted for muscle-endurance repetitions on the bench press ($p = .328$) or leg press ($p = .184$) or Wingate peak ($p = .343$) and mean power ($p = .679$; Table 2) between AI and PL groups.

Table 1 Bench-Press and Leg-Press One-Repetition-Maximum Values From Baseline Testing (T1) Through Week 8 (T3), kg

Group and time point	Bench press	Leg press
Aromatase and 5-α reductase inhibitor		
T1	108.55 ± 24.98	329.15 ± 61.20
T2	112.97 ± 24.84*	371.12 ± 68.20*
T3	114.04 ± 23.39*	398.53 ± 74.33*
Placebo		
T1	95.10 ± 26.89	295.80 ± 71.25
T2	100.17 ± 28.89*	332.17 ± 80.72*
T3	102.10 ± 29.29*	355.77 ± 81.53*

Note. Values are $M \pm SD$. No significant interactions ($p > .05$) occurred.
*Significant difference from baseline.

Table 2 Wingate Power Measures From Baseline Testing (T1) Through Week 8 (T3), W

Group and time point	Peak power	Mean power
Aromatase and 5-α reductase inhibitor		
T1	1,145 ± 185	599 ± 81
T2	1,178 ± 167	606 ± 79
T3	1,178 ± 167	605 ± 91
Placebo		
T1	1,311 ± 828	561 ± 76
T2	1,143 ± 182	556 ± 84
T3	1,151 ± 172	572 ± 79

Note. Values are $M \pm SD$. No significant interactions ($p > .05$) occurred.

Hormones

Significant Group × Time interaction effects were observed for serum total testosterone ($p = .018$; AI: T1 = 14.76 ± 3.97 ng/ml, T2 = 15.38 ± 3.19 ng/ml, T3 = 15.73 ± 3.62 ng/ml; PL: T1 = 15.80 ± 4.91 ng/ml, T2 = 14.38 ± 5.11 ng/ml, T3 = 13.70 ± 3.27 ng/ml; Figure 2) and bioavailable testosterone ($p = .049$; AI: T1 = 10.77 ± 4.11 ng/ml, T2 = 11.65 ± 3.59 ng/ml, T3 = 12.09 ± 4.16 ng/ml; PL: T1 = 11.80 ± 5.41 ng/ml, T2 = 10.99 ± 5.35 ng/ml, T3 = 10.11 ± 3.29 ng/ml; Figure 3) between AI and PL groups. No main effect for time was noted for total or bioavailable testosterone ($p > .05$). A significant main effect for time was noted for estradiol ($p < .001$; Figure 4). No significant interaction effects transpired over the 8-week study period for free testosterone ($p = .900$) or DHT ($p = .422$; Figure 5).

Discussion

The purpose of this study was to determine the effects of a commercially available product (T. foenum-graecum [standardized for Grecunin]) purported to inhibit aromatase and 5-α reductase activity on strength, body composition, and hormonal profiles in resistance-trained men during an 8-week resistance-training program. No adverse side effects were reported by any of the participants, nor were any clinical safety markers or hematological variables significantly altered ($p > .05$), demonstrating that within the study parameters and the experimental supplement dosage tested, the product appears safe when taken over an 8-week time period.

Over the allotted 8-week supplemental time frame, no changes were seen in any of the hormonal variables of interest in the PL group. It is noted, however, that the AI group underwent average increases of 6.57% and 12.26% for total testosterone and bioavailable testosterone, respectively ($p < .05$). Moreover, we did not see a decrease in serum estradiol and DHT levels, as would be expected from the AI; instead we observed nonsignificant increases ($p > .05$) of 26.62% and 6.10%, respectively. Even though our results demonstrate that the experimental AI increased endogenous testosterone levels, it did not completely block aromatase and 5-α reductase activity. Our results are in concurrence with those of others (Rohle et al., 2007; Willoughby et al., 2007) who found marginal increases in estradiol after supplementing with an aromatase-inhibiting supplement for 8 weeks.

Aromatase inhibits the conversion of testosterone to estradiol, which subsequently sends feedback to the hypothalamus and pituitary to promote testosterone production (Hayes et al., 2000). Therefore, estradiol levels would likely decrease to see testosterone levels inversely elevate. Our data agree with this; estradiol decreased 9.64% from Week 0 to Week 4 before rising above baseline values by the conclusion of the 8-week study. Because of a significant increase in total and bioavailable testosterone without a corresponding increase in estradiol and DHT, we conclude that the experimental

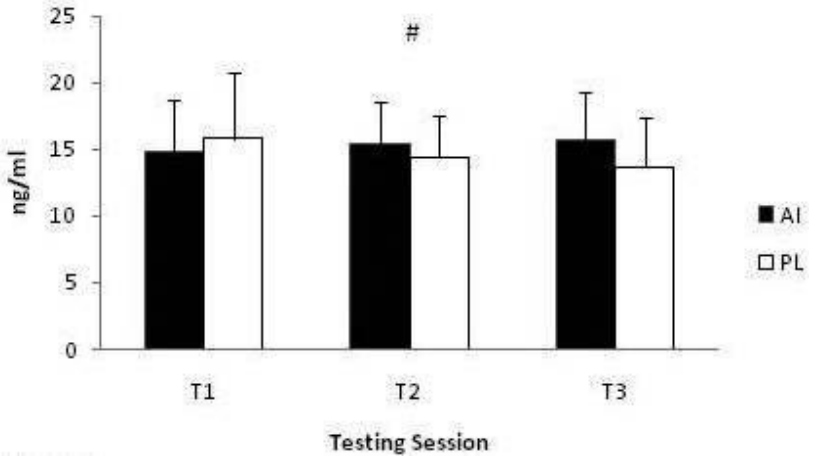


Figure 2 — Serum total testosterone changes from baseline testing (T1) through Week 8 (T), *M* ± *SD*. #Significant Group × Time interaction ($p < .05$).

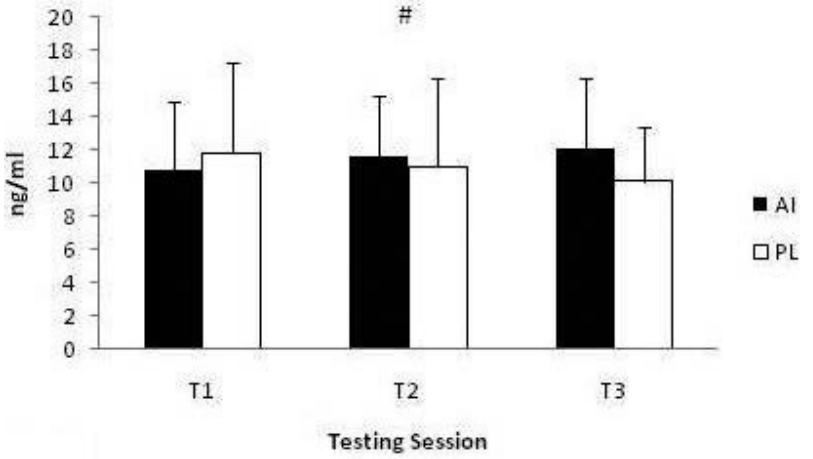


Figure 3 — Serum bioavailable testosterone changes from baseline testing (T1) through Week 8 (T), *M* ± *SD*. #Significant Group × Time interaction ($p < .05$).

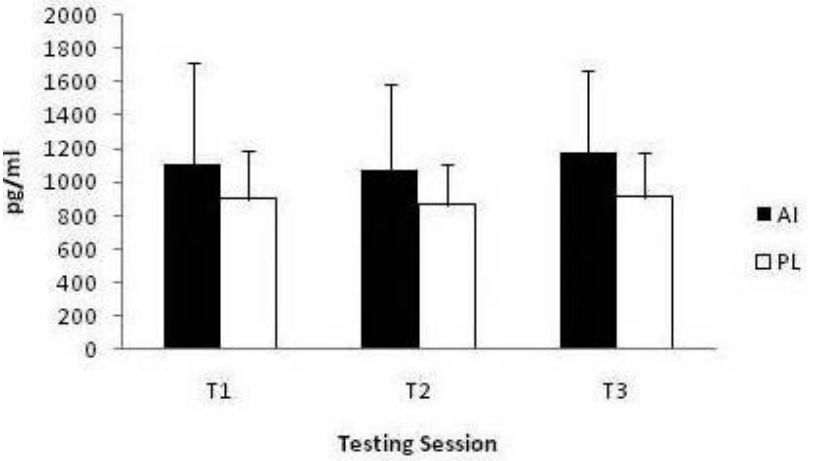


Figure 4 — Serum dihydrotestosterone changes from baseline testing (T1) through Week 8 (T), *M* ± *SD*. No significant changes were noted.

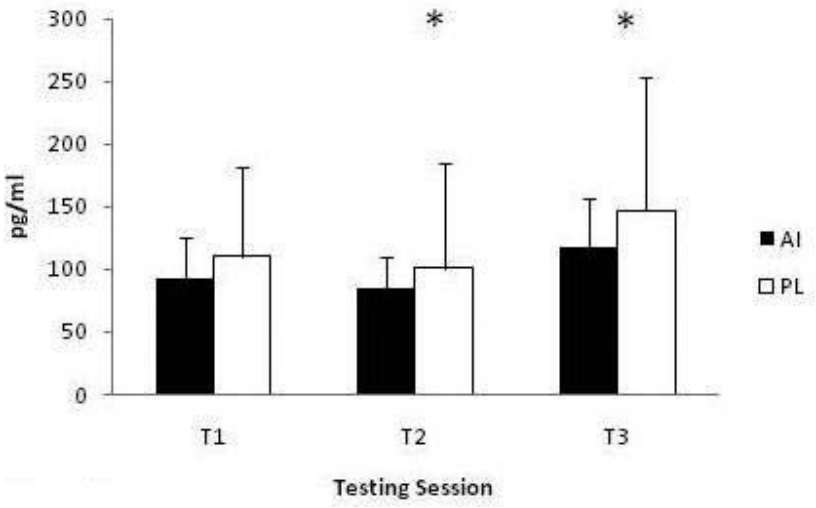


Figure 5 — Serum estradiol changes from baseline testing (T1) through Week 8 (T), $M \pm SD$. *Significant linear increase over baseline.

AI successfully, but incompletely, inhibited aromatase and 5-α reductase activity.

In the current study, the purported aromatase and 5-α reductase inhibitor had no effect on fat-free mass but was an effective stimulus for decreasing fat mass by 1.77%, compared with 0.55% in the PL group ($p < .05$). Our data are supported by our previous work (Willoughby et al., 2007), which also found a decrease in fat mass (3.5%) without changes in fat-free mass during an 8-week resistance-training program in conjunction with an aromatase-inhibiting supplement.

Increased serum androgen concentrations related to hypergonadism can accelerate lipolysis via activation of hormone-sensitive lipase (Hossain & Hornick, 1994), while a state of hypogonadism is correlated with a diminished fat-oxidation efficiency and a subsequent reduction in resting energy expenditure (Hayes, 2000). We observed significant increases in total and bioavailable testosterone levels, without any noticeable change in estradiol between AI and PL, thus indicating a possible connection between increased androgen levels and decreased fat mass, even though no markers of lipolysis were assessed.

For the evaluated performance measures, the AI group increased bench-press and leg-press 1RM strength 8.04% and 21.08%, respectively, but no differences were seen between groups ($p > .05$), which signifies that the experimental supplement had no effect on overall body strength. Previous research has shown that supplementation with anastrozole for 10 weeks did not affect strength, although total testosterone increased 58% and estradiol declined 50%. Our previous work (Rohle et al., 2007; Willoughby et al., 2007) experimenting with aromatase inhibitors marketed by nutritional supplement companies did not analyze strength in young, eugonadal men. However, the effects of testosterone derivatives coupled with resistance training vastly improve muscle strength across all populations (Bhasin et al., 1996; Bhasin et al., 1997; Bhasin et al., 2000; Ferrando et al., 2002; Schroeder et al., 2003; Snyder et al., 1999). These training adaptations would appear to be a result of supraphysiological doses of testosterone, because exercise-induced changes in testosterone do not appear to significantly affect muscle protein synthesis (West et al., 2009). After its release into the blood, testosterone can circulate to a desired muscle cell, translocate and bind to an androgen receptor, and promote intracellular transcriptional and translational events that ultimately increase fat-free mass (muscle hypertrophy). Because muscle cross-sectional area is linearly related to strength potential (force-production potential; Ratamess, 2008), the effects of supraphysiological doses of testosterone derivatives on muscle strength are clearly understood.

AIs marketed by nutritional supplement companies claim that these products increase androgen levels similarly to anabolic steroids while simultaneously suppressing estrogen levels. The current data, along with those from our previous work (Willoughby et al., 2007), support this notion to some extent, because we saw increases in total and bioavailable testosterone accompanied with minimal change in DHT and estradiol. Conversely, as our data suggest, an increase in endogenous testosterone levels does not always translate to an increase in muscle hypertrophy and strength. It is likely that the increase in endogenous testosterone levels from the experimental supplement did not affect androgen-receptor expression or the interaction between testosterone and an androgen receptor, which provides a possible explanation of why fat-free mass and strength did not increase more than in the PL group in our investigation. Thus, these data support the notion that elevated levels of testosterone within physiological levels have no influence on muscle strength in strength-trained young men.

AI drugs have been around for some time and have successfully been used as medicinal treatments for various types of cancer. However, AIs marketed as nutritional supplements are relatively new to the fitness industry, and there are limited data on their alleged benefits as

advertised by supplement companies. The results of this study indicate that 8 weeks of supplementation with a commercially available AI incompletely inhibited aromatase and 5-α reductase activity while significantly increasing total and bioavailable testosterone levels, as well as decreasing percent body fat, in conjunction with a resistance-training program. No changes between Al and PL were noted for upper and lower body strength, hematological variables, or clinical safety data.

Acknowledgments

This work was funded by Indus Biotech. We thank all participants and staff of the human performance laboratory for their contributions to this work. The results of the current study do not constitute an endorsement by IJSNEM.

References

Adams, G.M. (2002). *Exercise physiology laboratory manual* (4th ed.). New York: McGraw-Hill.

Baechle, T.R., & Earle, R.W. (Ed.). (2008). *Essentials of strength training and conditioning* (3rd ed.). Champaign, IL: Human Kinetics.

Bhasin, S., Storer, T.W., Berman, N., Callegari, C., Clevenger, B., Phillips, J., . . . Casaburi, R. (1996). The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *The New England Journal of Medicine*, *335*(1), 1–7.

Bhasin, S., Storer, T.W., Berman, N., Yarasheski, K.E., Clevenger, B., Phillips, J., . . . Casaburi, R. (1997). Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *The Journal of Clinical Endocrinology and Metabolism*, *82*(2), 407–413.

Bhasin, S., Storer, T.W., Javanbakht, M., Berman, N., Yarasheski, K.E., Phillips, J., . . . Beall, G. (2000). Testosterone replacement and resistance exercise in HIV-infected men with weight loss and low testosterone levels. *Journal of the American Medical Association*, *283*(6), 763–770.

Broeder, C.E., Quindry, J., Brittingham, K., Panton, L., Thomson, J., Appakondu, S., . . . Yarlagadda, C. (2000). The Andro Project: Physiological and hormonal influences of androstenedione supplementation in men 35 to 65 years old participating in a high-intensity resistance training program. *Archives of Internal Medicine*, *160*(20), 3093–3104.

Brown, G.A., Martini, E.R., Roberts, B.S., Vukovich, M.D., & King, D.S. (2002). Acute hormonal response to sublingual androstenediol intake in young men. *Journal of Applied Physiology (Bethesda, Md.)*, *92*(1), 142–146.

Brown, G.A., Vukovich, M.D., Reifenrath, T.A., Uhl, N.L., Parsons, K.A., Sharp, R.L., & King, D.S. (2000). Effects of anabolic precursors on serum testosterone concentrations and adaptations to resistance training in young men. *International Journal of Sport Nutrition and Exercise Metabolism*, *10*(3), 340–359.

Brown, G.A., Vukovich, M.D., Sharp, R.L., Reifenrath, T.A., Parsons, K.A., & King, D.S. (1999). Effect of oral DHEA on serum testosterone and adaptations to resistance training in young men. *Journal of Applied Physiology (Bethesda, Md.)*, *87*(6), 2274–2283.

Ferrando, A.A., Sheffield-Moore, M., Yeckel, C.W., Gilkison, C., Jiang, J., Achacosa, A., . . . Urban, R.J. (2002). Testosterone administration to older men improves muscle function: Molecular and physiological mechanisms. *American Journal of Physiology. Endocrinology and Metabolism*, *282*(3), E601–E607.

Hayes, F.J. (2000). Testosterone—Fountain of youth or drug of abuse? *The Journal of Clinical Endocrinology and Metabolism*, *85*(9), 3020–3023.

Hayes, F.J., Seminara, S.B., Decruz, S., Boepple, P.A., & Crowley, W.F., Jr. (2000). Aromatase inhibition in the human male reveals a hypothalamic site of estrogen feedback. *The Journal of Clinical Endocrinology and Metabolism*, *85*(9), 3027–3035.

Hossain, A., & Hornick, C.A. (1994). Androgenic modulation of lipid metabolism at subcellular sites in cholestatic rats. *Hormone and Metabolic Research. Hormon- und Stoffwechselforschung. Hormones et Metabolisme*, *26*(1), 19–25.

Kerksick, C.M., Wilborn, C.D., Campbell, B.I., Roberts, M.D., Rasmussen, C.J., Greenwood, M., & Kreider, R.B. (2009). Early-phase adaptations to a split-body, linear periodization resistance training program in college-aged and middle-aged men. *Journal of Strength and Conditioning Research*, *23*(3), 962–971.

Leder, B.Z., Rohrer, J.L., Rubin, S.D., Gallo, J., & Longcope, C. (2004). Effects of aromatase inhibition in elderly men with low or borderline-low serum testosterone levels. *The Journal of Clinical Endocrinology and Metabolism*, *89*(3), 1174–1180.

Mauras, N., Hayes, V., Welch, S., Rini, A., Helgeson, K., Dokler, M., . . . Urban, R.J. (1998). Testosterone deficiency in young men: Marked alterations in whole body protein kinetics, strength, and adiposity. *The Journal of Clinical Endocrinology and Metabolism*, *83*(6), 1886–1892.

Mauras, N., Lima, J., Patel, D., Rini, A., di Salle, E., Kwok, A., & Lippe, B. (2003). Pharmacokinetics and dose finding of a potent aromatase inhibitor, aromasin (exemestane), in young males. *The Journal of Clinical Endocrinology and Metabolism*, *88*(12), 5951–5956.

Quanjer, P.H. (1983). Standardized lung function testing. Report of working party on standardization of lung function tests of the European Community for Coal and Steel. *Bulletin Europeen de Physiopathologie Respiratoire*, *19*(5), 1–94.

Ratamess, N.A. (2008). Adaptations to anaerobic training programs. *Essentials of Strength Training and Conditioning*, *3*, 94–119.

Rohle, D., Wilborn, C., Taylor, L., Mulligan, C., Kreider, R., & Willoughby, D. (2007). Effects of eight weeks of an alleged aromatase inhibiting nutritional supplement 6-OXO (androst-4-ene-3,6,17-trione) on serum hormone profiles and clinical safety markers in resistance-trained, eugonadal males. *Journal of the International Society of Sports Nutrition*, *4*(13).

Schroeder, E.T., Terk, M., & Sattler, F.R. (2003). Androgen therapy improves muscle mass and strength but not muscle quality: Results from two studies. *American Journal of Physiology. Endocrinology and Metabolism*, *285*(1), E16–E24.

Siri, W.E. (Ed.). (1961). *Body volume measured by gas dilution.* Washington, DC: National Academy Press.

Siri, W.E. (1993). Body composition from fluid spaces and density: Analysis of methods. *Nutrition*, 9(5), 480–491; disc. 480, 492.

Snyder, P.J., Peachey, H., Hannoush, P., Berlin, J.A., Loh, L., Lenrow, D.A., . . . Strom, B.L. (1999). Effect of testosterone treatment on body composition and muscle strength

in men over 65 years of age. *The Journal of Clinical Endocrinology and Metabolism*, *84*(8), 2647–2653.

Taxel, P., Kennedy, D.G., Fall, P.M., Willard, A.K., Clive, J.M., & Raisz, L.G. (2001). The effect of aromatase inhibition on sex steroids, gonadotropins, and markers of bone turnover in older men. *The Journal of Clinical Endocrinology and Metabolism*, *86*(6), 2869–2874.

West, D.W., Kujbida, G.W., Moore, D.R., Atherton, P., Burd, N.A., Padzik, J.P., . . . Phillips, S.M. (2009). Resistance exercise-induced increases in putative anabolic hormones do not enhance muscle protein synthesis or intracellular signalling in young men. *The Journal of Physiology*, *587*(Pt. 21), 5239–5247.

Willoughby, D.S., Wilborn, C., Taylor, L., & Campbell, W. (2007). Eight weeks of aromatase inhibition using the nutritional supplement Novedex XT: Effects in young, eugonadal men. *International Journal of Sport Nutrition and Exercise Metabolism*, *17*(1), 92–108.

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 7, Suppl 2, 2014

ISSN - 0974-2441

Research Article

EFFECTS OF GLYCOSIDES BASED FENUGREEK SEED EXTRACT ON SERUM TESTOSTERONE LEVELS OF HEALTHY SEDENTARY MALE SUBJECTS: AN EXPLORATORY DOUBLE BLIND, PLACEBO CONTROLLED, CROSSOVER STUDY

MAHESH MOKASHI[1], RENU SINGH-MOKASHI[2], VISHWARAMAN MOHAN[2], PRASAD THAKURDESAI[2]

[1]Samiksha Hospital, 501-B, 2nd floor, Rasta Peth, Pune, Maharashtra, India, [2]Department of Scientific affairs, Indus Biotech Private Limited, 1, Rahul Residency, Off Salunke Vihar Road, Kondhwa, Pune-411 048, India. Email: prasad@indusbiotech.com

Received: 24 March 2014, Revised and Accepted: 21 April 2014

ABSTRACT

Objective: To evaluate acute effects of IND9 supplementation on serum testosterone levels in healthy sedentary male subjects. Methods: The study was designed as randomized, double blind, placebo controlled, two period, crossover study with 7 days of washout period using single study center. Sixteen healthy male subjects were randomized and received single dose of 600 mg (two capsules of 300 mg) of either IND9 or matching placebo capsules during each of the 2 study periods of 10 h each. Blood samples were collected three times at 3 h, 7 h and 10 h. The outcome measures were measurement of serum free testosterone (mFT) and total testosterone (TT), calculated levels of free testosterone (cFT), bioavailable testosterone (BT) levels and safety parameters. Results: During the study period, significant time-dependent interactions were found for mFT and cFT levels (within Placebo and IND9 supplemented arms), BT levels (within IND9 but not in Placebo arm) and TT levels (none of the arms). Two-way ANOVA of data of change from baseline at 10 h showed no significant interactions between the treatments and periods (absence of crossover effect) for all measures. Pairwise comparisons between change from baseline data (at 10 h) by unpaired 't' test showed significant increase in TT, BT and cFT but not in mFT levels in IND9 arm as compared to respective levels in placebo arm. The supplementation of IND9 and placebo was found to be safe and well-tolerated. All values were found within physiological limits. Conclusion: Acute administration of IND9 capsule supplementation to sedentary males showed potential androgenic benefits with good safety profile.

Keywords: Fenugreek seed extract, serum testosterone, Bioavailable, healthy sedentary subjects

INTRODUCTION

Hypogonadism has been assumed to be the important factor in the development of a condition variably termed partial androgen deficiency in the ageing male, androgen deficiency /decline in the ageing male, late onset hypogonadism or testosterone deficiency syndrome (TDS) [1]. These conditions are associated with a wide range of clinical symptoms ranging from loss of muscle mass, loss of muscular strength, reduced bone mineral density and a decrease in general well-being, depressed mood with mild cognitive impairment.

Testosterone (T), a potent anabolic hormone, plays an important role in antagonizing catabolic stress from daily physical challenges. Because of its hydrophobic nature, most of the circulating T is bound to plasma proteins, including sex hormone-binding globulin (SHBG) and albumin. The SHBG-bound fraction is biologically inactive because of the high binding affinity with T, whereas the albumin-bound T is readily dissociable and thus bioavailable as is the small percentage of FT that is normally circulating in the blood [2]. In middle-aged men, there is a rise in SHBG levels, which results in a more substantial age dependent decline in FT than TT [3, 4]. Existing data suggests a cause–effect relationship between low serum T levels and sexual dysfunction [5]. Moreover, a causal relationship between obesity or metabolic syndrome and sexual dysfunction also exists. [6-8]

In recent years, there has been significant interest in plant based natural medicines that are traditionally used to improve sexual function and performance. However, only few natural therapies have undergone evidence based scientific scrutiny and need to be scientifically evaluated for their efficacy and safety in relevant populations using standardized procedures. One such potential medicinal plant is Fenugreek (*Trigonella foenum-graecum* L.). Fenugreek seed, a spice and food grain, has traditional history of medicinal use in Egypt, Southern Europe, India, Asia, and North Africa [9].

Fenugreek seed extract is a component of many nutritional dietary products that are recommended for athlete and exercising male subjects. Fenugreek seed extract is reported to enhance endurance capacity and utilization of energy in male mice [10]. Furthermore, fenugreek derived products including extracts have been explored successfully for many exercise physiology applications involving healthy volunteers and patients [11, 12] including anabolic [13] and androgenic activities [14]. Safety of fenugreek seeds in human has also been established in many clinical trials and reviews [11-13]. Because of excellent safety profile, fenugreek seeds extracts are certified as GRAS (Generally recognized as safe) item under clause §182.20 (Essential oils, oleoresins and natural extractives including distillates) by US Food and Drug Administration (US FDA).

Physiological beneficial properties of wide range of plant derived glycosides have also been reported and reviewed extensively [15]. The potent androgenic activities are among the major health benefits associated with natural glycosides [16, 17].

The beneficial effects of a commercially available glycosides based standardized fenugreek seed extract (Testosurge) has been reported on muscular strength, improved body composition, and male hormonal profiles (serum testosterone levels) in resistance-trained men with excellent safety profile [14]. Recently, non-clinical safety profile of glycosides based standardized extract from fenugreek seed has been reported [18].

The correlation between serum T levels and resistance training or exercise is complex. The available evidences suggested that intensity and duration of exercise can alter serum T levels in complex manner. On one hand, serum T levels were virtually unaffected by 12-weeks of strength training in young and elderly subjects is reported [19]. On the other hand, few reports suggested that exercise can result in increased levels of circulating T [20, 21]. Acute exercise were found to increase the expression of FT and dihydrotestosterone (DHT) (bioactive metabolite of T) in the skeletal muscles in both male and

females suggesting that exercise stimulates local bioactive androgen metabolism leading to differential T levels depending on exercise amount or duration [22]. Therefore, effects of bioactive compounds need to be evaluated separately in sedentary and exercising population. However, such study of glycosides based fenugreek seed extract in sedentary (non-exercising) male subjects is lacking. Therefore, it was thought worthwhile to explore potential of glycosides based standardized fenugreek seed extract (IND9) for its possible androgenic effects in healthy sedentary male subjects.

METHODS

Participants

Sixteen healthy and non-exercising male subjects were enrolled in the study. Inclusion criteria consisted of healthy volunteers aged 18-45 years with normal health status on the basis of clinical and laboratory examination and willing to sign the written informed consent form. Subjects were excluded on the basis of any condition which in the opinion of the investigator makes the subject unsuitable for inclusion, obvious medical disorder on the basis of medical history, physical examination reveals any abnormality, obvious male sexual dysfunction, known hypersensitivity to herbal drugs/nutritional supplement/ foods, subjects who is consuming/ has received any medication during last 30 days that can have impact on male sex hormones, chronic alcoholics, drug abusers and participation in any other clinical trial during last 30 days. The study protocol was assessed and approved by the Independent Human Ethics Committee

Experimental design

This objective of the present study was to explore effects of acute (single dose) administration of IND9 supplementation on serum testosterone levels in sedentary male subjects. The study was performed as randomized, double blind, placebo controlled, two period (10 h each), crossover design with 7-days washout period using single study center (Samiksha Hospital, Pune). The overall design and conduct of the study in a form of flow-chart in Figure 1.

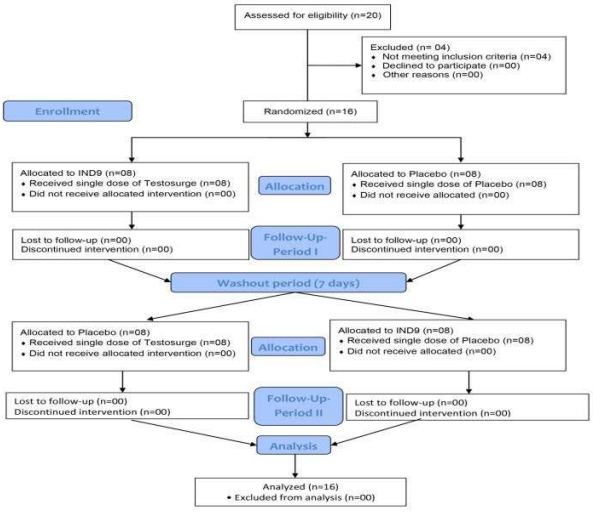


Fig.1: Study Flowchart (CONSORT diagram)

Screening and randomization

On day 0 of the study (i.e. period I), subjects were admitted to the site at 6.00 pm. Potential subjects were screened and requested to attend an information session. They were informed about the trial process and requested to provide consent for trial participation. This trial, being an exploratory study, no statistical method is applied to determine the sample size. A total of 16 healthy volunteers (on the basis of medical history and clinical examination) who met all inclusion and none of the exclusion criteria were included in the study. Demographic data, medical history, physical examination, vital signs and medications history (one month) were recorded in case report forms (CRFs) for consenting subjects.

Subjects were allocated a unique randomization number as per computer-generated randomization code which was available to investigator in case of serious adverse event (SAE) during the study. At time 8.00 pm (baseline), they were randomized to receive single dose of 600 mg (two capsules of 300 mg) in 1:1 ratio with 8 subjects each in IND9 or matching placebo supplementation arms. Subjects were not allowed to eat anything 1 h before and 2 h after consumption of the test supplementations.

The supplementation

After dispensing IND9 or matching placebo supplementations, baseline characteristics of subjects were recorded. Both IND9 and matching placebo products were enclosed in bottles containing capsules that were identical in appearance and individually coded. The IND9 and matching placebo (lactose, IP grade) capsules were manufactured and supplied by Indus Biotech Private Limited, Pune. Both IND9 and placebo were analyzed and complied with quality requirements related to microbial content and heavy metals.

Outcome measures

The primary efficacy outcome measures were serum free testosterone (directly measured (mFT) and calculated (cFT). The secondary (exploratory) efficacy outcome measures were measured serum total testosterone (TT), and calculated bioavailable testosterone (BT) levels at baseline (8.00 PM) and 3 h (11.00 PM), 7 h (3.00 AM) and 10 h (6.00 AM) after baseline. Calculation of cFT and BT was based on measurements of TT, SHBG and albumin assays in serum and applying the established formula [23]. The calculation was performed using website calculator of International Society for the Study of the Aging Male (ISSAM). The website (URL: http://www.issam.ch/freetesto.htm) uses the association constants (K_{SHBG} = 1 X 10^9 L/mol and $K_{albumin}$ = 3.6 X 10^4 L/mol) and considers average albumin concentrations equal to 4.3 g/dL in each sample.

Safety outcome measures such as vital signs and adverse events (AE). Subjects were asked to report at study center after 7 days of wash-out period (Period II). The procedure of period I was repeated with crossover supplementation schedule (reversed sequence of supplementation).

Statistics

The data was represented as mean ± standard deviation (SD). The data of each parameter of demographic and baseline measurements was analyzed by unpaired't' test to evaluate homogeneity between the subjects receiving different sequence namely sequence-AB (Active-Placebo) and sequence-BA (Placebo-Active). The data of serum testosterone (mFT, cFT, TT, and BT) values was analyzed by separate one-way repeated measure ANOVA to assess within the treatment effects. To assess crossover effects (interaction between two sequences), the data of change from baseline values for each type of serum T (mFT, cFT, TT, and BT) was analyzed by separate two-way ANOVA.

To assess between the group effects (IND9 vs placebo), the values of change of serum testosterone levels from baselines were compared by separate unpaired 't' tests for each time point (3 h, 7 h and 10 h). The P values less than 0.05 (two-sided) were considered significant.

Results

Demographics and baseline characteristics

Sixteen volunteers were enrolled and randomized between IND9 (n=8) and placebo group (n=8) per sequence. None of the subjects dropped out during the study. The unpaired 't' test showed uniformity in demography and baseline characteristics with no significant difference between sequence AB and BA with respect to age, weight, height, heart rate, respiratory rate, blood pressure (systolic and diastolic), body temperature and all efficacy outcome measures (Table 1).

Table 1. Demographic and baseline characteristics of study population

Characteristics	Sequence AB (n=8)	Sequence BA (n=8)	P value
Age (yrs)	24.50 ± 5.63	27.62 ± 4.40	0.237
Weight (kg)	63.59 ± 8.05	66.75 ± 8.18	0.449
Height (cm)	169.11 ± 6.58	164.75 ± 9.35	0.299
Heart Rate (bpm)	77.00 ± 1.51	76.25 ± 1.67	0.362
Respiratory rate (per min)	17.00 ± 0.76	17.63 ± 0.74	0.118
Systolic Blood pressure (mmHg)	113.75 ± 5.18	115.00 ± 9.26	0.744
Diastolic blood pressure (mmHg)	73.75 ± 5.18	75.00 ± 9.26	0.744
Body temperature (° F)	97.75 ± 0.46	97.63 ± 0.74	0.693
mFT (pg/ml)	11.59 ± 2.07	13.83 ± 6.46	0.365
cFT (pg/ml)	8.28 ± 1.95	8.99 ± 3.69	0.639
TT (ng/ml)	466.48 ± 133.08	435.70 ± 191.77	0.715
BT (ng/ml)	202.88 ± 45.45	228.38 ± 98.97	0.519

The data was presented as mean ± standard deviation (SD) and analyzed by unpaired "t" test between the sequences. FT - Free testosterone, TT - Total testosterone, BT - Bioavailable testosterone, mFT and cFT are serum free testosterone levels by direct measurement and calculated respectively.

Effect of treatments on serum testosterone levels (within the group)

The data of serum T levels at baseline, 3 h, 7 h and 10 h for IND9 and placebo is presented in Table 2. Moreover, the statistical measures obtained from separate one-way repeated measure ANOVAs (F, df and p-value) of each of outcome measure for IND9 and placebo are presented in Table 2.

Significant within the group (time dependent) difference in mFT levels was found within placebo (F = 5.11, df = 3, p = 0.003) or IND9 (F = 4.15, df = 3, p = 0.010). Significant within the group (time dependent) difference in cFT levels was found within placebo (F = 2.9, df = 3, p = 0.042) or IND9 (F = 4.26, df = 3, p = 0.009). However, no significant within the group (time dependent) difference was found in TT levels within placebo (F = 2.58, df = 3, p = 0.062) or IND9 (F = 2.14, df = 3, p = 0.104). Significant within the group (time dependent) difference was found in BT levels within IND9 (F = 2.97, df = 3, p = 0.039) but not within placebo arm (F = 2.05, df = 3, p = 0.116).

Table 2. Effect of treatments on serum testosterone levels (within the group effects)

Parameters	Treatment	Time from intervention				Statistics		
		Baseline	3 h	7 h	10 h	F	d.f.	P- value
mFT	Placebo	13.6 ± 4.8	9.1 ± 2.6	11.5 ± 2.7	13.2 ± 3.8	5.11	3	0.003
(pg/ml)	IND9	11.7 ± 2.6	9.2 ± 2.3	11.4 ± 3.2	13.5 ± 5.0	4.15	3	0.010
cFT	Placebo	8.4 ± 3.0	7.0 ± 3.5	9.3 ± 2.1	9.7 ± 2.3	2.9	3	0.042
(pg/ml)	IND9	7.2 ± 2.1	7.0 ± 2.4	9.1 ± 3.3	9.9 ± 2.8	4.26	3	0.009
TT (ng/ml)	Placebo	473.6 ± 185.3	361.9 ± 140.2	467.5 ± 133.7	501.1 ± 144.6	2.58	3	0.062
	IND9	405.5 ± 142.9	386.9 ± 149.3	439.8 ± 152.2	519.0 ± 189.3	2.14	3	0.104
BT (ng/ml)	Placebo	222.6 ± 81.9	180.8 ± 96.8	231.9 ± 54.3	242.1 ± 59.7	2.05	3	0.116
	IND9	184.5 ± 49.6	182.3 ± 67.0	218.6 ± 86.1	246.9 ± 77.7	2.97	3	0.039

The data was presented as mean ± standard deviation (SD) and was analyzed by one way repeated measure ANOVA for each parameter (within the treatments). FT - Free testosterone, TT - Total testosterone, BT - Bioavailable testosterone, mFT and cFT are serum free testosterone levels by direct measurement and calculated respectively.

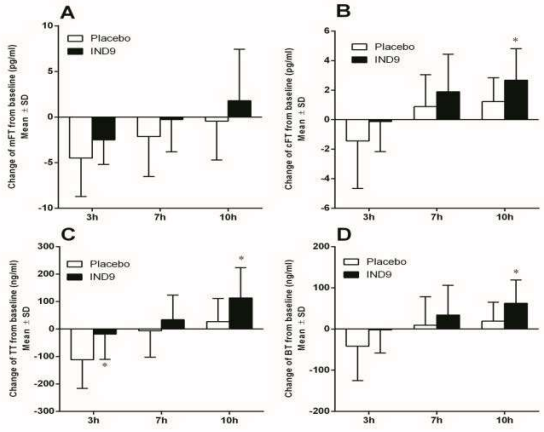


Fig.2. Effects of treatments on change in levels from baseline (between the group effects). (A) mFT) (B) cFT) (C) TT and (D) BT. The data for each parameter was presented as mean ± SD and analyzed by unpaired "t" test (comparisons between the treatments at corrosponding time) * P < 0.05 as compared to values of placebo group at corresponding time period. FT - Freetestosterone, TT - Total testosterone, BT - Bioavailable testosterone, mFT and cFT are serum free testosterone levels by direct measurement and calculated respectively.

Effects of treatments on change in levels at 10 h from baseline (between the groups)

The data of change from baseline values of serum testosterone levels is presented in Figure 2. Two-way ANOVA of change at 10 h from baseline data showed no significant interaction between the treatments and periods (crossover effect) for mFT (F = 1.386, df = 1, 31, p = 0.249), cFT (F = 0.132, df = 1, 31, p = 0.719), TT (F = 0.208, df = 1, 31, p = 0.652) and BT (F = 0.068, df = 1, 31, p = 0.796). The unpaired 't' test was used to compare changes from baseline between IND9 and placebo.

The mFT levels in IND9 supplemented subjects showed increase of 1.79 (± 5.65) whereas in placebo supplemented subjects showed decrease of 0.46 (± 4.24) with no significant difference between treatments. Increase in cFT levels showed by IND9 supplemented subjects (2.68 ± 2.14) were significantly (p = 0.038) higher than increase showed by placebo arm (1.22 ± 1.62). Increase in TT levels showed by IND9 supplemented subjects (113.55 ± 109.85) were significantly (p = 0.018) higher than increase showed by placebo arm (27.49 ± 83.65). Similarly, significant (p = 0.025) increase was found in BT levels in IND9 supplemented subjects (62.43 ± 56.78) as compared to increase showed by placebo supplemented subjects (19.5 ± 45.66).

SAFETY OUTCOME MEASURES

The supplementation of IND9 and placebo was found to be safe and well tolerated without any AEs during the study.

DISCUSSION

It is well recognized that circulating concentrations of testosterone in all forms (TT, BT and FT) are characterized by a diurnal rhythm, with highest levels in the morning and a nadir in the late afternoon [24]. Therefore, we have evaluated effects of acute administration of IND9 at 8.00 pm, when testosterone levels were at its nadir. The measurements were done during 10 h period with last measurement at 6.00 am (when testosterone levels were at the peak).

In the present study, significant increase in T levels (FT, TT and BT) on acute administration of IND9 supplementation as compared with placebo group was observed. There was no significant crossover effect (treatment X sequence effect) found which further substantiate efficacy of IND9 on testosterone levels.

It is important to confirm low T concentrations in men with an initial T level in the mildly hypogonadal range, because 30% of such patients may have a normal T level on repeat measurement [25]. Also, 15% of healthy young men may have a T level below the normal range in a 24-h period [26]. These variations make it difficult to draw conclusions based on direct T measurement alone. Therefore, FT levels were calculated based on reported method [23]. In the present study, acute administration of IND9 supplementation showed statistically significant increase in cFT but not in terms of mFT. These results are attributed to the large variations in mFT in healthy young men as reports earlier [26].

Serum total T concentrations, representing the sum of unbound and protein-bound testosterone in circulation. Most of the T is bound to SHBG or albumin with only 0.5–3% of circulating T is unbound or "free" [27, 28]. The term BT refers to FT plus albumin-bound T. BT reflects the view that albumin-bound testosterone is readily dissociable and thus bioavailable. Calculated BT is the reported as the best marker for the androgen status in males [29]. Therefore, increased FT and BT levels by IND9 in the present study might be mediated through displacement of T from binding domains of SHBG thereby making it bioavailable. However, more studies will be needed for confirmation.

In the present study, single dose of IND9 capsule supplementation was found to increase BT as compared to placebo group. In the past, 8-week supplementation of fenugreek seed extracts showed to increase strength and body composition by increasing TT and BT with decreased serum estradiol levels in resistance-trained men [30]. These effects are purported to be medicated through increasing BT levels via blocking conversion of T to estrogen and dihydrotestosterone (DHT). Further, increased BT levels are suggested to be responsible for increased protein synthesis and strength. The results of present study provided additional support to androgenic activity profile of fenugreek seed extract in sedentary male.

Recently, hypogonadism was found to increase risk of CVD. On one hand, low T levels not only correlate significantly with CVD risk factors, CHD events and mortality independent of age [31]. On the other hand, lower T and SHBG levels are reported to have inverse relationship with markers of metabolic syndrome such as triglycerides, HDL-cholesterol and hypertension in young male population [32]. Furthermore, strong link between abnormal lipid profile and CVD risk is well established. In increased T levels shown in the present study suggest possible beneficial effects of IND9 in reducing CVD risk in aging males with hypogonadism. However, detailed study of IND9 in aging male will be required to evaluate this hypothesis.

The acute administration of IND9 was well tolerated and showed excellent safety profile with no adverse events during the study. Furthermore, all T levels were found to be within physiological limits.

CONCLUSION

In conclusion, acute administration of IND9 capsule supplementation showed promising androgenic activity with good safety profile in healthy sedentary male subjects.

ACKNOWLEDGEMENTS

Authors would like to acknowledge Mr. Sunil Bhaskaran, MD, Indus Biotech Private Limited, Pune, India for providing financial support.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

REFERENCES

1. Morales A, Schulman CC, Tostain J, F CWW. Testosterone Deficiency Syndrome (TDS) needs to be named appropriately--the importance of accurate terminology. Eur Urol. 2006;50(3):407-9.
2. Bhasin S, Matsumoto AM. Patient information page from The Hormone Foundation. Patient guide to testosterone therapy in adult men with androgen deficiency syndromes. J Clin Endocrinol Metab. 2010;95(6):3085-7.
3. Harman J, Ward M. The role of nutritional therapy in the treatment of equine Cushing's syndrome and laminitis. Altern Med Rev. 2001;6 Suppl:S4-16.
4. Yeap BB, Almeida OP, Hyde Z, Norman PE, Chubb SA, Jamrozik K, et al. In men older than 70 years, total testosterone remains stable while free testosterone declines with age. The Health in Men Study. Eur J Endocrinol. 2007;156(5):585-94.
5. Marberger M, Roehrborn CG, Marks LS, Wilson T, Rittmaster RS. Relationship among serum testosterone, sexual function, and response to treatment in men receiving dutasteride for benign prostatic hyperplasia. J Clin Endocrinol Metab. 2006;91(4):1323-8.
6. Esposito K, Giugliano D. Obesity, the metabolic syndrome, and sexual dysfunction. Int J Impot Res. 2005;17(5):391-8.
7. Mulhall J, Teloken P, Brock G, Kim E. Obesity, dyslipidemias and erectile dysfunction: a report of a subcommittee of the sexual medicine society of North America. J Sex Med. 2006;3(5):778-86.
8. Corona G, Mannucci E, Forti G, Maggi M. Hypogonadism, ED, metabolic syndrome and obesity: a pathological link supporting cardiovascular diseases. Int J Androl. 2009;32(6):587-98.
9. World Health Organization. WHO monographs on selected medicinal plants. Geneva: World Health Organization; 2007.
10. Ikeuchi M, Yamaguchi K, Koyama T, Sono Y, Yazawa K. Effects of fenugreek seeds *(Trigonella foenum greaecum*) extract on endurance capacity in mice. J Nutr Sci Vitaminol (Tokyo). 2006;52(4):287-92.
11. Basch E, Ulbricht C, Kuo G, Szapary P, Smith M. Therapeutic applications of fenugreek. Altern Med Rev. 2003;8(1):20-7.
12. Thompson Coon JS, Ernst E. Herbs for serum cholesterol reduction: a systematic view. J Fam Pract. 2003;52(6):468-78.
13. Poole C, Bushey B, Foster C, Campbell B, Willoughby D, Kreider R, et al. The effects of a commercially available botanical supplement on strength, body composition, power output, and hormonal profiles in resistance-trained males. J Int Soc Sports Nutr. 2010;7:34.
14. Wilborn C, Taylor L, Poole C, Foster C, Willoughby D, Kreider R. Effects of a purported aromatase and 5alpha-reductase inhibitor on hormone profiles in college-age men. Int J Sport Nutr Exerc Metab. 2010;20(6):457-65.
15. Rao AV, Gurfinkel DM. The bioactivity of saponins: triterpenoid and steroidal glycosides. Drug Metabol Drug Interact. 2000;17(1-4):211-35.
16. Michael HN, Salib JY, Eskander EF. Bioactivity of Diosmetin Glycosides Isolated from the Epicarp of Date Fruits, Phoenix dactylifera, on the Biochemical Profile of Alloxan Diabetic Male Rats. Phytother Res. 2012.
17. Aswar U, Bodhankar SL, Mohan V, Thakurdesai PA. Effect of furostanol glycosides from *Trigonella foenum-graecum* on the

reproductive system of male albino rats. Phytother Res. 2010;24(10):1482-8.

18. Thakurdesai PA, Mohan V, Bhaskaran S. Toxicological evaluation of flavonoid glycosides based standardized fenugreek seed extract. 46th Annual Conference of Indian Pharmacological Society and International Conference on Translational Medicine; 2013 December 16-18, 2013; Bangalore, India. 2013.
19. Craig BW, Brown R, Everhart J. Effects of progressive resistance training on growth hormone and testosterone levels in young and elderly subjects. Mech Ageing Dev. 1989;49(2):159-69.
20. Kuoppasalmi K, Naveri H, Rehunen S, Harkonen M, Adlercreutz H. Effect of strenuous anaerobic running exercise on plasma growth hormone, cortisol, luteinizing hormone, testosterone, androstenedione, estrone and estradiol. J Steroid Biochem. 1976;7(10):823-9.
21. Zmuda JM, Thompson PD, Winters SJ. Exercise increases serum testosterone and sex hormone-binding globulin levels in older men. Metabolism. 1996;45(8):935-9.
22. Aizawa K, Iemitsu M, Maeda S, Otsuki T, Sato K, Ushida T, et al. Acute exercise activates local bioactive androgen metabolism in skeletal muscle. Steroids. 2010;75(3):219-23.
23. Vermeulen A. Hormonal cut-offs of partial androgen deficiency: a survey of androgen assays. J Endocrinol Invest. 2005;28(3 Suppl):28-31.
24. Cooke RR, McIntosh JE, McIntosh RP. Circadian variation in serum free and non-SHBG-bound testosterone in normal men: measurements, and simulation using a mass action model. Clin Endocrinol (Oxf). 1993;39(2):163-71.
25. Swerdloff RS, Wang C, Cunningham G, Dobs A, Iranmanesh A, Matsumoto AM, et al. Long-term pharmacokinetics of transdermal testosterone gel in hypogonadal men. J Clin Endocrinol Metab. 2000;85(12):4500-10.
26. Spratt DI, O'Dea LS, Schoenfeld D, Butler J, Rao PN, Crowley WF, Jr. Neuroendocrine-gonadal axis in men: frequent sampling of LH, FSH, and testosterone. Am J Physiol. 1988;254(5 Pt 1):E658-66.
27. Bhasin S, Cunningham GR, Hayes FJ, Matsumoto AM, Snyder PJ, Swerdloff RS, et al. Testosterone therapy in adult men with androgen deficiency syndromes: an endocrine society clinical practice guideline. J Clin Endocrinol Metab. 2006;91(6):1995-2010.
28. Giton F, Guechot J, Fiet J. Comparative determinations of non SHBG-bound serum testosterone, using ammonium sulfate precipitation, Concanavalin A binding or calculation in men. Steroids. 2012;77(12):1306-11.
29. Elmlinger MW, Kuhnel W, Doller PC. Evaluation of direct and indirect markers to assess the androgen status in healthy males during aging. Clin Lab. 2006;52(9-10):491-6.
30. Wilborn C, Taylor L, Poole C, Foster C, Willoughby D, Kreider R. Effects of a Purported Aromatase and 5 α-Reductase Inhibitor on Hormone Profiles in College-Age Men. Int J Sport Nutr. 2010;20(6):457.
31. Corona G, Rastrelli G, Monami M, Guay A, Buvat J, Sforza A, et al. Hypogonadism as a risk factor for cardiovascular mortality in men: a meta-analytic study. Eur J Endocrinol. 2011;165(5):687-701.
32. Firtser S, Juonala M, Magnussen CG, Jula A, Loo BM, Marniemi J, et al. Relation of total and free testosterone and sex hormone-binding globulin with cardiovascular risk factors in men aged 24-45 years. The Cardiovascular Risk in Young Finns Study. Atherosclerosis. 2012;222(1):257-62.