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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,*]

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.

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

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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

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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).

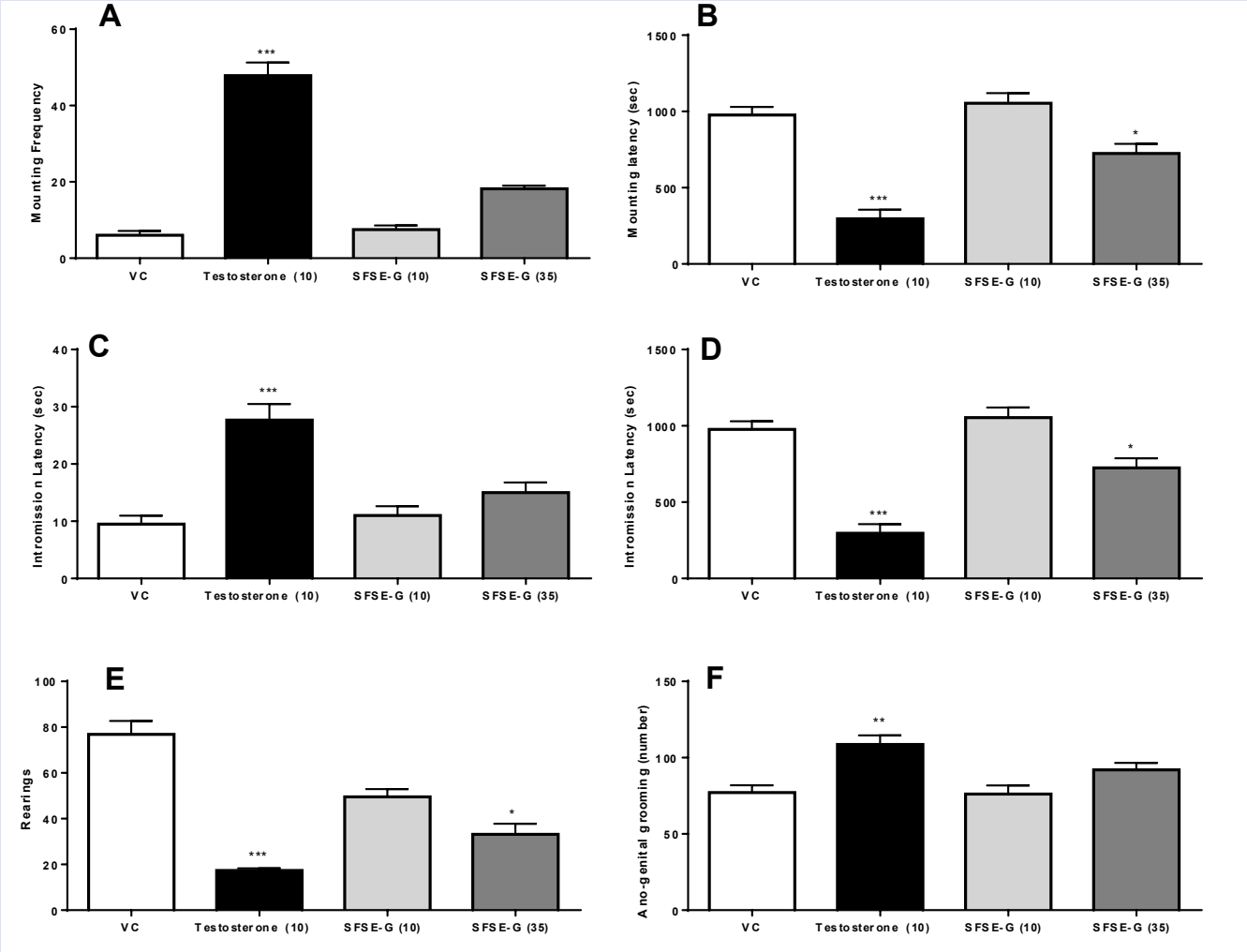

Figure 1: Effect of SFSE-G (10 and 35 mg/kg, p. o. daily) on sexual behavior-related parameters in male rats. n= 6, Data represented are as the mean ± SEM. Each parameter was analyzed using a separate one-way ANOVA followed by Dunnett's test. * P < 0.05, ** P < 0.01, *** P < 0.001 as compared to VC group.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

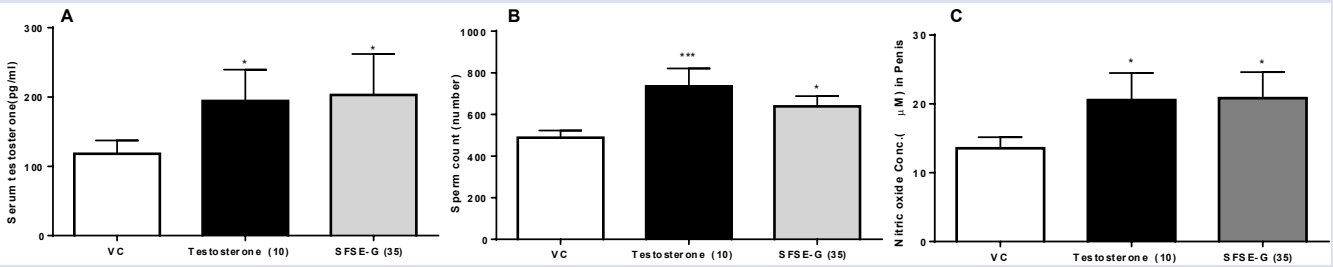

Figure 2: Effect of subacute oral administration SFSE-G (10 and 35 mg/kg) on (A) serum testosterone, (B) sperm count, (C) Penile nitric oxide (NO) concentration in male rats. n= 5, Data represented are mean ± SEM. Each parameter was analyzed using a separate one-way ANOVA followed by Dunnett's test. * 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).

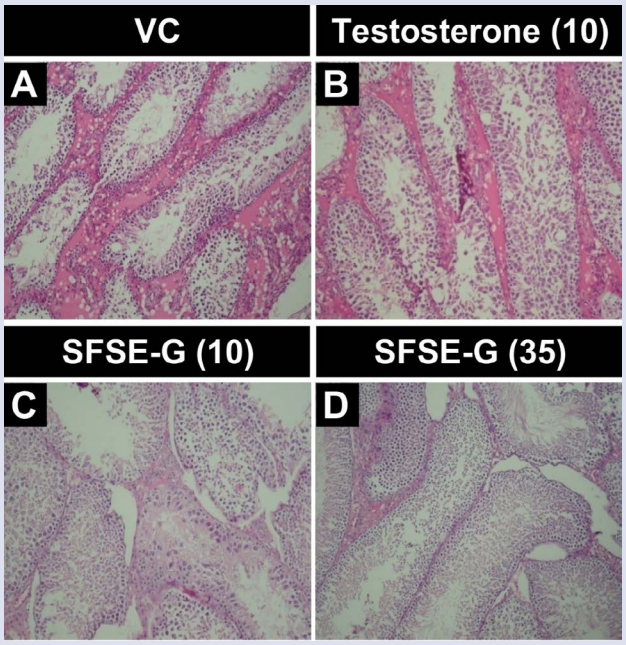

Figure 3: Photomicrographs of sections of representative testes of male rats showing the effects of subacute administration of (A) VC (vehicle control)) (B) testosterone (10 mg/kg, biweekly, subcutaneous), (C) SFSE-G (10 mg/kg, oral), and (D) SFSE-G (25 mg/kg, oral). H and E stain, ×100 magnifications.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

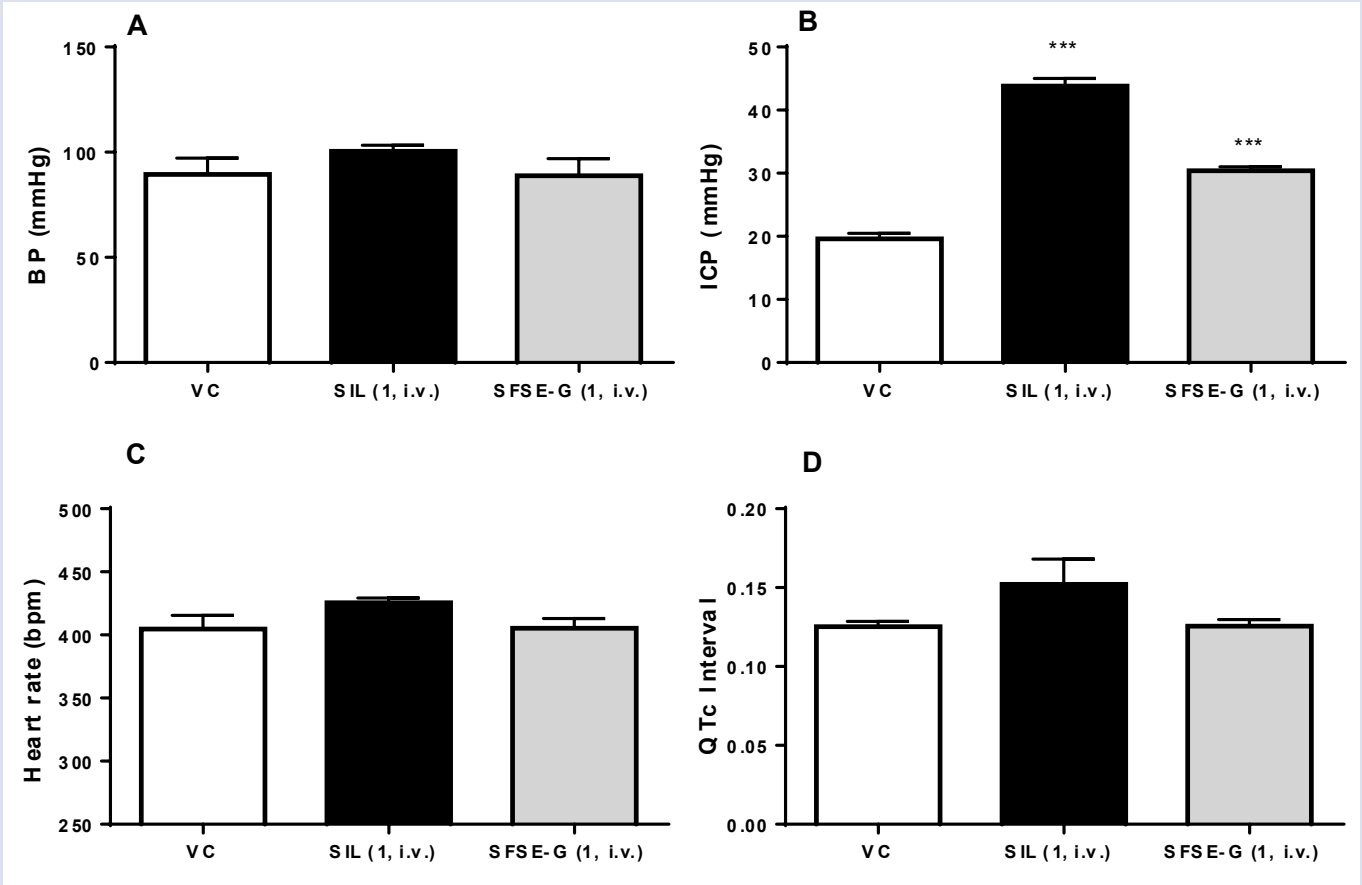

Figure 4: Effect of intravenous administration of SIL and SFSE-G at dose of 1 mg/kg on (A) blood pressure, (B) intracavernous pressure, (C) heart rate, (D) and QTc (corrected QT interval from ECG in male rats. n= 5, Data represented are mean ± SEM. Each parameter was analyzed using a separate one-way 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).

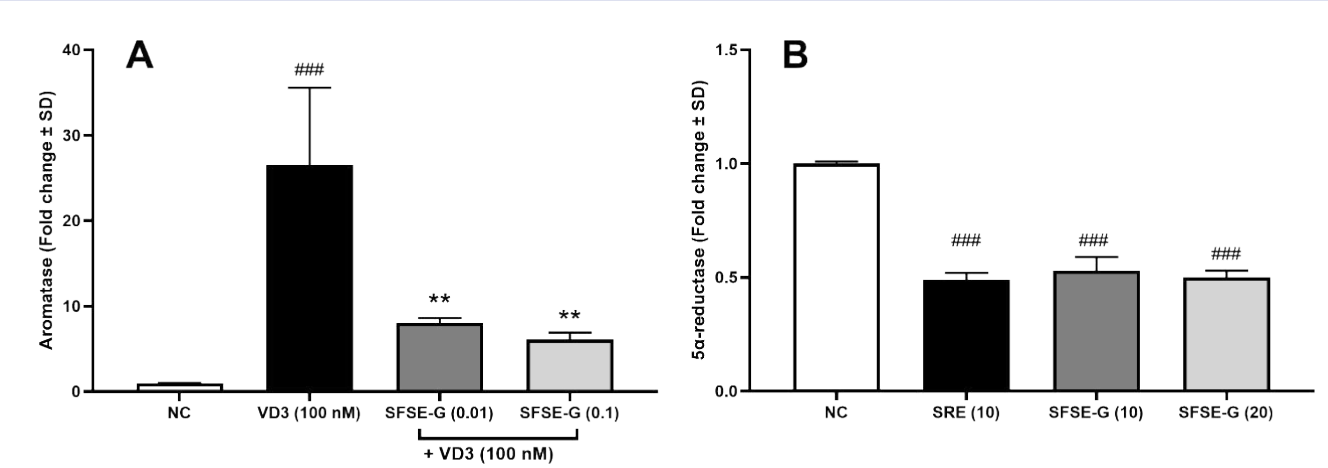

Figure 5. Effect of SFSE-G on gene expression in Fold change of enzymes (A) aromatase in human breast epithelial cells (MCF7 cell line) and (b) 5-alpha-reductase in human prostate epithelial cells (PNT2 cell line). The figures in parentheses indicate the dose in (A) ng/ml and (B) µg/ml. VD3, Vitamin 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

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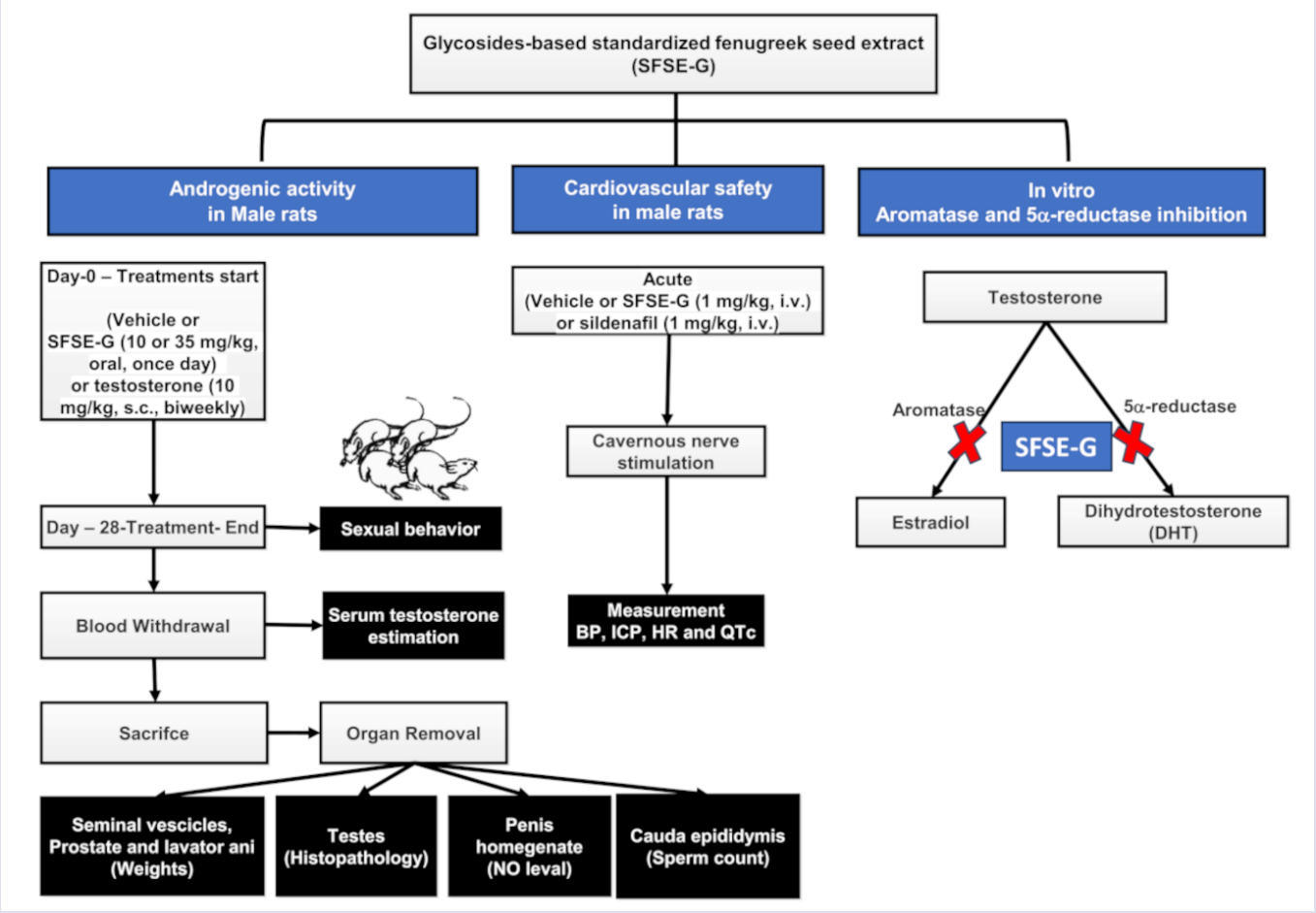
GRAPHICAL ABSTRACT



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.

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