**Sphaeranthus indicus** Attenuates Testosterone induced Prostatic Hypertrophy in Albino Rats

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The present study reports the attenuating effect of *Sphaeranthus indicus* extracts (SI) on prostatic hyperplasia induced by testosterone in albino rats. *In vitro* studies were conducted to assess the 5α-reductase inhibitory potential of the petroleum ether, ethanolic and aqueous extracts of SI. A biochemical marker, β-sitosterol, was isolated and extracts were characterized utilizing HPTLC. Testosterone (3 mg/kg s.c.) was administered to the rats along with the test extracts and isolated β-sitosterol for a period of 28 days. The weight of the rats, the urine output, serum testosterone concentrations and prostate-specific antigen (PSA) levels were recorded. The prostate/body weight ratio (P/BW) was calculated and histological studies were performed to observe the changes in the histoarchitecture of the prostate. Finasteride was used as a positive control (1 mg/kg p.o.). *Sphaeranthus indicus* extracts attenuated the increase in the P/BW ratio induced by testosterone in the treated groups. The petroleum ether extract exhibited the best activity, although the ethanol and aqueous extracts also exhibited significant activity. Urine output was also improved significantly, demonstrating the clinical implications of the study. Histological studies, testosterone levels which were measured weekly and PSA levels measured at the end of the study also support claims for the potential use of *Sphaeranthus indicus* in the treatment of prostatic hyperplasia. Copyright © 2011 John Wiley & Sons, Ltd.

**Keywords:** *Sphaeranthus indicus*; benign prostatic hyperplasia; testosterone; prostate-specific antigen; prostate/body weight ratio.

**INTRODUCTION**

Benign prostatic hyperplasia (BPH) is the result of gradual overgrowth of the prostate gland; a gland that lies at the base of the bladder and encircles the urethra. The enlarged prostate impinges on the urethra and therefore BPH is generally associated with impairment in urinary function (McMinn, 1994; Page et al., 2002). It is reported that 80% of men above an age of 80 suffer from BPH (Dull et al., 2002).

*Sphaeranthus indicus* Linn. (SI) (Compositae) is a herb found mostly in southern India. It is also known as ‘gorakhmundi’ (MHFW, 2001) and ‘East Indian globe thistle’. It is a bitter stomachic, stimulant, alternative, pectoral and demulcent, and externally emollient (Nadkarni, 1976). It is a multi-branched herb with round purple flowers that grows plentifully in rice fields (Kirtikar and Basu, 1935; Sadaf et al., 2006) and is distributed throughout India, Ceylon, Malay, China and Africa. It is used indigenously in the Indian system of traditional medicine as a remedy for various ailments, being used as a tonic, laxative, digestive, anthelmintic, and for the treatment of insanity, tuberculosis, diseases of the spleen, anaemia, bronchitis, elephantiasis, pain of the uterus and vagina, piles, asthma, leucoderma and hemicrania (Kirtikar and Basu, 1935). Practitioners of the Ayurvedic system of medicine employ this herb for constricting the enlarged prostate. The herb is an ingredient of certain proprietary marketed preparations in India and USA, namely ‘prostabliss’ (Bliss Ayurveda, USA) which is used for the management of benign prostatic hyperplasia. It is used traditionally to reduce nodular growth of the urogenital system and to reduce burning during urination caused by inflammation and infection (MHFW, 2001). Previous studies have reported the presence of stigmasterol, β-sitosterol (Gupta et al., 1967), hentriacontane, sesquiterpene lactone (Gogte et al., 1986) sesquiterpene glycoside, sphaeranthanolide (Shekhani et al., 1990), flavone and isoflavone glycosides (Yadav and Kumar, 1998) in the powdered caputula of SI.

In the prostate, dihydrotosterone (DHT) is produced from testosterone (T) by the enzyme 5α-reductase (5α-R). DHT is the more potent androgen that promotes growth of the prostate. Although the pathogenesis of BPH is not completely defined, these androgens have been identified as playing an integral part in the disease process. Inhibition of the production or actions of DHT can result in the inhibition of the growth of the prostate gland.

There is substantial clinical evidence that androgens and dihydrotosterone play a key role in the development of BPH. Androgens are also involved in the development and progression of prostate cancer. Various approaches involving attenuation of androgenic stimulation of prostatic growth and the use of 5α-reductase inhibitors such as finasteride and epristeride have been employed, but their use is limited by multiple side effects. Finasteride can cause adverse effects such as gynecomastia, impairment of muscle growth and severe myopathy due to the structural similarity to steroidal hormones. However, the magnitude of therapeutic effect produced by finasteride is relatively small, and a clinically significant benefit is observed in less than half of the...
treated patients (Uygur et al., 1998). Thus, it may be rewarding to look into traditional herbal medicines for the management of BPH.

This study was undertaken with a view to determining the antiandrogenic potential of SI on testosterone induced hypertrophy of the prostate in albino rats in vivo. Thereby, the 5α-reductase inhibitory activity of the extracts was examined in vitro, in order better to establish the mechanism(s) of action involved in the antihyperplastic effect of extracts.

MATERIALS AND METHODS

Plant material. Flower heads of SI were collected from the area adjoining the university campus. The plant was authenticated by the Department of Botany, Dr Hari Singh Gour Vishwavidyalaya, Sagar. A herbarium specimen has been deposited in the Department of Botany with an accession number of Bot/Herbarium/1011.

Preparation of extracts. Flower heads of SI were shade dried and coarsely powdered. The powdered flower heads were packed in soxhlet extractors and extracted with petroleum ether (60–80°C) until complete extraction. The solvent from the petroleum ether extract (SIP) was eliminated under reduced pressure (yield 3.63% w/w). The defatted marc was extracted with ethanol (95% v/v) to obtain the ethanol extract (SIE) (yield 17.30% w/w). The marc left after the ethanol extraction was macerated with distilled water for 24 h and the aqueous extract (SIA) was finally obtained by vacuum drying (yield 6.14% w/w).

Drugs and chemicals. Testosterone was obtained as a gift from Sun Pharma Advanced Research Center (SPARC), Vadodara, Gujarat, India. Finasteride was purchased from Sigma Aldrich, St Louis, Missouri, USA. Ethylenediamine tetracetic acid (EDTA), sodium phosphate and sucrose were purchased from Himedia Pvt. Ltd, Mumbai. Methanol, ethyl acetate and petroleum ether (60–80°C) were purchased from Qualigens Fine Chemicals Pvt Ltd, Mumbai. Testosterone ELISA kit (UBI Magiwel) was purchased from United Biotech Inc., Mountain View, California, USA and the PSA ELISA kit was purchased from Cusabio Biotech Co. Ltd, Newark, Delaware, USA. All other chemicals used in the study were of analytical grade.

Characterization of extract and selection of marker. Chromatographic profile of SIP in toluene: ethyl acetate (8:2) revealed the presence of ten spots. One of the spots showed an identical Rf value with the standard compound β-sitosterol at Rf 0.95 when visualized under UV at 254 nm. The TLC profile of SIE in chloroform: methanol: toluene (8:2:1) and SIA in butanol: acetic acid: water (4:1:3) also showed the presence of this compound on co-chromatography.

HPTLC analysis and isolation of marker compound. Precoated and preactivated TLC plates (E. Merck No. 5548) of silica gel 60 F254+366 with the support of aluminium sheets 0.1 mm thick and 20 × 20 cm were used. The SIP (10 mg) was weighed accurately and dissolved in 10 mL of petroleum ether. The extract sample was applied in the form of a band using CAMAG LINOMAT V, an automatic sample application device, maintaining a band width 6 mm, space 10 mm, 250 nL/s. The quantity of sample applied was 10 μL. The mobile phase used was toluene: ethyl acetate (8:2) (Nahata and Dixit, 2011). β-Sitosterol (10 mg) was dissolved in petroleum ether (10 mL). The solution was analysed in the same manner as described above. The AUC for β-sitosterol at 254 nm was found to be 8634.3 (Rf 0.95). The AUC for the compound at same Rf in SIP was found to be 10170.7.

For isolation of this compound, column chromatography was performed. Column was packed in methanol and was eluted with methanol. A white crystalline material melting at 140°C was isolated. It gave a positive Liebermann Burchard test indicating the presence of sterol. It was characterized as β-sitosterol after co-chromatography with the standard using TLC and HPTLC. The percentage of isolated compound in the extract was found to be 46.76%. The melting point of the isolated compound was determined using the superflit melting point determination apparatus and found to be 140°C which was identical to the standard sample (140°C). Further superimposable FTIR analysis with the standard confirmed the identity of the isolated compound as β-sitosterol. The NMR analysis of the isolate further confirmed its identity as β-sitosterol. The 1H-NMR spectrum of the isolated compound exhibited a one-proton doublet at δ 5.35 (J = 1.0 Hz) assigned to H-6 proton. A broad one-proton multiplet at δ 3.51 (J = 1.15 Hz) is ascribed to α-oriented H-3 methine proton (axial) interacting with C-2 equatorial, C-2 axial, C-4 axial and C-4 equatorial protons. Three doublets, integrating three protons each, at δ 0.94 (J = 6.34 Hz), 0.84 (J = 4.49 Hz) and 0.82 (J = 3.83 Hz) are due to C-21, C-26 and C-27 secondary methyls, respectively. A three proton triplet at δ 0.81 (J = 2.74) is ascribed to C-29 primary methyl protons. The remaining two tertiary C-18 and C-19 methyl signals appeared as singlets at δ 0.67 (J = 3.08) and δ 1.00 (J = 4.13), respectively. The presence of all the methyls in the region δ 0.67 to δ 1.00 suggests that these functionalities are attached to saturated carbons. The remaining methylene and methine protons resonated in the region δ 2.28–1.02.

Similarly SIE and SIA were also analysed for their HPTLC profiles and the presence of marker compound. The procedure was the same as followed for the case of SIP. The solvent system for SIE and SIA were chloroform: methanol: toluene (8:2:1) and butanol: acetic acid: water (4:1:3), respectively. The percentage of β-sitosterol came out to be 22.26% in SIE (Rf 0.93, 254 nm) and 13.16% in SIA (Rf 0.71, 254 nm).

Preparation of enzyme solution. Human prostate (about 200 mg) procured from the local hospital of Sagar was minced in small pieces and homogenized in 10 mL of medium A (20 mM sodium phosphate, pH 6.5, containing 0.32 mM sucrose and 1 mM EDTA) as described previously (Pandit et al., 2008; Nandecha et al., 2010; Nahata and Dixit, 2011). The homogenate was centrifuged at 4000 rpm (716 × g) for 15 min. The supernatant was used as a source of enzyme, 5α-reductase. The concentration of enzyme in the supernatant was determined by Bradford method of protein estimation (Bradford, 1976).
Preparation of test materials. Testosterone (1 mM solution in ethanol), extracts (1 mg/mL) were prepared in ethanol (95%) with gentle heating wherever necessary. EDTA solution (10 mg/mL) was made in distilled water. Finasteride (10 mg/mL) was prepared in ethanol.

Determination of optimum concentration of enzyme. It was determined by keeping testosterone concentration constant and varying the concentration of the prostate homogenate (containing the enzyme). Testosterone solution (1 mM) was prepared in ethanol. The reaction mixture (1 mL) was prepared by adding testosterone solution (0.1 mL), enzyme solution (0.1–0.9 mL) and sodium phosphate buffer (20 mM). The reaction mixture was incubated at 37 °C for 1 h. The reaction was terminated by the addition of 2 mL of ethyl acetate. The reaction mixture was then shaken vigorously for 1 min and the ethyl acetate layer was separated. It was evaporated to dryness, and the residue dissolved in 2 mL of methanol. The testosterone content in the methanol solution was estimated by HPLC (Shimadzu AT10, Kyoto, Japan, fitted with a diode-array detector). The volume of sample injected in the column was 20 μL. The detection wavelength was 245 nm and the retention time for testosterone was 6.35 min. The column was eluted isocratically with a mobile phase of methanol:water (80:20) at a flow rate of 1.0 mL/min (Purdon and Lehman-McKeeman, 1997). The optimum amount of enzyme solution which is required for the conversion of testosterone to dihydrotestosterone was found to be 0.8 mL.

Determination of inhibitory concentrations of extract. The reaction mixture (1.5 mL) was made by adding 0.1 mL of testosterone solution, 0.1 mL of EDTA solution, 0.1–0.5 mL of extract solutions (in their respective solvents) for separate groups, optimum amount of enzyme solution (i.e. 0.8 mL) and sodium phosphate (20 mM), to a final volume of 1.5 mL. The reaction mixture was incubated at 37 °C for 60 min, and the reaction was terminated by the addition of 2 mL of ethylacetate. The rest of the procedure is the same as that described in the section above.

In vivo studies. The results of the in vitro studies were encouraging as appreciable 5α-reductase inhibitory activity was found in the test extracts. Henceforth to assess their in vivo effects and to validate the findings of in vitro studies, in vivo studies were performed.

Animals. Male Sprague-Dawley rats weighing 100–250 g (2–3 months old) were housed in polypropylene cages at room temperature (25 ± 2 °C) and were fed on standard pellet diet (Brooke Bond, Lipton, India) and water ad libitum. The protocol for animal experimentation was approved by the Institutional Animal Ethics Committee of B. R. Nahata College of Pharmacy, Contract Research Center, Mandsaur, Madhya Pradesh, India (Reg. No. 918/ac/05/CPCSEA).

Acute toxicity studies. Acute toxicity studies were performed following OECD guidelines (2001) (OECD 423, Acute Toxic Class Method) (Roll et al., 1986). 2000 mg/kg oral dose of the test extract (SIP, SIE and SIA) was found to be safe as no mortality was observed during the study. On the basis of these studies, the doses of 10, 20 and 50 mg/kg p.o. were selected for all the extracts of SI. Preparation of extracts. The SIP, SIE and SIA were suspended in Tween-80 solution (0.2% v/v) for oral administration. Rats were given an oral dose of 10, 20 and 50 mg/kg p.o. once daily for 28 days (Carabajal et al., 2004). Testosterone was dissolved in arachis oil for s.c. injection (3 mg/kg s.c.). Finasteride was suspended in Tween 80 (0.2% v/v) and administered per orally (1 mg/kg p.o.). Isolated β-sitosterol was also suspended in Tween-80 solution for oral dosing.

Experimental design. The rats were divided into 14 groups with six rats in each group. Vehicle treated control group received arachis oil (s.c) and Tween 80 (0.2% v/v p.o.). Testosterone treated group was administered testosterone (3 mg/kg s.c) and Tween 80 (0.2% v/v p.o.). The finasteride treated group received finasteride (1 mg/kg p.o.) along with testosterone (3 mg/kg s.c). The extract treated groups were administered SI extracts (10, 20 and 50 mg/kg p.o.) along with testosterone (3 mg/kg s.c). Isolated β-sitosterol was also administered (10 and 20 mg/kg p.o.) along with testosterone (3 mg/kg s.c) to separate groups of animals.

Body and prostatic weights. Body weights were taken a day before starting of the treatment (baseline) and thereafter weekly until the completion of the study, i.e. on day 28 of treatment. On day 29, the animals were anaesthetized under light ether anesthesia and killed. The prostates were immediately dissected out and weighed. Mean body weights and prostatic/body weight ratios were calculated for each group. On the basis of mean prostatic weights and P/BW ratios, the % recovery in P/BW ratio by test groups was also calculated compared with the testosterone treated group. The increase induced by testosterone was considered 100% and all other test groups were compared with this reading taken as control. The reduction in weight induced by test extracts was compared with the testosterone treated group. The formula used for calculation of % recovery was

\[
\% \text{ recovery by the test sample} = \frac{A - B}{A} \times 100
\]

where A is the % increase in prostatic weight induced by testosterone (considered 100%), and B is the % increase in prostatic weight induced by test sample.

Measurement of urine output. The urine output of individual animals was monitored at the beginning of the study, i.e. on day 0 and thereafter once weekly until the completion of the studies, i.e. day 28 of the study. Metabolic cages were used for the purpose of urine collection. The animals were kept for 24 h in the cages and the urine volume was recorded for each individual animal of each group. During this period, the animals had free access to food and water.

Measurement of serum testosterone concentration. Testosterone levels of individual animals of each group were measured weekly using a testosterone ELISA kit. After every 7 days, the effect of the test samples on the serum testosterone levels was measured using an ELISA reader (Bioline BPR08). Blood was collected from the retro-orbital plexus of the rats and centrifuged at 2000 × g for 20 min to separate the serum. This serum was tested for its testosterone content using the procedure supplied with the kit (UBI Magiwel total

testosterone kit). The UBI Magiwel testosterone quantitative test is based on the principle of competitive solid phase enzyme immunoassay. The test sample competes with enzyme-labeled-testosterone for a fixed and limited number of antibody sites on the microtiter wells. In the assay procedure, the testosterone standard or test serum is incubated with the testosterone antibody and the testosterone-horseradish peroxidase conjugate in the antirabbit IgG coated well. In this solid-phase system, the antibody bound testosterone will remain on the well while unbound testosterone will be removed by washing. A color is developed when TMB substrate is mixed with the antibody testosteronhorseradish peroxidase enzyme conjugate. After a short incubation, the enzyme reaction is stopped and the intensity of the color is measured with a microreader at 450 nm.

Measurement of prostate-specific antigen. Prostate-specific antigen (PSA) levels were measured for individual rats of each group to find the severity of hyperplasia induced in the prostate by testosterone treatment. For this purpose a PSA ELISA kit was utilized. The PSA ELISA kit is intended for the quantitative determination of total PSA (prostate-specific antigen). This kit was obtained from Cusabio Biotech Co. Ltd, Newark, Delaware, USA. The PSA was quantitated by the method of Nilsson et al. (1997). The PSA ELISA is a solid-phase, noncompetitive immunoassay based upon the direct sandwich technique. Calibrators, controls and samples were incubated together with biotinylated anti-PSA monoclonal antibody and horseradish peroxidase (HRP) labelled anti-PSA monoclonal antibody in streptavidin-coated microtiter strips. After washing, buffered substrate (TMB-HRP substrate) which contains hydrogen peroxide and chromogen reagent (3,3′,5,5′ tetramethyl benzidine) was added to each well and the enzyme reaction was allowed to proceed. The color intensity was determined in the microtiter plate spectrophotometer at 620 nm. Calibration curves were constructed for each assay by plotting absorbance versus the concentration of each calibrator. The PSA concentration of samples was then read from the calibration curve.

Histological studies. After prostatic weight measurement, the tissues were fixed in 10% formalin (in normal saline). After 24 h, the tissues were subjected to histological studies using a microtome followed by haematoxylin and eosin (H–E) staining. The slides were observed under a microscope (Labovision trinocular microscope) and the images recorded. One of the authors, Professor V. K. Dixit, who read the histology specimens, was kept blind to the treatment groups.

Statistical analysis. All results are expressed as mean ± SEM (n = 6). Comparisons between groups were performed using the Dunnett’s test using Graph pad Prism statistical software. Values of p and F values and the degrees of freedom were calculated. A value of p < 0.05 was considered to be statistically significant.

RESULTS

High-performance TLC

Co-chromatography of SI extracts along with β-sitosterol as a biochemical marker revealed the presence of β-sitosterol in the extract, with an Rf value of 0.95, 0.93 and 0.71, respectively, for SIP, SIE and SIA when analysed at 254 nm. The identity of marker was confirmed by melting point determination and overlain FTIR analysis with the standard and NMR analysis. As β-sitosterol is a well known steroidal molecule established clinically for the treatment of benign prostatic hyperplasia (Wilt et al., 1999), the presence of β-sitosterol as a major constituent in all the extracts support our claims of the antihyperplastic activity of SI.

In vitro studies

The optimum concentration of the enzyme was found to be 270.0 µg (present in 0.8 mL of enzyme solution calculated by the Bradford method of protein estimation) (Bradford, 1976). Varying concentrations of test samples (extracts and finasteride) were incubated with a constant amount of testosterone and enzyme in the reaction mixture, and the residual testosterone content was determined after termination of the reaction with ethyl acetate. The residual testosterone content in the reaction mixture increased with increasing concentrations of SIP, SIE, SIA and finasteride. The IC50 value, i.e. the concentration of test compound required for 50% inhibition of the control conversion of 1 mM testosterone, was calculated by regression analysis (Table 1).

In vivo studies

The results of the in vitro studies paved the way for the pharmacological screening of the extracts to evaluate their potential against hyperplasia induced by testosterone in rats. The results obtained are discussed as follows.

Determination of body weight, prostatic weight and prostate/body weight (P/BW) ratio of test groups. In the testosterone treated group, mean body weight and mean prostatic weight showed a considerable increase after 28 days of treatment. In the case of the vehicle treated group, no appreciable increase in body weight was observed as noted in Table 2. While in the case of the finasteride treated group, a decrease was observed. Table 2 summarizes the effects of SIP (10, 20 and 50 mg/kg p.o.), SIE (10, 20 and 50 mg/kg p.o.), SIA (10, 20 and 50 mg/kg p.o.) and finasteride (1 mg/kg p.o.) on prostatic hyperplasia induced with testosterone. The P/BW ratio calculated in the case of the vehicle treated group was 1.47 ± 0.15. It was 7.27 ± 0.38 for the testosterone treated group (p < 0.01 compared with the finasteride treated group) and 2.62 ± 0.27 for the finasteride treated group (p < 0.01 compared with the

Table 1. 5α-Reductase inhibitory concentrations (IC50) of treated groups

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum ether extract</td>
<td>0.132 mg</td>
</tr>
<tr>
<td>2</td>
<td>Ethanol extract</td>
<td>0.317 mg</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous extract</td>
<td>0.387 mg</td>
</tr>
<tr>
<td>4</td>
<td>Finasteride</td>
<td>1.06 µg</td>
</tr>
</tbody>
</table>
Table 2. Effect of test extracts of Sphaeranthus indicus and isolated β-sitosterol on prostatic weight

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Body weight (g)</th>
<th>Prostatic weight (mg)</th>
<th>Prostatic/body weight ratio (P/BW ratio)</th>
<th>Treatment effect on P/BW ratio (P2 - P1)</th>
<th>% increase in prostate weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank control (vehicle only)</td>
<td>110.75 ± 9.21</td>
<td>117.50 ± 14.93</td>
<td>170.15 ± 22.08</td>
<td>1.47 ± 0.15</td>
<td>0</td>
</tr>
<tr>
<td>Testosterone (T) (3 mg/kg s.c.)</td>
<td>113.75 ± 36.36</td>
<td>138.75 ± 38.31</td>
<td>1028.41 ± 21.83</td>
<td>7.27 ± 0.38</td>
<td>5.80</td>
</tr>
<tr>
<td>T + Finasteride (1)</td>
<td>127.50 ± 12.50</td>
<td>126.25 ± 7.46</td>
<td>337.67 ± 56.19</td>
<td>2.62 ± 0.27</td>
<td>1.15</td>
</tr>
<tr>
<td>SIP 10 + T</td>
<td>152.00 ± 36.17</td>
<td>153.75 ± 33.62</td>
<td>785.95 ± 251.62</td>
<td>4.73 ± 0.57</td>
<td>3.26</td>
</tr>
<tr>
<td>SIP 20 + T</td>
<td>177.50 ± 27.50</td>
<td>173.75 ± 26.72</td>
<td>683.80 ± 122.51</td>
<td>3.88 ± 0.12</td>
<td>2.41</td>
</tr>
<tr>
<td>SIP 50 + T</td>
<td>97.50 ± 14.36</td>
<td>110.00 ± 21.60</td>
<td>339.40 ± 65.83</td>
<td>3.01 ± 0.28</td>
<td>1.54</td>
</tr>
<tr>
<td>SIE 10 + T</td>
<td>105.00 ± 11.90</td>
<td>120.00 ± 5.774</td>
<td>635.78 ± 66.80</td>
<td>6.60 ± 0.17</td>
<td>5.13</td>
</tr>
<tr>
<td>SIE 20 + T</td>
<td>160.00 ± 43.39</td>
<td>187.50 ± 33.26</td>
<td>916.87 ± 229.06</td>
<td>5.25 ± 0.30</td>
<td>3.78</td>
</tr>
<tr>
<td>SIE 50 + T</td>
<td>202.50 ± 8.53</td>
<td>221.25 ± 7.18</td>
<td>1464.60 ± 64.98</td>
<td>4.71 ± 0.49</td>
<td>3.24</td>
</tr>
<tr>
<td>SIA 10 + T</td>
<td>122.50 ± 7.50</td>
<td>120.00 ± 8.66</td>
<td>511.00 ± 42.52</td>
<td>4.08 ± 0.19</td>
<td>2.61</td>
</tr>
<tr>
<td>SIA 20 + T</td>
<td>112.50 ± 2.50</td>
<td>120.00 ± 4.08</td>
<td>492.4 ± 13.14</td>
<td>4.10 ± 0.14</td>
<td>2.63</td>
</tr>
<tr>
<td>SIA 50 + T</td>
<td>220.00 ± 23.45</td>
<td>240.00 ± 18.70</td>
<td>909.5 ± 80.77</td>
<td>3.79 ± 0.13</td>
<td>2.32</td>
</tr>
<tr>
<td>Isolate 10 + T</td>
<td>95.00 ± 5.00</td>
<td>105.00 ± 6.00</td>
<td>475.95 ± 20.45</td>
<td>4.31 ± 0.15</td>
<td>2.84</td>
</tr>
<tr>
<td>Isolate 20 + T</td>
<td>105.00 ± 5.00</td>
<td>110.00 ± 0.00</td>
<td>424.50 ± 15.82</td>
<td>4.18 ± 0.16</td>
<td>2.71</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6). ANOVA followed by Dunnett's test. Df = 13, 103

*p < 0.05 versus testosterone treated group; **p < 0.01 versus finasteride treated group.*

†Recovery in P/BW ratio by test groups compared with the testosterone treated group.

P1, increase in P/BW ratio of blank group; P2, increase in P/BW ratio of the test group.

testosterone treated group). In the case of SIP, the P/BW ratios were 4.73 ± 0.57, 3.88 ± 0.12 and 3.01 ± 0.28 for 10, 20 and 50 mg/kg doses, respectively. Similarly in the case of the SIE treated groups, the ratios were 6.60 ± 0.17, 5.25 ± 0.30 and 4.71 ± 0.49 for 10, 20 and 50 mg/kg doses, respectively. In the case of the SIA treated groups, the ratios were 4.18 ± 0.16 for 10 and 20 mg/kg, respectively.

The % recoveries compared with the testosterone treated group showed a recovery of 51.07% (10 mg/kg) and 53.31% (20 mg/kg). Recovery with standard finasteride (1 mg/kg) was 80.10%.

Measurement of urine output. The urethra is pressed by the overgrowth of the prostate which results in the obstruction in urine flow. The mean urine output was measured to denote the clinical implications of the study as urine flow is seriously obstructed in the case of hyperplasia of the prostate. In the case of the vehicle treated control group, there was practically no change in urine output. As the hyperplasia progressed with testosterone treatment the urine output was reduced and the output was drastically reduced after 28 days of treatment in the testosterone treated control group animals. When SI extracts and isolated compound were administered along with testosterone, significant improvements in urine output over the testosterone control group were observed. The p and F values and the degrees of freedom were calculated and are listed below:

\[ p = 0.0001, F = 3.318, Df = 13, 103 \]
\[ p = 0.0001, F = 2.989, Df = 13, 103 \]
\[ p = 0.0001, F = 7.676, Df = 13, 103 \]
\[ p = 0.0001, F = 7.979, Df = 13, 103 \]

Treated groups showed significant results (p < 0.01) compared with the testosterone treated group. The results are depicted in Fig. 1.

Measurement of serum testosterone concentration. The 5α-reductase inhibitory activity found in the extracts during in vitro studies was validated during in vivo studies by measuring the serum testosterone concentration of various groups. The extracts as well as isolated and finasteride treatment increased the testosterone levels in the serum of the test animals as seen by the levels determined by using testosterone ELISA kit. In the normal vehicle treated group the levels were unchanged during the study as measured on days 0, 7, 14, 21 and 28 of the study. The testosterone treated group showed a decrease in these levels as the study progressed and this decrease continued until the end of the study which implicates the activity of the enzyme.
(5α-reductase) in the prostate of testosterone treated animals. Treatment of animals with exogenous testosterone caused its elevation in serum. Simultaneous administration of extracts and isolate along with testosterone led to a further increase in serum testosterone levels suggesting inhibition of 5α-reductase activity of the extracts and isolate. The results are depicted in Fig. 2. The \( p \) and \( F \) values and the degrees of freedom were calculated \( (p < 0.0001, F = 76.908, Df = 13, 155) \). In the test groups, the \( p \) values were found to be significant \( (p < 0.01) \) compared with the testosterone and finasteride treated control groups.

**Measurement of prostate-specific antigen.** The PSA serum levels are abnormally elevated in patients with prostate cancer, benign prostatic hypertrophy (BPH) and patients with prostate inflammatory conditions (Catalona et al., 1995). The effect of the administration of test extracts, isolate and finasteride along with testosterone on the PSA levels of rats is an indication of the hypertrophy of the prostate induced by testosterone. This parameter was measured in the serum of the test animals of various groups using the PSA ELISA kit following the procedure supplied with the kit. The normal PSA level in the vehicle treated group was found to be \( 0.140 \pm 0.087 \) µg/L. The PSA level increased to \( 1.533 \pm 0.731 \) µg/L in the testosterone treated group. The finasteride treated group showed a decrease in the PSA level to \( 0.900 \pm 0.378 \) µg/L \( (p < 0.05 \) compared with the testosterone treated group). The SIP in doses of 10, 20 and 50 mg/kg p.o. showed PSA levels of \( 0.425 \pm 0.675 \) \( (p < 0.01) \), \( 0.183 \pm 0.693 \) \( (p < 0.01) \) and \( 0.163 \pm 0.025 \) \( (p < 0.01) \) µg/L, respectively, which indicate the protective effects of SIP on testosterone induced hyperplasia. The SIE also caused a decrease in the PSA levels with values of \( 0.375 \pm 0.311 \) \( (p < 0.01) \), \( 0.250 \pm 0.025 \) \( (p < 0.01) \) and \( 0.205 \pm 0.121 \) \( (p < 0.01) \) µg/L for 10, 20 and 50 mg/kg p.o., respectively. The SIA showed PSA levels of \( 0.360 \pm 0.036 \) \( (p < 0.01) \), \( 0.233 \pm 0.145 \) \( (p < 0.01) \) and \( 0.205 \pm 0.001 \) \( (p < 0.01) \) µg/L for 10, 20 and 50 mg/kg p.o., respectively. The decreases observed were significant compared with the testosterone treated group. The isolate treated groups also exhibited significant effects with PSA levels of \( 0.880 \pm 0.325 \) \( (p < 0.05) \) and \( 0.445 \pm 0.126 \) \( (p < 0.01) \) µg/L for 10 and 20 mg/kg p.o., respectively. These observations indicate the protective effects of the extracts on testosterone induced hyperplasia. The observations are depicted in Fig. 3.

**Histological examinations**

*Control group (arachis oil).* Normal histological features of prostate gland were visible showing tubules of variable diameter and irregular lumen. The lumens were filled with prostatic secretions. In the connective tissue, blood vessels and lymph vessels, the matrix was normal. In some places, an aggregation of columnar cells was observed. The prostate gland is surrounded by capsule; a thick layer of involuntary muscles with distinct nucleus and normal sarcoplasmic texture was visible (Fig. 4A).
Testosterone treated group (3 mg/kg s.c.). The tubules became wider compared with the control. The walls of tubules were thickened and every tubule almost had developed large involutions projecting into the lumen, reducing the volume of the lumen compared with the control. The amount of the secretion in some tubules increased. The connective tissue was compressed and the blood vessels were dilated compared with the control. The distinct nucleus and normal sarcoplasmic texture were not visible. The shape of the tubules was obliterated. The lumen was narrow, but at most places, the transitional nature of the epithelium persisted (Fig. 4B).

Testosterone + finasteride (1 mg/kg, p.o.). Normal distribution of stroma was seen. The projections were not prominent as seen in the testosterone treated group. Although finasteride antagonized the effects of testosterone to a good degree, several cells with an increased volume were present throughout the transitional epithelium. Cells with swollen nuclei were prominent in many places. Reduced involutions in lesser number were observed (Fig. 4C).

Testosterone + SIP (10, 20 and 50 mg/kg p.o.). Vacuolization in the cells was clear. The lumen of the tubules was normal and in some places the epithelium was slightly thicker than the control. The lumen was filled with more eosinophilic secretions. The squamous cells were scanty in number, but more cuboidal and pear shaped cells were present in the transitional epithelium. Involutions were
few in number and even less than that observed in the control. The connective tissue between the tubules was reduced. Tubules had a large lumen. End secretory parts of the prostate gland were easily visible. The stroma is composed of smooth muscles and connective tissue. A significant improvement compared with the testosterone treated group could be identified easily (Fig. 4D, 4E, 4F).

**Testosterone + SIE (10, 20 and 50 mg/kg p.o.).** With the better intraluminal secretions, the tubules showed morphological improvement in texture. The epithelium was wide and thicker. Compared with the testosterone treated group, the stroma (composed of connective and smooth muscle cells) was normal. The end secretory parts of the gland were more maintained than the testosterone treated group. The appearance of the transitional epithelium resembled that of the control. The involutions had become thicker than those seen in the control and fewer in number. Some tubules still had a wider lumen with smooth transitional epithelium. Minor curvature in the epithelium was also observed (Fig. 4G, 4H, 4I).

**Testosterone + SIA (10, 20 and 50 mg/kg p.o.).** The shape and size of the tubules were recovered. The epithelium was more maintained compared with the testosterone treated group. End secretory parts of the gland were easily visible and prostatic concretions could be seen. The stroma was normal composed of smooth muscles and connective tissues. The prostatic parts of the urethra were also visible. Compared with the testosterone treated group, there was a significant improvement in the histoarchitecture (Fig. 4J, 4K, 4L).

**Testosterone + isolated β-sitosterol (10 and 20 mg/kg p.o.).** The lumen of the tubules was normal and in some places the epithelium was slightly thicker than the control. The stroma was normal. The end secretory parts of the gland were more maintained than in the testosterone treated group. The appearance of the transitional epithelium resembled that of the control. Overall the effects induced by testosterone were effectively antagonized in these groups (Fig. 4M, 4N).

**DISCUSSION**

Testosterone is converted to the more potent dihydrotestosterone by the enzyme 5α-reductase present in prostate homogenates (Steers, 2001; Dhanotiya et al., 2009). Addition of SIP, SIE and SIA in the reaction mixture showed increased levels of unchanged testosterone in the reaction mixture, suggesting an inhibition...
of enzyme action by these test materials. Furthermore, the inhibition of conversion by these materials clearly reflected that enzyme activity was blocked and, therefore, more testosterone remained unchanged in the reaction mixture. The results of the present investigations suggest that SIP, SIE and SIA inhibit prostatic hyperplasia induced with an exogenous supply of testosterone in a rat model.

The etiology of BPH in humans is heterogeneous, and no other species shows the same complexity of this disorder. Animal models of BPH studied to date do not appear to fully mimic the stromal and epithelial changes with BPH in humans (Mahapokai et al., 2000). Therefore, in vitro and animal models are of limited value for the study of BPH events. Spontaneous animal models are limited to ‘nonhuman’ primates and canines (hormone-induced BPH in canines appears to be an especially replicate model of human BPH), but ethical and economic problems have reduced the applicability of these models (Carson and Rittmaster, 2003). In particular, BPH induced with testosterone or dihydrotestosterone does not reproduce all findings of BPH in humans because the pathogenesis of BPH is dependent on a functional androgenic signal involving several components (e.g. testosterone synthesis in the testes, conversion of testosterone to dihydrotestosterone, transportation of dihydrotestosterone to target prostate tissues, binding of dihydrotestosterone to androgenic receptor and the subsequent gene modulation) (Carson and Rittmaster, 2003; Deslypere et al., 1992; Zhou et al., 1995). BPH in humans also involves prostatic estrogens and α-adrenergic receptors not fully reproducible in other models. Thus, the model used in the present study has limitations in predicting the effects of any treatment in the management of BPH in humans.

Nevertheless, the effects of testosterone and dihydrotestosterone on prostatic growth in rodents have previously been documented and used to assess the effects of drugs used for prostatic hyperplasia treatment, including saw palmetto fruit lipid extract (Bombardelli et al., 1997; Paubert-Braquet et al., 1996). In the present study, in rats administered with SIP, SIE and SIA along with testosterone, the increase in prostatic weight and P/BW ratio were attenuated after 28 days of oral treatment at different doses compared with the only testosterone treated group. The weekly measurement of testosterone concentration in serum also supports our findings. The levels of testosterone were increased significantly after 14 days and this increase is a result of the inhibition of the enzyme 5α-reductase by the test extracts, the enzyme being responsible for the conversion of testosterone to dihydrotestosterone which is more potent than testosterone in causing inflammation of the prostate. Our extracts proved to be inhibitors of 5α-reductase and hence retained less harmful testosterone in the body.

Urinary output was measured once weekly to assess the clinical implications of the study. It was found that urine output was decreased drastically in the testosterone treated group. A significant increase was observed in urine output in the extract, isolate and finasteride treated groups. In most of the cases urine output returned to the normal values as on day 0 of the study. This finding is significant as prostatic hyperplasia directly causes urinary problems such as painful micrturition, reduced urine flow, urinary urgency etc. The results of the study could provide a solution to the problem if adopted clinically.

Further prostate-specific antigen levels were measured at the end of the study, i.e. on day 28. Prostate-specific antigen (PSA) is a protein produced by the cells of the prostate gland. The PSA test measures the level of PSA in the blood. PSA serum levels are abnormally elevated in patients with prostate cancer, benign prostatic hypertrophy (BPH) and patients with prostate inflammatory conditions. If a decrease in PSA levels is observed, it can be considered that the test sample in question is having protective effects on the inflammatory conditions and hypertrophy of the prostate induced by testosterone. Testosterone treatment increased the PSA levels which is an indication of hyperplasia, whereas finasteride reduced the PSA levels significantly suggesting its protective effects. All the extracts as well as the isolated compound significantly reduced the PSA levels which are an indication of their 5α-reductase activity and efficacy in the treatment of prostatic hyperplasia.

The results of the study suggest that SIP prevented prostatic hyperplasia significantly in a dose dependent manner with 50 mg/kg showing the best activity. SIE, SIA and isolated β-sitosterol also showed significant activity in a dose dependent manner. The in vitro studies cleared the mechanism of prevention of prostatic hyperplasia induced by testosterone. It is evident that SI extracts have 5α-reductase inhibitory activity. The weekly serum testosterone levels are suggestive of the mechanism of action of the extracts and finasteride. The recovery in urine output also suggests that the extracts have a positive effect on hypertrophy of the prostate. As β-sitosterol is a well known molecule established clinically for the treatment of benign prostatic hyperplasia (Wilt et al., 1999), the presence of β-sitosterol as a major constituent in all the extracts further support our claims. The histological findings showed a recovery in the prostatic histoarchitecture particularly in the cuboidal epithelial cells, intracellular lumen, tubular latency and shape which lend further support for SI as a strong candidate for the management of prostatic hyperplasia. Further studies are necessary to confirm the effect of the drug on BPH in humans. The study shows that SI holds sufficient promise to be used as a drug for the prevention of benign prostatic hyperplasia and is a firm candidate for further clinical research in this area.

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Conflict of interest

The authors have no potential conflicts of interest.
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