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Ameliorative effects of stinging nettle (*Urtica dioica*) on testosterone-induced prostatic hyperplasia in rats

A. Nahata & V. K. Dixit

Department of Pharmaceutical Sciences, Doctor Hari Singh Gour Vishwavidyalaya, Sagar, Madhya Pradesh, India

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Correspondence
Prof. Dr. Vinod Kumar Dixit, Department of Pharmaceutical Sciences, Doctor Hari Singh Gour Vishwavidyalaya, Sagar 470 003, Madhya Pradesh, India.
Tel.: +917582264582;
Fax: +917582403051;
E-mail: dixitvk2011@rediffmail.com; aloknahata@gmail.com

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Summary

The present study investigated the effects of stinging nettle (*Urtica dioica* L.) (UD) on benign prostatic hyperplasia (BPH) induced by testosterone. *In vitro* studies were conducted to assess the 5α-reductase inhibitory potential of UD. Two biochemical markers viz., *β*-sitosterol and scopoletin, were isolated and characterised in the extracts utilising High-performance thin layer chromatographic, FTIR, NMR and overlain UV spectral studies. Hyperplasia was induced in rats by subcutaneous administration of testosterone (3 mg kg^{-1} s.c.) for 28 days in all the groups except the vehicle-treated group. Simultaneous administration of petroleum ether and ethanolic extracts (10, 20 and 50 mg kg^{-1} p.o.) and isolated *β*-sitosterol (10 and 20 mg kg^{-1} p.o.) was undertaken. Finasteride was used as a positive control (1 mg kg^{-1} p.o.). Measurement of prostate/body weight ratio, weekly urine output and serum testosterone levels, prostate-specific antigen levels (on day 28) and histological examinations carried out on prostates from each group led us to conclude that UD can be used as an effective drug for the management of BPH.

Introduction

Benign hyperplasia of the prostate gland (BPH) is a condition experienced by a majority of ageing men. Although not usually life threatening, it results in a variety of symptoms related to blockage of urine outflow by the enlarged prostate gland (Birkhoff, 1983). Of men, 50–80% over 50 years of age suffer from BPH. Treatment for this condition is usually surgery to remove as much of the enlarged gland as possible, thereby restoring urinary flow. Currently, there are few medical treatments for BPH. Although not approved for use in this condition, alpha blockers have been used in treatment of BPH, and it is hypothesised that reduction of the alpha adrenergic stimulation gives symptomatic relief (Lepor, 1989). A specific inhibitor of the 5α-reductase enzyme, finasteride (also known as MK-906 and Proscar®), has recently been approved by the FDA for the treatment of BPH. Inhibiting 5α-reductase in men causes a significant decrease in intraprostatic dihydrotestosterone (DHT) by decreasing the conversion of testosterone to DHT (McConnell *et al.*, 1989). This decrease in intraprostatic DHT is thought to cause the decrease in prostate volume seen in men treated with finasteride (Stoner, 1990).

Phytotherapeutics are very popular for the treatment of BPH, especially in Europe, where numerous plant extracts have been used. One of the most popular plants is stinging nettle (*Urtica dioica*, family Urticaceae) (UD). Various water extracts from UD roots are widely used for treatment of benign prostatic hyperplasia (BPH) (Bondarenko *et al.*, 2003; Schneider & Rubben, 2004; Lopatkin *et al.*, 2005; Safarinejad, 2005). Urtica has also been used for many years as a traditional herbal medicine in China for the treatment of eczema, rheumatism and inflammation. *Urtica fissa* has also been reported to possess inhibitory effects on prostatic hyperplasia (Zhang *et al.*, 2008).

Urtica dioica L. (Urticaceae), stinging nettle, is a widespread, common, medicinal plant often used in folk medicine against various diseases. A large number of compounds of different polarity and belonging to various chemical classes, including fatty acids, terpenes, phenylpropanes, lignans, coumarins, triterpenes, ceramides,

sterols and lectins, have been isolated from UD. Among these are oxalic acid, linoleic acid, 14-octacosanol, 13-hydroxy-9-cis,11-trans-octadecadienoic acid, α-dimorphecolic acid (9-hydroxy-10-trans,12-cis-octadecadienoic acid), scopoletin, p-hydroxybenzaldehyde, homovanillyl alcohol, β-sitosterol, stigmasterol, 24-R-ethyl-5α-cholestan- 3β,6α-diol, campesterol, daucosterol (and related glycosides), secoisolariciresinol- 9-O-β-D-glucoside, neoolivil, oleanolic acid, ursolic acid, UD agglutinin and polysaccharides RP1–RP5 (WHO, 2002). Lichius *et al.* (1999) reported that the polysaccharide fraction of the 20% methanolic extract of UD showed an inhibitory effect on the growth of prostatic lymph node carcinoma cells. *Urtica dioica* agglutinin (UDA), a lectin from stinging nettle roots, was reported to directly inhibit cell proliferation of Hela cells and block the binding of EGF to its receptor and was regarded to be responsible for inhibiting effects in BPH treatment (Wagner *et al.*, 1994).

Urtica dioica of Indian origin was studied for its effects on testosterone-induced prostatic hyperplasia. Petroleum ether, ethanolic and aqueous extracts of UD were tested for their *in vitro* 5α-reductase inhibitory potential, and it was found that the petroleum ether and ethanolic extracts possess appreciable activity. Aqueous extract was on a back foot as compared to the other two extracts. Hence petroleum ether and ethanolic extracts were selected for *in vivo* studies using testosterone-induced prostatic hyperplasia model in rats. Further presence of β-sitosterol was noted in both the extracts, and scopoletin was found in ethanolic extract. Both of these compounds have a preventive effect on prostatic disorders viz., BPH and prostate cancer (Braeckman, 1994; Berges *et al.*, 1995; Klippel *et al.*, 1997; Wilt *et al.*, 1998, 1999; Li *et al.*, 2001). During the study, the prostate/body weight (BW) ratios were noted for all the groups. Urine output of individual rats was measured weekly, and % obstruction in urinary flow was calculated. Weekly serum testosterone levels were recorded, and prostate-specific antigen (PSA) levels were noted at the end of the study to find out the effectiveness of the extracts against prostatic hyperplasia induced by testosterone. Histological studies performed after euthanising the animals on the last day of the study provided an insight into the changes in the histoarchitecture of the prostate of various groups.

Materials and methods

Plant material

Roots of UD were collected from Nainital and identified by Prof. Kumud Upadhyay, Department of Pharmaceutical Sciences, Kumaon University, Nainital. A herbarium has been deposited at the Department of Pharmaceutical Sciences for future reference (Voucher Specimen no. – KU/Herb/08/1005).

Preparation of extracts

UD roots were ground to a coarse powder, packed in soxhlet extractors and extracted with petroleum ether (60–80 °C) till complete extraction. The solvent from the petroleum ether extract (UDP) was eliminated under reduced pressure (yield- 11.01% w/w). The defatted marc was extracted with ethanol (95% v/v) to obtain the ethanolic extract (UDE) (yield- 5.07% w/w). The marc left after the ethanolic extraction was macerated with distilled water for 24 h, and the aqueous extract (UDA) was finally obtained by vacuum drying (yield- 2.90% w/w).

Drugs and chemicals

Testosterone was obtained as a gift from Sun Pharma Advanced Research Center (SPARC), Vadodara, Gujarat, India. Finasteride, β-sitosterol and scopoletin were purchased from Sigma Aldrich (St Louis, MO, USA). Petroleum ether (60–80 °C) was purchased from Qualigens Fine Chemicals Pvt. Ltd. (Mumbai, India). Testosterone ELISA kit (UBI MAGIWEL) was purchased from United Biotech Inc. (Mountain View, CA, USA), and PSA ELISA kit was purchased from Cusabio Biotech Co. Ltd. (Newark, DE, USA). All other chemicals used in the study were of analytical grade.

In vitro studies

With a view to explore the possibility that the extracts may have some action on prostatic hyperplasia, the extracts were screened for 5α-reductase activity, the key enzyme involved in hyperplasia of the prostate. The *in vitro* studies measured the 5α-reductase inhibitory potential of the extracts and finasteride by determining the concentration of testosterone in the reaction mixture using HPLC (Nahata & Dixit, 2011a,b; Nandecha *et al.*, 2010).

Characterisation of extract and selection of markers

The *in vitro* studies led us to conclude that petroleum ether and ethanolic extract of UD possess appreciable inhibitory potential against 5α-reductase. Hence, thin layer chromatographic profiling was carried out to find out the presence of various phytoconstituents. Aqueous extract being less active *in vitro* was not included in further studies. UDP and UDE were co-chromatographed with standard marker compounds viz., β-sitosterol and scopoletin.

When visualised under UV at 254 nm, both the extracts revealed the presence of β-sitosterol, whereas scopoletin was found only in UDE. The solvent system used for UDP was toluene: ethyl acetate (8 : 2) and that for UDE was Chloroform : Methanol : Toluene (8 : 2 : 1).

High-performance thin layer chromatographic (HPTLC) analysis and isolation of markers

High-performance thin layer chromatographic was performed to develop the characteristic fingerprint profile for petroleum ether and ethanolic extracts of UD. UDP and β-sitosterol, a biochemical marker, were dissolved in petroleum ether. Ten microlitres of the sample solutions was applied, and the plate was developed in toluene: ethyl acetate (8 : 2). Developed plates were scanned densitometrically using a Camag TLC scanner 3 (CAMAG, Muttenz, Switzerland) at 254 nm and documented. Similarly, UDE was also analysed for its HPTLC profile and the presence of marker compounds viz. β-sitosterol and scopoletin. The procedure was the same as followed in the case of UDP. The solvent system for UDE was Chloroform : Methanol : Toluene (8 : 2 : 1). The percentage of β-sitosterol in UDP and UDE was calculated to be 27.28% and 19.96%, respectively. The percentage of scopoletin in UDE was 16.98%.

For isolation of these compounds, column chromatography was performed. UDP was used for the isolation of β-sitosterol and UDE for scopoletin. For isolation of β-sitosterol, 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 characterised 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 27.28%. The melting point of the isolated compound was determined using the superfit melting point determination apparatus and found to be 140 °C which was identical to standard sample (140 °C). Further overlain FTIR analysis with the standard confirmed the identity of isolated compound as β-sitosterol. The NMR analysis of 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–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.

For isolation of scopoletin, UDE was subjected to column chromatography using silica gel (80–120 mesh) as adsorbent and chloroform : methanol : toluene (8 : 2 : 1) as eluent. A crystalline material melting at 204 °C was isolated. It gave blue fluorescence in UV and was characterised as scopoletin by mixed melting point, UV absorption maxima and overlain FTIR spectral analysis. The FTIR spectrum of the compound is characteristic of 6-methoxy, 7-hydroxy coumarin i.e. scopoletin. Overlain FTIR spectrum with reference standard proved this fact. NMR analysis of the isolate further confirmed its identity as scopoletin. 1H-NMR spectrum showed a three proton singlet at 3.9δ corresponding to –O-CH_3, two pairs of doublets centred at 6.1δ and 7.7δ corresponding to C_3-H and C_4-H with J = 9 cps indicating these protons on adjacent carbon atoms of a double bond, two aromatic protons (singlets) C_8-H and C_5-H at 6.8δ and 6.9δ, respectively, stretching at 9.9δ indicative of a −OH that shows one exchangeable proton with D_2O.

Moreover, the overlain UV spectra (recorded from HPTLC studies) of standard β-sitosterol and the β-sitosterol present in the extract were found to be superimposable on each other confirming the presence of β-sitosterol in UD extracts. Same was the case with standard scopoletin and scopoletin present in UDE.

In vivo studies

The results of the *in vitro* studies were encouraging as appreciable 5α-reductase inhibitory activity was found in the UDP and UDE. 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 (OECD, 2001) (OECD 423- Acute Toxic Class Method) (Roll *et al.*, 1986; OECD, 2001). In all cases, 2000 mg kg^{-1} oral dose of the test extract 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^{-1} p.o. were selected for UDP and UDE.

Preparation of extracts

UDP and UDE 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^{-1} p.o. once daily for 28 days (Carbajal *et al.*, 2004; Nahata & Dixit, 2011a,b). Testosterone was dissolved in arachis oil for s.c. injection (3 mg kg^{-1} s.c.). Finasteride was suspended in tween – 80 (0.2% v/v) and administered per orally (1 mg kg^{-1} p.o.). β-sitosterol was also included in the study in doses of 10 and 20 mg kg^{-1} p.o. and was suspended in tween-80 (0.2% v/v) for p.o. administration.

Experimental design

Eleven groups containing six rats per group were created for this study. Hyperplasia was induced by subcutaneous administration of testosterone (3 mg kg^{-1}) for 28 days in all the groups except the vehicle-treated group. Rats were treated with vehicle [tween-80 (0.2% v/v p.o.)] or finasteride (1 mg kg^{-1}, p.o.), UDP (10, 20 or 50 mg kg^{-1}, p.o.), UDE (10, 20 or 50 mg kg^{-1}, p.o.) or β-sitosterol (10 or 20 mg kg^{-1}, p.o.) before administration of arachis oil (s.c.) or testosterone (3 mg kg^{-1} s.c).

Body and prostatic weights

Body weights were taken a day before the starting of the treatment (baseline) and on the completion of the study i.e. on 28th day of treatment. On day 29, animals were anesthetised under light ether anaesthesia and sacrificed. The prostates were immediately dissected out and weighed. Mean BWs and prostatic/BW ratios were calculated for each group. On the basis of mean prostatic weights and P/BW ratios, we also calculated the % recovery in P/BW ratio by test groups as compared to 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 testosterone-treated group. The formula used for calculation of % recovery (Nahata & Dixit, 2011a) was:

$$\%\ \text{recovery by the test sample} = A - B$$

where A = % increase in prostatic weight induced by testosterone (considered 100%).

B = % 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 weekly till the completion of the studies i.e. 28th day of the study. Metabolic cages were used for the purpose of urine collection. Animals were kept for 24 h in the cages, and the urine volume was recorded for each individual animal of each group. The reduction in urine volume and subsequent % obstruction in urine flow was calculated for each group. During this period, 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 testosterone ELISA kit. After every 7 days, the effect of the test samples on the serum testosterone levels was measured using ELISA reader (BIOLINE BPR08). Blood was collected from the retro orbital plexus of the rats, and it was 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, United Biotech Inc.). The UBI Magiwel testosterone quantitative test is based on the principle of competitive solid-phase enzyme immunoassay. The test sample competes with enzyme-labelled-tetsosterone 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 anti-rabbit 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 colour is developed when TMB substrate is mixed with the antibody-bound testosterone-horseradish peroxidase enzyme conjugate. After a short incubation, the enzyme reaction is stopped, and the intensity of the colour is measured with microreader at 450 nm.

Measurement of prostate-specific antigen (PSA)

Prostate-specific antigen levels were measured for individual rats of each group to find the extent of

hyperplasia induced in the prostate by testosterone treatment. For this purpose, PSA ELISA kit was utilised. The PSA ELISA kit is intended for the quantitative determination of total PSA. This kit was obtained from Cusabio Biotech Co. Ltd., Newark, DE, USA. PSA was quantified 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 stripes. After washing, buffered substrate (TMB-HRP substrate) that contains hydrogen peroxide and chromogen reagent (3, 3′, 5, 5′tetra methyl benzidine) was added to each well, and the enzyme reaction was allowed to proceed. The colour 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 concentration of PSA in samples was then read from the calibration curve.

Histological studies

After prostatic weight measurements, the tissues were fixed in 10% formalin (in normal saline). After 24 h, the tissues were subjected to histological studies using microtome followed by Haematoxylin & Eosin (H&E) staining. The slides were observed under a microscope (Labovision trinocular microscope) and the images recorded. One of the authors, Prof. V.K. Dixit, who read the histology specimens, was kept blind to the treatment groups. The observations are discussed in the section of histological examinations later in the manuscript.

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 (Graphpad Software Inc., La Jolla, CA, USA). P and F values and degrees of freedom were calculated. $P < 0.05$ was considered to be statistically significant.

Results

HPTLC and characterisation of marker

Co-chromatography of petroleum ether (UDP) and ethanolic (UDE) extracts along with β-sitosterol and scopoletin as marker compounds revealed the presence of β -sitosterol in both the extracts, with an Rf value of 0.95 for UDP (toluene : ethyl acetate/8 : 2) and 0.93 for UDE (Chloroform : Methanol : toluene/8 : 2 : 1) when analysed at 254 nm (Figs 1 and 2). Overlain UV spectral interpretation further confirmed the presence of β-sitosterol in the extracts (Fig. 3). Scopoletin was found to be present in UDE at an Rf value of 0.79 (Chloroform : Methanol : toluene/8 : 2 : 1) when analysed at 254 nm. Overlain UV spectral interpretation also confirmed its presence in the extract (Fig. 3). Further β-sitosterol and scopoletin were isolated from UDP and UDE, respectively, and their identities confirmed by melting point, FTIR and NMR analysis.

In vitro studies

The IC50 values calculated for UDP, UDE, UDA and standard 5α-reductase inhibitor i.e. finasteride were 0.19, 0.12, 0.32 mg and 1.06 μg, respectively. Although UDE and UDP extracts showed good activity, the activity of finasteride is about 10 times greater. Relative inhibitory potency of the test material is thus Finasteride > UDE > UDP > UDA (Table 1).

In vivo studies

The results of the *in vitro* studies led us to perform the pharmacological screening of the extracts to evaluate their potential against testosterone-induced hyperplasia in rats. Results are discussed as follows.

Determination of body weight, prostatic weight and prostate/body weight (P/BW) ratio of test groups

In testosterone-treated group, mean BW and mean prostatic weight showed a considerable increase after 28 days of treatment. In case of vehicle-treated group, no appreciable increase in BW was observed as noted in the Table 2. While in case of finasteride-treated group, a decrease was observed. Table 2 summarises the effects of UDP (10, 20 and 50 mg kg^{-1} p.o.), UDE (10, 20 and 50 mg kg^{-1} p.o.) and finasteride (1 mg kg^{-1} p.o.) on prostatic hyperplasia induced with testosterone. The P/BW ratio calculated in case of vehicle-treated group was 1.47 ± 0.15. It was 7.27 ± 0.38 for testosterone-treated negative control group and 2.62 ± 0.27 for finasteride-treated positive control group. In case of UDP, P/BW ratios were 5.55 ± 0.23, 5.33 ± 0.11 and 3.03 ± 0.08 for 10, 20 and 50 mg kg^{-1} doses, respectively. Similarly in case of UDE-treated groups, the ratios were 5.44 ± 0.31, 4.36 ± 0.39 and 3.19 ± 0.14 for 10, 20 and 50 mg kg^{-1} doses, respectively. Similarly β-sitosterol-treated groups showed a P/BW ratio of 4.31 ± 0.15 and 4.18 ± 0.16 for 10 and 20 mg kg^{-1} dose, respectively. Most of the values

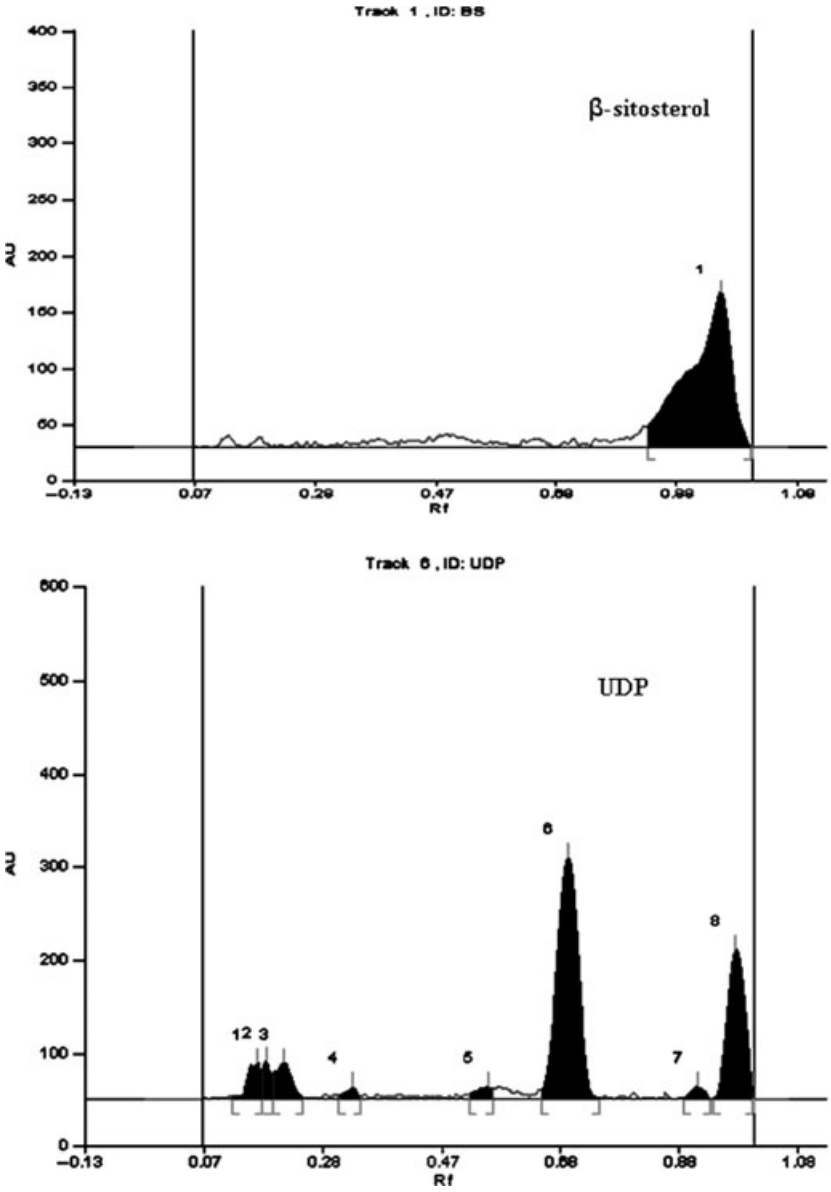

Fig. 1 High-performance thin layer chromatographic Profile of UDP and standard β-sitosterol in toluene : ethyl acetate (8 : 2) at 254 nm.

were significant as compared to testosterone-treated group and finasteride-treated groups. On the basis of mean prostatic weights and P/BW ratios, we also calculated the % recovery in P/BW ratio by test groups as compared to testosterone-treated group.

The % recoveries thus calculated for UDP-treated group at doses of 10, 20 and 50 mg kg^{-1} were 29.61%, 33.37% and 73.12%, respectively. In case of UDE, these recoveries were 31.51%, 50.17% and 70.23% for 10, 20 and 50 mg kg^{-1}, respectively. β-sitosterol-treated groups showed a recovery of 51.07 (10 mg kg^{-1}) and 53.31% (20 mg kg^{-1}). Recovery with standard finasteride (1 mg kg^{-1}) was 80.10% (Table 2). Thus, UDP proved to be better than UDE in this aspect.

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 case of hyperplasia of the prostate. In case of vehicle-treated control group, there was practically no change in urine output. As hyperplasia progressed with testosterone treatment, urine output was reduced, and a drastic reduction was observed after 28 days of treatment in testosterone-treated control group animals. When UD extracts were administered along with testosterone, significant improvement in urine output over testosterone-treated

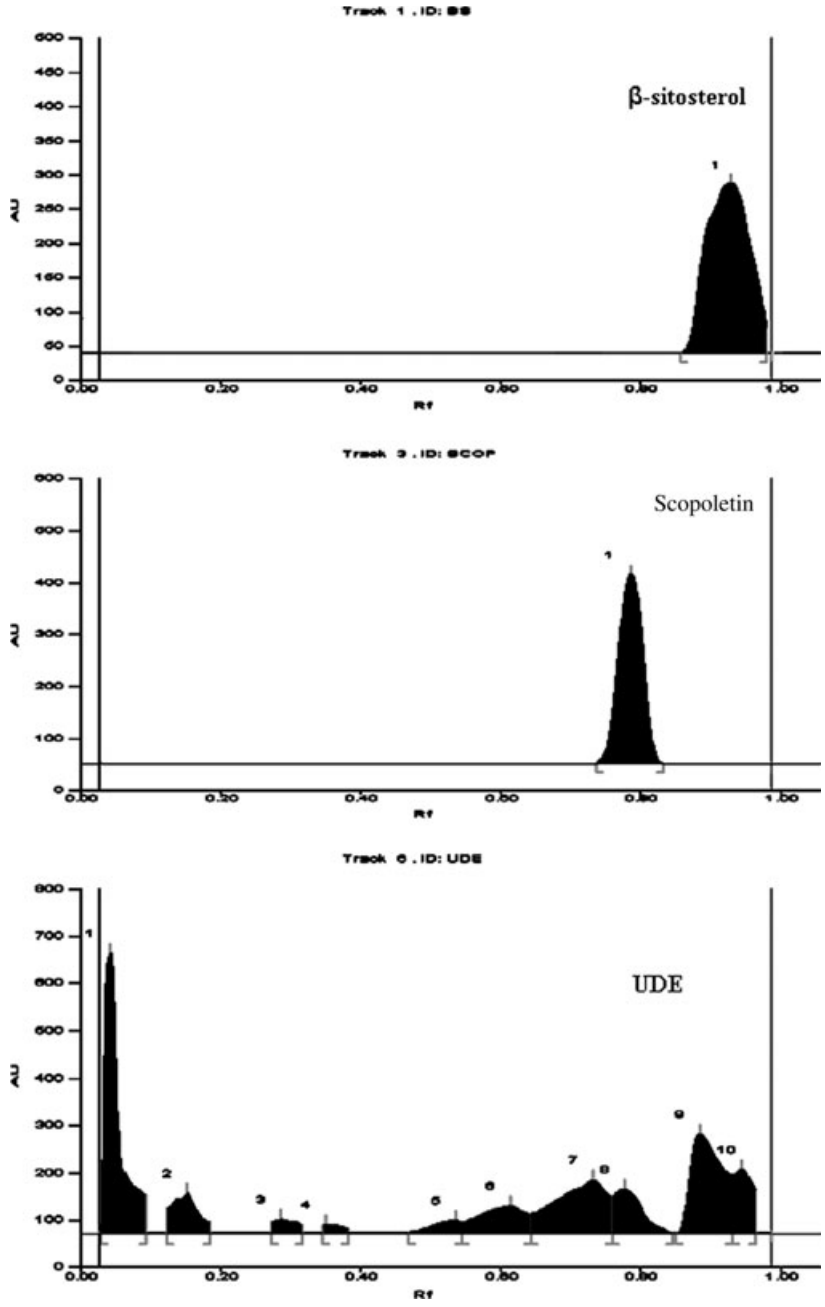

Fig. 2 High-performance thin layer chromatographic profile of standard β- sitosterol, scopoletin and UDE in Chloroform : Methanol : Toluene (8:2:1) at 254 nm.

control group was observed. The % obstruction in urine output for UDP-treated groups in doses of 10, 20 and 50 mg kg^{-1} was 8.00, 2.02 and 4.90%, respectively, as against 77.15% in testosterone-treated group. Similarly, when UDE was administered in graded doses of 10, 20 and 50 mg kg^{-1} along with testosterone, significant improvement in urine output was recorded as compared to testosterone-treated group. The % obstruction in urine output for UDE-treated groups was 5.15, 2.17 and 1.96%, respectively, for doses of 10, 20 and 50 mg kg^{-1}. In β-sitosterol-treated group, the % obstruction recorded was 7.53% (10 mg kg^{-1} p.o.) and 11.42% (20 mg kg^{-1} p.o.). In finasteride-treated positive control group, practically no obstruction in urinary output was recorded. The relative efficacy of the extracts in reducing the obstruction caused by testosterone can be stated in the following order:

Finasteride > UDE50 > UDP20 > UDE20 > UDP50 > UDE10 > UDP 10 > β-sitosterol (10) > β-sitosterol (20). The results are depicted in Table 3.

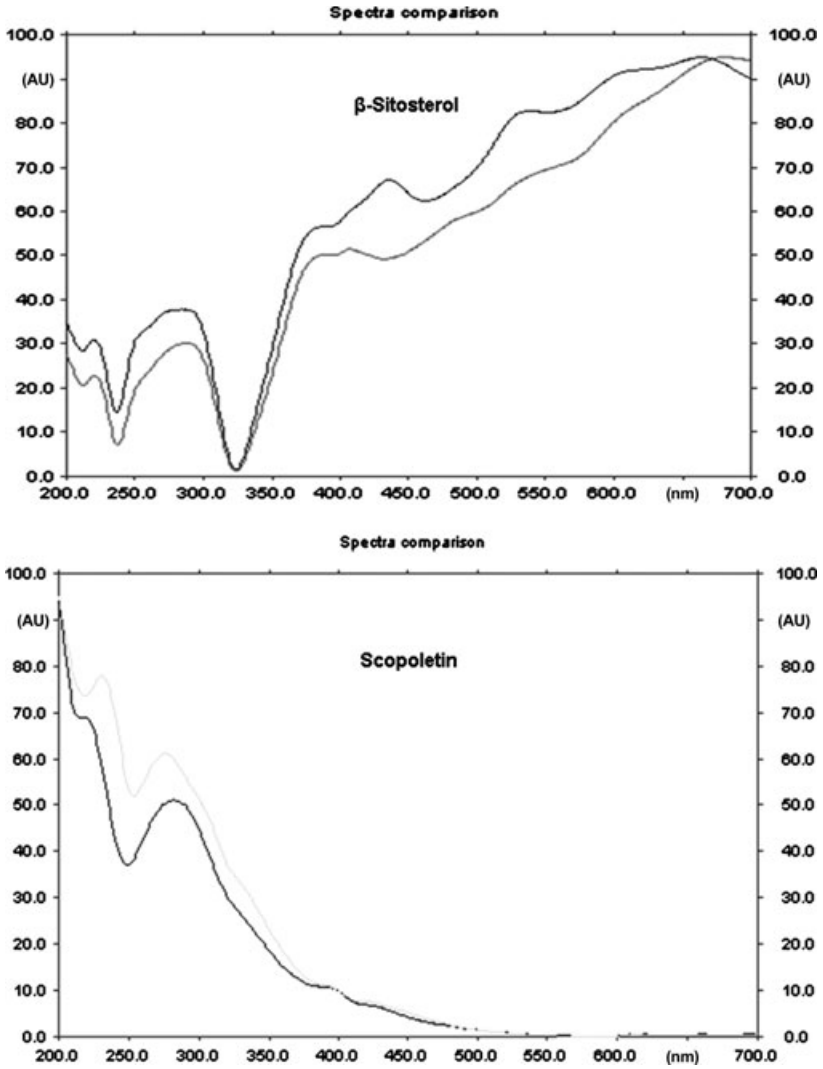

Fig. 3 Overlain UV spectra of isolated β- sitosterol and scopoletin with standard markers using high-performance thin layer chromatographic.

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 serum testosterone concentration of various groups. In normal vehicle-treated group, the levels were unchanged during the study as measured on 0, 7, 14, 21 and 28th day of the study. Testosterone-treated group showed a decrease in these levels as the study progressed and this decrease continued till 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, finasteride and β-sitosterol along with testosterone led to further increase in serum testosterone levels suggesting inhibition of 5α-reductase activity of the extracts, finasteride and β-sitosterol. The results are depicted in Fig. 4.

Table 1 5α-reductase inhibitory concentrations (IC-50) of treated groups

S. No	Group	IC 50
1	Petroleum ether extract	0.19 mg
2	Ethanolic extract	0.12 mg
3	Aqueous extract	0.32 mg
4	Finasteride	1.06 μg

Measurement of prostate-specific antigen

Prostate-specific antigen serum levels are abnormally elevated in patients with prostate cancer, BPH and patients with prostate inflammatory conditions (Catalona *et al.*, 1995). The effect of the administration of test extracts

Table 2 Effect of test extracts of *Urtica dioica* and *β*-sitosterol on prostatic weight

Treatment (mg kg^{-1})	Body weight (g)		Prostatic weight (mg)	Prostatic/body weight ratio (P/BW ratio)	Treatment effect on P/BW (P_2–P_1)	% increase in prostate weight	% recovery[a]
	Day 0	Day 28					
Blank control (vehicle only)	110.75 ± 9.21	117.50 ± 14.93	170.15 ± 22.08	1.47 ± 0.15	0	0	0
Testosterone (T) (3 mg kg^{-1} s.c.)	113.75 ± 36.36	138.75 ± 38.31	1028.41 ± 21.83	7.27 ± 0.38[b]	5.80	100	0
T +Finasteride (1 mg kg^{-1} p.o.)	127.50 ± 12.50	126.25 ± 7.46	337.67 ± 56.19	2.62 ± 0.27[c]	1.15	19.90	80.10
UDP 10 + T	103.75 ± 8.98	122.50 ± 8.29	560.30 ± 155.62	5.55 ± 0.23[b,c]	4.08	70.39	29.61
UDP 20 + T	105.00 ± 2.88	112.50 ± 4.33	545.45 ± 19.47	5.33 ± 0.11[b,c]	3.86	66.63	33.37
UDP 50 + T	105.00 ± 9.57	111.25 ± 13.44	366.32 ± 43.76	3.03 ± 0.08[c]	1.56	26.88	73.12
UDE 10 + T	136.00 ± 15.81	131.25 ± 13.28	599.98 ± 96.11	5.44 ± 0.31[b,c]	3.97	68.49	31.51
UDE 20 + T	117.50 ± 7.50	127.50 ± 7.77	429.42 ± 16.25	4.36 ± 0.39[b,c]	2.89	49.83	50.17
UDE 50 + T	142.50 ± 17.01	153.75 ± 15.99	408.30 ± 112.08	3.19 ± 0.14[c]	1.72	29.77	70.23
BS 10 + T	95.00 ± 5.00	105.00 ± 5.00	475.95 ± 20.45	4.31 ± 0.15[b,c]	2.84	48.93	51.07
BS 20 + T	105.00 ± 5.00	110.00 ± 0.00	424.50 ± 15.82	4.18 ± 0.16[b,c]	2.71	46.69	53.31
				$P < 0.0001$			
				$F = 20.387$			

BW, body weight.

Values are mean ± SEM ($n = 6$) anova followed by Dunnett's test. Df = 10 103.

UDP 10, UDP 20, UDP 50: Petroleum ether extract of *U. dioica* (10, 20 and 50 mg kg^{-1} p.o., respectively); UDE 10, UDE 20, UDE 50: Ethanolic extract of *U. dioica* (10, 20 and 50 mg kg^{-1} p.o., respectively); BS 10, BS 20: *β*-sitosterol (10 and 20 mg kg^{-1} p.o., respectively); P_1: P/BW ratio of blank group; P_2: P/BW ratio of test group.

[a]% recovery in P/BW ratio by test groups as compared to testosterone treated group.

[b]$P < 0.01$ versus finasteride treated group.

[c]$P < 0.01$ versus testosterone treated group (Dunnett's test).

Table 3 Mean urine output of various groups measured weekly using metabolic cage

Treatment (mg kg^{-1})	Urine output (ml)					Obstruction in urine output (%)$(U_0–U_{28})/U_0 \times 100$
	Day 0	Day 7	Day 14	Day 21	Day 28	
Blank (vehicle only)	1.02 ± 0.05	1.05 ± 0.06	1.10 ± 0.04	1.05 ± 0.09[a]	1.02 ± 0.02[a]	–
Testosterone (3 mg kg^{-1} s.c.)	0.98 ± 0.09	0.85 ± 0.06	0.75 ± 0.08	0.37 ± 0.08[c]	0.22 ± 0.02[c]	77.15
Finasteride (1 mg kg^{-1} p.o.) + T	1.01 ± 0.07	0.97 ± 0.01	0.95 ± 0.02	0.97 ± 0.07[a]	1.00 ± 0.04[a]	0.99
UDP 10 + T	1.00 ± 0.12	1.00 ± 0.10	0.82 ± 0.085	0.67 ± 0.085	0.92 ± 0.04[b]	8.00
UDP 20 + T	0.99 ± 0.08	0.97 ± 0.04	0.80 ± 0.040	0.47 ± 0.075[c]	0.97 ± 0.11[b]	2.02
UDP 50 + T	1.02 ± 0.02	1.00 ± 0.04	1.05 ± 0.028	0.70 ± 0.070[a]	0.97 ± 0.047[b]	4.90
UDE 10 + T	0.97 ± 0.09	0.95 ± 0.02	0.92 ± 0.062	0.62 ± 0.047	0.92 ± 0.045[b]	5.15
UDE 20 + T	0.92 ± 0.08	0.92 ± 0.10	0.82 ± 0.125	0.40 ± 0.070[d]	0.90 ± 0.070[b]	2.17
UDE 50 + T	1.02 ± 0.11	1.02 ± 0.02	1.02 ± 0.085	0.50 ± 0.070[d]	1.00 ± 0.040[b]	1.96
BS 10 + T	0.99 ± 0.84	0.97 ± 0.12	0.90 ± 0.07	0.60 ± 0.13	0.92 ± 0.09[b]	7.53
BS 20 + T	0.875 ± 0.10	0.725 ± 0.18	0.62 ± 0.17	0.22 ± 0.07[d]	0.77 ± 0.11[b]	11.42
		$F = 3.318$	$F = 2.989$	$F = 7.676$	$F = 7.979$	
		$P < 0.0001$	$P = 0.001$	$P < 0.0001$	$P < 0.0001$	

U_0: Urine output on day 0; U_{28}: Urine Output on Day 28; UDP 10, UDP 20, UDP 50: Petroleum ether extract of *U. dioica* (10, 20 and 50 mg kg^{-1} p.o., respectively); UDE 10, UDE 20, UDE 50: Ethanolic extract of *U. dioica* (10, 20 and 50 mg kg^{-1} p.o., respectively).

Values are mean ± SEM ($n = 6$) anova followed by Dunnett's test. Df = (10, 78)103.

[a]$P < 0.01$ as compared to testosterone.

[b]$P < 0.05$ as compared to finasteride.

[c]$P < 0.01$ as compared to finasteride.

[d]$P < 0.05$ as compared to testosterone.

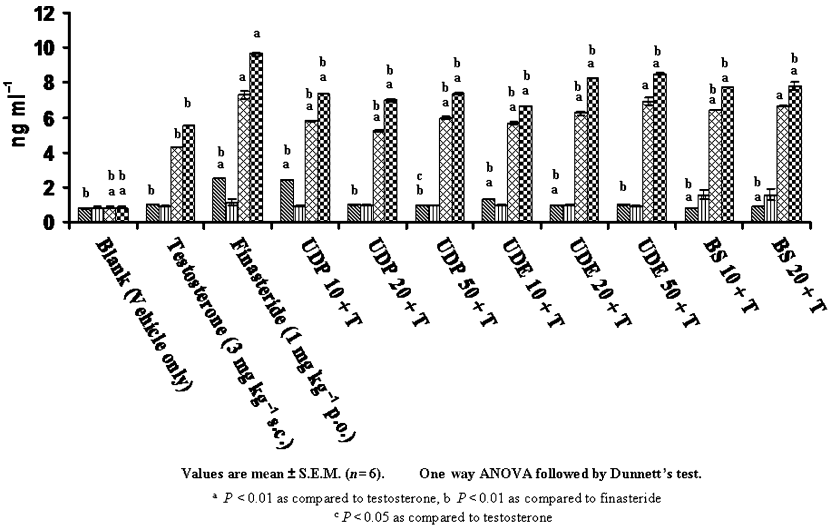

Fig. 4 Mean serum testosterone levels of various groups measured weekly using testosterone ELISA Kit.

and finasteride along with testosterone on the PSA level in 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 PSA ELISA kit following the procedure supplied with the kit. The normal PSA level in vehicle-treated group was found to be 0.140 ± 0.087 μg l^{-1}. This level increased to 1.533 ± 0.731 μg l^{-1} in testosterone-treated group. Finasteride-treated group showed a decrease in PSA level to 0.900 ± 0.378 μg l^{-1} ($P < 0.05$ as compared to testosterone-treated group). UDP in doses of 10, 20 and 50 mg kg^{-1} p.o. showed levels of 1.463 ± 0.472 ($P < 0.05$), 0.915 ± 0.622 ($P < 0.05$) and 0.260 ± 0.026 ($P < 0.01$) μg l^{-1}, respectively, which indicate the protective effects of UDP on testosterone-induced hyperplasia. Decrease in PSA levels was also observed with UDE treatment which exhibited levels of 2.250 ± 0.500 ($P < 0.05$), 1.783 ± 0.753 ($P < 0.05$) and 0.325 ± 0.025 ($P < 0.01$) μg l^{-1} for 10, 20 and 50 mg kg^{-1}, respectively. The decreases observed were significant as compared to testosterone-treated group. These observations indicate that UDP was more effective than UDE in counteracting testosterone-induced hyperplasia. β-sitosterol-treated group also showed a decrease in PSA levels to 0.880 ± 0.325 ($P < 0.05$) (10 mg kg^{-1} p.o.) and 0.445 ± 0.126 ($P < 0.05$) (20 mg kg^{-1} p.o.). The observations are depicted in Fig. 5.

Histological examinations

Control group (arachis oil)

Normal histological features of prostate gland are visible showing the tubules of variable diameter and irregular lumen. Lumens are filled with prostatic secretions. In connective tissue, blood vessels and lymph vessels, matrix is normal (Fig. 6a).

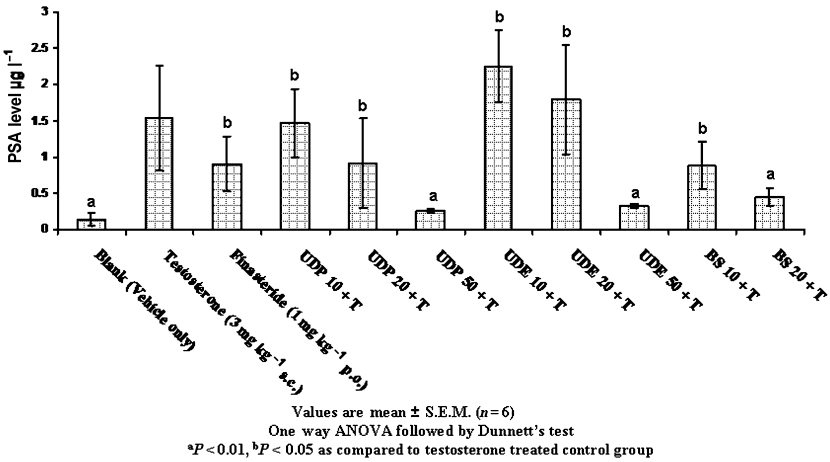

Fig. 5 Mean prostate-specific antigen (PSA) levels of various groups measured using PSA ELISA kit.

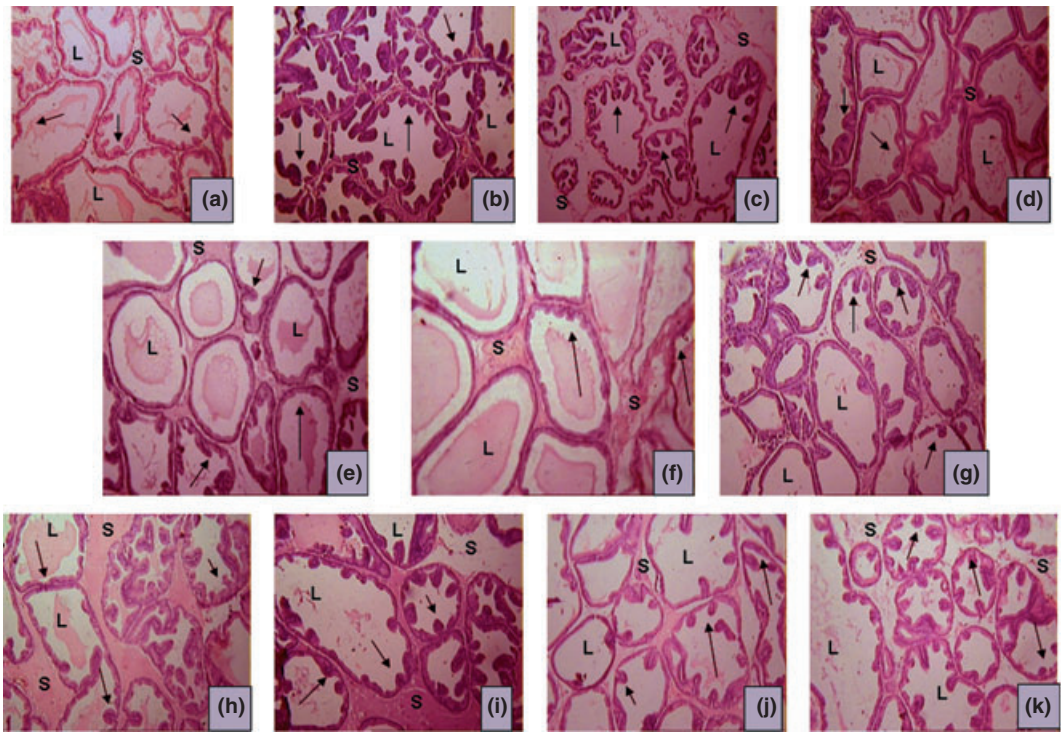

Fig. 6 Histopathological observations of the effect of test extracts of *Urtica dioica* and standard β-sitosterol on testosterone-induced hyperplasia (×100). L, Lumen, S, the stromal compartment and arrows indicate the luminal involutions. (a) Vehicle-treated control group. (b) Testosterone-treated group (3 mg kg^{-1} s.c.). (c) Finasteride-treated group 1 mg kg^{-1} p.o. (d) Petroleum ether extract–treated group 10 mg kg^{-1}. (e) Petroleum ether extract–treated group 20 mg kg^{-1}. (f) Petroleum ether extract–treated group 50 mg kg^{-1}. (g) Ethanolic extract–treated group 10 mg kg^{-1}. (h) Ethanolic extract–treated group 20 mg kg^{-1}. (i) Ethanolic extract–treated group 50 mg kg^{-1}. (j) β-sitosterol-treated group 10 mg kg^{-1}. (k) β-sitosterol-treated group 20 mg kg^{-1}.

Testosterone-treated group (3 mg kg^{-1} s.c.)
Tubules have become wider as compared to control. The walls of tubules have thickened, and every tubule almost has developed large involutions projecting into the lumen, reducing the volume of the lumen as compared to control. The connective tissue has been compressed, and blood vessels have dilated as compared to control. Shape of the tubules has become obliterated (Fig. 6b).

Testosterone + UDP (10, 20 and 50 mg kg^{-1} p.o.)
Lumen of the tubules is normal and at some places slightly obliterated. Involutions are few in number and even less than what were observed in control. Connective tissue between the tubules is reduced. Stroma is composed of smooth muscles and connective tissue. A significant improvement as compared to testosterone-treated group can be easily identified (Fig. 6d–f).

Testosterone + UDE (10, 20 & 50 mg kg^{-1} p.o.)
Although tubules have shown morphological improvement in the texture, still the epithelium is wide and thicker. As compared to testosterone-treated group, the stroma is normal. The appearance of the transitional epithelium resembles that of control. Involutions in the epithelium are fewer and thick (Fig. 6g–i).

Testosterone + finasteride (1 mg kg^{-1}, p.o.)
Normal distribution of stroma is seen. The projections are not prominent as seen in the testosterone-treated group. Although Finasteride has antagonised the effects of testosterone appreciably, still, several cells with their increased volume are present throughout the transitional epithelium. Cells with swollen nuclei are prominent at many places. Involutions are less. (Fig. 6c).

Testosterone + β-sitosterol (10 and 20 mg kg^{-1} p.o.)
Lumen of the tubules is normal, and at some places, epithelium is slightly thicker than control. Stroma is normal. The appearance of the transitional epithelium resembles that of control. (Fig. 6j,k).

Discussion

A densitometric high-performance TLC analysis was performed to develop the characteristic fingerprint profile for the petroleum ether and alcoholic extract of UD. This can

be used as a tool for evaluation and standardisation of the drug. The presence of β-sitosterol in petroleum ether and ethanolic extracts and scopoletin in the ethanolic extract was ascertained using this technique. This led us to isolate the two compounds from the extracts and their structures elucidated via FTIR, mixed melting point and NMR analysis.

Testosterone is converted to more potent DHT by the enzyme 5α-reductase present in prostate homogenates (Steers, 2001; Dhanotiya *et al.*, 2009). During *in vitro* studies, the addition of UDP, UDE and UDA in reaction mixture showed increased levels of unchanged testosterone in the reaction mixture, suggesting inhibition of enzyme action by these test materials. Furthermore, the inhibition of conversion by these materials clearly reflects that enzyme activity is blocked and, therefore, more testosterone remains unchanged in the reaction mixture. It was noted that finasteride is about 10 times more potent in inhibiting 5α-reductase activity in *in vitro* studies. As least activity was shown by UDA, it was not included in the studies thereafter, and only UDP and UDE were taken up for *in vivo* studies.

The effects of testosterone and DHT 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 (Paubert-Braquet *et al.*, 1996; Bombardelli *et al.*, 1997; Kumar *et al.*, 2010). In the present study, in rats administered with UDP and UDE along with testosterone, the increase in prostatic weight and P/BW ratio was attenuated after 28 days of oral treatment at different doses as compared to testosterone-treated negative control group. The weekly measurement of testosterone concentration in serum also supports our findings. The levels of testosterone are 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 DHT that 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.

It was found that urine output was decreased drastically in testosterone-treated group due to enlargement of prostate gland. In extract and finasteride-treated groups, significant decrease in prostatic weight was noted with concominant increase in urinary output. In most of the cases, urine output returned to the normal values as on day 0 of the study. Obstruction in urine output shown by testosterone-treated group was significantly greater than extract and finasteride-treated groups. The extracts UDP as well as UDE, finasteride and β-sitosterol exhibited a significant improvement in urine output as compared to the testosterone-treated group. Obstruction of urinary discharge, painful micturition, reduced urine flow, urinary urgency, etc. are major pathological problems of clinical significance in patients with BPH. The results of the study indicate possible use of the drug in stated conditions.

Further PSA levels were measured at the end of the study, i.e. on 28th day. 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, BPH and patients with prostate inflammatory conditions. If a decrease in PSA levels is observed, it can be assessed 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.

Both the extracts and β-sitosterol 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 UDP prevented prostatic hyperplasia significantly with 50 mg kg^{-1} showing the best activity. UDE also showed significant results. The *in vitro* studies cleared the mechanism of prevention of prostatic hyperplasia induced by testosterone. It is evident that UD extracts have 5α reductase inhibitory activity. The weekly serum testosterone levels are suggestive of the mechanism of action of the extracts and finasteride. The decrease in urinary obstruction also suggests that the extracts have a positive effect on hypertrophy of the prostate. As β-sitosterol is a well-known molecule established for the treatment of BPH (Martindale, 1989; The Merck Index, 2006), the presence of β-sitosterol as a major constituent in the extracts further supports our observations. A number of clinical studies undertaken by different scientific groups have supported clinical efficacy of β-sitosterol in prostate disorders (Braeckman, 1994; Berges *et al.*, 1995; Klippel *et al.*, 1997; Wilt *et al.*, 1998, 1999). Our studies with β-sitosterol are indicative of its protective effects on testosterone-induced hyperplasia. The percentage of β-sitosterol in UDP and UDE was calculated to be 27.28% and 19.96%, respectively. The amount of β-sitosterol present in the extract does not seem to be sole contributor of the activity as 20 mg kg^{-1} dose of standard β-sitosterol is not as effective as the extract (50 mg kg^{-1}). The effects of β-sitosterol might be potentiated by the presence of other constituents present in the extract. Synergistic mechanism of action along with the other phytoconstituents present in the extract can be suggested for these preventive effects. Moreover, scopoletin might be

potentiating the effects of β-sitosterol as it has been reported to possess antiproliferative and apoptotic effects against prostate cancer cell line PC-3 (Li *et al.*, 2001). Scopoletin has been reported as an anti-inflammatory molecule, and hence it should be useful in inflammatory conditions of the prostate (Delporte *et al.*, 1998; Ding *et al.*, 2008). The histological findings have shown significant recovery in the prostatic histoarchitecture which further support the utility of UD in the management of prostatic hyperplasia. The studies provide support to clinical studies carried out on UD for BPH. On the basis of above studies, it can be concluded that UD can be used as a potential candidate for the treatment and management of BPH and is a potential candidate for further clinical research in this area. β-sitosterol and scopoletin present in the active extracts can be considered clinically effective biomolecules from UD.

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