**Ganoderma lucidum** is an inhibitor of testosterone-induced prostatic hyperplasia in rats

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**Introduction**

Nowadays, androgen-mediated diseases such as prostate cancer, hirsutism, acne, androgenic alopecia and benign prostatic hyperplasia (BPH) have become serious problems (Bartsch et al., 2002). Above all, BPH is one of the most common ailments seen in older men; 40% of men 50–60 years of age and 90% of men 80–90 years of age have been diagnosed with BPH. The principal prostatic androgen is dihydrotestosterone, which is formed by the steroid enzyme 5α-reductase from its substrate testosterone (Russell & Wilson, 1994). 5α-reductase is a membrane-bound NADPH-dependent enzyme that catalyses the reduction of testosterone to the more potent androgen, dihydrotestosterone. The effect of dihydrotestosterone is purely androgenic in that, unlike testosterone, it cannot be transformed into oestrogen. As the weight of the seminal vesicles depends on the 5α-reduced androgens, it is important to regulate the level of dihydrotestosterone.

Two isoforms of 5α reductase have been cloned, expressed and characterised (types 1 and 2) that display different tissue expression patterns, enzyme kinetic parameters and chromosomal localisation (Jenkins et al., 1991). Both isoforms are overexpressed in BPH tissue (Iehle et al., 1999). Because BPH therapy can reduce dihydrotestosterone levels by blocking its conversion from testosterone, 5α-reductase inhibitors could be useful in the treatment (Bartsch et al., 2000). In rats, the type 1 isozyme predominates in tissues such as liver, kidney, brain, lung and skin but also exists in the prostate, whereas the type 2 isozyme is more abundant in genital tissues such as the prostate. A number of synthesised 5α-reductase inhibitors with steroidal moiety have been reported. However, it should be noted that these inhibitors have the potential to cause adverse effects such as those reported for finasteride (Uygur et al., 1998)—i.e., gynecomastia, impairment of muscle growth and severe myopathy—due to their structural similarity to steroidal hormones. Hence, the
emergence of therapeutic materials having fewer side effects—preferably, edible natural products—would be highly desirable if their safety could be guaranteed.

Although there is no clear evidence that patients who develop BPH will ultimately have prostate cancer, androgens do influence the development of prostate cancer (Ross et al., 1992; Giovannucci et al., 1997; Hsing et al., 2002). The use of finasteride, the 5α-reductase inhibitor, can lower the androgen levels in the prostate and reduce the risk of prostate cancer (Thompson et al., 2003).

For thousands of years, mushrooms have been known as a source of medicine. The fungi *Ganoderma lucidum* (GL) has been used for centuries in East Asia. Its fruiting body is called as ‘Reishi’ in Japan and ‘Lingzhi’ in China. In these areas, GL has been a popular folk or oriental medicine to cure various human diseases such as hepatitis, hypertension, hypercholesterolemia, gastric cancer (Wasser & Weis, 1999; Yun, 1999) and lung cancer (Tang et al., 2006). However, the precise mechanism and active compounds of GL against these biological activities have remained unclear.

Although the inhibitory effects on the proliferation and migration of prostate cancer cells by GL (Jian et al., 2004) and 5α-reductase inhibition and suppression of androgen-induced prostate cell growth by GL (Fujita et al., 2005; Liu et al., 2007) have been reported, the clinical implications such as the urine output and the testosterone levels and prostate-specific antigen levels (PSA) have not been determined till date. Noguchi et al. (2008) carried out a randomised, placebo-controlled study in men with lower urinary tract symptoms (LUTS) to evaluate safety and efficacy of an extract of GL and also suggested a need for large-scale evaluation of phytotherapy in the treatment of these diseases. We have demonstrated the basis for the future use of *Ganoderma* in therapy by measuring the weekly urine output and testosterone levels. PSA levels were also measured as it is the major determinant of diseased state of the prostate. Along with these parameters, effect of GL extracts on hyperplastic prostate was also tested using testosterone-induced hyperplasia model. Petroleum ether extract that remained untouched in the studies undertaken previously by above-mentioned workers was included in the present study, and it came out to be the best inhibitor of prostatic hyperplasia induced by testosterone.

### Materials and methods

#### Materials

Powdered fruiting body of GL was supplied by kind courtesy of Dr. Prabhat Chouhan, Gano Excel Industries, Kedah Darul Aman, Malaysia.

Preparation of extracts

GL powder was packed in soxhlet extractors and extracted with petroleum ether (60–80 °C) till complete extraction. The solvent from the petroleum ether extract (GLP) was eliminated under reduced pressure (yield- 0.51% w/w). The defatted marc was extracted with ethanol (95% v/v) to obtain the ethanolic extract (GLE) (yield- 3.05% w/w). The marc left after the ethanolic extraction was macerated with distilled water for 24 h, and the aqueous extract (GLA) was finally obtained by vacuum-drying (yield- 0.62% w/w).

Drugs and chemicals

Testosterone was obtained as a gift from Sun Pharma Advanced Research Center (SPARC), Vadodara, Gujarat, India. Finasteride and β-sitosterol were purchased from Sigma Aldrich, St Louis, MO, USA. Ethylenediamine tetraacetic acid (EDTA), sodium phosphate and sucrose were purchased from HIMEDIA Pvt. Ltd., Mumbai, India. Methanol, ethyl acetate and petroleum ether (60–80 °C) were purchased from Qualigens Fine Chemicals Pvt. Ltd., Mumbai, India. Testosterone ELISA kit 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 (Pandit et al., 2008; Nandecha et al., 2010). The detailed methodology is described in following sections.

Preparation of enzyme solution

Human prostate (about 200 mg) procured from the local hospital of Sagar was minced into small pieces and homogenised in 10 ml of medium A (20 mM sodium phosphate, pH 6.5, containing 0.32 mM sucrose and 1 mM EDTA). The homogenate was centrifuged at 716 g for 15 min. The supernatant was used as a source of the enzyme viz., 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) and extracts (1 mg ml\(^{-1}\)) were prepared in ethanol (95%) with gentle heating wherever necessary. EDTA solution (10 mg ml\(^{-1}\)) was made in distilled water. Finasteride (10 µg ml\(^{-1}\)) was prepared in ethanol.

Determination of optimum concentration of enzyme
It was determined by keeping the concentration of substrate constant and varying the concentration of enzyme. Testosterone solution (1 mM) was prepared in ethanol. 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 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. Testosterone content in methanolic solution was estimated by HPLC (AT10; Shimadzu Corp., Kyoto, Japan). The column was eluted isocratically with a mobile phase of methanol:water (80:20) at a flow rate of 1.0 ml min\(^{-1}\) (Purdon & Lehman-McKeeman, 1997). The volume of sample injected in the column was 20 µl, and the wavelength was 245 nm. 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 for separate groups, optimum amount of enzyme solution (i.e., 0.8 ml) and sodium phosphate buffer (20 mM) to a final volume of 1.5 ml. Reaction mixture was incubated at 37 °C for 60 min, and reaction was terminated by addition of 2 ml of ethyl acetate. Rest of the procedure is same as described in the section above.

Characterisation of extract and selection of marker
The in vitro studies led us to conclude that petroleum ether and ethanolic extract of GL is having 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 was not included in further studies. GLP and GLE were co-chromatographed with a standard marker compound viz. β-sitosterol. When visualised under UV at 254 nm, both the extracts revealed the presence of β-sitosterol. The solvent system used for GLP was toluene : ethyl acetate (8 : 2) and that for GLE was chloroform : methanol : toluene (8 : 2 : 1).

High-performance thin-layer chromatographic (HPTLC) analysis
High-performance thin-layer chromatographic analysis was performed to develop the characteristic fingerprint profile for petroleum ether and ethanolic extracts of GL. GLP 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, GLE was also analysed for its HPTLC profile and the presence of marker compound. The procedure was the same as followed in the case of GLP. The solvent system for GLE was chloroform : methanol : toluene (8 : 2 : 1). The percentage of β-sitosterol in GLP and GLE was calculated to be 28.30% and 18.82% respectively.

Moreover, the overlain UV spectra 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 GL extracts.

Further HPTLC studies reported in American Herbal Pharmacopoeia for Reishi mushroom GL were performed with our samples to validate their identity. The HPTLC profile of ethanolic extract in dichloromethane : methanol (9 : 1) was found to be identical to that reported by the pharmacopoeial text (American Herbal Pharmacopoeia, 2006).

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 (OECD, 2001) (OECD 423 Acute Toxic Class
GLP (10, 20 or 50 mg kg\(^{-1}\)) or testosterone (3 mg kg\(^{-1}\)) for this study. Hyperplasia was induced by subcutaneous administration of 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.). \(\beta\)-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.), GLP (10, 20 or 50 mg kg\(^{-1}\) p.o.), GLE (10, 20 or 50 mg kg\(^{-1}\) p.o.) or \(\beta\)-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 anesthesia and sacrificed. The prostates were immediately dissected out and weighed. Mean body weights and prostatic/body weight ratios were calculated for each group.

**Measurement of urine output**

The urine output of individual animals were monitored at the beginning of the study i.e. on day 0 and thereafter weekly till the completion of the study i.e. 28th day. 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. 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 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 purchases from United Biotech Inc., Mountain View, CA, USA). The UBI MAGIWEL testosterone quantitative test is based on the principle of competitive solid-phase enzyme immunoassay. The test sample competes with enzyme-labelled testosterone for a fixed and limited number of antibody sites on the microtitre 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 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. PSA was quantified by the method of Nilsson et al. (1997). The PSA ELISA is a solid-phase, non-competitive 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 microtitre 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 microtitre 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 and 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 blinded 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 GL extracts along with β-sitosterol as a biochemical marker revealed the presence of β-sitosterol in the extracts, with an Rf value of 0.95 for GLP (toluene : ethyl acetate/8 : 2) and 0.93 for GLE (chloroform : methanol : toluene/8 : 2 : 1) when analysed at 254 nm (Figs 1 and 2). Overlaid UV spectral interpretation further confirmed the presence of β-sitosterol in the extracts (Fig. 3).

In vitro studies

The optimum concentration of the enzyme was found to be 0.8 ml (270.0 µg protein calculated by Bradford
Varying concentrations of test samples were incubated with a constant amount of testosterone and enzyme in reaction mixture, and the residual testosterone content was determined after termination of reaction with ethyl acetate. The residual testosterone content in reaction mixture increased with increasing concentrations of GLP, GLE, GLA 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. The IC50 values calculated for GLP, GLE, GLA and standard 5α-reductase inhibitor i.e. finasteride were 0.164 mg, 0.01 mg, 0.29 mg and 1.06 μg respectively. Although GLE and GLP extracts showed good activity, the activity of finasteride is about 10 times greater. Relative inhibitory potency of the test material is thus Finasteride > GLE > GLP > GLA (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 testosterone-induced hyperplasia in rats. Results obtained are discussed in the following sections.

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

In testosterone-treated group, mean body weight and mean prostatic weight showed a considerable increase after 28 days of treatment. In case of vehicle-treated group, no
An appreciable increase in body weight was observed as noted in Table 2. While in case of finasteride-treated group, a decrease was observed. Table 2 summarises the effects of GLP (10, 20 and 50 mg kg\(^{-1}\) p.o.), GLE (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 GLP, P/BW ratios were 4.59 ± 0.34, 3.30 ± 0.31 and 3.12 ± 0.30 for 10, 20 and 50 mg kg\(^{-1}\) doses respectively. Similarly in case of GLE-treated groups, the ratios were 5.12 ± 0.44, 3.79 ± 0.16 and 3.27 ± 0.21 for 10, 20 and 50 mg kg\(^{-1}\) doses respectively. Similarly, \(\beta\)-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 were significant when compared to testosterone-treated group and finasteride-treated positive control group. In case of GLP, P/BW ratios were 4.59 ± 0.34, 3.30 ± 0.31 and 3.12 ± 0.30 for 10, 20 and 50 mg kg\(^{-1}\) doses respectively. Similarly in case of GLE-treated groups, the ratios were 5.12 ± 0.44, 3.79 ± 0.16 and 3.27 ± 0.21 for 10, 20 and 50 mg kg\(^{-1}\) doses respectively. Similarly, \(\beta\)-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 were significant when compared to testosterone-treated group and finasteride-treated groups. On the basis of mean prostatic weights and P/BW ratios, we calculated the % recovery in P/BW ratio by test groups when compared to testosterone-treated group. The formula used for calculation of % recovery was as follows:

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

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

B = % increase in prostatic weight induced by test sample

The % recoveries thus calculated for GLP-treated group at doses of 10, 20 and 50 mg kg\(^{-1}\) were 46.10%, 68.35% and 71.57% respectively. In case of GLE, these recoveries were 36.99%, 59.94% and 68.94% for 10, 20 and 50 mg kg\(^{-1}\) respectively. \(\beta\)-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, GLP proved to be better than GLE 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 the hyperplasia progressed with testosterone treatment, the urine output was reduced, and output was drastically reduced after 28 days of treatment in testosterone-treated control group. When GL extracts were administered along with testosterone, significant improvement in urine output over testosterone-treated control group were observed. The % obstruction in urine output was significantly lower in GL extracted groups.

**Table 1** \(\beta\)-reductase inhibitory concentrations (IC-50) of treated groups

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>IC 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum ether extract</td>
<td>0.164 mg</td>
</tr>
<tr>
<td>2</td>
<td>Ethanolic Extract</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous Extract</td>
<td>0.29 mg</td>
</tr>
<tr>
<td>4</td>
<td>Finasteride</td>
<td>1.06 μg</td>
</tr>
</tbody>
</table>

Fig. 3 Overlain UV spectra of standard \(\beta\)-sitosterol with \(\beta\)-sitosterol present in the extract using high-performance thin-layer chromatographic.
output for GLP-treated groups in doses of 10, 20 and 50 mg kg^{-1} were 3.94, 9.60 and 1.47% respectively as against 77.15% in testosterone-treated group. Similarly, when GLE was administered in graded doses of 10, 20 and 50 mg kg^{-1} along with testosterone, significant improvement in urine output was recorded when compared to testosterone-treated group. The % obstruction in urine output for GLE-treated groups was 3.23, 3.46 and 0.48% respectively for doses of 10, 20 and 50 mg kg^{-1}. In \( \beta \)-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: 

GLE50 > Finasteride > GLP50 > GLE10 > GLE20 > GLP10 > GLP20 > \( \beta \)-sitosterol (10) > \( \beta \)-sitosterol (20).

The results are depicted in Table 3.

**Measurement of serum testosterone concentration**

The 5\( \alpha \)-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. The extracts as well as 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 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 (5\( \alpha \)-reductase) in the prostate of testosterone-treated animals. Treatment of animals with exogenous testosterone caused its elevation in serum. Simultaneous administration of extracts and \( \beta \)-sitosterol along with testosterone led to further increase in serum testosterone levels suggesting inhibition of 5\( \alpha \)-reductase activity of the extracts and \( \beta \)-sitosterol. The results are depicted in Fig. 4.

**Measurement of prostate-specific antigen**

PSA 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 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 $0.140 \pm 0.087$ μg/ml. This level increased to $1.533 \pm 0.067$ μg/ml in testosterone-treated group. Finasteride-treated group showed a decrease in PSA level to $0.900 \pm 0.378$ μg/ml ($P < 0.05$ compared to testosterone-treated group). GLP in doses of 10, 20 and 50 mg kg$^{-1}$ p.o. showed levels of $0.896 \pm 0.558$ ($P < 0.05$), $0.507 \pm 0.338$ ($P < 0.05$) and $0.200 \pm 0.002$ ($P < 0.01$) μg/ml respectively, which indicate the protective effects of GLP on testosterone-induced hyperplasia. Decrease in PSA levels was also observed with GLE treatment, which exhibited levels of $1.395 \pm 1.305$ ($P < 0.05$), $0.782 \pm 0.452$ ($P < 0.01$) and $0.210 \pm 0.021$ ($P < 0.01$) μg/ml for 10, 20 and 50 mg kg$^{-1}$ respectively. The decreases observed were significant compared to testosterone-treated group. These observations indicate that GLP was more effective than testosterone.

<table>
<thead>
<tr>
<th>Treatment (mg kg$^{-1}$)</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (vehicle only)</td>
<td>$1.02 \pm 0.05$</td>
<td>$1.05 \pm 0.06$</td>
<td>$1.10 \pm 0.04$</td>
<td>$1.05 \pm 0.09$</td>
<td>$1.02 \pm 0.02$</td>
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<tr>
<td>Testosterone (3 mg kg$^{-1}$ s.c.)</td>
<td>$0.98 \pm 0.09$</td>
<td>$0.85 \pm 0.06$</td>
<td>$0.75 \pm 0.08$</td>
<td>$0.37 \pm 0.08$</td>
<td>$0.22 \pm 0.02$</td>
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<tr>
<td>Finasteride (1 mg kg$^{-1}$ p.o.) + T</td>
<td>$1.01 \pm 0.07$</td>
<td>$0.97 \pm 0.01$</td>
<td>$0.950 \pm 0.02$</td>
<td>$0.970 \pm 0.07$</td>
<td>$1.000 \pm 0.04$</td>
</tr>
<tr>
<td>GLP 10 + T</td>
<td>$1.01 \pm 0.07$</td>
<td>$1.00 \pm 0.05$</td>
<td>$0.77 \pm 0.06$</td>
<td>$0.82 \pm 0.02$</td>
<td>$0.97 \pm 0.04$</td>
</tr>
<tr>
<td>GLP 20 + T</td>
<td>$0.88 \pm 0.25$</td>
<td>$0.85 \pm 0.02$</td>
<td>$0.60 \pm 0.09$</td>
<td>$0.67 \pm 0.04$</td>
<td>$0.80 \pm 0.05$</td>
</tr>
<tr>
<td>GLP 50 + T</td>
<td>$1.01 \pm 0.05$</td>
<td>$1.00 \pm 0.19$</td>
<td>$0.82 \pm 0.10$</td>
<td>$0.95 \pm 0.06$</td>
<td>$1.00 \pm 0.14$</td>
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<tr>
<td>GLE 10 + T</td>
<td>$1.02 \pm 0.08$</td>
<td>$1.01 \pm 0.40$</td>
<td>$1.07 \pm 0.11$</td>
<td>$0.97 \pm 0.11$</td>
<td>$0.98 \pm 0.04$</td>
</tr>
<tr>
<td>GLE 20 + T</td>
<td>$1.02 \pm 0.02$</td>
<td>$1.00 \pm 0.08$</td>
<td>$0.98 \pm 0.04$</td>
<td>$0.92 \pm 0.06$</td>
<td>$0.97 \pm 0.04$</td>
</tr>
<tr>
<td>GLE 50 + T</td>
<td>$1.03 \pm 0.08$</td>
<td>$1.02 \pm 0.02$</td>
<td>$0.90 \pm 0.08$</td>
<td>$0.95 \pm 0.05$</td>
<td>$1.02 \pm 0.04$</td>
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<tr>
<td>BS 10 + T</td>
<td>$0.99 \pm 0.84$</td>
<td>$0.97 \pm 0.12$</td>
<td>$0.90 \pm 0.07$</td>
<td>$0.60 \pm 0.13$</td>
<td>$0.92 \pm 0.09$</td>
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<tr>
<td>BS 20 + T</td>
<td>$0.875 \pm 0.10$</td>
<td>$0.725 \pm 0.18$</td>
<td>$0.62 \pm 0.17$</td>
<td>$0.22 \pm 0.07$</td>
<td>$0.77 \pm 0.11$</td>
</tr>
</tbody>
</table>

Values are mean ± SEM ($n = 6$) anova followed by Dunnett’s test. Df = (8, 78) 103.
GLP 10, GLP 20, GLP 50 – petroleum ether extract of *Ganoderma lucidum* (10, 20 and 50 mg kg$^{-1}$ p.o. respectively).
GLE 10, GLE 20, GLE 50 – ethanolic extract of *G. lucidum* (10, 20 and 50 mg kg$^{-1}$ p.o. respectively).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (vehicle only)</td>
<td>$1.02 \pm 0.05$</td>
<td>$1.05 \pm 0.06$</td>
<td>$1.10 \pm 0.04$</td>
<td>$1.05 \pm 0.09$</td>
<td>$1.02 \pm 0.02$</td>
</tr>
<tr>
<td>Testosterone (3 mg kg$^{-1}$ s.c.)</td>
<td>$0.98 \pm 0.09$</td>
<td>$0.85 \pm 0.06$</td>
<td>$0.75 \pm 0.08$</td>
<td>$0.37 \pm 0.08$</td>
<td>$0.22 \pm 0.02$</td>
</tr>
<tr>
<td>Finasteride (1 mg kg$^{-1}$ p.o.) + T</td>
<td>$1.01 \pm 0.07$</td>
<td>$0.97 \pm 0.01$</td>
<td>$0.950 \pm 0.02$</td>
<td>$0.970 \pm 0.07$</td>
<td>$1.000 \pm 0.04$</td>
</tr>
<tr>
<td>GLP 10 + T</td>
<td>$1.01 \pm 0.07$</td>
<td>$1.00 \pm 0.05$</td>
<td>$0.77 \pm 0.06$</td>
<td>$0.82 \pm 0.02$</td>
<td>$0.97 \pm 0.04$</td>
</tr>
<tr>
<td>GLP 20 + T</td>
<td>$0.88 \pm 0.25$</td>
<td>$0.85 \pm 0.02$</td>
<td>$0.60 \pm 0.09$</td>
<td>$0.67 \pm 0.04$</td>
<td>$0.80 \pm 0.05$</td>
</tr>
<tr>
<td>GLP 50 + T</td>
<td>$1.01 \pm 0.05$</td>
<td>$1.00 \pm 0.19$</td>
<td>$0.82 \pm 0.10$</td>
<td>$0.95 \pm 0.06$</td>
<td>$1.00 \pm 0.14$</td>
</tr>
<tr>
<td>GLE 10 + T</td>
<td>$1.02 \pm 0.08$</td>
<td>$1.01 \pm 0.40$</td>
<td>$1.07 \pm 0.11$</td>
<td>$0.97 \pm 0.11$</td>
<td>$0.98 \pm 0.04$</td>
</tr>
<tr>
<td>GLE 20 + T</td>
<td>$1.02 \pm 0.02$</td>
<td>$1.00 \pm 0.08$</td>
<td>$0.98 \pm 0.04$</td>
<td>$0.92 \pm 0.06$</td>
<td>$0.97 \pm 0.04$</td>
</tr>
<tr>
<td>GLE 50 + T</td>
<td>$1.03 \pm 0.08$</td>
<td>$1.02 \pm 0.02$</td>
<td>$0.90 \pm 0.08$</td>
<td>$0.95 \pm 0.05$</td>
<td>$1.02 \pm 0.04$</td>
</tr>
<tr>
<td>BS 10 + T</td>
<td>$0.99 \pm 0.84$</td>
<td>$0.97 \pm 0.12$</td>
<td>$0.90 \pm 0.07$</td>
<td>$0.60 \pm 0.13$</td>
<td>$0.92 \pm 0.09$</td>
</tr>
<tr>
<td>BS 20 + T</td>
<td>$0.875 \pm 0.10$</td>
<td>$0.725 \pm 0.18$</td>
<td>$0.62 \pm 0.17$</td>
<td>$0.22 \pm 0.07$</td>
<td>$0.77 \pm 0.11$</td>
</tr>
</tbody>
</table>

Values are mean ± SEM ($n = 6$). One way anova followed by Dunnett’s test.

Fig. 4 Mean serum testosterone levels of various groups measured weekly using testosterone ELISA kit.
GLE 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. At some places, aggregation of columnar cells has been observed (Figs 6a and 7a).

*Testosterone-treated group (3 mg kg\(^{-1}\) s.c.)*

Tubules have become wider when 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 compared to control. The amount of the secretion in some tubules has increased. The connective tissue has been compressed, and blood vessels have dilated compared to control. The distinct nucleus and normal sarcoplasmic texture is not visible. Shape of the tubules has become obliterated (Figs 6b and 7b).

Testosterone + GLP (10, 20 and 50 mg kg\(^{-1}\) p.o.)

Vacuolisation in the cells is clear. Nucleus is picnotic (vacuole formation in outer layer of nucleus). Chromatin material in nucleus is normally distributed. Lumen of the tubules is normal and at some places slightly obliterated. Involutions are few in number and even less than what are observed in control. Connective tissue between the tubules is reduced. Stroma is composed of smooth muscles and connective tissue. A significant improvement compared to testosterone-treated group can be easily identified (Figs 6d–f and 7d).

Testosterone + GLE (10, 20 and 50 mg kg\(^{-1}\) p.o.)

With the better intraluminal secretions, tubules have shown morphological improvement in the texture. Still the epithelium is wide and thicker. When compared to testosterone-treated group, the stroma (composed of connective and smooth muscle cells) is normal. The appearance of the transitional epithelium resembles that of control. Connective tissue has increased appreciably as the dose is increased, and at some places it resembles the normal control group. Minor curvature in the epithelium is also observed. Involutions in the epithelium are fewer and thick (Figs 6g–i and 7e).

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 is antagonising the effects of testosterone up to a good degree, several cells with their increased volume are present throughout the transitional epithelium. Cells with swollen nuclei are prominent at many places. Reduced involutions in lesser number are observed (Figs 6c and 7c).

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. Overall, the effects induced by testosterone are effectively antagonised in these groups (Figs 6j, k and 7f).
**Discussion**

A densitometric high-performance TLC analysis was performed to develop the characteristic fingerprint profile for the petroleum ether and alcoholic extract of GL. This can be used as a tool for evaluation and standardisation of the drug.

GL has been used as a remedy for a wide variety of ailments, and its hidden therapeutic potentials are being explored for various diseases. The present study is an approach in this direction where we have identified the possible clinical implications of GL in BPH and its major clinical symptoms viz. urinary problem, which is a major cause of discomfort for most of the patients suffering from this disease.

Testosterone is converted to more potent dihydrotestosterone by the enzyme 5α-reductase present in prostate homogenates (Steers, 2001; Dhanotiya et al., 2009; Nandecha et al., in press). During in vitro studies, addition of GLP, GLE and GLA 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 GLA, it was not included in the studies thereafter, and only GLP and GLE were taken up for in vivo studies.

The results of the present investigations suggest that GLP and GLE at different dose levels inhibit prostatic hyperplasia induced with an exogenous supply of testosterone in a rat model.

The aetiology 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. In particular, BPH induced with testosterone or dihydrotestosterone do 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) (Deslypere et al., 1992; Zhou et al., 1995; Carson & Rittmaster, 2003). BPH in humans also involves prostatic oestrogens 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 on 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 (Paubert-Braquet et al., 1996; Bombardelli et al., 1997). In the present study, in rats administered with GLP and GLE along with testosterone, the increase in prostatic weight and P/BW ratio were attenuated after 28 days of oral treatment at different doses when 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 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.

Obstruction of urinary discharge is the major pathological problem of clinical significance in BPH patients. It was found that urine output was decreased drastically in testosterone-treated group. Significant increase is observed in urine output in extract- and finasteride-treated groups. 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 GLP as well as GLE, finasteride and β-sitosterol exhibited a significant improvement in urine output compared to the testosterone-treated group. This finding is significant as prostatic hyperplasia directly causes urinary problems like painful micturition, reduced urine flow, urinary urgency, etc. 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 abnor-
mally 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 GLP prevented prostatic hyperplasia significantly in a dose-dependent manner with 50 mg kg\(^{-1}\) showing the best activity. GLE also showed significant activity in a dose-independent manner. The \textit{in vitro} studies cleared the mechanism of prevention of prostatic hyperplasia induced by testosterone. It is evident that GL extracts have 5α reductase inhibitory activity. The weekly serum testosterone levels are suggestive of the mechanism of action of the extracts and finasteride. The recoveries in urine output also suggest 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; Wilt et al., 1999). Our studies with β-sitosterol are indicative of its protective effects on testosterone-induced hyperplasia. The histological findings have shown the recovery in the prostatic histoarchitecture particularly in the cuboidal epithelial cells, intracellular lumen, tubular latency and shape which further support to make GL a strong candidate for the management of prostatic hyperplasia. Further studies are necessitated to confirm the effect of the drug on BPH in humans. The study is the first attempt to analyse the effects of petroleum ether extract of GL, and the attempt proved successful as GLP showed better results than GLE in our experiments. The study shows that GL holds sufficient promise to be used as a drug for the prevention of BPH and is a firm candidate for further clinical research in this area. It is suggested, on the basis of these studies, to further undertake clinical trails on GL to develop an effective nutraceutical for the treatment of BPH.

The overall results clearly reflect the utility of extract in BPH. Because of conversion of testosterone to dihydrotestosterone, the prostate size is increased, thereby causing obstruction in urinary output. The observed effect that extracts do not allow the increase as reflected by urinary output, P/BW ratios and histoarchitecture showed that activity of administered testosterone was blocked by the extract and resulted in recovery.

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### References


A. Nahata and V. K. Dixit

Ganoderma lucidum attenuates prostatic hyperplasia


