

ORIGINAL ARTICLE

Preventive effect of *Pueraria mirifica* on testosterone-induced prostatic hyperplasia in Sprague Dawley rats

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Summary

Pueraria mirifica (PM) extract contains phytoestrogen daidzein and genistein. In this study, we investigated the protective effect of PM extract, daidzein and genistein on a testosterone-induced prostatic hyperplasia in rats. Testosterone was administered at 3 mg kg⁻¹ to rats followed by the PM extract, daidzein and genistein for a period of 30 days with finasteride as positive control. The testosterone level was increased, indicating inhibition of 5 α -reductase converting testosterone to dihydrotestosterone. This was confirmed by prostate-specific antigen level that significantly decreased when treated with PM extract, daidzein and genistein. The PM extract, daidzein and genistein reduced the increase in the prostate/body weight ratio in testosterone-induced rats. This gives indication that PM extract, daidzein and genistein possessed protective activity for the treatment of benign prostatic hyperplasia. The analysis of histoarchitecture of the prostate has also shown that there was a significant improvement in prostatic cells of the testosterone-induced rats when treated with PM extract, daidzein and genistein.

Introduction

The development of benign prostate hyperplasia (BPH) is due to the uncontrolled growth of the prostate gland (Arruzabala *et al.*, 2006), which causes outflow obstruction of the bladder and results in lower urinary tract symptoms. It is the most common ailment in elderly men at the age of over 50 with an estimated prevalence of up to 40%. Almost 90% of men above 80 years old suffer from the ailment (Thorpe & Neal, 2003; Bhargava *et al.*, 2004).

The aetiology of BPH still remains to be solved. It influences the hormonal changes in ageing men. The prostatic androgen, dihydrotestosterone (DHT), is formed in the prostate from testosterone by 5 α -reductase (Russell & Wilson, 1994). Then, DHT binds to androgen receptors and promotes protein synthesis and cellular growth of the prostate cells. This will lead to the increased formation and accumulation of DHT in the prostate, along with ageing, and may encourage cell growth and hyperplasia induction (Carson & Rittmaster, 2003).

Conventionally, antiandrogenic drugs such as 5 α -reductase inhibitor (finasteride and dutasteride) and α -adrenoreceptor antagonist (alfuzosin, doxazosin, tamsulosin and terazosin) are used for BPH treatment, although they have various side effects (Patel & Chapple,

2008). In addition to conventional therapy, an alternative therapy is also possible to treat BPH, as it may have fewer side effects.

Phytoestrogen is a compound from plants such as soya. It has been reported to provide beneficial effects on preventive or therapeutic action in carcinogenesis, atherosclerosis and osteoporosis (Cooke, 2006; Kim, 2008; Patisaul & Jefferson, 2010). It has an ability to influence testosterone levels and may also exert a protective effect against development of prostate cancer. Huang *et al.* (2008) reported that 20 and 100 mg kg⁻¹ daidzein showed some preventive effects on prostate hyperplasia induced by testosterone in rats. Genistein was reported to act as an oestrogen agonist in the stimulation of the prostate gland of adult rodents (Santti *et al.*, 1998). It is believed that genistein and daidzein exert an antioestrogenic effect indirectly by reducing androgen receptor expression (Fritz *et al.*, 2002). Genistein and daidzein have also been reported to be associated in lowering the risk of prostate cancer (Nelles *et al.*, 2011).

Pueraria mirifica (PM) is a Thai herb containing high amount of phytoestrogen. It belongs to the same family as soyabean and *Pueraria lobata*. It has been reported to contain 13 phytoestrogens, namely isoflavones (puerarin, daidzein, daidzin, genistein and genistin) and others such

as miroestrol, β -sitosterol, stigmasterol, coumestrol, puerarin, mirificin, mirificoumestan and kwakhurin (Chansakaow *et al.*, 2000; Urasopon *et al.*, 2007). It has been exhibited in various reproductive organs mainly in females. Jaroernporn *et al.* (2006) reported that PM gives no overt toxicity and abnormal clinical sign in male mice. PM has been used extensively as an alternative medicine for the oestrogen effect and age rejuvenation in humans, not only by women. Its high content of phytoestrogen triggered us to find out whether it could prevent prostate hyperplasia induced by testosterone.

Enlargement of the prostate that is induced by testosterone could be used to assess the potential treatment effects of BPH by inhibiting the formation of DHT using plant extracts. In this study, we investigated the preventive effect of *P. mirifica* on BPH. Prostate-specific antigen (PSA) levels were measured to evaluate the protective effect of PM extract, daidzein and genistein on testosterone-induced prostatic hyperplasia in a rat model.

Materials and methods

Animals

Twelve-week-old male Sprague Dawley rats, weighing 200 – 250 g, were obtained from Faculty of Veterinary, Universiti Putra, Malaysia. They were kept in polypropylene cages at room temperature ($25 \pm 2^\circ \text{C}$) with light/dark cycles of 12 h in Animal House, Universiti Malaya. The rats were fed a nonphytoestrogen pellet diet (Altromin, Lage, Germany) and water *ad libitum*. After 7 days of acclimatisation, the rats were divided into 10 experimental groups. Animal handling was conducted in accordance with the Institutional Animal Ethics Committee of Universiti Malaya.

Preparation of extracts

The *P. mirifica* tuberous root was purchased from Guangzhou, China, in January 2012. The powdered sample of 50 g PM was extracted with 250 ml of distilled water. Then, the solution was incubated in a water bath at 40°C for 12 h. After the incubation, the solution was filtered and the extract was dried in the freeze drier. The constituents of daidzein and genistein in the extract were 5.1 mg/100 g dry weight and 2.3 mg/100 g dry weight respectively.

Drugs and chemicals

Testosterone propionate, finasteride, daidzein and genistein were purchased from Sigma-Aldrich, St Louis, MO, USA. A testosterone ELISA kit and a PSA ELISA kit were purchased from Cusabio Biotech Co. Ltd., Wuhan, China. All other chemicals used in the study were of analytical grade.

Acute toxicity studies

Acute toxicity studies were carried out following OECD guidelines (OECD 423; Acute Toxic Class Method). In all cases, 2000 mg kg^{-1} oral dose of the *P. mirifica* extract and 200 mg kg^{-1} daidzein and genistein were found to be safe as no mortality was observed during the study. On the basis of this study, the doses of 10, 100, 1000 mg kg^{-1} were selected for *P. mirifica*, whereas doses of 10 and 100 mg kg^{-1} for daidzein and genistein respectively.

Administration and dosage

Powdered PM extract was suspended in distilled water for oral administration. Rats were given oral doses of 10, 100 and 1000 mg kg^{-1} once daily for 30 days. Testosterone propionate was diluted in corn oil and injected subcutaneously at 3 mg kg^{-1} , daily for 30 days as described previously (Nahata & Dixit, 2012). Finasteride was suspended in Tween-20 (0.2% v/v) and administered orally (1 mg kg^{-1} , p.o.). Daidzein and genistein were administered orally at doses of 10 and 100 mg kg^{-1} by suspending in Tween-20 (0.2% v/v).

Rats were randomly divided into 10 groups of six rats. Prostate hyperplasia was induced by subcutaneous administration of testosterone (3 mg kg^{-1}) for 30 days in all groups except the control group. The rats were treated with vehicle (Tween-20 (0.2% v/v, p.o.), finasteride (1 mg kg^{-1} , p.o.), PM (10, 100 or 1000 mg kg^{-1} , p.o.), daidzein (10 or 100 mg kg^{-1} , p.o.) or genistein (10 or 100 mg kg^{-1} , p.o.) before administration of corn oil or testosterone (3 mg kg^{-1}), subcutaneously.

Body and prostatic weights

Animals were weighed a day before the starting of the treatment (baseline), on the day before drug administration started and weekly thereafter. On day 31, animals were anaesthetised with a ketamine and xylazine cocktail and killed. Blood was collected, and the prostates were immediately dissected out and weighed. Prostate weight (PW) and PW to bodyweight ratios (P/BW) were calculated. Percentage of inhibition was calculated as follows:

Inhibition Percentage

$$= 100 - \left[\frac{\text{treated group} - \text{negative control}}{\text{positive control} - \text{negative control}} \times 100 \right]$$

Measurement of serum testosterone concentration

Testosterone levels of individual animals of each group were measured every 15 days using testosterone ELISA kit. Blood was collected from the tail of the rats and

centrifuged at 2000 g for 20 min to separate the serum. This serum was tested for testosterone content using the procedure supplied with the kit (CUSABIO Total Testosterone kit). The intensity of the colour was 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. The PSA ELISA kit was intended for the quantitative determination of total PSA. This kit was obtained from Cusabio Biotech Co. Ltd. The PSA ELISA is a solid-phase, noncompetitive immunoassay based upon the direct sandwich technique. Calibrators, controls and samples were treated with biotinylated anti-PSA monoclonal antibody and horseradish peroxidase (HRP)-labelled anti-PSA monoclonal antibody in streptavidin-coated microtitre stripes and then incubated. After washing, a buffered substrate (TMB-HRP substrate) was added to each well, and the enzyme reaction was allowed to proceed. Colour intensity was determined from the microplate reader at 450 nm, with wavelength correction at 540 nm. Calibration curves were drawn for each assay by plotting absorbance versus concentration of each calibrator. The concentration of PSA in samples was then determined from the calibration curve.

Histological studies

The prostate gland tissues were fixed in 10% formalin (in normal saline) for 24 h. Then, slide histology was prepared using microtome followed by haematoxylin and eosin (H&E) staining. The structural arrangement of the prostate cells was observed under a microscope and the image recorded.

Statistical analysis

All results are expressed as mean \pm SEM ($n = 6$). Comparisons between groups were performed using the ANOVA test, Duncan's and Dunnett's test by SPSS statistical software New York, USA. $P < 0.05$ was considered to be statistically significant.

Results

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

The mean body weight of all animals in this experiment showed a considerable increase after 30 days of treatment. Table 1 summarises the effect of PM (10, 100, 1000 mg kg⁻¹, p.o.), daidzein (10, 100 mg kg⁻¹, p.o.),

genistein (10, 100 mg kg⁻¹, p.o.) and finasteride (1 mg kg⁻¹, p.o.), on prostatic hyperplasia induced by testosterone. The mean body weight of the PM-treated and (T+HP)-treated animals decreased after 30 days compared with the control and testosterone control, but the decrease of P/BW ratio was not induced by toxicity. The decrease in the mean body weight could be attributed to the suppression of the animals' appetite by the extract leading to reduced food intake.

The P/BW ratio calculated for negative control group was 3.85 ± 0.09 . Administration of testosterone significantly elevated the P/BW ratio of testosterone-treated group (9.49 ± 0.27) when compared with the negative control rats. The percentage inhibition on elevation of P/BW for finasteride-treated group was 81.03%, while for PM extract in testosterone-treated rats, the percentage inhibition was 41.31%, 52.66% and 67.55% by 10, 100 and 1000 mg kg⁻¹ PM extract when compared to negative control rats. Similarly, daidzein-treated groups showed a percentage inhibition on elevation of P/BW of 72.70% and 78.01% for 10 and 100 mg kg⁻¹ doses respectively. Similarly, genistein-treated groups showed a percentage inhibition on elevation of P/BW of 72.87% and 68.62% for 10 and 100 mg kg⁻¹ doses respectively. Most of the values were significant when compared to testosterone-treated group and negative control group.

The percentages of the recovery of the groups treated with PM extract, daidzein and genistein as compared with the testosterone-treated group were calculated based on the mean prostatic weights and P/BW ratios. The increase induced by testosterone was considered to be 100%, and this was compared with other treated groups. The formula used for calculation of % recovery was as follows:

$$\% \text{ Recovery} = A - B,$$

where $A =$ % increase in prostatic weight in testosterone-treated group (considered 100%), $B =$ % increase in prostatic weight induced by test sample.

The percentage recovery calculated for PM extract in testosterone-treated rats was 49.89%, 56.43% and 86.20% by 10, 100 and 1000 mg kg⁻¹ when compared to negative control animals respectively. While daidzein-treated groups showed a percentage recovery of 72.70% and 78.01% for 10 and 100 mg kg⁻¹ doses, respectively, similarly, genistein-treated groups showed a recovery of 72.87% and 68.62% for 10 and 100 mg kg⁻¹ doses respectively. The recovery percentage for finasteride-treated group was 91.09%.

Measurement of serum testosterone concentration

The rats treated with PM extract, daidzein, genistein and finasteride showed increased testosterone serum levels (Fig. 1). In the finasteride testosterone-induced group,

Table 1 Effect of treatments on body weights and prostate enlargements in testosterone-treated rats

Treatment (mg kg ⁻¹)	Body weight (g)		Prostatic weight, PW (mg)	Prostatic/body weight ratio (P/BW)	% Inhibition on P/BW Ratio	% Increase in PW	% Recovery
	Day 0	Day 30					
Negative control (vehicle only)	236.83 ± 1.54	394.83 ± 6.87 ^b	1521.52 ± 46.93 ^a	3.85 ± 0.09 ^{ab}	0.00	0.00	0.00
Testosterone (T)	231.67 ± 2.16	362.17 ± 9.04	3427.80 ± 76.12 ^b	9.49 ± 0.27 ^b	0.00	100.00	0.00
T+Finasteride	242.00 ± 2.96	344.00 ± 0.89	1691.33 ± 16.11 ^a	4.92 ± 0.05 ^a	81.03	8.91	91.09
T+LP	228.00 ± 1.13	344.67 ± 8.87	2476.73 ± 146.49 ^{ab}	7.16 ± 0.26 ^{ab}	41.31	50.11	49.89
T+MP	227.83 ± 1.54	360.33 ± 13.45	2352.00 ± 95.06 ^{ab}	6.52 ± 0.08 ^{ab}	52.66	43.57	56.43
T+HP	228.00 ± 3.57	313.83 ± 5.46 ^a	1784.63 ± 55.00 ^a	5.68 ± 0.09 ^a	67.55	13.80	86.20
T+LD	231.00 ± 0.78	319.17 ± 10.19 ^a	1707.17 ± 94.10 ^a	5.39 ± 0.36 ^a	72.70	9.74	90.26
T+HD	229.00 ± 1.03	369.67 ± 10.40	1889.40 ± 151.93 ^a	5.09 ± 0.30 ^a	78.01	19.30	80.70
T+LG	225.00 ± 4.33	322.67 ± 10.80 ^a	1729.50 ± 66.51 ^a	5.38 ± 0.20 ^a	72.87	10.91	89.09
T+HG	229.00 ± 3.28	358.67 ± 9.39	2010.43 ± 27.29 ^a	5.62 ± 0.10 ^a	68.62	25.65	74.35

Values are mean ± SEM ($n = 6$) ANOVA followed by Dunnett's test.

LP, MP, HP – Aqueous extract of *Pueraria mirifica* (10, 100, 1000 mg kg⁻¹, p.o. respectively).

LD, HD – daidzein (10, 100 mg kg⁻¹, p.o. respectively).

LG, HG – genistein (10, 100 mg kg⁻¹, p.o. respectively).

^a $P < 0.05$ compared to testosterone group.

^b $P < 0.05$ compared to finasteride group.

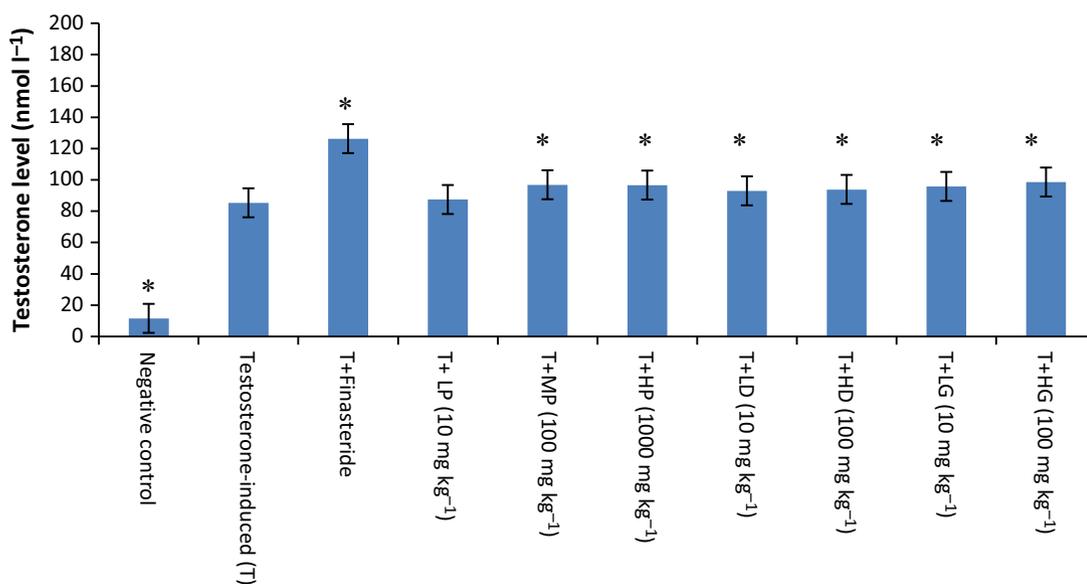


Fig. 1 Mean serum testosterone levels of various groups. * $P < 0.05$ compared to testosterone-induced group.

testosterone serum levels were highly increased during 30 days of the study. Administration of the PM extract, daidzein and genistein, along with testosterone, caused an increase in the serum testosterone levels, indicating inhibition of 5 α -reductase to form DHT.

Measurement of prostate-specific antigen

The administration of PM extract, daidzein and genistein reduced the PSA level in testosterone-induced rats. The normal PSA level in negative control group was

0.75 ± 0.02 ng ml⁻¹. The PSA level in testosterone-treated group was significantly increased to 5.53 ± ng ml⁻¹. The finasteride group showed a significant decrease in PSA level (1.09 ± 0.08 ng ml⁻¹) compared to testosterone-treated group. PM extract, doses 10, 100 and 1000 mg kg⁻¹, also showed a decrease in level of PSA of 1.92 ± 0.13, 1.71 ± 0.15 and 1.26 ± 0.03 ng ml⁻¹, respectively, which indicates the protective effects of PM on testosterone-induced hyperplasia. Daidzein (10 and 100 mg kg⁻¹) and genistein (10, 100 mg kg⁻¹) also showed a decrease in PSA levels

of 1.72 ± 0.09 , 1.50 ± 0.06 , 1.51 ± 0.07 and 1.39 ± 0.02 ng ml^{-1} respectively. The observations are depicted in Fig. 2.

Histopathology of prostate

Normal histological features of prostate gland were visible in the negative control group (Fig. 3a). The tissue was tightly packed; the epithelium was cuboidal and regular in size. The tubules were variable in diameter with irregular lumen. Connective tissue, blood vessels and lymph vessels, and matrix were normal.

In positive control group which is testosterone-treated group, there was disruption in the histoarchitecture of the prostate tissue (Fig 3b). The tubules become wider compared to negative control. Every tubule developed large involutions and projection into the lumen, reducing the volume of the lumen. The shapes of the tubules also become obliterated. The amount of connective tissue was well marked with increased oval acini size. Stromal proliferation and glandular hyperplasia with epithelial proliferation and nuclear stratification were observed. In the finasteride-treated group, the normal distribution of stroma was seen (Fig 3c). The projection was not prominent. The histoarchitectures were improved compared to testosterone-treated group.

PM extract treatment groups showed mild glandular hyperplasia (Fig 3d–f). Vacuolisation in the cells is clear. Lumens of the tubules are normal, and at some places, few slightly obliterated involution and projection were found.

The histoarchitecture was improved with high dose of PM extract. The connective tissue increased appreciably as the dose increased, and at some places, it resembles the negative control appearances. For the daidzein- and genistein-treated group, the lumina of the tubules were normal, and at some, places the epithelium was slightly thicker than the negative control. Stromal appearances were normal (Fig 3g–j).

Discussion

In this study, treatment of BPH in testosterone-induced rats with *P. mirifica* for 30 days significantly inhibited the development of benign prostatic hyperplasia, as evidenced by a reduction in elevated prostate weight and prostate weight per body weight ratio, serum testosterone, and PSA level and by histopathological analysis.

It is well-established that the conversion of testosterone to DHT is catalysed by 5α -reductase enzyme, which occurred abundantly in the nuclear membrane microsomes of prostatic epithelial cells. Thus, an increased production of DHT will result in the development of BPH (McConnell *et al.*, 1992). The use of 5α -reductase inhibitors will reduce the concentration of DHT in prostate gland tissue without disrupting the sexual function as it is only involved in inhibiting the formation of DHT.

Pueraria mirifica has been used widely for oestrogen effect and age rejuvenation. It is known to have high phytoestrogen contents. Previous studies reported that phytoestrogen had beneficial effects on carcinogenesis (Patisaul & Jefferson, 2010) and BPH (Huang *et al.*,

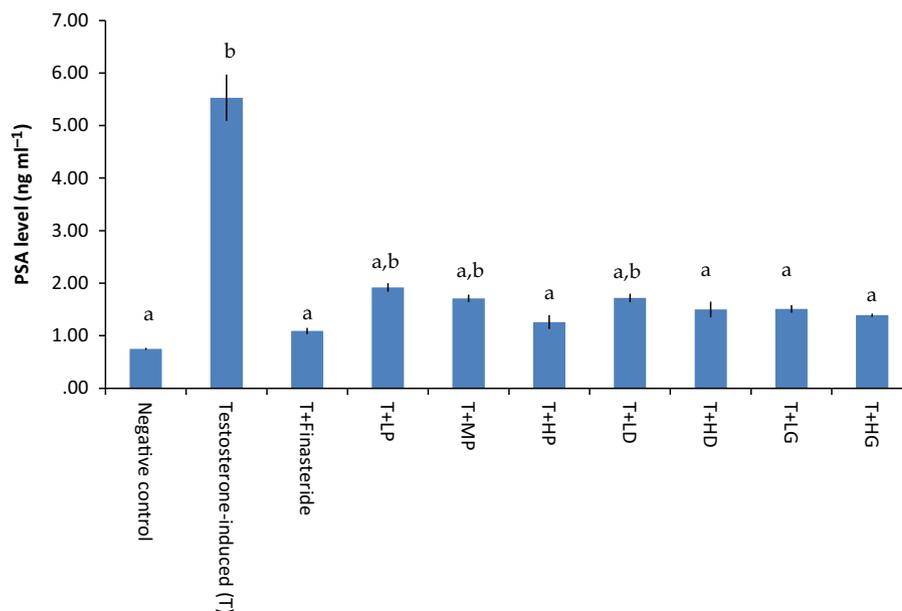


Fig. 2 Mean prostate-specific antigen (PSA) levels of various groups. ^a $P < 0.05$ compared to testosterone group; ^b $P < 0.05$ compared to finasteride group.

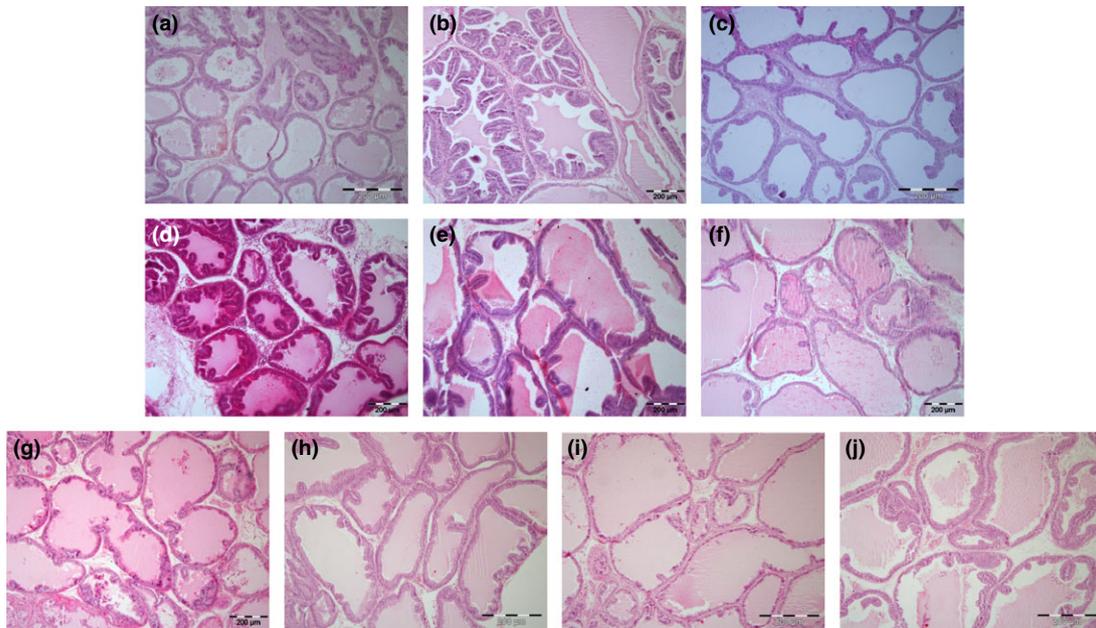


Fig. 3 Light micrograph of prostate (10 \times). (a) Negative control. (b) Testosterone group. (c) T+Finasteride group. (d) T+LP. (e) T+MP. (f) T+HP. (g) T+LG. (h) T+HG. (i) T+LD. (j) T+HD.

2008). The present study was carried out to determine the possibilities of using *P. mirifica* for BPH treatment.

Dihydrotestosterone is formed from testosterone by the enzyme 5α -reductase which is present in prostate homogenates (Dhanotiya *et al.*, 2009; Nandecha *et al.*, 2010). Nahata & Dixit (2012) reported that finasteride is about 10 times more potent in inhibiting 5α -reductase activity *in vitro*. The results of the present study suggest that PM at different dose levels inhibits prostatic hyperplasia induced with an exogenous supply of testosterone in a rat model.

In this study, PM at doses of 10, 100 and 1000 mg kg⁻¹ administered orally for 30 days significantly and dose dependently inhibited the increase in prostate weight by 49.89%, 56.43% and 86.20%, respectively, while the PW/BW ratio decreased by 41.31%, 52.66% and 67.55%, respectively, in induced testosterone rats. The effect achieved at doses of 1000 mg kg⁻¹ was comparable with that obtained with finasteride, a drug used to treat BPH (Aliaev *et al.*, 2002).

As PM significantly prevented the increase in the PW/BW ratio, this gives indications that it possessed preventive effects on BPH in rats.

The prostate weight gain induced by testosterone was accompanied by histological changes of BPH and reduced the formation of BPH. A similar observation was also reported by Noa *et al.* (2005), Arruzazabala *et al.* (2006) and Nahata and Dixit (2012).

The PW/BW ratio is used as the main marker of the effects of treatments of BPH in the testosterone-induced rat model, but it is also related to body weight changes.

Therefore, PW/BW ratio is directly related to the prostate weight gain.

In the present study, the administered PM, daidzein and genistein, along with testosterone, showed a decrease in the prostatic weight and P/BW ratio after 30 days of oral treatment at different doses when compared to testosterone-treated group. The levels of testosterone were increased significantly when treated with PM, daidzein and genistein as a result of the inhibition of the 5α -reductase that catalysed the conversion of testosterone to DHT, which caused inflammation of the prostate. This gives strong indications that PM, daidzein and genistein act as inhibitors of 5α -reductase and attenuate the development of BPH.

The PSA is used to measure the formation of BPH in prostate gland. The level of PSA serum is abnormally elevated in patients with prostate cancer, BPH and patients with prostate inflammatory conditions. The decrease in PSA levels indicates that the test sample possessed a protective effect 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. The PM extract, daidzein and genistein significantly reduced the PSA levels, which are an indication of their 5α -reductase activity and efficacy in the treatment of prostatic hyperplasia. This suggested that PM extract, daidzein and genistein provide prevention from BPH in a dose-dependent manner with maximum activity at a dose of 1000 mg kg⁻¹.

The histological analysis has shown that the recovery in the prostatic histoarchitecture, especially in the cuboidal epithelial cells, intracellular lumen, tubular latency and shape, which provide further evidence to support PM extract, daidzein and genistein, is effective in the prevention and management of prostatic hyperplasia.

Further studies are required to validate the effect of the PM extract, daidzein and genistein on BPH. This is the first study attempting to analyse the effects of PM extract, daidzein and genistein, which strongly indicated that PM extracts possessed protective effect on BPH. The study shows that PM extract holds sufficient promise to be used as a drug for the prevention of BPH and can undergo further clinical research in BPH.

The overall results clearly indicate the protective effect of the PM extract for BPH treatment due to its ability to inhibit 5 α -reductase to form DHP and to reduce the PSA level. This will result in decreased P/BW ratios and improved histoarchitecture of the prostate gland cells.

Conclusion

Oral administration of PM extract, daidzein and genistein for 30 days showed dose-dependent reduction of prostate enlargement in testosterone-induced rats. The preventive effect is likely due to 5 α -reductase inhibitory activity of the PM extract, daidzein and genistein. Further experimental studies are required to validate the present findings before deciding whether they are meaningful enough to be explored in humans with BPH.

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