Effect of *Veratrum maackii* on testosterone propionate-induced benign prostatic hyperplasia in rats

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Abstract

*Veratrum maackii* (VM), a perennial plant in the Melanthiaceae family, has anti-hypertensive, anti-cholinergic, anti-asthmatic, anti-tussive, anti-fungal, anti-melanogenesis, and anti-tumor activities. Here, we investigated the therapeutic effect of VM on benign prostatic hyperplasia (BPH) in human normal prostate cell line (WPMY-1) and a testosterone propionate-induced BPH animal model. WPMY-1 cells were treated with VM (1-10 µg/mL) and testosterone propionate (100 nM). BPH in rats was generated via daily subcutaneous injections of testosterone propionate (3 mg/kg) dissolved in corn oil, for 4 weeks. VM (150 mg/kg) was administered daily for 4 weeks by oral gavage concurrently with the testosterone propionate. All rats were sacrificed and the prostates were dissected, weighed, and subjected to histological, immunohistochemical, and biochemical examinations. Immunoblotting experiments indicated that WPMY-1 cells treated testosterone propionate had increased expression of prostate specific antigen (PSA) and androgen receptor (AR), and treatment with VM or finasteride blocked this effect. In rat model, VM significantly reduced prostate weight, prostatic hyperplasia, prostatic levels of dihydrotestosterone (DHT), and expression of proliferation markers such as proliferating cell nuclear antigen (PCNA) and cyclin D1, but increased the expression of pro-apoptotic Bcl-2-associated X protein (Bax) and the cleavage of caspase-3. VM administration also suppressed the testosterone propionate-induced activation of nuclear factor-κB (NF-κB). Our results indicate that VM effectively represses the development of testosterone propionate-induced BPH, suggesting it may be a useful treatment agent for BPH.

**Keywords:** *Veratrum maackii*, Testosterone propionate, Benign prostatic hyperplasia, NF-κB
Introduction

Benign prostatic hyperplasia (BPH) is a common pathologic condition in older men.\textsuperscript{1)\textsuperscript{1}} The human prostate has two major cell types: glandular cells (secretory acini), which consist of columnar and basal cells, and dense stromal cells, which mainly consist of collagen and smooth muscle cells.\textsuperscript{2)\textsuperscript{2}} BPH is characterized by the progressive growth of prostatic glandular epithelial and stromal elements, culminating in uncontrolled hyperplasia.\textsuperscript{3)\textsuperscript{3}} As the prostate enlarges, it tightens the urethra, prompting a weak urinary stream, incomplete bladder emptying, dysuria, nocturia, and bladder outlet obstruction.\textsuperscript{4)\textsuperscript{4}}

Although the pathogenesis of BPH has not yet been totally explained, aging and elevated androgens are established risk factors for BPH.\textsuperscript{5)\textsuperscript{5}} Dihydrotestosterone (DHT) is a potent androgen that has a critical role in prostate growth. The prostate synthesizes DHT from circulating testosterone via 5\textsubscript{\alpha}-reductase (5\textsubscript{\alpha}R). DHT then binds to androgen receptors (ARs), and promotes protein synthesis and growth of prostate cells.\textsuperscript{6)\textsuperscript{6}} The serum concentrations of testosterone and DHT diminish with age in old men, however DHT production is remarkably increased in men with BPH.\textsuperscript{7,8)\textsuperscript{7}} Recent studies reported that a higher serum DHT level and DHT/testosterone ratio were associated with larger prostate volume and increased prevalence of BPH.\textsuperscript{9)\textsuperscript{9}} Given the importance of DHT in the advancement of BPH, particular inhibitors of 5\textsubscript{\alpha}-reductase, for example, dutasteride and finasteride, are utilized to treat BPH.\textsuperscript{10,11)\textsuperscript{10}} These medications repress the transformation of testosterone to DHT, and accordingly suppress prostate growth and hyperplasia.\textsuperscript{7)\textsuperscript{7}} However, 5\textsubscript{\alpha}-reductase inhibitors may have serious side-effects, such as loss of libido, ejaculatory or erectile disorders, and dizziness.\textsuperscript{12,13)\textsuperscript{12}} Therefore, there is increasing interest in the use of phytotherapies for BPH, because plant-derived compounds may have fewer adverse effects.

*Veratrum maackii* (VM), a perennial plant in the Melanthiaceae family, contains several bioactive steroidal alkaloids, including cevanine, veratramine, jervine, solanidine, and
verazine. These compounds have been proven to be effective against several disorders (coughs, wounds, epilepsy, and aphasia), making it popular among Americans, Europeans, and Asians. In addition, the dried roots and rhizomes of several Veratrum species, including V. nigrum and V. maackii, have traditionally been used to treat apoplexy-related aphasia, wind-type dysentery, jaundice, headache, scabies, and chronic malaria. Recent studies of several species in this genus have documented anti-hypertensive, anti-cholinergic, cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitory, anti-asthmatic, anti-tussive, anti-fungal, anti-melanogenesis, and anti-tumor activities. However, no studies have yet investigated the protective effect of VM against BPH. The present study used a rat model of testosterone-induced BPH to investigate the therapeutic potential of VM against BPH and the underlying molecular mechanism.
Materials and Methods

Plants, chemicals, and reagents

VM was provided from the Plant Extract Bank of the Korea Research Institute of Bioscience & Biotechnology (KRIIBB, Daejeon, Republic of Korea). Two reference standard alkaloids, jervine, and veratramine (Fig. 1A), were purchased from Biopurify Phytochemicals (Sichuan, China) and their purity was more than 99.0% based on analysis by high-performance liquid chromatography (HPLC). HPLC-grade solvents (methanol, acetonitrile, and water) were obtained from J.T. Baker (NJ, USA). The anhydrous acetic acid (99.8%) was provided by Merck KGaA chemicals (Darmstadt, Germany).

HPLC analysis of two marker alkaloids in VM

HPLC analysis was performed using a Shimadzu Prominence LC-20A series system (Shimadzu, Kyoto, Japan), which had two pumps (LC-20AT), an online degasser (DGU-20A3), a column oven (CTO-20A), an auto-sampler (SIL-20A), and an evaporative light scattering detector (ELSD, ELSD-LTII). Separation of the two alkaloids was performed on a Phenomenex Gemini C_{18} column (250 × 4.6 mm; particle size: 5 µm, CA, USA) and the column was maintained under constant temperature (40°C). The mobile phases were composed of distilled water with 1.0% (v/v) acetic acid and acetonitrile with 1.0% (v/v) acetic acid. The gradient flows were as follows: 10% B, 0–5 min; 10–80% B, 5–30 min; 80% B, 30–40 min; 80–10% B, 40–50 min; and 10% B, 50–60 min. The solvents were delivered at a flow rate of 1.0 mL/min with an injection volume of 10 µL. The drift tube in the ELSD detector had a temperature of 70°C and a nitrogen pressure of 360 KPa. For quantitative analysis of the two alkaloids in the VM, 1.0 g of a ground sample powder was dissolved in 50 mL of 70% methanol, and then sonicated for 60 min at room temperature using an ultrasonic wave extractor. The resulting solution was filtered through a 0.2 µm membrane filter (Pall
Life Sciences, MI, USA) before HPLC injection.

**Cell culture and treatments**

The human normal prostate cell line (WPMY-1, ATCC #CRL-2854) was from the American Type Culture Collection (ATCC, VA, USA). This cell line was maintained in DMEM medium (Gibco, CA, USA), supplemented with 100 µg/mL streptomycin/penicillin and 10% heat inactivated fetal bovine serum (FBS; Gibco). WPMY-1 cells were maintained in a controlled environment at 37°C in a humidiified 5% CO₂ incubator.

WPMY-1 cells were seeded overnight at a density of 500,000 cells per well in 6–well plates. Then, the cells were treated with 10 µM of testosterone propionate (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan). Some cells were also treated with 30 µM of finasteride (Sigma, MO, USA) or VM (1-10 µg/mL). The cells were washed briefly with phosphate-buffered saline (PBS), and cell lysates were stored at -70°C and utilized to evaluate the levels of prostate specific antigen (PSA) and androgen receptor (AR).

**Cell viability assay**

Viability of WPMY-1 cells were assessed using a modified 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, 100 µL of cell suspensions (DMEM) were plated (96-wells) at a density of 10,000 cell per well. Then, the VM was treated at various concentrations ranging from 1 to 10 µg/mL. After 3 days, MTT solution (10 µL/well; Daeillab Service, Seoul, Republic of Korea) was added. Optical density was determined at 450 nm using an Infinite 200 PRO microplate reader (Tecan Treading AG, Mannedorf, Switzerland). The percentage of cell viability was calculated as: (OD_{450nm treated group})/(OD_{450nm control group}) × 100%. 

*Biological and Pharmaceutical Bulletin Advance Publication*
**Animals**

7-week old male SD rats weighing 170-200g (Orient Bio Inc, Seongnam-si, Republic of Korea) were used in this study. All animal experiments were conducted under standard laboratory conditions such as temperature (23 ± 2°C) and 12 h light-dark cycles. Rats were fed with a standard laboratory diet and given water *ad libitum*. The animal experiment was approved by the Animal Experimental Ethics Committee of Chungnam National University (Daejeon, Republic of Korea; Approval No. CNU-00589).

**Experimental Design**

Rats were separated into four groups and treated daily for 4 weeks. Rats given oral injection of PBS and corn oil subcutaneously (s.c.) were considered as control group (CONT). The BPH group (BPH) was given oral injection of PBS and testosterone propionate (Tokyo Chemicals Ins. Co.) at 3mg/kg Body weight (BW) s.c. The finasteride group (FINA) received finasteride (10 mg/kg BW, Sigma) orally and s.c. injection of testosterone propionate, and the VM group received VM (150 mg/5mL PBS/kg BW) orally and s.c. injection of testosterone propionate. We conducted a preliminary trial with three doses of VM (50, 150, and 300 mg/kg) to identify the optimum dose for treating BPH. Based on the results of these experiments (data not shown), we chose a dose of 150 mg/kg as being able to reduce BPH-related parameters. (data not shown).

Following the final injection, rats were sacrificed and blood was collected. Blood was centrifuged, and serum was stored at -70°C until analysis. Prostates were carefully dissected by removing surrounding connective tissues and weighed. The absolute prostate weights were considered in order to calculate the percent hyperplasia inhibition indicated as follows: 100 − [(FINA or VM − CONT)/(BPH − CONT)] × 100. A part of tissue obtained from ventral prostate lobe was fixed in a 10% buffered formalin solution. The remaining tissue of every
Biological and Pharmaceutical Bulletin Advance Publication

prostate was frozen at -70°C.

**Histological analysis**

Prostate tissues were fixed in 10% buffered formalin, proceed in a tissue processor (Leica Bio systems, Wetzlar, Germany), and embedded at the tissue embedding console system (Sakura Finetek Japan Co., Ltd, Tokyo, Japan). The tissue sections obtained from a rotary microtome (Sakura Finetek Japan Co., Ltd, Tokyo, Japan) were dried overnight on a slide warmer and stained with hematoxylin and eosin (H&E; Sigma). Ten fields from each rat were randomly photographed using a light microscope (Nikon ECLIPSE Ni-U, Tokyo, Japan). Thickness of epithelial cell layers was measured in millimeters (mm) using Image J software (ver. 46a; NIH, MD, USA).

**Immunohistochemistry**

Immunohistochemistry was performed using antibody against proliferative cell nuclear antigen (PCNA; Abcam, Cambridge, UK), and then treated with a biotinylated secondary antibody for 1 h. Immune complexes were detected using diaminobenzidine (DAB; Vector laboratories, CA, USA), followed by counter-staining with hematoxylin. For each rat, five prostate sections that were randomly distributed within the ventral prostate lobe were analyzed. The cells with brown nuclei were considered as PCNA-positive, counted, and expressed as a percentage of total cells.

**Determination of prostatic apoptotic cells**

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using a commercially available kit (Merck Millipore Corporation, MA, USA). The apoptotic (TUNEL-positive) cells were analyzed in at least five randomly selected fields and
expressed as a percentage of total cells.

**Determination of prostatic DHT and serum testosterone**

The serum and prostate levels of testosterone and DHT were measured using commercially available enzyme-linked immunosorbent assay (ELISA; ALPCO Diagnostics, NH, USA) kits as instructed by the manufacturer.

**Western blot analysis**

Equal amounts (30 µg) of total, cytosolic, or nuclear proteins were separated by SDS-PAGE. Gels were transferred on to nitrocellulose membranes (40 V/350mA) and blotted with respective primary antibodies as follows: anti-PCNA, anti-cyclin D1, anti-caspase-3, anti-caspase-8, anti-NF-κB, anti-β-actin (Cell Signaling Technology, MA, USA), anti-B-cell lymphoma 2 (Bcl-2), and anti-Bcl-2-associated X protein (Bax) (Santa Cruz, CA, USA). Following additional washing with PBS containing 0.1% Tween 20 (PBS-T), the protein signals were detected using chemiluminescence detection kit and quantified using Image Lab Software (Ver. 6.0, Bio-rad Laboratories, Inc, CA, USA).

**Quantitative real-time PCR (RT-qPCR)**

Total RNA was extracted from the frozen tissues using an RNeasy mini RNA isolation kit (Qiagen, MD, USA) according to the manufacturer’s protocol. Equal amounts of total RNA (1 µg) from each rat were subjected to reverse transcription (RT) by a QuantiTect® RT kit (Qiagen). Then, quantitative real-time PCR was performed using SYBR Green Master Mix (Thermo Scientific, MA, USA). The utilized primer sequences were as follows (forward and reverse, respectively): 5α-reductase type2 (5αR2, 5′-GGGGATGCAAGCCTTTTACC-3′
and 5’-TCCCTGGGCAGAAAGGAGTA-3’) and β-actin (5’-GTCGTACCACTGGCATTGTG-3’ and 5’-GCCATCTTGCTCGAAGTC-3’).

**Statistical analysis**

Values are expressed as means ± standard error of the mean (SEM). Multiple comparisons were performed using one-way ANOVA, followed by the Tukey–Kramer post hoc test (P value < 0.05). Statistical analyses were performed using GraphPad Prism (ver. 6.02, GraphPad Software, Inc., CA, USA).
Results

HPLC analysis of two alkaloids (jervine and veratramine) in VM

We optimized the HPLC-ELSD method for quantitative analysis of two alkaloids (jervine and veratramine) in the VM sample. Jervine and veratramine had retention times of 16.77 and 17.08 min, respectively. Figure 1B shows representative HPLC-ELSD chromatograms of each standard and of the VM sample. We established calibration curves for jervine and veratramine by plotting the logarithm of the peak area versus the logarithm of concentration. For jervine, the regression equation was: \( y = 1.67x + 1.67 \) (Coefficient of determination \( r^2 \) = 0.9988) and for veratramine, the regression equation was: \( y = 1.66x + 1.72 \) \( r^2 = 0.9986 \). The VM sample had 4.42 ± 0.07 mg/g jervine and 10.72 ± 0.05 mg/g veratramine.

VM reduces expression of PSA and AR levels in WPMY-1 cells

PSA is an indicator of prostate cancer progression in human.\(^{18}\) In addition, DHT binds to the AR and promotes protein synthesis and prostate growth, so blockage of this signaling can decrease prostate volume.\(^{19}\) Hence, we examined the effect of VM on the expression of PSA and AR in WPMY-1 cells. Immunoblotting showed that testosterone propionate treatment significantly increased the expression of PSA and AR, but finasteride (30 µM) or VM (1-10 µg/mL) blocked the effect of testosterone propionate in a concentration dependent manner (Fig. 2A). MTT assay was further carried out in order to determine the cytotoxic effect of VM on WPMY-1 cells. VM had no significant effect on cell viability at concentrations up to 10 µg/mL (Fig. 2B).

VM reduces prostatic weights in BPH induced rats

Increased prostate weight is a key indicator of BPH.\(^{20}\) Rats in the BPH group had
significantly increased prostate weight. However, treatment with finasteride or VM reduced the prostate weight by 40.87% and 43.62%, respectively (Table 1).

**VM reduces testosterone, DHT, and expression of 5αR2 levels in rats with BPH**

During the pathogenesis of BPH, 5αR catalyzes the conversion of testosterone to DHT, which accelerates hyperplasia of prostate. Thus, we determined the effect of VM on serum testosterone, prostatic DHT, and mRNA expression of 5αR2 in rats. The results indicated that testosterone propionate treatment significantly increased the levels of serum testosterone and prostatic DHT. Finasteride or VM significantly decreased testosterone and DHT levels in serum and prostate, respectively. Moreover, mRNA expression of 5αR2 increased by testosterone propionate was down-regulated by finasteride or VM (Fig. 3).

**VM restores histological changes in rats with BPH**

The prostate in CONT rats showed normal cell morphology, with single layer of epithelial cell layer formation. In contrast, rats treated with testosterone propionate had notable glandular hyperplasia accompanied by decreased glandular luminal area (Fig. 4A). However, finasteride or VM significantly reduced the epithelial thickness of prostates similar to the rats in CONT group (Fig. 4A and B).

**VM decreases cell proliferation in rats with BPH**

PCNA is a well-known marker of cell proliferation. Thus, we evaluated the effect of VM on cell proliferation by measuring prostatic PCNA. The immunohistochemical results indicate that testosterone propionate significantly increased the number of PCNA-positive cells, and that finasteride or VM treatment significantly decreased prostatic PCNA (Fig. 5A and B). Immunoblotting also indicated that testosterone propionate increased the expression
of PCNA and cyclin D1, but these increases were significantly reduced by finasteride or VM (Fig. 5C and D).

**VM increases apoptosis in rats with BPH**

Increased cell proliferation and decreased apoptosis of prostate is related with BPH development.24) Thus, we examined the effect of VM on apoptosis in the prostate. The number of apoptotic (TUNEL-positive) cells in the prostate was greatly decreased in rats given testosterone propionate, and this effect was significantly reduced by finasteride or VM (Fig. 6A and B). In agreement, Western blotting showed greater expression of cleaved caspase-3 and -8 in the finasteride or VM groups than in the BPH group (Fig. 6C). In addition, Bcl-2 expression was lower and Bax expression was greater in the finasteride or VM groups, so they had lower Bcl-2/Bax ratios than the BPH group (Fig. 6D).

**VM inhibits NF-κB translocation to the nuclei in rats with BPH**

Next, we explored the possible mechanism by which VM treatment reduces cell proliferation and increases apoptosis by measuring the nuclear translocation of NF-κB. Testosterone propionate administration notably increased the nuclear translocation of NF-κB in the BPH group, and finasteride or VM treatment reversed this effect (Fig. 7).
Discussion

This study evaluated the efficacy of VM on BPH using in vitro (WPMY-1 cells) model and in vivo BPH rat model. VM suppressed the testosterone propionate-induced elevation of PSA and AR levels in WPMY-1 cells. In addition, rats with BPH had increased prostate weight, and elevated serum or prostatic levels of testosterone, DHT, and mRNA expression of 5αR2. However, treatment of rats with VM led to significant reductions in prostate weight and in the levels of serum testosterone, prostatic DHT, and mRNA expression of 5αR2. Histopathological examination of prostate tissues showed that VM attenuated testosterone propionate-induced prostatic hyperplasia.

Prostatic 5αR2 converts testosterone into DHT, which promotes prostate growth. More specifically, DHT increases hyperplasia of prostate epithelial and stromal cells, leading to increase overall prostate size. Finasteride is a 4-aza-steroid inhibitor of 5αR. The action mechanism of finasteride is based on its ability to preferentially inhibit 5αR2 by forming a stable complex with the enzyme. Inhibition of 5αR2 prevents the peripheral conversion of testosterone to DHT, resulting in significant decreases in serum and tissue DHT concentrations, and a subsequent decrease in prostatic size. Previous studies found that the prostate weight was increased in rats injected with testosterone for 4 weeks, and that finasteride treatment suppressed the DHT level in serum and the 5αR2 mRNA level in prostate tissues of BPH-induced rats. However, finasteride also produces serious side effects which has led researchers to investigate alternative materials that can be used to treat BPH with fewer side effects. The present study found that VM treatment significantly reduced the mRNA expression of 5αR2 and consequently reduced the prostatic DHT level and prostatic size in a rat model. However, further study is needed to determine how VM regulates 5αR2.

Rats with BPH had significantly increased prostate weight compared with control rats,
and administration of VM significantly reduced prostate weight. These results are in agreement with our histomorphological analysis of the prostates. The prostates of rats with BPH had epithelial hyperplasia and increased epithelial thickness, whereas rats treated with VM only had mild epithelial hyperplasia and greatly reduced epithelial thickness. We also determined the expression of cyclin D1 and PCNA in the prostate to further examine the effects of VM on epithelial cell proliferation. PCNA is an important marker of cell proliferation that is synthesized in the G1/S phase of the cell cycle, and cyclin D1 controls the G1/S transition. Thus, increased expression of PCNA and cyclin D1 correlates with cell proliferation, and expression of each marker in the prostate is increased significantly in patients and animal models with BPH. We found that VM treatment significantly suppressed PCNA and cyclin D1 expression in the prostates of rats. These findings suggest that the beneficial effects of VM may be due to its anti-proliferative activity.

In the normal prostate, epithelial cell growth is balanced by a tightly regulated control of cell proliferation and apoptosis. However, suppression of apoptosis in the prostate can promote BPH. The Bcl-2 family proteins, which include anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as Bax, are important regulators of apoptosis. Bax promotes apoptosis by stimulating the release of cytochrome c from the mitochondria and downstream activation of caspase-3; Bcl-2 inhibits apoptosis by limiting the pro-apoptotic impacts of Bax and by obstructing the release of cytochrome c. Caspase-8 also initiate the proteolytic activation of procaspase-3 by a multi-step mechanism. Normal prostatic tissue has weak or undetectable expression of Bcl-2 and caspase-3 in epithelial cells. However, hyperplastic prostatic tissue has increased expression level of Bcl-2, and the expression level of Bax and activation of caspase-3 and caspase-8 cleavage are decreased. In agreement with the previous reports, our findings showed that rats with BPH had increased expression level of Bcl-2, but decreased expression levels of Bax. However, VM markedly reduced the
expression level of Bcl-2, and increased the expression level of Bax, and thereby decreased the Bcl-2/Bax ratio. In addition, the cleavage-mediated activation of caspase-3 and caspase-8 was greater in rats treated with VM. These results suggest that VM-induced apoptosis is responsible for its effects on BPH, and the underlying mechanism appears to involve a decreasing ratio of Bcl-2/Bax.

The pro-apoptosis effect of VM may be due to the alkaloids found Veratrum, which include jervine, cevanines, veratramines, ussurienines, and pingbeinones. Ghezali et al. showed that jervine induced COX-2 overexpression to trigger pro-apoptotic effects in the human erythroleukemia cell lines, HEL and TF1a. In addition, veratramine was shown to selectively and directly bind a specific site in the target DNA sequence for the transcription factor, activator protein-1 (AP-1), which regulates cell proliferation and apoptosis. To further examine the mechanism by which VM promotes apoptosis, we analyzed the effects of VM on NF-κB. Previous work showed that NF-κB stimulates cyclin D1 transcription in G1 phase to affect pRB phosphorylation and the G1-to-S-phase transition. NF-κB is also known to inhibit apoptosis via the activation of several anti-apoptotic proteins, including members of the Bcl-2 family. In the present work, we found that administration of testosterone propionate to rats increased the nuclear translocation (activation) of NF-κB, but VM significantly inhibited this activation of NF-κB along with parallel decreases in cyclin D1 and the ratio of Bcl-2/Bax. These results suggest that a mechanism involving NF-κB may underlie the VM-induced inhibition of cell proliferation and induction of apoptosis.

Species of Veratrum are toxic rangeland plants that have been linked to teratogenicity and hyposensitive activity. However, VM has long been applied as the Chinese traditional medicine, “Lilu”, which is used to treat apoplexy-associated aphasia, wind-type dysentery, jaundice, headache, scabies, chronic malaria, and other disorders. In the present work, rats treated with VM showed lower body weights compared to the control group (Table 1).
However, previous studies showed that daily injection of testosterone propionate can reduce body weight gain\textsuperscript{45} and we did not find any significant difference between the VM-treated group and those treated with other versions of testosterone propionate (the BPH and FINA groups). In addition, VM-treated rats did not exhibit any difference in clinical signs/symptoms, mortality, or gross pathology relative to the other groups, nor did VM treatment have any significant effect on the weights of the liver, lung, kidney, or spleen, or the blood chemistry parameters (ALT and AST) of these rats (data not shown). H&E staining of the organs revealed that there was no histopathological abnormality associated with VM treatment (data not shown). Our data indicate that VM does not cause any evident toxicity in rats at a dose of 150 mg/kg. However, further studies of potential chronic toxicity and genotoxicity are needed to establish the safe oral dose of VM.

In conclusion, we showed that administration of VM to rats prevented the progression of testosterone propionate-induced BPH, by reducing prostate weight and prostatic hyperplasia. This effect can be attributed, at least in part, to a VM-mediated decrease of DHT levels in the prostate, and to the pro-apoptotic and anti-proliferative activities of VM. These findings suggest that VM has potential as a novel therapeutic agent for BPH treatment. Further studies will be needed to determine the clinical utility of VM.

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Declaration of Conflicting Interests

The authors declare no conflict of interest.


Figure legends

**Fig. 1.** The representative chemical structures and HPLC-ELSD chromatograms of standard alkaloids in VM. (A) Chemical structures of jervine and veratramine. (B) HPLC-ELSD chromatogram of (I: jervine (peak 1)), II: veratramine (peak 2) and III: jervine and veratramine (peak 1 and 2) VM extract.

**Fig. 2.** The effect of VM on cell viability and protein expression of PSA and AR in human normal prostate cell line WPMY-1. (A) The cells were plated overnight and treated with testosterone propionate (10 µM), finasteride (30 µM) or VM at the indicated concentrations for 72h, and cell lysates (30 µg) were subjected to western blot analysis using antibody against PSA and AR. Abbreviations: CONT, normal control; TP, testosterone propionate treated; FINA, testosterone propionate and finasteride treated; VM, testosterone propionate and VM treated; (B) Cell viabilities of WPMY-1 cells treated with VM at the indicated concentrations for 72h was measured by MTT assay. Values are presented as the means ± SEMs. Significant difference at *# P<0.05 or ## P<0.01 versus the CONT group and *P <0.05 or **P <0.01 versus the TP group.

**Fig. 3.** VM inhibited the levels of serum testosterone, prostatic DHT and mRNA expression of 5αR2 in rats subjected to testosterone propionate-induced BPH. (A) The levels of testosterone in serum. (B) The levels of prostatic DHT. (C) The mRNA expression levels of 5αR2 in prostate tissue. The fold change in the expression of the target gene relative to that of the endogenous control (β-actin) was calculated using the 2-ΔΔCt method. Abbreviations: CONT, control rats administered with PBS and corn oil; BPH, rats administered with testosterone propionate (3 mg/kg) and PBS; FINA, rats administered with testosterone propionate and finasteride (10 mg/kg); VM, rats administered with testosterone propionate and VM.
propionate and VM (150 mg/kg). Values are presented as the means ± SEMs. Significant difference at *$P<0.05$ or **$P<0.01$ versus the CONT group and *$P<0.05$ or **$P<0.01$ versus the BPH group.

**Fig. 4.** VM restored the testosterone propionate-induced histopathological alterations in prostates of rats subjected to testosterone propionate-induced BPH. (A) The prostate tissues were stained with Hematoxylin & Eosin and observed under an optical microscope at ×200 magnification. (B) Mean epithelial thickness of prostate (mm). Abbreviations: CONT, control rats administered with PBS and corn oil; BPH, rats administered with testosterone propionate (3 mg/kg) and PBS; FINA, rats administered with testosterone propionate and finasteride (10 mg/kg); VM, rats administered with testosterone propionate and VM (150 mg/kg). Values are presented as the means ± SEMs. Significant difference at *$P<0.05$ or **$P<0.01$ versus the CONT group and *$P<0.05$ or **$P<0.01$ versus the BPH group. (Color figure can be accessed in the online version.)

**Fig. 5.** VM suppressed the prostate epithelial cell proliferation in rats subjected to testosterone propionate-induced BPH. (A) Prostate epithelial cells stained with PCNA antibody (×400). (B) The number of PCNA-positive cells as a percentage. (C) PCNA and (D) cyclin D1 expression in prostate tissue were detected using western blotting. The protein densities were compared with β-actin as an internal control. Abbreviations: CONT, control rats administered with PBS and corn oil; BPH, rats administered with testosterone propionate (3 mg/kg) and PBS; FINA, rats administered with testosterone propionate and finasteride (10 mg/kg); VM, rats administered with testosterone propionate and VM (150 mg/kg). Values are presented as the means ± SEMs. Significant difference at *$P<0.05$ or **$P<0.01$ versus the CONT group and *$P<0.05$ or **$P<0.01$ versus the BPH group.
Fig. 6. VM promotes the prostate epithelial cell apoptosis in rats subjected to testosterone propionate-induced BPH. (A) Prostate tissue was subjected to TUNEL staining, and arrows indicate the TUNEL-positive cells (×400). (B) The number of TUNEL-positive cells as a percentage. (C) Caspase3, 8 and (D) Bcl-2, Bax expression in prostate tissue were detected using western blotting. The protein densities were compared with β-actin as an internal control. Abbreviations: CONT, control rats administered with PBS and corn oil; BPH, rats administered with testosterone propionate (3 mg/kg) and PBS; FINA, rats administered with testosterone propionate and finasteride (10 mg/kg); VM, rats administered with testosterone propionate and VM (150 mg/kg). Values are presented as the means ± SEMs. Significant difference at *$P<0.05$ or **$P<0.01$ versus the CONT group and *$P<0.05$ or **$P<0.01$ versus the BPH group.

Fig. 7. VM prevents the nuclear translocation of NF-κB in rats subjected to testosterone propionate-induced BPH. Western blotting of cytosolic and nuclear protein fractions of prostate for NF-κB expression. Abbreviations: CONT, control rats administered with PBS and corn oil; BPH, rats administered with testosterone propionate (3 mg/kg) and PBS; FINA, rats administered with testosterone propionate and finasteride (10 mg/kg); VM, rats administered with testosterone propionate and VM (150 mg/kg). Values are presented as the means ± SEMs. Significant difference at *$P<0.05$ or **$P<0.01$ versus the CONT group and *$P<0.05$ or **$P<0.01$ versus the BPH group.
Fig. 1

A

B

(I)

(II)

(III)

Biological and Pharmaceutical Bulletin Advance Publication
Fig. 2

A

<table>
<thead>
<tr>
<th></th>
<th>CONT</th>
<th>TP</th>
<th>FINA</th>
<th>1</th>
<th>5</th>
<th>10</th>
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<tbody>
<tr>
<td>PSA</td>
<td></td>
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<tr>
<td>AR</td>
<td></td>
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<tr>
<td>β-actin</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

VM (µg/mL)

B

Cell viability (%)

Concentration (µg/mL)
Fig. 3
Fig. 4

A

B

Epithelial thickness of prostate (mm)

CONT  BPH  FINA  VM

##  **  **
Fig. 5

A

B

C

D

Biological and Pharmaceutical Bulletin Advance Publication
Fig. 7
Table 1. Effect of *Veratrum maackii* on prostatic enlargement in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Prostate weight (g)</th>
<th>Prostate/Body weight</th>
<th>% inhibition of prostate weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>Corn oil/PBS</td>
<td>398.4±8.666</td>
<td>0.560±0.044</td>
<td>0.141±0.012</td>
<td></td>
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<tr>
<td>BPH</td>
<td>Testosterone propionate/PBS</td>
<td>352.5±6.695</td>
<td>1.593±0.083##</td>
<td>0.448±0.016##</td>
<td></td>
</tr>
<tr>
<td>FINA</td>
<td>Testosterone propionate/Finasteride</td>
<td>357.7±8.680</td>
<td>1.171±0.056**</td>
<td>0.328±0.016**</td>
<td>40.87</td>
</tr>
<tr>
<td>VM</td>
<td>Testosterone propionate/VM</td>
<td>335.0±25.80</td>
<td>1.142±0.095**</td>
<td>0.341±0.011**</td>
<td>43.62</td>
</tr>
</tbody>
</table>

CONT, corn oil injection and phosphate buffered saline (PBS)-treated rats; BPH, testosterone propionate (3mg/kg) and PBS-treated rats; FINA, testosterone propionate (3mg/kg) and finasteride (10 mg/kg)-treated rats; VM, testosterone propionate (3mg/kg) and *Veratrum maackii* (150 mg/kg)-treated rats. The results were expressed as mean ± SEM. ##P<0.01 when compared with the CONT group. **P<0.01 when compared with the BPH group.