



The Extract of *Couroupita guianensis* Aubl. Ameliorates Benign Prostatic Hyperplasia *In Vitro* and *In Vivo*

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Abstract – The therapeutic effects of the leaves of *Couroupita guianensis*, a large tropical tree in the family of Lecythidaceae improving testosterone-induced Benign Prostatic Hyperplasia (BPH) were tested *in vitro* and *in vivo*. In BPH rats induced by castration and testosterone treatment, the prostate index was improved in groups administered with the extracts of *C. guianensis* extracted with 50%-, 100%-ethanol or boiling water, which was comparable with positive control, finasteride. The extract *C. guianensis* leaves showed significant inhibition on the expressions of type 2 5-alpha reductase (5αR) in RWPE-1 human prostatic epithelial cells, and effectively attenuated the expressions of androgen receptor, type 2 5αR and proliferating cell nuclear antigen in LNCap human prostatic adenocarcinoma cells. The leaves of *C. guianensis* that exerted evident suppression on BPH-related biomarkers *in vitro* and improvement of prostate index *in vivo* has a potential therapeutic use for the treatment of BPH.

Keywords – *Couroupita guianensis*, benign prostatic hyperplasia, RWPE-1, LNCap, testosterone, *in vivo*

Introduction

Benign Prostatic Hyperplasia (BPH) is an age-related disease which is common in men over the age 60 with approximately 50% of 60s experiences the characteristic clinical symptoms of BPH.¹ In patients with BPH, the stromal and epithelial proliferation of the prostate surrounding the urethra causing various uncomfortable lower urinary tract symptoms such as frequent urination, urgency, weak urine flow, and urinary retention. Drug

options for the treatment of BPH include: 1) 5-alpha reductase (5αR) inhibitors, 2) alpha-1 adrenergic antagonists, and 3) The combinations of 5-alpha reductase inhibitors and alpha-1 adrenergic antagonist. 5αR inhibitors, finasteride and dutasteride are effective in reducing prostate volume and improving lower urinary tract symptoms by locally inhibiting the production of dihydrotestosterone (DHT) within the prostate.² As the aging population increases worldwide, the market demand for active materials that prevent or treat BPH is increasing. In the course of searching for new plant materials that improve BPH, it was found that the methanolic extract of *Couroupita guianensis* collected from Bangladesh or Laos effectively attenuated the activity of 5αR enzyme *in vitro*.

C. guianensis Aubl. (Lecythidaceae) also known as cannonball tree is a large tropical tree growing to a height 20 m. The fruits are spherical with a diameter of 15-24 cm larger than a cricket ball, and has a woody capsule, reminiscent of a cannonball. The leaves of this tree have been traditionally used in Eastern Asia for the treatment of alopecia, inflammation, fever and malaria. Also, juice

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extracted from the leaves is used to treat skin disease. Recently, antimicrobial, analgesic, antioxidant, anti-inflammatory and skin fibroblast stimulating activities of *C. guianensis* have been reported.³⁻⁶ Previous phytochemical investigations on this plant reported the isolation of triterpenoid, triterpenoid ester of fatty acid, flavonoids, alkaloids.⁶⁻¹³

In this study, the effects of the extracts of *C. guianensis* leaves on BPH-related biomarkers using human prostatic cell lines, LNCap and RWPE-1. The regulative activities of the extract on the expressions of type 2 5 α R, prostate specific antigen (PSA) in RWPE-1, and on the expressions of androgen receptor (AR), type 2 5 α R and proliferating cell nuclear antigen (PCNA) in LNCap cells were measured. Also, the therapeutic effects of the extract of *C. guianensis* were evaluated *in vivo* on BPH rats induced by castration and testosterone treatment.

Experimental

Plant materials – Leaves of *C. guianensis* Aubl. were collected in Zobra village, Hathazari Upazila district, Chittagong, Bangladesh in 2015 (voucher specimen accession number: KRIB 0069205) and collected in Nonsomboun Village, Sikhodthabong District, Vientiane, Laos in 2019 (voucher specimen accession number: KRIB 0089170). The retained material is preserved at the herbarium of Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Extraction and sample preparation – For *in vitro* study, the dried leaves of *C. guianensis* (15 g) collected from Bangladesh or Laos were extracted with 80% MeOH three times for 3 h each in an ultrasonic apparatus. The filtrate was evaporated under reduced pressure to give a methanolic extract, which was suspended in water and then successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol, respectively. The extraction ratio of *C. guianensis* leaves from Bangladesh or Laos were given in Table 1. For *in vivo* study, the dried leaves of *C. guianensis* (15 g) from Bangladesh were extracted with 50%- or 100%-ethanol three times for 3 h each in an ultrasonic apparatus, or extracted with boiling water for 3 h. The extraction ratio was given in Table 2.

Cell cultures – RWPE-1 (normal human prostatic epithelial cell line) and LNCap (human prostatic adenocarcinoma cell line) were purchased from the American Type Culture Collection (Manassas, VA, United States). The RWPE-1 cells were cultured in keratinocyte serum-free medium (K-SFM; Gibco BRL, Grand Island, NY, USA) supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor and 1% (v/v) antibiotics (100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin). LNCap cells were cultured in RPMI1640 (GenDEPOT, CM059-050) containing 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) antibiotics (100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin) (Sigma-Aldrich Inc. P4333).

Western blot – RWPE-1 and LNCaP cells were seeded at a density of 2×10^5 cells/well and 6×10^5 cells/well in 6-well plates overnight, respectively. The cells were treated with Testosterone Propionate (TP) (0.5 μ M) for 1 h, followed by treated with finasteride (10 μ M) or each compound at various concentrations indicated for further 24 h. The cells were washed three times with cold-phosphate-buffered saline (PBS) and cell lysates were extracted with a lysis buffer (M-PER™ Mammalian Protein Extraction Reagent 78501, Thermo Scientific) containing protease inhibitor cocktail (Thermo Scientific, USA, Prod # 78425). Protein extracts were centrifuged at 13000 rpm for 20 min at RT. The protein content of cell lysate was quantified by Bradford assay. Thirty microgram of harvested protein was separated in 8-10% Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) at 100 V and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skim milk for 1 h in room temperature. Anti-AR (5153T, Cell Signaling Technology), anti-PSA (5365S, Cell Signaling Technology), anti-SRD5A2 (sc-293232, Santa Cruz Biotechnology), anti-PCNA (13110S, Cell Signaling Technology), anti- β -actin (4967S, Cell Signaling Technology) were employed in 1% skim milk. Then, the membrane was incubated with each primary antibody at 4°C overnight. After washing three times with Tris Buffered Saline with Tween (TBST) buffer, the immunoreactive bands were visualized by using immunopure peroxidase conjugated mouse anti-rabbit IgG or goat anti-

Table 1. The yield of methanolic extract and its organic fractions of *C. guianensis* leaves collected from Bangladesh or Laos

	methanolic extract	<i>n</i> -hexane fraction	chloroform fraction	ethyl acetate fraction	<i>n</i> -butanol fraction
	(w/w%)				
Bangladesh	23.63 \pm 0.6	24.02 \pm 2.1	3.30 \pm 0.3	2.29 \pm 0.1	5.87 \pm 0.6
Laos	18.02 \pm 0.9	7.63 \pm 1.2	14.37 \pm 2.3	3.92 \pm 0.5	15.60 \pm 2.0

mouse IgG (1:10000 dilution; Santa Cruz Biotechnology). Membrane was incubated with secondary antibody for 1 h at room temperature. Blots were washed three times with TBST buffer. Protein bands were visualized using ECL solution (bio-rad clarity Max western ECL substrate) and calibrated using the Chemidoc Imaging System (Fusion FX5, Vilber Lourmat, France).

Experimental Animals – A total of 36 Male Wistar rats (7 weeks old) of an average body weight of 250 ± 10 g were purchased from Orient Bio (Seoul, South Korea). Animals were acclimatized for two weeks under a 12 h:12 h light-dark cycle at $20 \pm 2^\circ\text{C}$ and $50 \pm 5\%$ room humidity with ad lib access to food and water. Animal experiments were carried out according to the guidelines issued by the Gyeongnam Department of Environment & Toxicology, Korea Institute of Toxicology on the Care and Use of Laboratory Animals. The animal care and protocol were reviewed and approved by the IACUC (Institutional Animal Care and Use Committee) at the Korea Institute of Toxicology Gyeongnam Department of Environmental Toxicology and Chemistry (approval No. 1803-0005).

Induction of BPH and measurement of prostate index – To exclude the influence of testosterone, 30 rats were castrated by removing the testes and epididymis. The rats in sham group were given the sham surgery. For castration surgery, animals were anesthetized by intramuscular injection of Zoletile 20 mg/kg + Rompun 10 mg/kg body weight. Preoperative antibiotics, Cephazolin 20 mg/kg were administered subcutaneously. One week after the surgical operation, the animals were orally administered with test materials or positive control, and subcutaneously administered with TP (Tianjin Jinyao Amino Acid Co. Ltd.) that induces prostatic hyperplasia daily for 6 weeks. Castrated animals were randomly allocated to six groups ($n = 6$, per group), as detailed below. After a treatment period of 6 weeks, body weight and prostate weight were accurately measured. The prostate index were calculated dividing prostate weight (mg) by body weight (100 g).

1. Sham group: non-BPH-induced and received oral 0.5% CMC-Na
2. VC (Vehicle Control) group: BPH-induced and received oral 0.5% CMC-Na
3. PC (Positive Control) group: BPH-induced and received finasteride (1 mg/kg body weight, *p.o.*)
4. T1 group: BPH-induced and received 50% ethanolic extract of *C. guianensis* (100 mg/kg body weight, *p.o.*)
5. T2 group: BPH-induced and received 100% ethanolic extract of *C. guianensis* (100 mg/kg body weight, *p.o.*)
6. T3 group: BPH-induced and received boiling water

extract of *C. guianensis* (100 mg/kg body weight, *p.o.*)

Statistical Analysis – All statistical analyzes were performed using SPSS statistics 17.0 program (SPSS Inc., Chicago, IL, USA), one-way ANOVA followed by Dunnett's post hoc test was for the data satisfied with Levene's test. Otherwise, a nonparametric Kruskal-Wallis test with Dunn's test was applied. $P < 0.05$ was considered as significant. Data was converted into a graph using GraphPad Prism 5 (San Diego, CA, USA).

Result and Discussion

The therapeutic effects of *C. guianensis* to improve BPH are evaluated in LNCaP, human prostatic adenocarcinoma cells and in RWPE-1, human prostatic epithelial cells. LNCaP is androgen sensitive cell lines that needs androgens for cell growth and survival. LNCaP expresses AR and PSA mRNA/proteins in response to androgens and widely used in prostate cancer research.¹⁴ RWPE-1 is a non-tumorigenic human prostate epithelial cell and is known as a good model to elucidate the molecular mechanisms involved in the proliferation of benign prostate epithelial cells.¹⁵ *C. guianensis* used in this study were collected from Bangladesh and Laos. Prior to assays, the extraction ratio and activities two samples of *C. guianensis* collected from two regions were compared. The leaves of *C. guianensis* collected from Bangladesh or Laos were extracted with methanol and partitioned by polarity using organic solvents. The yield percentage of methanol extract and each fractions are shown in Table 1. The yield of methanol extract of Bangladesh and Laos were 23.63 and 18.02, respectively. Although the yields of organic fractions of the Bangladesh and Laos samples were slightly different, it was observed that the ratio of nonpolar fraction (combined with the *n*-hexane and chloroform soluble fraction) was similar; 27.32 for Bangladesh and 22.00 for Laos, and the ratio of *n*-butanol soluble fraction was relatively high in Laos sample. The effects of the extract and fractions on AR, type 2 5 α R and PCNA in RWPE-1 cells were tested. In cells treated with Bangladesh *C. guianensis* leaves, the reduction of AR expression was observed in all groups treated with extract or fractions of *C. guianensis* whereas the inhibition of 5 α R or PCNA were not significant (Fig. 1A). In cells treated with Laos *C. guianensis* leaves, though the inhibitory activities of each groups were slightly different from those of Bangladesh, the attenuation AR expression was most remarkable as well (Fig. 1B). A further *in vitro* and *in vivo* study was carried out using the Bangladesh *C. guianensis*, of which the enough

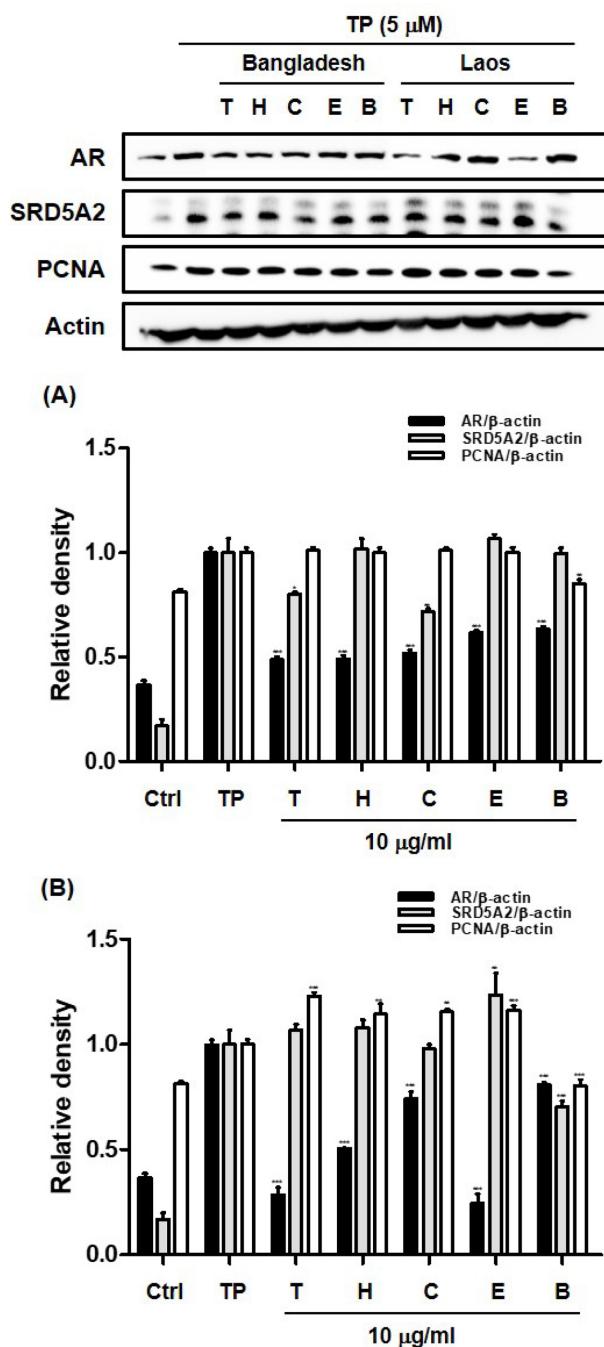


Fig. 1. The effects of methanolic extract and its organic fractions of *C. guianensis* leaves from Bangladesh (A) or Laos (B) on the expressions of androgen receptor (AR), type 2 5- α reductase (5 α R) and proliferating cell nuclear antigen (PCNA) in RWPE-1 cells. Cells were treated with test materials (extract or organic fractions of *C. guianensis* leaves, 10 μ g/ml) and then treated with testosterone propionate (TP, 0.5 μ M) for 24 h. The expression levels of AR, type 2 5 α R, PCNA in cells were analyzed by western blot. Results are presented as the mean \pm S.D. of triplicate experiments; *** p < 0.001 compared to TP-treated cells. T: total methanolic extract *C. guianensis* leaves, H: *n*-hexane fraction, C: chloroform fraction, E: ethyl acetate fraction, B: *n*-butanolic fraction of *C. guianensis* extract.

Table 2. The yield of 50%-, 100%-ethanol and boiling water extracts of *C. guianensis* leaves collected from Bangladesh

	50%-ethanol	100%-ethanol	Boiling water
	(w/w%)		
Bangladesh	27.45 \pm 2.6	10.67 \pm 1.9	24.80 \pm 1.6

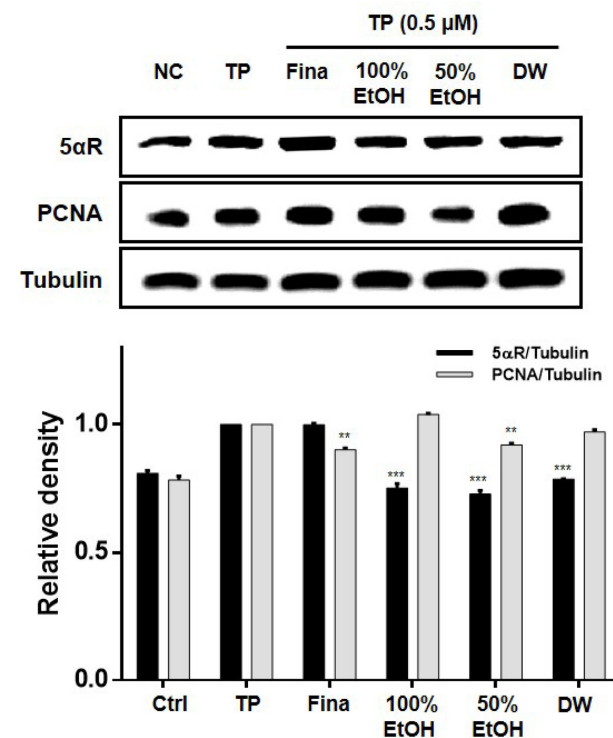


Fig. 2. The effects of 50%-, 100%-ethanol and boiling water extracts of *C. guianensis* leaves from Bangladesh on the expressions of type 2 5- α reductase (5 α R) and proliferating cell nuclear antigen (PCNA) in RWPE-1 cells. Cells were treated with each extract (10 μ g/ml), and then treated with testosterone propionate (TP, 0.5 μ M) for 24 h. The expression levels of type 2 5 α R, PCNA in cells were analyzed by western blot. Results are presented as the mean \pm S.D. of triplicate experiments; ** p < 0.01 and *** p < 0.001 compared to TP-treated cells. Fina: finasteride, a selective type 2 5 α R inhibitor.

amount of sample required for animal experiments is secured.

The extracts of *C. guianensis* leaves were prepared using ethanol/water as extraction solvents that are most commonly used in commercial processes. The yield percentage of 50%-, 100%-ethanol and boiling water extracts were shown in Table 2. The therapeutic effects of the three extracts of *C. guianensis* on BPH-related biomarkers are evaluated in RWPE-1 and LNCap cells. The enzyme 5 α R converts testosterone to DHT, an active androgen, which is known to be involved in the development of BPH by binding to androgen receptors and stimulating the production of various growth

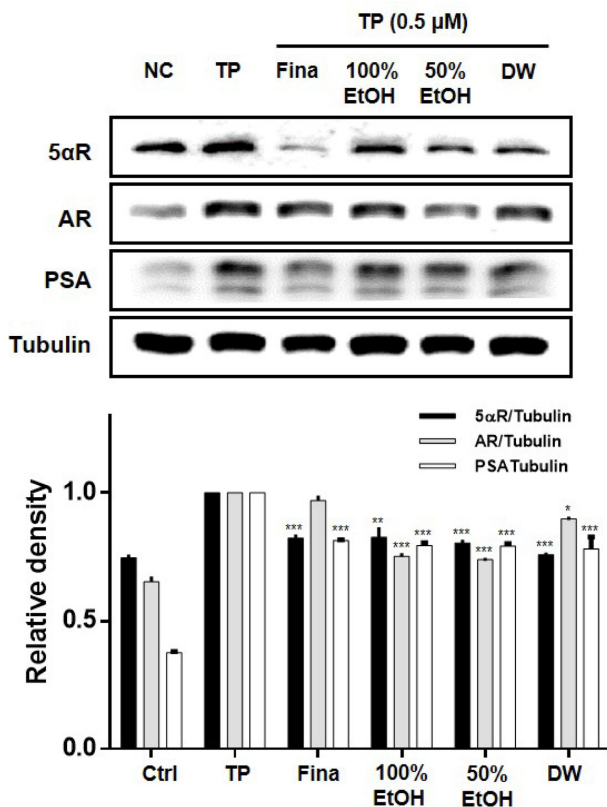


Fig. 3. The effects of 50%-, 100%-ethanol and boiling water extracts of *C. guianensis* leaves from Bangladesh on the expressions of androgen receptor (AR), type 2 5- α reductase (5 α R) and prostate specific antigen (PSA) in LNCap cells. Cells were treated with each extract (10 μ g/ml), and then treated with testosterone propionate (TP, 0.5 μ M) for 72 h. The expression levels of AR, type 2 5 α R, PSA in cells were analyzed by western blot. Results are presented as the mean \pm S.D. of triplicate experiments; ** p < 0.01 and *** p < 0.001 compared to TP-treated cells. Fina: finasteride, a selective type 2 5 α R inhibitor.

factors.¹⁶ Hence, searching for new substances that inhibit the expression or activity of 5 α R is recognized as general strategy for the development of therapeutics for BPH.^{17,18} In androgen-responsive prostate cells, AR plays an important role in the initiation and progression of androgen-dependent gene transcription and is therefore essential for cell development, growth and function.¹⁹ Moreover, a significant increase of PCNA gene expression in prostatic tissues of BPH rats,²⁰ and the increased secretion of PSA are observed in BPH patients.²¹ In RWPE-1 cells, the suppression of type 2 5 α R expression was observed in all groups treated with the extracts of *C. guianensis* (Fig. 2). In LNCap cells, the expression levels of AR, type 2 5 α R and PSA were increased by the treatment of TP, whereas the treatment of cells with finasteride, a selective type 2 5 α R inhibitor, attenuated

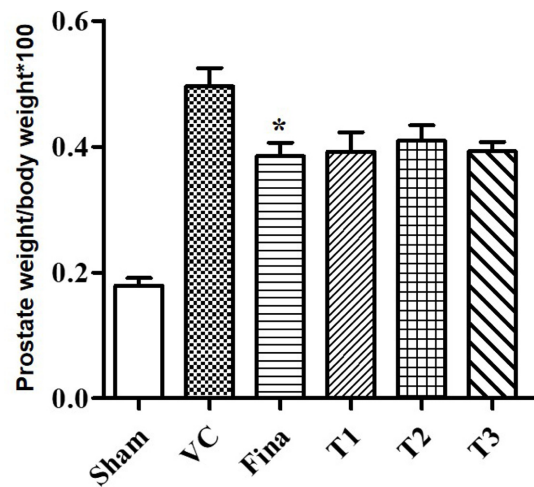


Fig. 4. The effects of 50%-, 100%-ethanol and boiling water extracts of *C. guianensis* leaves on prostate index (PI) in testosterone propionate (TP)-induced BPH rats. Results were expressed as the mean \pm S.D. of animals in each group. * p < 0.05 vs. the VC group. Sham, non-BPH-induced and received 0.5% CMC-Na; VC, BPH-induced and received 0.5% CMC-Na; PC, BPH-induced and finasteride (1 mg/kg body weight)-treated group. T1, BPH-induced and 50%-ethanolic extract of *C. guianensis* (100 mg/kg body weight)-treated group; T2, BPH-induced and 100%-ethanolic extract of *C. guianensis* (100 mg/kg body weight)- treated group; T3, BPH-induced and boiling water extract of *C. guianensis* (100 mg/kg body weight)-treated group.

type 2 5 α R and PSA expressions (Fig. 3). The treatment of cells with *C. guianensis* extracts significantly inhibited the increased expressions of AR, type 2 5 α R and PSA induced by TP (Fig. 3). Based on the inhibitory effects of *C. guianensis* on the expressions of AR, type 5 α R and PSA *in vitro*, the therapeutic effects of *C. guianensis* extract on BPH rats were evaluated *in vivo* (Fig. 4). BPH rats were induced by castration by removing the testes and epididymis, and the treatment of TP. Six weeks after the induction of BPH by TP and the co-treatment of *C. guianensis* extracts, it was found that prostate index (PI) in TP-only treated rats (VC group) was considerably increased as compared to sham group. PI is calculated by expressing prostate weight as a percentage of body weight, which has been proposed to be a risk factor of BPH and prostate cancer.²² The increased PI was lowered in rats treated with finasteride, a positive control (PC group). Though no statistical significance was found, the relative reduction in PI were found in all *C. guianensis* extract-administered groups (T1~T3). Taken together, *C. guianensis* that exerted evident suppression on BPH-related biomarkers *in vitro* and improvement of PI *in vivo* has a potential therapeutic use for the treatment of BPH.

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