

Standardized Seed Extract of *Quisqualis indica* (HU-033) Attenuates Testosterone Propionate-Induced Benign Prostatic Hyperplasia *via* α 1-Adrenergic Receptors and Androgen/Estrogen Signaling.

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ABSTRACT: Benign prostatic hyperplasia (BPH) is an age-related disease characterized by prostatic enlargement and is the most common urologic symptoms in elderly men 60 years of age and older. Previously, we documented that 70% ethanol (EtOH) seed extract of *Quisqualis indica* (QI) attenuates pathological condition of testosterone propionate (TP)-induced BPH rat model *via* modulation of proliferation and apoptosis of prostate cells. Based on this potential of QI, we produced standardized seed extract of QI (HU-033) in order to prove further mechanisms. In this study, we aimed to suggest further mechanisms underlying the relationship between BPH and HU-033. Through not only cellular and nuclear receptor functional assays, but TP-mediated BPH rat model treated with HU-033, we demonstrated that HU-033 exerted antagonist effect on α 1_A- and α 1_D-adrenergic receptors *in vitro* and inhibitory effect on protein expression of androgen receptor and estrogen receptor alpha *in vivo*. Taken together, these results suggest that HU-033 is a novel candidate for the management of BPH.

Keywords: HU-033, benign prostate hyperplasia, α 1-adrenergic receptor, estrogen receptor α , androgen receptor

INTRODUCTION

The rapid increase in the age of the general population has been a pivotal factor in the increased prevalence of senescence-related disorders, including cancer, cardiovascular disease, and neurodegenerative symptoms; subsequently, these have emerged as a significant public health issue (Brunet et al., 2014). Benign prostatic hyperplasia (BPH), a representative gender-dependent disease, is the most common pathological condition in elderly men 60 years of age and older (Lepor, 2005; Woodard et al., 2016).

BPH, the result of gradual overgrowth of the prostate gland that lies at the base of the bladder and encircles the urethra, is generally associated with impairment in urinary tract function, including urinary intermittency, frequency, weak stream, and nocturia (Shabbir et al., 2004). Drug therapy using α 1-adrenergic receptor antagonist (α 1-

blockers), 5 α -reductase inhibitors, muscarinic receptor antagonists, and phosphodiesterase 5 inhibitors is common for the treatment of BPH. However, these drugs may cause drug-related adverse effects such as erectile dysfunction, sexual desire disorder, ectopic disorder, and cardiovascular effects such as orthostatic hypotension (Lepor, 2011; Huri et al., 2014). Because natural materials derived from plants are generally observed to lead to fewer side effect, many patients with BPH are interested in the use of complementary and alternative medicine.

Previously, we disclosed that seed extract of *Quisqualis indica* (QI) protected against testosterone propionate (TP)-mediated BPH in rats by decreasing expression level of dihydrotestosterone (DHT) and modulating proliferation and apoptosis of prostate cancer cells (Ub Wijerathne et al., 2017). As the standardized seed extract of QI, we turned out HU-033 on a mass production basis by adding dextrin as an excipient under the process

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of material standardization. By utilizing this substance, we examined further molecular mechanisms underlying the anti-BPH activity of HU-033.

MATERIALS AND METHODS

Preparation of HU-033

The seeds of QI were obtained from a local herbal market (Ansan, Korea), authenticated by Dr. Yeon, and deposited in the herbarium of the HUONS Research Center (Voucher no. HU033/SKJA150427, Ansan, Korea). The dried seeds of QI were homogenized to a fine powder (50 kg) and extracted by reflux with 500 L of 70% ethanol at 80°C for 6 h. The solution was concentrated under vacuum until elimination of the organic solvent was complete. Maltodextrin was then mixed with QI seed extract (total soluble solids content) in a 1:1 mass ratio. The final product (HU-033) obtained after mixing with maltodextrin and spray-drying (ODA-25, SeoGang Engineering, Cheonan, Korea) was collected and stored at room temperature.

High-performance liquid chromatography (HPLC) assay

For the identification of quisqualic acid from other amino acids, all types of amino acids were subjected to pre-column derivatization with *o*-phthalaldehyde and ethanethiol, as described previously (Fernstrom et al., 1981). Free amino acids were measured by HPLC performed using an Agilent 1200 series system (Agilent Technologies, Santa Clara, CA, USA). The separation of HU-033 was performed at 30°C with a ZORBAX Eclipse Plus C18 column (4.6×250 mm, 5 µm particle size; Agilent Technologies). Sodium acetate buffer (solvent A; 0.01 M, pH 4.6) with methanol (solvent B) was used as the mobile phase, at a flow rate of 1 mL/min, with a programmed gradient of: 0~5 min, 20% B; 5~14 min, 30% B; 30 min, 50% B; and 35 min, 100% B; this was followed by equilibration to 20% B for 5 min. The absorbance at 338 nm was measured. The administered HU-033 was standardized to a quisqualic acid content of 1% by using a validated HPLC assay, as described by the Hong Kong Chinese Materia Medica Standards office.

Experimental animals

Eight-week-old male Sprague-Dawley (SD) rats were purchased from Orient Bio Inc. (Seongnam, Korea). Animals were maintained in an environment of 20~24°C and 45~55% humidity under a 12-h light/dark cycle and allowed *ad libitum* access to a standard rodent chow and sterilized tap water. All experiments were conducted according to the guidelines of the Animal Experimental Ethics Committee of Chungnam National University (Daejeon, Korea; Approval number: CNU-00961).

Establishment of TP-induced BPH rat model

The rats were acclimatized for 1 week prior to randomized division into 6 groups (6 rats/group): the normal control (NC) group, which received an oral administration of PBS and a subcutaneous injection of corn oil; the BPH group, which received an oral administration of PBS and a subcutaneous injection of 3 mg/kg TP; the finasteride (Fina) group, used as a positive control, which received an oral administration of 10 mg/kg Fina and a subcutaneous injection of 3 mg/kg TP; and three HU-033 groups, which received an oral administration of 50, 150, and 300 mg/kg HU-033, respectively, and a subcutaneous injection of 3 mg/kg TP. All rats received the indicated treatments daily for 4 weeks and body weight was measured once per week. At the end of the experiment, the rats were fasted overnight, anesthetized by the intraperitoneal injection of pentobarbital (100 mg/kg), and dissected to obtain blood samples from the caudal vena cava. The whole blood samples were centrifuged at 1,000 *g* for 10 min to obtain serum, which was stored at -80°C until further analysis. The prostate of each rat was also carefully recovered and weighed. The prostatic index and percentage inhibition were calculated from the following equation:

$$\text{Percentage inhibition (\%)} = 100 - \frac{\text{Treated group} - \text{Normal control}}{\text{Positive control} - \text{Negative control}} \times 100$$

(Patil et al., 2016). The ventral prostate of each rat was fixed overnight in 10% neutral buffered formalin (NBF) and the rest of the prostate was snap-frozen in liquid nitrogen for protein assays.

Cell counting kit-8 (CCK-8) assay

The cytotoxic effect of HU-033 on LNCaP cells was evaluated by using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The cells were plated at 1×10^4 cells/well in 96-well plates in 100 µL Roswell Park Memorial Institute-1640 medium, to which HU-033 (25~3,200 µg/mL) was added. After incubation at 37°C for 72 h, the medium was replaced with 10 µL WST-8 reagent and incubated under the same conditions for a further 2 h. The absorbance at 450 nm was determined by using a microscope reader, from which the cell viability was calculated from the following equation:

$$\text{Absorbance (\%)} = 100 - \frac{\text{OD}_{450 \text{ nm}} \text{ of the HU-033 group}}{\text{OD}_{450 \text{ nm}} \text{ of the control group}} \times 100$$

Evaluation of antagonist effect against human $\alpha 1$ -adrenergic receptors

The antagonist effect of HU-033 on $\alpha 1_A$ -, $\alpha 1_B$ -, and $\alpha 1_D$ -adrenergic receptors was determined using Eurofins Cerep (Celle-L vescault, France). Briefly, Chinese hamster ovary (CHO) cells expressing the $\alpha 1_A$ and $\alpha 1_B$ receptors, and rat basophil leukemia cells expressing the $\alpha 1_D$ receptor were used to measure the effect on $\alpha 1_A$ agonist-induced cytosolic Ca^{2+} ion mobilization, $\alpha 1_B$ agonist-induced cyclic adenosine monophosphate (cAMP) production, and $\alpha 1_D$ agonist-induced cytosolic Ca^{2+} ion mobilization, respectively.

Western blotting

Tissues were homogenized in radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA, USA). For western blotting, 30~40 μ g of protein was separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. For experiments using prostate tissue, the membranes were probed with anti-androgen receptor (AR; Invitrogen, Carlsbad, CA, USA), anti-estrogen receptor alpha (ER α ; Abcam, Cambridge, MA, USA), or anti- β -actin (housekeeping gene; Sigma-Aldrich Co., St. Louis, MO, USA) antibodies diluted in Tris-buffered saline [0.05% Tween-20 (TBS-T) and 5% skim milk]. After washing in TBS-T, the membranes were developed by using an enhanced chemiluminescence kit (Thermo Fisher Scientific).

Immunohistochemical (IHC) analysis

IHC staining was performed by using a Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) in accordance with the manufacturer's instructions. After antigen retrieval, the sections were blocked in normal serum and then incubated with anti-AR (Invitrogen) or

ER α (Abcam) antibodies at 4°C. After incubation overnight, the sections were then incubated in biotinylated secondary antibody and developed by using a diaminobenzidine peroxidase substrate kit (Vector Laboratories Inc.).

Statistical analysis

Experiments were conducted at least three times and the data were expressed as the mean \pm standard deviation (SD) or standard error of the mean (SEM). All statistical analyses were computed by using Statistical Package for the Social Sciences 16.0 software (IBM Corp., New York, NY, USA). Student's *t*-test was used to compare the parameters between two groups, whereas analysis of variance followed by Tukey's *post hoc* test was used to compare the parameters between three groups; in all analyses, $P < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

HPLC analysis of HU-033

We verified the test substance, HU-033 through validated HPLC analysis. As shown in Fig. 1, HU-033 was standardized by the content of quisqualic acid (left) and several amino acids contained in HU-033 (1, asparagine; 2, quisqualic acid; 3, arginine; 4, glutamic acid) were determined (right).

Antagonistic effect of HU-033 on $\alpha 1$ -adrenergic receptors

Prior to beginning *in vitro* studies, we evaluated the cytotoxicity of HU-033 in different concentrations (from 0.4 mg/mL up to 3.2 mg/mL). As a result, we confirmed that HU-033 did not affect cell viability at all concentrations (Fig. 2A). And then, we disclosed the interaction between HU-033 and subtypes of $\alpha 1$ -adrenergic receptors including $\alpha 1_A$, $\alpha 1_B$, and $\alpha 1_D$. As shown in Fig. 2B and 2C, we found that expression of $\alpha 1_A$ and $\alpha 1_D$ -adrenergic recep-

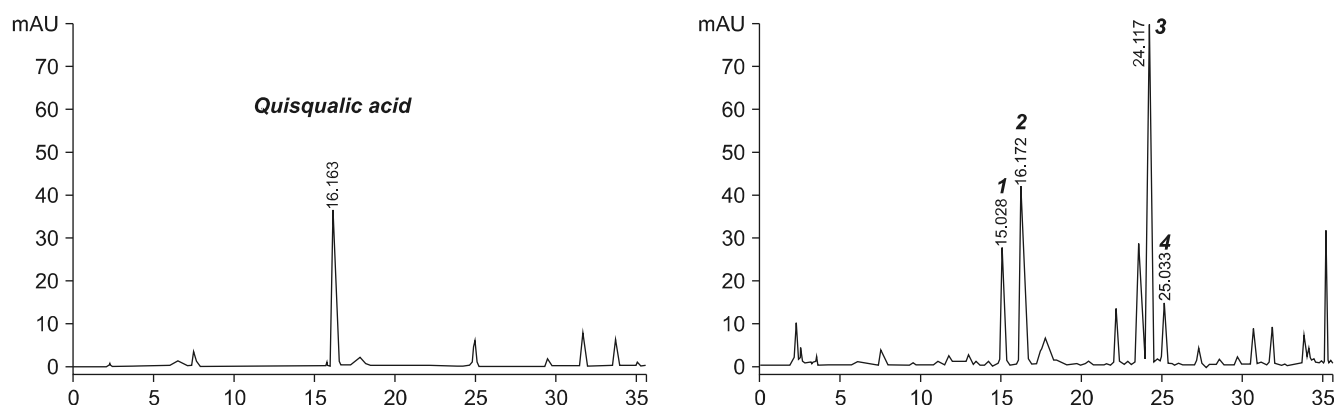


Fig. 1. High-performance liquid chromatography (HPLC) analysis of standardized seed extract of *Quisqualis indica* (HU-033). Chromatogram of quisqualic acid standard (left) and HPLC profile of amino acids in standardized HU-033 (right). 1, asparagine; 2, quisqualic acid; 3, arginine; 4, glutamic acid.

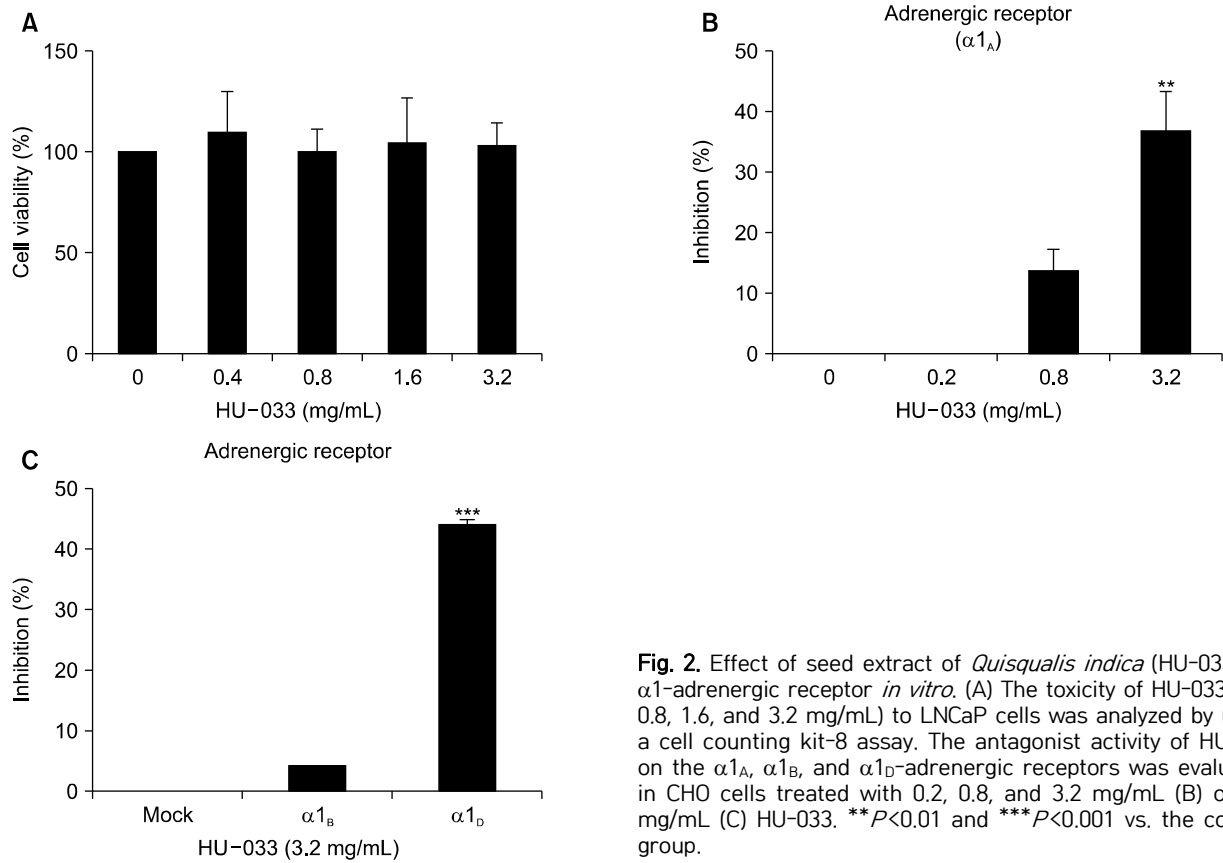


Fig. 2. Effect of seed extract of *Quisqualis indica* (HU-033) on α_1 -adrenergic receptor *in vitro*. (A) The toxicity of HU-033 (0.4, 0.8, 1.6, and 3.2 mg/mL) to LNCaP cells was analyzed by using a cell counting kit-8 assay. The antagonist activity of HU-033 on the α_{1A} , α_{1B} , and α_{1D} -adrenergic receptors was evaluated in CHO cells treated with 0.2, 0.8, and 3.2 mg/mL (B) or 3.2 mg/mL (C) HU-033. ** $P < 0.01$ and *** $P < 0.001$ vs. the control group.

tors were selectively inhibited by HU-033 *in vitro*. It is well established that 5α -reductase is an essential enzyme in the formation of DHT, which is a key factor in BPH. An increase in the level of this principle prostatic androgen triggers the occurrence of BPH (Bartsch et al., 2002; Carson and Rittmaster, 2003). Hence, 5α -reductase inhibitors, such as finasteride and dutasteride, are widely used for the treatment of BPH and act through a reduction in prostate volume and the consequent improvement of lower urinary tract symptoms (Kim et al., 2018). In addition to the androgen-signaling axis, alpha adrenergic

receptors including doxazosin, tamsulosin, and alfuzosin, induce the relaxation of the smooth muscle in the prostate and around the neck of the bladder, effectively relieving BPH symptoms (Janknegt et al., 1993; Chon et al., 1999; Shelbaia et al., 2013; Chapple, 2005). Among the subfamilies of alpha adrenergic receptors, α_{1A} receptors are linked with relaxation of prostate smooth muscle and α_{1D} receptors are linked with weak urine flow and acute retention (Forray et al., 1994). Thus, these results suggest the potential of HU-033 as the inhibitor of BPH pathogenesis-associated α_{1A} and α_{1D} except for α_{1B} .

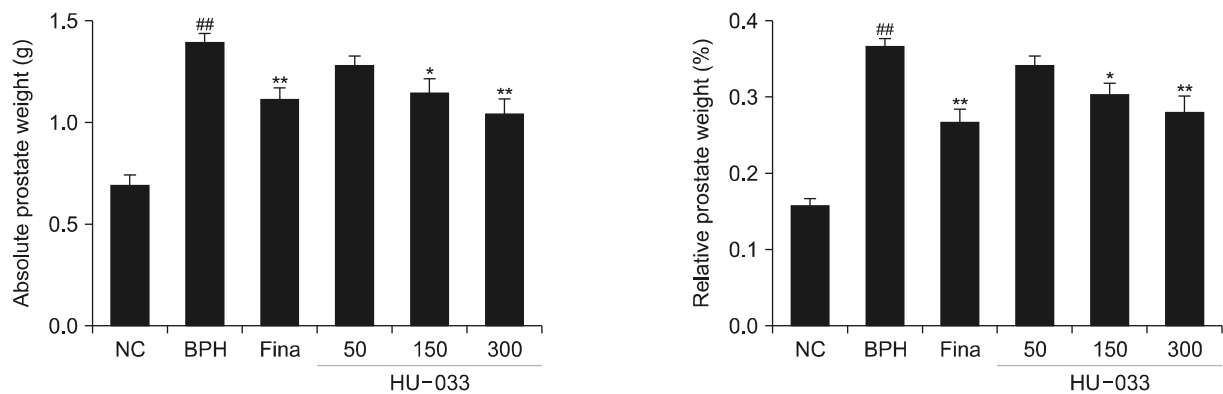


Fig. 3. Effect of seed extract of *Quisqualis indica* (HU-033) on the weight of prostate tissues isolated from testosterone propionate (TP)-induced benign prostatic hyperplasia (BPH) rats. The absolute and relative prostate weights were calculated. NC, normal control, corn oil injection and PBS treatment; BPH, TP (3 mg/kg) injection and PBS treatment; Fina, TP (3 mg/kg) injection and finasteride (10 mg/kg) treatment; HU-033-50, 150, and 300, TP (3 mg/kg) injection and HU-033 (50, 150, and 300 mg/kg) treatment, respectively. ## $P < 0.01$ vs. the NC group and * $P < 0.05$ and ** $P < 0.01$ vs. the BPH group.

Therapeutic effect of HU-033 administration on prostate weight of rats

At the end of the TP-induced BPH rat model study, all rats were sacrificed and we measured the prostate weight isolated from them treated with finasteride or HU-033 (50, 150, and 300 mg/kg). As shown in Fig. 3, treatment of finasteride that is a selective competitive inhibitor of 5 α -reductase type 2 reduced weight of enlarged prostate caused by TP injection. In addition, administration of HU-033 decreased prostate weight with concentration of 150 and 300 mg/kg in comparison with BPH group (neg-

ative control).

Inhibitory effect of HU-033 administration on AR and ER α expression in prostate tissues

Our previous study revealed that 70% EtOH seed extract of QI suppressed protein expression of AR *in vitro* (Ub Wijerathne et al., 2017). But, *in vivo* effect of QI seed extract on AR and ER α expression has been unknown. Hence, molecular mechanisms underlying HU-033-mediated BPH treatment were investigated through IHC analysis with AR and ER α antibody. HU-033 significantly

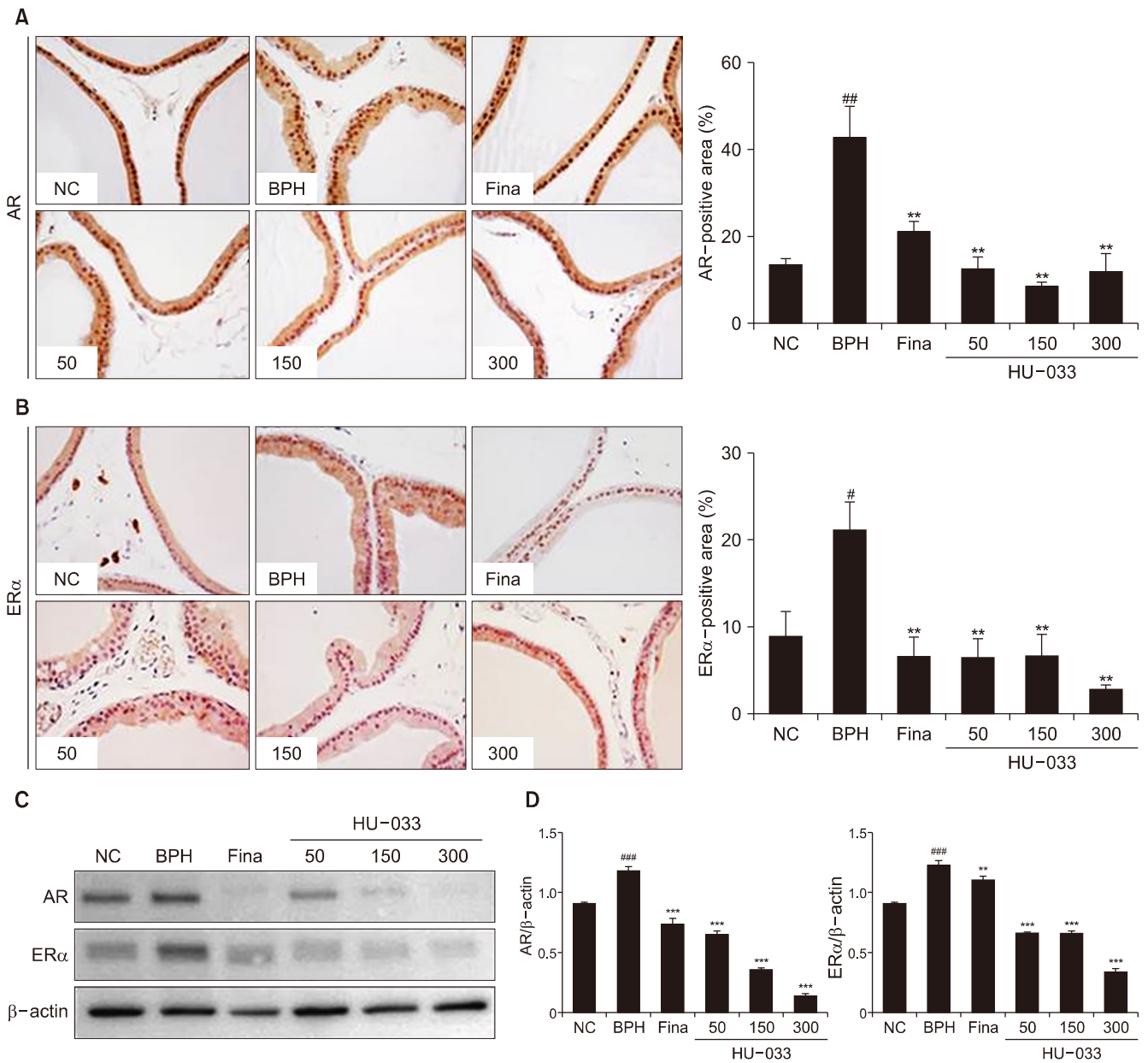


Fig. 4. Effect of seed extract of *Quisqualis indica* (HU-033) on the expression of androgen receptor (AR) and estrogen receptor (ER) α *in vivo*. (A) Representative photomicrographs of prostate tissues (magnification $\times 400$, left). The percentage of AR-positive area in the prostate tissue (right). (B) Representative photomicrographs of prostate tissues (magnification $\times 400$, left). The percentage of ER α -positive area in the prostate tissue (right). (C) The protein expression of AR, ER α , and β -actin in rat prostate tissues was analyzed by using western blotting. (D) The relative ratio of band intensity was calculated for AR/ β -actin and ER α / β -actin. [#] $P < 0.05$, ^{##} $P < 0.01$, and ^{###} $P < 0.001$ vs. the normal control (NC) group and ^{**} $P < 0.01$ ^{***} $P < 0.001$ vs. the benign prostatic hyperplasia (BPH) group.

down-regulated the degree of elevated AR-positive region in prostate tissues from BPH group (Fig. 4A). HU-033 also negatively regulated expression of ER α in prostate tissues (Fig. 4B). This observation was reaffirmed by western blotting using protein lysates of prostate tissues from experimental rat groups (Fig. 4C and 4D). As the key molecular mechanisms for BPH modulation, androgen/AR signaling induces the development of BPH development *via* control of the interaction between epithelial and stromal cells, and their subsequent proliferation. The selective knockout of AR in stromal smooth-muscle cells negatively regulated BPH development *in vitro* and blockade of this signaling reduced BPH volume *in vivo* (Izumi et al., 2013). ER α also contributed to bladder impairment in the pathogenesis of BPH. It was proven that ER α conditional-knockout mice did not suffer from BPH despite the co-treatment of TP and 17 β -estradiol, which suggested a stimulatory role for ER α in BPH (Nicholson et al., 2015). In line with *in vitro* study, administration of HU-033 on TP-induced BPH rat model blocked pathological condition of prostate tissues by regulating the expression of AR and ER α *in vivo*.

Previously, we reported the first documented case of seed extract of QI in BPH. In BPH rat models, serum concentration of DHT, and mRNA level of 5 α -reductase type 2 were significantly inhibited following the treatment with QI. Also, QI exerted anti-proliferative and pro-apoptotic activities of prostate cells through regulating proliferating cell nuclear antigen, cyclin D1, caspase-3, and -9 (Ub Wijerathne et al., 2017). Based on this research, we tried to disclose further mechanisms by using standardized material of QI, HU-033. In this study, we demonstrated additional preclinical data against BPH through usage of HU-033 as the standardized material. We confirmed not only antagonistic effect on α 1-adrenergic receptors *in vitro* but also inhibitory effect on AR and ER α *in vivo*. This preclinical research disclosed that HU-033 is a dietary supplement for overcoming prostate disease.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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