

# Ethanol Extract of *Chaenomeles sinensis* Inhibits the Development of Benign Prostatic Hyperplasia by Exhibiting Anti-oxidant and Anti-inflammatory Effects

Jong Su Kang<sup>1,\*\*</sup>, Xin Yi Zhao<sup>2,\*\*</sup>, Jeong Ho Lee<sup>3</sup>, Jeong-Sang Lee<sup>2,\*</sup>, Young-Sam Keum<sup>1,\*</sup>

<sup>1</sup>College of Pharmacy and Integrated Research Institute for Drug Development, Dongguk University, Goyang, <sup>2</sup>Department of Biotechnology and Functional Foods, College of Medical Sciences, Jeonju University, Jeonju, <sup>3</sup>Sunchang Research Institute of Health and Longevity, Sunchang, Korea

*Chaenomeles sinensis* is known to inhibit the development and progression of many age-related diseases, but the underlying molecular mechanisms are largely unclear. In the present study, we observed that the ethanol extract of *Chaenomeles sinensis* scavenged 2,2'-diphenylpicrylhydrazyl and 2,2'-azinobis diammonium radicals in vitro. The ethanol extract of *Chaenomeles sinensis* activated antioxidant response element-luciferase activity and induced expression of NRF2 target genes in HaCaT cells. The ethanol extract of *Chaenomeles sinensis* also suppressed LPS-induced expression of COX-2 and iNOS proteins, and mRNA expression of TNF- $\alpha$  and IL-2 in RAW264.7 cells. Finally, the ethanol extract of *Chaenomeles sinensis* significantly suppressed testosterone propionate-induced benign prostatic hyperplasia in mice. Together, our study provides the evidence that the ethanol extract of *Chaenomeles sinensis* inhibits the development of benign prostatic hyperplasia by exhibiting anti-oxidant and anti-inflammatory effects.

**Key Words** Benign prostatic hyperplasia, *Chaenomeles sinensis*, NF-E2-related factor 2, Lipopolysaccharide, Oxidative stress

## INTRODUCTION

Benign prostatic hyperplasia (BPH) is a common disease among old-aged men [1]. Epidemiological studies have demonstrated that various etiological factors are responsible for increasing the risk of the development and the progression of BPH [2]. BPH occurs when both stromal and epithelial cells of the prostate in the transitional zone proliferate by cellular processes that are thought to be influenced by sex hormones and inflammation, resulting in the lower urinary tract syndromes (LUTS) [3]. Treatment options for men with BPH include the oral administration of 5 $\alpha$ -reductase inhibitors and  $\alpha_1$ -adrenergic receptor antagonists [4]. Finasteride and dutasteride are clinically available 5 $\alpha$ -reductase inhibitors, which attenuate the development of BPH by suppressing the conversion of testosterone into dihydrotestosterone [5]. On the other hand, doxazosin and tamsulosin are widely used

$\alpha_1$ -adrenergic receptor antagonists, which relieve the LUTS by relaxing the neck of the bladder and smooth muscles in the prostate [6]. However, the use of these medications is restricted due to the adverse effects, such as erectile dysfunction, loss of libido, dizziness, and upper respiratory infection [7].

Plants have been the most utilized natural sources of pharmacologically active products due to the abundance and the accessibility [8]. Therefore, exploring plant ingredients or plant extracts has been proposed as a strategy to inhibit or delay many age-related diseases [9]. *Chaenomeles* plants originating from eastern Asia are adapted to the ecological zones particularly in the temperate areas of Korea, Japan and China; they are widely used for both ornamental decoration and medical purposes. Major species of *Chaenomeles* include *Chaenomeles japonica*, *Chaenomeles. Speiosa*, and *Chaenomeles sinensis* [10], and the analysis of phytochem-

Received February 5, 2022, Revised February 19, 2022, Accepted February 25, 2022

**Correspondence to** Jeong-Sang Lee, E-mail: jslee11@jj.ac.kr, <https://orcid.org/0000-0002-2080-2065>

Young-Sam Keum, E-mail: keum03@dongguk.edu, <https://orcid.org/0000-0002-9348-466X>

\*These authors contributed equally to this work as co-correspondence authors.

\*\*These authors contributed equally to this work as co-first authors.



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icals existing in the *Chaenomeles* plants demonstrated the presence of diverse types of flavonoids, lignins, aroma-active compounds [11-13]. While *Chaenomeles sinensis* (also known as Chinese quince) is known to exhibit various beneficial pharmacological effects [14], it is still unclear whether *Chaenomeles sinensis* could exert suppressive effects on the development of BPH. The present study was undertaken to address this issue.

## MATERIALS AND METHODS

### Cell culture, chemicals, and reagents

HaCaT and RAW264.7 cells were purchased from the American Type Cell Collection (ATCC, Manassas, VA, USA). Testosterone propionate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol extract of *Chaenomeles sinensis* was provided by Sunchang Institute of Health and Longevity (Sunchang, Korea). Dulbecco's modified Eagle medium (DMEM), heat-inactivated FBS, PBS, and penicillin/streptomycin (Pen/Strep) were purchased from Welgene (Daegu, Korea). Polyclonal antibody against NF-E2-related factor 2 (NRF2) was purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal antibodies against 8-hydroxydeoxyguanosine (8-OHdG) and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibody against 4-hydroxynonenal (4-HNE) was purchased from Abcam (Cambridge, MA, USA). 2,2'-diphenylpicrylhydrazyl (DPPH) was purchased from Cayman (Ann Arbor, MI, USA) and 2,2'-azinobis diammonium salt (ABTS) was purchased from Roche Diagnostics (Mannheim, Germany). Monoclonal antibodies against iNOS and COX-2 were purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA).

### DPPH and ABTS assay

DPPH and ABTS assays were conducted as previously described [15,16].

### Measurement of antioxidant response element (ARE)-luciferase activity

Establishment of human keratinocyte HaCaT-ARE-GFP-luciferase cells were previously described [17]. In brief, HaCaT-ARE-luciferase cells were seeded in six-well plates, cultured until 70% confluence, and exposed to ethanol extract of *Chaenomeles sinensis*. After treatment, cells were lysed with a luciferase lysis buffer (0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM DTT, 2 mM EDTA) and the resulting luciferase activity was measured by the GLOMAX Multi-system (Promega, Madison, WI, USA). The data is depicted as a fold ratio of the firefly luciferase activity compared with the control after normalization with the protein concentration.

### MTT assay

HaCaT cells were plated in 96-well culture plates. After exposure of ethanol extract of *Chaenomeles sinensis*, the media was removed and HaCaT cells were mixed with 200  $\mu$ L MTT stock solution (2 mg/mL) for 1 hour. HaCaT cells were washed with 1x PBS and lysed with 100  $\mu$ L DMSO, followed by spectrophotometer measurement at the wavelength of 570 nm. The percentage of viable cells was plotted in comparison with the control group.

### Western blot analysis

After acquisition, samples were incubated with 200  $\mu$ L RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and protease inhibitors cocktail) for 1 hour on ice. Lysates were collected by centrifugation, and protein concentrations were measured by use of the BCA protein assay kit (Thermo Fisher, Pittsburgh, PA, USA). Equal amounts of lysates were resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was incubated in blocking buffer (5% skim milk in 1x PBS-0.1% Tween-20, PBST) for 1 hour and hybridized with appropriate primary antibodies in 1x PBS containing 3% bovine serum albumin (BSA) overnight at 4°C. After washing three times with 1x PBST for 30 minutes, the membrane was hybridized with appropriate HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature and washed three times with 1x PBST solution for 30 minutes. The membrane was visualized using an enhanced chemiluminescence (ECL) detection system. The image of  $\beta$ -actin was used as a control for equal loading of samples.

### Real-time RT-PCR assay

Total RNA was isolated using Hybrid-R RNA extraction kit (GeneAll, Seoul, Korea). One  $\mu$ g of total RNA was subject to cDNA synthesis, using PrimeScript RT-PCR kit (TaKaRa Korea, Seoul, Korea). Real-time PCR was performed on CFX96 instrument (Bio-Rad, Hercules, CA, USA) using EvaGreen Supermix (Bio-Rad). The primer sequences used for the quantitation of target genes are illustrated in Table 1. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) (human) or  $\beta$ -actin (mouse) was used as an internal control.

### Induction of BPH by testosterone propionate in mice

The animal experiment was carried under the Institutional Animal Care and Use Committee-approved Protocol (IA-CUC-2019-002-1) of Dongguk University (Seoul, Korea). Six-week-old Balb/c mice were purchased from Daehan Bioblink (Eumseong, Korea), housed in the sterile filter-capped microisolator cages, and provided with water and diet ad libitum. Mice were separated and intraperitoneally injected with testosterone alone for 35 days to induce BPH or together with intraperitoneal injection of finasteride (positive control)

**Table 1.** List of real-time PCR primers

	Accession no.	Gene	Sequence
Human	NM_010442	HO-1	Forward:5'-AGCCCCACCAAGTTCAAACA-3' Reverse :5'-TCTCTGCAGGGGCAGTATCT-3'
	NM_000903	NQO1	Forward:5'- CTCAACCGCTTGCTGTATGC-3' Reverse :5'- TTCAACTGGTCCTGCCCATC-3'
	NM_002046	GAPDH	Forward:5'- CATCAATGGAAATCCCATCA-3' Reverse :5'- GGCAGAGATGATGACCCTTT-3'
Mouse	NM_011198	COX-2	Forward:5'- CTTCTGGGAGCACAACAGAGT-3' Reverse :5'- AAGTGGTAACCGTCCAGGTG-3'
	U 43428	iNOS	Forward:5'- ACATCGACCCGTCCACAGTAT-3' Reverse :5'- CAGAGGGGTAGGCTTGTCTC-3'
	NM_013693	TNF- $\alpha$	Forward:5'- CAGGCGGTGCCTATGTCTC-3' Reverse :5'- CGATCACCCCGAAGTTCA-3'
	NM_008366	IL-2	Forward:5'- CCTGAGCAGGATGGAGAATTACA-3' Reverse :5'- TCCAGAACATGCCGCAGAG-3'
	NM_007393	$\beta$ -actin	Forward:5'- CTAAGCCAACCGTAAAAAG-3' Reverse :5'- ACCAGAGGCATACAGGGACA-3'

HO-1, heme oxygenase-1; NQO1, NAD[P]H:quinone oxidoreductase; GAPDH glyceraldehyde-3-phosphate dehydrogenase; IL-2, interleukin-2.

or with oral administration of ethanol extract of *Chaenomeles sinensis* to evaluate the pharmacological activities.

### Hematoxylin & Eosin (H&E) staining

At sacrifice, mouse prostate tissues were excised and fixed in 10% formalin solution overnight. Tissue dehydration was performed by serially immersing the tissues into 75%, 80%, 85%, 90%, 95%, 100% ethanol, and xylene solution for 1 hour at each step. Dehydrated tissues were embedded in the paraffin block. Paraffin-embedded tissues were sectioned at 5  $\mu$ m, mounted on the slide, and deparaffinized. Tissues were stained with Mayer's hematoxylin solution for 5 minutes at room temperature and rinsed in tap water until the water becomes clear. In the bluing step, the tissues were stained with repeated cycles of eosin Y ethanol solution for 70 seconds, 5 dips in 95% ethanol, and 5 dips in 100% ethanol at room temperature. The tissues were rinsed with distilled water and the images were taken on the microscope (Olympus, Tokyo, Japan).

### Immunohistochemistry staining

Tissues on the slide were incubated with 1% BSA blocking solution for 30 minutes. After washing three times with 1x PBS, the tissues were hybridized with primary antibodies overnight at 4°C. The slides were washed with 1x PBS three times and incubated with anti-rabbit and anti-mouse UltraTEK HRP antibodies (ScyTek Inc., Logan, UT, USA). Development of the slides was performed with 3',3'-diaminobenzidine (DAB) (GBI Labs, Bothell, WA, USA). The slides were then sealed with mounting medium and the images were taken on the microscope (Olympus).

### Statistical analysis

Statistical analyses were performed using Microsoft Excel (Mi-

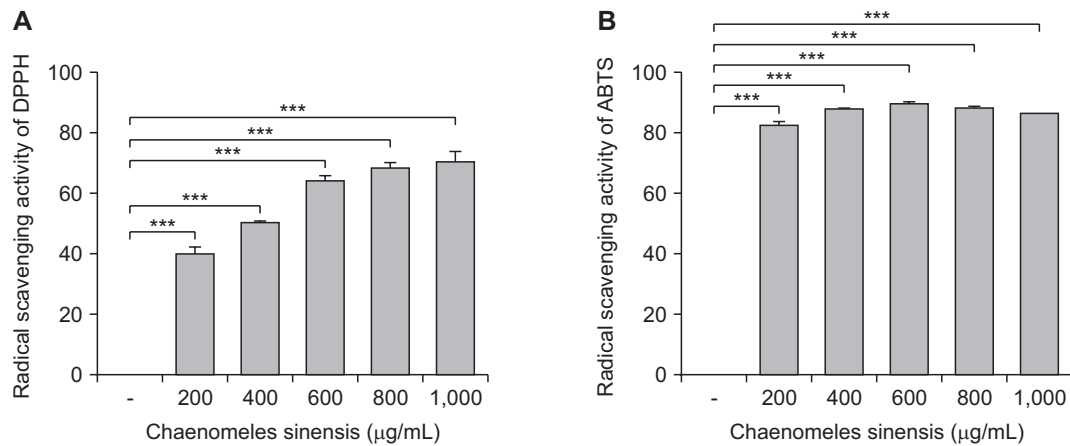
crosoft, Redmond, WA, USA). Statistical analysis was conducted using Student's *t*-tests. Asterisks indicate a statistical significance of \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## RESULTS

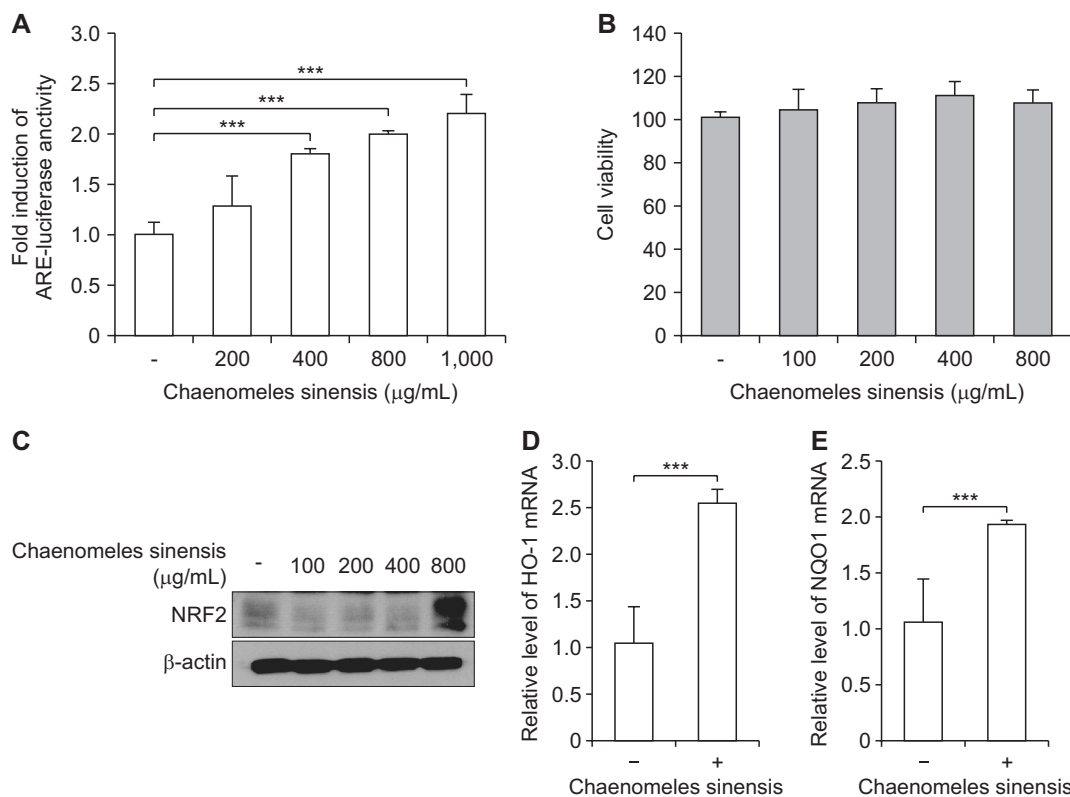
### Ethanol extract of *Chaenomeles sinensis* scavenges free radicals in vitro and induces the expression of NRF2 target genes in HaCaT cells

Aberrant production of reactive oxygen species (ROS) is responsible for the generation of oxidative stress [18]. Oxidative stress can be relieved by treatment with antioxidants or by the induction of cellular phase II cytoprotective enzymes [19], the latter of which is under the control of transcription factor, NRF2 [20]. In a normal condition, NRF2 is constantly poly-ubiquitinated by Kelch-like ECH-associated protein 1 (KEAP1), an adaptor of Cullin 3 E3 ubiquitin ligase complex. Upon exposure to oxidants or electrophiles, NRF2 translocates into the nucleus and activates the ARE, a cis-acting DNA element located in the promoter of NRF2 target genes [21]. We first examined whether ethanol extract of *Chaenomeles sinensis* possesses free radical scavenging effects in vitro. DPPH and ABTS are synthetic compounds that can generate colored radical and redox-active compounds, and the ability of a test sample of interest to reduce the colored compound is monitored by spectrophotometer [22]. Our results show that ethanol extract of *Chaenomeles sinensis* exhibited significant DPPH (Fig. 1A) and ABTS (Fig. 1B) free radical scavenging effects in vitro.

We next examined whether ethanol extract of *Chaenomeles sinensis* could upregulate ARE-dependent NRF2 gene expression in human keratinocyte HaCaT cells. Our results show that ethanol extract of *Chaenomeles sinensis* enhanced



**Figure 1. Ethanol extract of *Chaenomeles sinensis* possesses radical scavenging effects in vitro as examined by DPPH (A) and ABTS (B) assays.** DPPH, 2,2'-diphenylpicrylhydrazyl; ABTS, 2,2'-azinobis diammonium salt. Asterisks indicate a statistical significance with  $***P < 0.001$  ( $n = 3$ ).

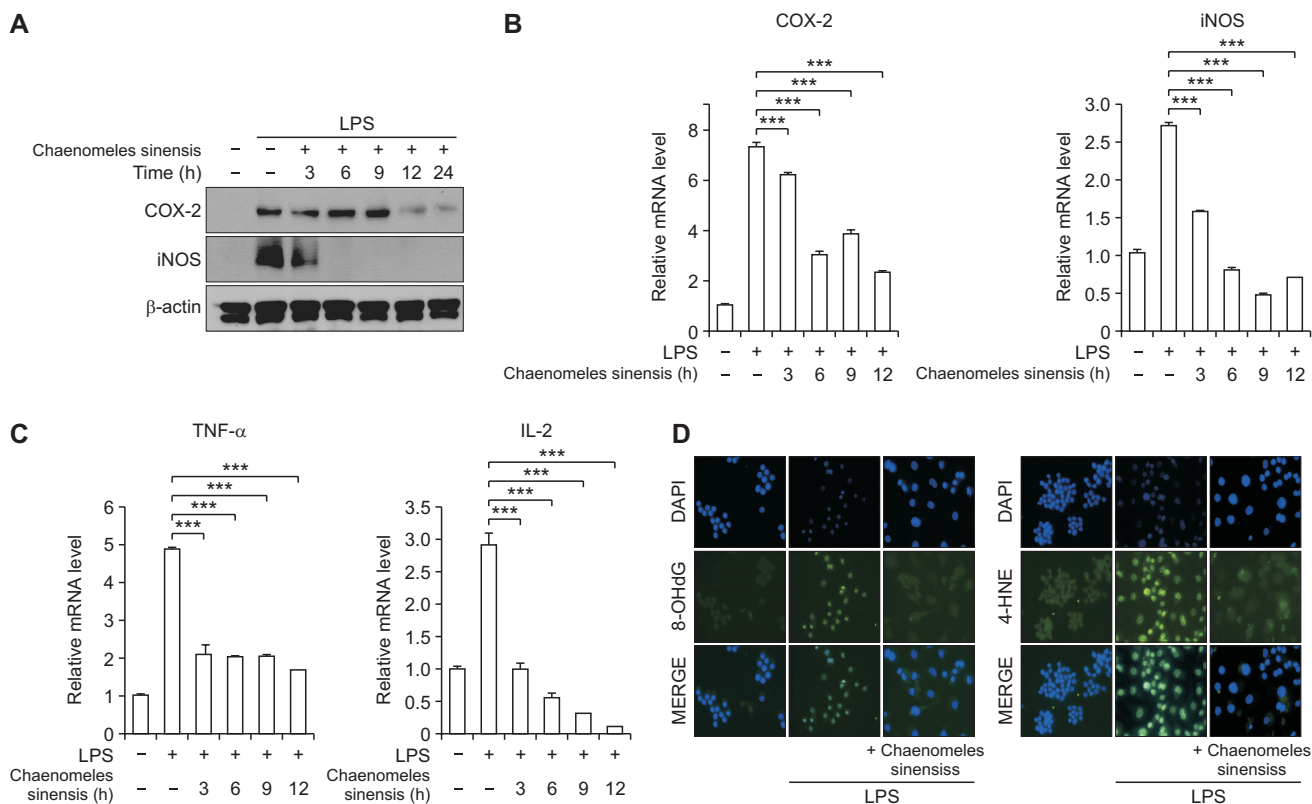


**Figure 2. Ethanol extract of *Chaenomeles sinensis* upregulates ARE-dependent NRF2 target gene expression in HaCaT cells.** (A) Ethanol extract of *Chaenomeles sinensis* were treated to HaCaT-ARE-GFP-luciferase cells at various concentrations for 24 hours, and the luciferase activity was measured. Asterisks indicate a statistical significance with  $***P < 0.001$  ( $n = 3$ ). (B) Ethanol extract of *Chaenomeles sinensis* does not affect the viability of HaCaT cells. Ethanol extract of *Chaenomeles sinensis* was treated to HaCaT cells at various concentrations for 24 hours, and the MTT assay was conducted. (C) Ethanol extract of *Chaenomeles sinensis* induces NRF2 expression in HaCaT cells. Ethanol extract of *Chaenomeles sinensis* were treated to HaCaT cells at various concentrations for 24 hours, and Western blot analysis was conducted against NRF2. (D) Ethanol extract of *Chaenomeles sinensis* activates transcription of NRF2 target genes (HO-1 and NQO1). Ethanol extract of *Chaenomeles sinensis* (800 µg/mL) was treated to HaCaT cells for 24 hours, and real-time RT-PCR was conducted using HO-1 and NQO1 specific primers. Asterisks indicate a statistical significance with  $***P < 0.001$  ( $n = 4$ ). ARE, antioxidant response element; NRF2, NF-E2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase.

ARE-dependent luciferase activity in HaCaT-ARE-GFP-luciferase cells (Fig. 2A) without affecting the viability of these cells (Fig. 2B). Ethanol extract of *Chaenomeles sinensis* also induced NRF2 expression (Fig. 2C) and caused transcriptional activation of NRF2 target genes such as heme oxygenase-1 (HO-1) and NADP(H):quinone oxidoreductase (NQO1) (Fig. 2D). Together, our results demonstrate that ethanol extract of *Chaenomeles sinensis* exhibits antioxidant effects as evidenced by scavenging free radicals and the induction of ARE-dependent NRF2 target genes.

**Ethanol extract of *Chaenomeles sinensis* inhibits the expression of pro-inflammatory enzymes and cytokines, mitigating oxidative stress damages in RAW264.7 cells**

Because oxidative stress is closely associated with pro-inflammatory responses [23], we examined whether ethanol extract of *Chaenomeles sinensis* could inhibit lipopolysaccharide (LPS)-induced pro-inflammatory responses in RAW264.7 cells. Our results show that ethanol extract of *Chaenomeles sinensis* suppressed LPS-induced expression of COX-2 and iNOS (Fig. 3A). Suppression of COX-2 and iNOS by ethanol extract of *Chaenomeles sinensis* was regulated at the transcriptional level (Fig. 3B). In addition, ethanol extract of *Chaenomeles sinensis* inhibited LPS-induced transcriptional activation of TNF- $\alpha$  and interleukin-2 (IL-2) (Fig. 3C). Finally, ethanol extract of *Chaenomeles sinensis* suppressed the formation of LPS-induced oxidative damage markers, 8-OHdG and 4-HNE in RAW264.7 cells (Fig. 3D).



**Figure 3. Ethanol extract of *Chaenomeles sinensis* inhibits LPS-induced pro-inflammatory responses and protects against oxidative damages in RAW 264.7 cells.** (A) Ethanol extract of *Chaenomeles sinensis* suppresses LPS-induced COX-2 and iNOS in RAW264.7 cells. RAW264.7 cells were exposed to LPS (2  $\mu$ g/mL) alone or together with ethanol extract of *Chaenomeles sinensis* (800  $\mu$ g/mL) for various times and Western blot analysis was performed against COX-2 and iNOS. (B) Ethanol extract of *Chaenomeles sinensis* suppresses LPS-induced transcriptional activation of iNOS and COX-2 in RAW264.7 cells. RAW264.7 cells were exposed to LPS (2  $\mu$ g/mL) alone or together with ethanol extract of *Chaenomeles sinensis* (800  $\mu$ g/mL) for various times and real-time RT-PCR analysis was conducted using specific primers against COX-2 (left panel) and iNOS (right panel). Asterisks indicate a statistical significance with  $***P < 0.001$  (n = 4). (C) Ethanol extract of *Chaenomeles sinensis* suppresses LPS-induced transcriptional activation of TNF- $\alpha$  and IL-2 in RAW264.7 cells. RAW264.7 cells were exposed to LPS (2  $\mu$ g/mL) alone or together with ethanol extract of *Chaenomeles sinensis* (800  $\mu$ g/mL) for various times and real-time RT-PCR analysis was conducted against specific primers against TNF- $\alpha$  (left panel) and IL-2 (right panel). Asterisks indicate a statistical significance with  $***P < 0.001$  (n = 4). (D) Ethanol extract of *Chaenomeles sinensis* protects against LPS-induced oxidative damages in RAW264.7 cells. RAW264.7 cells were exposed to LPS (2  $\mu$ g/mL) alone or together with ethanol extract of *Chaenomeles sinensis* (800  $\mu$ g/mL) for 24 hours, and the oxidative damages were monitored by conducting immunofluorescence staining using antibodies against 8-OHdG (left panel) and 4-HNE (right panel). DAPI staining was conducted to visualize the nucleus. LPS, lipopolysaccharide; IL-2, interleukin-2; DAPI, 4'-6-diamidino-2-phenylindole; 4-HNE, 4-hydroxynonenal; 8-OHdG, 8-hydroxydeoxyguanosine.



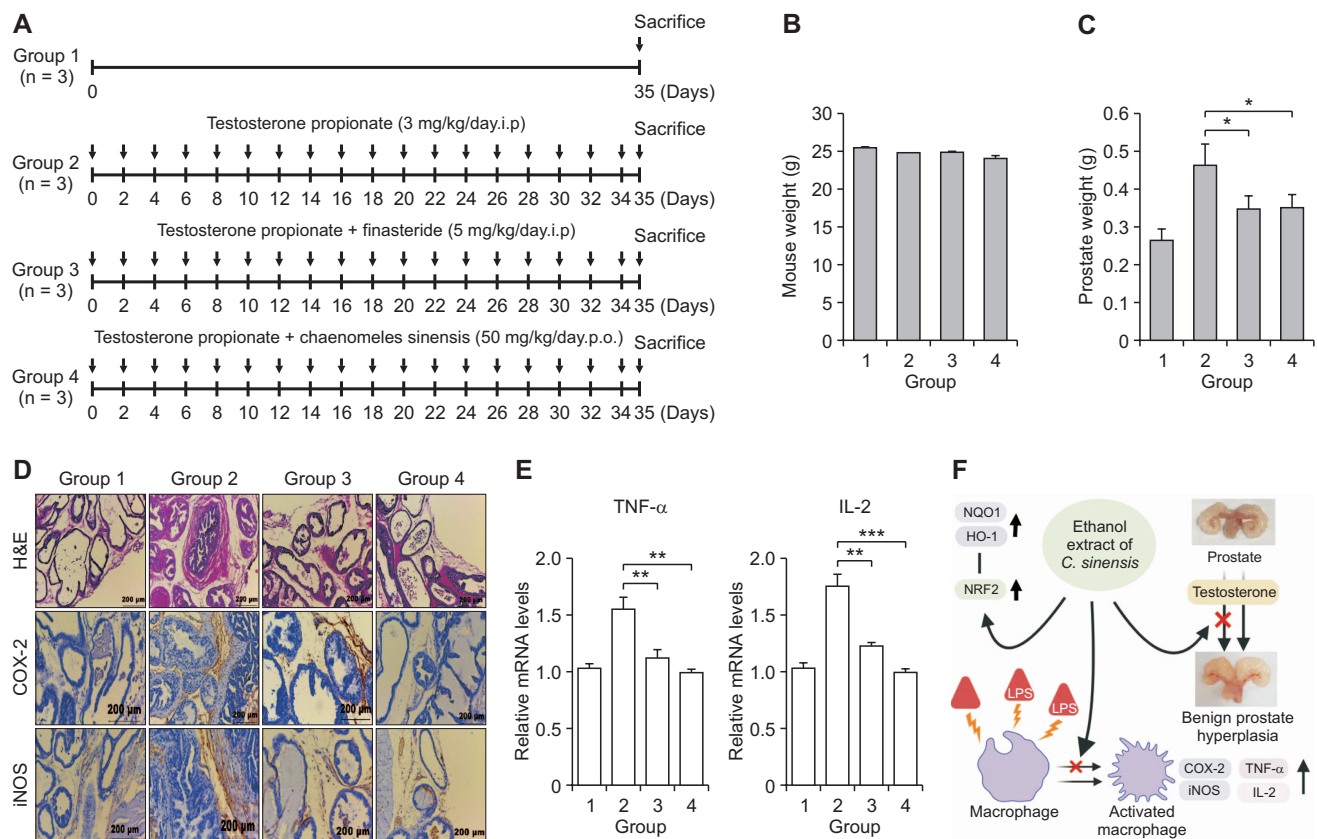
### Ethanol extract of *Chaenomeles sinensis* suppresses testosterone propionate-induced BPH in mice

We examined whether ethanol extract of *Chaenomeles sinensis* could inhibit the progression of BPH in vivo. We treated mice with testosterone propionate for 35 days to induce BPH, in which ethanol extract of *Chaenomeles sinensis* was orally administered to evaluate its suppressive effects (Fig. 4A). Finasteride was included as a positive control (Fig. 4A). We observed that oral administration of *Chaenomeles sinensis* did not affect the weight of mice at the end of study (Fig. 4B). However, ethanol extract of *Chaenomeles sinensis* significantly suppressed an increase in the prostate weight induced by testosterone propionate (Fig. 4C). Immunohistochemistry of the mouse prostate showed that the induction of COX-2 and iNOS by testosterone propionate was significantly attenuated by ethanol extract of *Chaenomeles sinensis* (Fig. 4D).

Likewise, transcriptional activation of TNF- $\alpha$  and IL-2 by testosterone propionate was significantly suppressed by ethanol extract of *Chaenomeles sinensis* (Fig. 4E). Together, these results indicate that ethanol extract of *Chaenomeles sinensis* possesses inhibitory effects on testosterone propionate-induced BPH, and this event is associated with suppression of COX-2, iNOS, TNF- $\alpha$ , and IL-2.

### DISCUSSION

*Chaenomeles sinensis* is rarely taken as a raw fruit since it is astringent and hard due to a high amount of lignin [24]. However, *Chaenomeles sinensis* extract has been utilized in traditional medicine, and a number of preclinical studies have been recently performed to evaluate the in-depth pharmacological effects of *Chaenomeles sinensis* extract. For example, Kim and colleagues showed that WS-5, a 45% ethanol



**Figure 4.** Ethanol extract of *Chaenomeles sinensis* inhibits the development of BPH induced by testosterone propionate in mice. (A) Experimental scheme of testosterone propionate-induced BPH in mice. (B) The body weight of mice did not change at the end of study. The body weight of mice was measured at the end of study. (C) Oral administration of ethanol extract of *Chaenomeles sinensis* inhibits the induction of BPH by testosterone propionate in mice. The weight of the prostate in mice was measured at sacrifice, and asterisks indicate a statistical significance with  $*P < 0.05$ . (D) Ethanol extract of *Chaenomeles sinensis* suppresses testosterone propionate-induced expression of COX-2 and iNOS in the prostate of mice. The prostates of mice were stained with specific antibodies against COX-2 and iNOS by immunostaining. (E) Ethanol extract of *Chaenomeles sinensis* suppresses the induction of TNF- $\alpha$  (left panel) and IL-2 (right panel) mRNAs induced by testosterone propionate in the prostate of mice. Total RNA was extracted from the prostate of mice, and real-time RT-PCR was conducted using specific primers against mouse TNF- $\alpha$  (left panel) and IL-2 (right panel). Asterisks indicate a statistical significance with  $**P < 0.01$  and  $***P < 0.001$ . (F) Schematic diagram demonstrating how ethanol extract of *Chaenomeles sinensis* exerts beneficial effects. BPH, Benign prostatic hyperplasia; IL-2, interleukin-2; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase.

extract of a mixture of three traditional plants with *Curcuma longa* L. (turmeric), the fruits of *Chaenomeles sinensis*, and the dried rhizome of *Zingiber officinale* Roscoe (ginger) improved  $\beta$ -amyloid-induced memory impairment by inhibiting acetylcholine esterase (AChE) [25].

Lee and colleagues [26] have characterized phytochemicals existing in the twig of *Chaenomeles sinensis* by nuclear magnetic resonance (NMR) and demonstrated that selected compounds exhibited significant anti-carcinogenic, neuro-protective, and anti-inflammatory effects [26]. Zhang and colleagues have demonstrated that the ethyl acetate fraction of the *Chaenomeles sinensis* extract exhibited anti-hyperuricemic and nephroprotective effects [27]. However, we are unaware of whether ethanol extract of *Chaenomeles sinensis* used in our study possesses analogous pharmacological effects since chemical composition in *Chaenomeles sinensis* extract will vary depending on the cultivation and extraction condition.

In the present study, we have demonstrated that ethanol extract of *Chaenomeles sinensis* induced NRF2-dependent gene expression in HaCaT cells, inhibited LPS-induced pro-inflammatory enzymes and cytokines in RAW264.7 cells, and suppressed the development of BPH induced by testosterone propionate in vivo (Fig. 4F). Considering the implication of oxidative stress and inflammation in pathogenesis, it is possible to speculate that *Chaenomeles sinensis* extract could be applicable to treatment of other chronic diseases. Further studies will be needed to examine whether ethanol extract of *Chaenomeles sinensis* could decrease the level of dihydrotestosterone in the blood and/or interferes with the androgen receptor signaling pathway in the prostate since dihydrotestosterone, a metabolite of testosterone formed by 5 $\alpha$ -reductase, promotes BPH by activating the androgen receptor signaling pathways [28].

## FUNDING

This work was supported by the grant of the Sunchang Research Institute of Health and Longevity.

## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

## ORCID

Jong Su Kang, <https://orcid.org/0000-0002-5655-2475>  
Xin Yi Zhao, <https://orcid.org/0000-0003-0897-7310>  
Jeong Ho Lee, <https://orcid.org/0000-0002-1052-1497>  
Jeong-Sang Lee, <https://orcid.org/0000-0002-2080-2065>  
Young-Sam Keum, <https://orcid.org/0000-0002-9348-466X>

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