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Evaluation of estrogenic potential by herbal formula, HPC 03 for *in vitro* and *in vivo*

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Abstract

HPC 03 is herbal formula that consists of extracts from *Angelica gigas*, *Cnidium officinale* Makino and *Cinnamomum cassia* Presl. The present study evaluated the estrogenic potential of HPC 03 by using *in vitro* and *in vivo* models. The regulatory mechanisms of HPC 03 in estrogen-dependent MCF-7 cells were assessed. HPC 03 induced the proliferation of estrogen receptor-positive MCF-7 cells, and the proliferation was blocked by the addition of the estrogen antagonist tamoxifen. The estrogen receptor$_{\alpha/\beta}$ luciferase activities were significantly increased by HPC 03 treatment, which also increased the mRNA expression of the estrogen-responsive genes *Psen2*, *Pgr* and *Ctsd*. Also, we evaluated the ameliorative effects of HPC 03 on menopausal symptoms in ovariectomized rats. HPC 03 treatment in OVX rats significantly affected the uterine weight, increased the expression of estrogen-responsive genes *Pgr* and *Psen2* in uterus, increased bone mineral density loss in the femur and inhibited body weight increase. Serum E2, collagen type 1 and osteocalcin were significantly increased, while serum LH, FSH and ALP were decreased compared with OVX rats. HPC 03 may be a promising candidate for the treatment of menopause, but further research is necessary to determine whether the observed effects also occur in humans.

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Introduction

Postmenopausal women experience estrogen deficiency-related menopausal symptoms, which include hot flashes, mood swings and sweating and exhibit a dramatic increase in the risk of metabolic syndrome, cognitive deficits, cardiovascular disease, dyslipidemia and osteoporosis. As the general population ages and life expectancy increases, the importance of preventing and/or improving menopause-related changes has become paramount (Hill *et al.* 2016, Roberts & Hickey 2016, Zhu *et al.* 2016).

Hormone replacement therapy (HRT) is widely used for the treatment of menopause symptoms and osteoporosis after menopause. Although HRT may lower the rates of osteoporosis and fracture, large-scale clinical research has shown that HRT is a factor in the growth of ovarian cancer and the increased incidence of breast cancer, cardiovascular disease and stroke (Anagnostis *et al.* 2016, Hill *et al.* 2016). Therefore, increased interest in complementary therapies using natural products, which have good effectiveness and fewer side effects, has arisen from both patients and physicians (Taylor 2015, Zhu *et al.* 2016).

Phytoestrogens, the name given to plant-derived estrogens, occur naturally in a diverse range of foods and include isoflavones, lignans, coumestans and flavonoids (e.g. quercetin and kaempferol). As the structure of phytoestrogens is similar to that of human estrogen, phytoestrogens can bind to estrogen receptors (ERs). Many researchers have searched for additional phytoestrogens that show estrogen-like activity, which has led to the continuous discovery of novel phytoestrogens in nature (Dittfeld *et al.* 2015, Franco *et al.* 2016).

Various plants have traditionally been used to treat menopausal patients. Some herbal plants, such as *Actaea racemosa*, *Caulerpa racemosa*, *Angelica sinensis*, *Angelica gigas*, *Panax ginseng*, *Piper methysticum* and *Oenothera biennis*, have a potent effect on menopausal symptoms (Huntley & Ernst 2003, Borrelli & Ernst 2008, Ross 2014, Franco *et al.* 2016). A reduction in hot flashes serves as a good indicator of the hormonal activity of these herbs, and there is increasing evidence that they confer a variety of protective effects on the body, such as an anti-osteoporotic effect. To develop a herbal medicine-derived remedy for the treatment of menopausal symptoms and osteoporosis after menopause, we surveyed the list of medicinal plants traditionally used in clinical practice and conducted an experimental screening, which focused on the regulation of ER-mediated proliferation, using the human breast cancer cell line MCF7. Finally, we selected a formula, HPC 03 that consisted of three herbal plants: *Angelica*

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gigas, *Cnidium officinale* Makino and *Cinnamomum cassia* Presl.

A. gigas (Umbelliferae) root has been traditionally used in Korean folk medicine as a female balancing agent and a panacea for gynecologic complaints. *A. gigas* has been reported to exert estrogenic effects in ovariectomized rats (Choi *et al.* 2012). *Angelica* spp. is not used alone in Chinese medicine formulations, but must be given in concert with other botanicals to promote the synergies needed for therapeutic effects (Huntley & Ernst 2003, Chang *et al.* 2012, Choi *et al.* 2012). *C. officinale* Makino has been used in Asia for centuries as a medicinal plant for the treatment of pain and inflammation (Lee *et al.* 2013, 2016*b*). *C. officinale* contains a variety of volatile phthalide derivatives (Leon *et al.* 2017). Ferulic acid is one of the major compounds found in *C. officinale*. Recent reports have suggested that various phytochemicals with antioxidant and anti-inflammatory properties, such as ferulic acid, confer bone protective effects and suppress bone resorption, which results in increased bone strength (de la Cruz *et al.* 2014, Sagar *et al.* 2016). *C. cassia* Presl. (Lauraceae) is widely cultivated in China: the dry bark has been regarded as a folk medicine and a food spice in daily diet. The total polyphenolic contents (cinnamic, flavonic and anthocyanic derivatives) are found in the extract of *C. cassia*. All the aforementioned compounds are polyphenols, which have antioxidant, antiproliferative and antiangiogenic biological activities. Some of these compounds displayed weak estrogenic activity, which may confer a protective effect against breast cancer (Asif 2012, Sun *et al.* 2016).

The purpose of this study was to assess the estrogenic activity of HPC 03 by using various *in vitro* assays. We also treated OVX rats, which exhibit most of the characteristics of human menopausal symptoms, with HPC 03 for 12 weeks and then assessed the metabolic parameters related to menopausal symptoms.

Materials and methods

Plant material and extraction

The dried fruits of *A. gigas*, *C. officinale* Makino and *C. cassia* Presl were purchased from the local herbal market in Jeonbuk, Korea. The herbs were identified and authenticated by Dr Hyoung-Kwon Cho of the Hanpoong Pharm. Co. Ltd, where the voucher specimens (#HPS-23, HPS-20, HPS-78) were deposited. The herb materials were identified and tested for harmful substance (pesticides: BCH, Aldrin, Dieldrin, Endrin, DDT: heavy metals: Pb, As, Hg, Cd) by Hanpoong Pharm & Food (Jeonju, Korea). The three herbs were mixed in a 2:1:1 (w/w) ratio of *A. gigas*:*C. officinale* Makino:*C. cassia* Presl. The mixture was extracted with a tenfold volume of 30% ethanol for 3 h at 80°C. The solvent was removed under reduced pressure in a rotary evaporator (N-1000S, EYELA, Japan) to yield the extract (26.67%, w/w). The extracted powder was used for the preparation of HPC 03. The content of the markers decursin, ferulic acid or cinnamic acid in HPC 03 was quantitated using high-performance liquid chromatography (HPLC).

HPLC analysis of HPC 03

The quality of HPC 03 was controlled by monitoring the amounts of decursin, ferulic acid or cinnamic acid, which were marker compounds of *A. gigas*, *C. officinale* Makino and *C. cassia* Presl., respectively. HPC 03 was used for further experiments only when the concentrations of decursin (including decursinol angelate), ferulic acid or cinnamic acid were 107.4 ± 0.91, 0.82 ± 0.01, and 0.29 ± 0.05 mg/g, respectively (Table 1). For HPLC analysis, HPC 03 powder (0.5 g) was hydrolyzed in 1 M HCl in water (20 mL). The hydrolysis was performed in triplicate. The acid-hydrolyzed sample was refluxed at 100°C for 1 h, cooled to room temperature (~20 °C), diluted to 200 mL and sonicated for 5 min. The extract was filtered through a 0.45 µm filter prior to injection into a Waters HPLC system (Waters, Milford, MA, USA), which comprised a Waters 1525 pump, a 2707 autosampler and a 2998 PDA detector. The chromatic separation was achieved at 35°C on a Waters Sunfire C18 column (250 mm × 4.6 mm I.D., 5 µm particle size). The flow rate was 1.0 mL/min and the injection volume was 10 µL. The sample was injected twice to analyze each marker compound, and the eluents from the column were monitored at 330 nm for decursin, 320 nm for ferulic acid and 280 nm for cinnamic acid. The HPLC chromatogram of HPC 03 is given in Fig. 1.

Cell culture

MCF-7, an ER-positive human breast cancer cell line, was purchased from American Type Culture Collection (ATCC) and cultured in DMEM medium supplemented with 10% fetal bovine serum and penicillin–streptomycin solution (100 units/mL penicillin and 100 µg/mL streptomycin: Hyclone Laboratories Inc., South Logan, UT, USA). The cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2. The medium was renewed 2–3 times per week, and

Table 1 Effects of HPC 03 on alkaline phosphate (ALP), bone alkaline phosphate (BALP), collagen type 1 (CTx) and osteocalcin (OC) in OVX rats.

Factor	SHAM	OVX	OVX + E2	OVX + HPC 03		
				50 mg/kg	100 mg/kg	200 mg/kg
ALP (U/L)	46.5 ± 4.0^a	89.3 ± 4.1^b	50.0 ± 0.9^a	82.3 ± 1.2^b	72.3 ± 1.2^c	63.0 ± 4.1^c
BALP (U/L)	102.0 ± 8.1^a	156.3 ± 10.9^b	98.2 ± 5.2^a	148.2 ± 4.2^b	123.3 ± 8.3^c	117.5 ± 2.3^c
CT_x (pg/mL)	80.1 ± 2.1^a	120.4 ± 6.0^b	92.4 ± 2.5**	118.9 ± 4.0^b	107.9 ± 3.8^c	105.8 ± 3.1^c
OC (pg/mL)	62.1 ± 3.5^a	87.6 ± 1.2^b	65.3 ± 4.0^a	82.0 ± 3.9^b	75.3 ± 2.2^b	71.9 ± 5.2^c

Serum levels of ALP, BALP, CTx and OC were measured at the end of the treatment period. Values with different letters (a, b, c) are significantly different one from another (one-way ANOVA followed by Newman–Keuls multiple range test, $P < 0.05$).

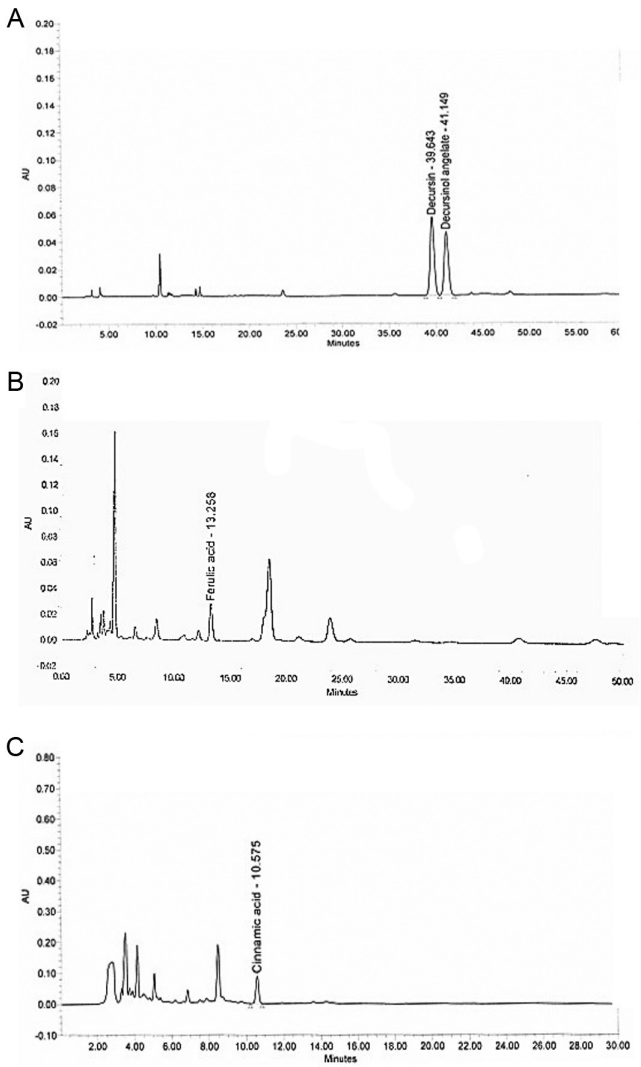

Figure 1 HPLC chromatograms showing peaks corresponding to the marker compounds of HPC 03.

before reaching confluence, the cells were subcultured every 3–4 days in a 1:4 ratio.

Proliferation assay of MCF-7 cells (E-screen assay)

Confluent MCF-7 cells were washed twice with phosphate-buffered saline (PBS) (Hyclone Laboratories) and 0.05% trypsin-EDTA solution (Invitrogen) was added for 1 min. After trypsin-EDTA was removed, the culture was left at room temperature for 5–10 min: subsequently, the cells were detached, resuspended in DMEM medium, counted and seeded into 96-well plates at a density of 2×10^4 cells/well. After 24 h, the cells were completely attached to the well bottom: the medium was then aspirated and estrogen-free medium containing both phenol-red-free DMEM (Invitrogen) and 5% charcoal-dextran-stripped human serum (Hyclone Laboratories) was added. MCF-7 cells were treated with different concentrations of HPC 03 and were cultured for 24 h. In addition, HPC 03 was added to the medium at concentrations at which it showed estrogenic activity, either with or without the ER-antagonist tamoxifen (Sigma-Aldrich). 17β-Estradiol and PBS were used as the positive and negative controls, respectively.

MTT proliferation assay

Cell proliferation was assessed using the MTT assay on cells which had been cultured for 24 h. After an incubation period, 100 µL of 5 mg/mL thiazolyl blue tetrazolium bromide (Sigma) solution was added to cells in each well and cells were incubated further for 4 h in a humidified atmosphere at 37°C in an atmosphere of 5% CO_2. The medium was replaced with 1 mL dimethyl sulfoxide. The absorbance was measured at 540 nm by using a microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA), and the cell proliferation values were expressed as a percentage compared with the negative control, which was considered to represent 100% cell proliferation.

ER reporter assays

To assess the activation of human $ER_{\alpha/\beta}$, nuclear receptor reporter assays were performed (Indigo Biosciences, State College, PA, USA). HPC 03 were prepared and diluted in medium provided by the manufacturer. The cell recovery medium provided in the assay kit was thawed, warmed to 37°C and added to the tube of frozen reporter cells. The cell suspension (100 µL) was dispensed into the wells of a 96-well assay plate and incubated for 4 h to allow the cells to attach. HPC 03 (100 µL) was added to the cells at the indicated concentrations and incubated for 24 h. Luciferase activity was quantified using a BioTek Synergy plate reader (Biotek Instruments, Winooski, VT, USA).

Gene expression analysis

Prior to reverse transcription polymerase chain reaction (RT-PCR), total RNA was extracted using a total RNA extraction kit (easy-BLUE, iNtRON Biotechnology, Korea). The RNA isolation protocol included a DNase I treatment step. RNA samples were quantified by the measurement of the optical density at 260 nm. All reaction mixtures contained approximately 100 ng RNA in a reaction volume of 25 µL. The primer and probe concentrations were 300 nM and 200 nM, respectively. The conditions used for real-time quantitative RT-PCR were as follows: 30 min at 48°C (RT, inactivation), 10 min at 95°C (initial activation) and then 40 cycles of amplification for 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension). The following TaqMan Gene Expression Assays (Applied Biosystems) were used as the primers and probes for amplification: *Psen2* (Hs01577197_m1), *Pgr* (Hs015456702_m1), *Ctsd* (Hs00157205_m1) and *Gapdh* (Hs02786624_g1). Data analysis was performed with SDS 2.1.1 software, gene expression levels were normalized to the expression of the *Gapdh* housekeeping gene and relative expression level and PCR efficiency were evaluated (Pfaffl 2001).

Animals

Six-week-old female Sprague–Dawley (SD) rats were purchased from Orientbio (Gyeonggi-do, Korea). The animals were housed in the following conditions: two rats per cage, air-conditioned room at 23 ± 1°C and 55–60% relative humidity, 12-h light/dark cycle and were fed an AIN 76A diet. After a 1-week acclimatization period, 7-week-old female SD rats were anesthetized with 2% isoflurane and the ovaries were removed bilaterally. SHAM surgery ligation and excision of a piece of adipose tissue of the same size was performed near the ovary and was medicated separately. The study was approved by the Wonkwang University Animal Care Committee (WKU16-06). We performed a pilot study with reference to previously published studies to determine the optimal sample size (Choi *et al.* 2012, Zhao *et al.* 2013, Zhang *et al.* 2016, Takakura *et al.* 2017). The pilot study was conducted on groups of SHAM, OVX, OVX + E2 (positive control) and OVX + HPC 03 (200 mg/kg/day) rats for 12 weeks. After killing, the BMD and hormone levels of the OVX + E2 and OVX + HPC 03 groups were determined, which were significantly higher than those of the OVX group. In the present study, we chose to use five animals per group, which was the minimal number of experimental animals that could demonstrate significant differences in the pilot study.

Animal experiment protocol

After an adaptation period of 6 weeks, the rats were divided into six groups SHAM (sham-operated control), OVX (ovariectomized, but untreated), OVX + HPC 03 (ovariectomized rats administered 50, 100 and 200 mg/kg/day HPC 03, respectively) and OVX + E2 (ovariectomized rats administered 10 µg/kg estradiol). All rats were fed daily by gavage for 12 consecutive weeks. The body weight of each rat was measured once a week until the final day of administration. On the last day of the study, serum samples were collected from abdominal aortic artery and stored at −70°C until examination. The uteruses were dissected out and immediately weighed.

Bone density

After anesthetization with ketamine and xylazine (100 mg/kg body weight and 10 mg/kg body weight, respectively), the bone mass density (BMD) of the right femur were measured using a dual-energy X-ray absorptiometer (DEXA: InAlyzer, Medikors, Seungnam, Korea), which was equipped with the appropriate software for the assessment of bone density in small animals.

Hormone assay

Blood samples were collected from each rat before killing. To separate serum, blood was kept at 4°C for 1 h, followed by centrifugations for 300 min at 15,000 ***g*** at 4°C. Serum was kept at −80°C until hormonal assay was performed. The serum estradiol (E2), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations were determined by indirect ELISA method. The kits used for the experiments included Estradiol Kit (Enzo Life Sciences, Inc. NY, USA: minimum detectable concentration (minDC) = 14 pg/mL), LH (Antibodies-Online, Atlanta, GA, USA: minDC = 98.77 pg/mL) and FSH kits (Antibodies-Online, minDC = 2.47 ng/mL). All experiments were performed in accordance with the manufacturer's instructions (Shelton *et al.* 2013, Sewani-Rusike *et al.* 2016, Tomaz *et al.* 2016). Serum samples were added to the appropriate micro ELISA plate wells and combined with the specific antibody. A biotinylated detection antibody specific to E2 and avidin-horseradish peroxidase (HRP) conjugate was then added to each microplate well successively and incubated. Free components were washed away. The substrate solution was added to each well. Only the wells that contained E2, biotinylated detection antibody and avidin-HRP conjugate appeared blue in color. The enzyme–substrate reaction was terminated by the addition of a sulfuric acid solution, and the color turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The OD value was proportional to the concentration of E2. The concentration of serum E2 (pg/mL), LH (pg/mL) and FSH (ng/mL) was then calculated using kit provided standards. LH and FSH measurement were performed in a similar manner using a different ELISA kit.

Biochemical markers of bone

Serum levels of total alkaline phosphatase (ALP), bone-specific ALP (BALP) activities, osteocalcin (OC) and C-telopeptide of type I collagen (CTX-I) were measured using sandwich ELISA kits (ALP: Kamiya Biomedical Company, Tukwila, WA, USA. minDC = 1.24 U/L pg/mL; BALP: Cusabio Biotech, MD, USA. minDC = 3.9 U/L pg/mL; OC: LifeSpan Biosciences, Inc., Seattle, WA, USA. minDC = 15.63 pg/mL; CTX-I: Cusabio Biotech, Baltimore MD, USA. minDC = 31.25 pg/mL). All experiments were performed in accordance with the manufacturer's instructions (Chen *et al.* 2014, Conte Neto *et al.* 2014, Wei *et al.* 2014). Capture antibody were coated onto the wells of microplates. Samples, including a standard containing protein, were pipetted into these wells. During the first incubation, the protein antigen binds to the capture antibody. After washing, a detection antibody is added to the wells, and this antibody binds to the immobilized protein captured during the first incubation. After removal of excess detection antibody, an HRP conjugate was added and bound to the detection antibody. After a third incubation and washing to remove the excess HRP conjugate, a substrate solution is added and is converted by the enzyme to a detectable form. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The concentrations of ALP, BALP, OC and CTX-I in the samples were calculated by comparing the OD of the samples with the standard curve.

Statistical analysis

Data were expressed as mean ± S.D. Significant differences were compared using repeated measures ANOVA followed by the Newman–Keuls multiple range test. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using GraphPad Software.

Results

Effects of HPC 03 on ER-mediated cell proliferation

The proliferative effects of HPC 03 were evaluated relative to negative control cells. E2, which significantly stimulated proliferation of ER-positive MCF-7 cells, was used as the positive control. HPC 03 (25–100 µg/mL) also stimulated MCF-7 proliferation, the maximum effect (131%), which was equivalent to the proliferative effect of 0.1 nM E2 in this cell line, was observed at a concentration of 50 µg/mL HPC 03 and E2-induced proliferation was blocked by 6.4 µM tamoxifen (Fig. 2).

Effects of HPC 03-induced $ER_{\alpha/\beta}$-mediated luciferase activity

HPC 03 exhibited estrogenic activity in human ERα reporter assays at 25–100 µg/mL ($P<0.01$) (Fig. 3A) and human ERβ reporter assays at 50–100 µg/mL ($P<0.01$) (Fig. 3B), which indicated that these effects were ER mediated.

Effects of HPC 03 on the mRNA levels of estrogen-dependent genes in cell in OVX rats

Three estrogen-dependent genes (*Psen2*, *Pgr* and *Ctsd*) in MCF7 cells were selected for the investigation of the HPC 03-induced transcriptional response through ER binding. Real-time PCR analysis showed that the mRNA levels of *Psen2*, *Pgr* and *Ctsd* were significantly increased under these conditions. Estrogen-dependent genes expression induced by HPC 03 and E2 was blocked by addition of tamoxifen indicating an ER-dependent mechanism for the estrogenic effect on MCF-7 cell proliferation (Fig. 4).

Effects of HPC 03 on body weight change and uterine weight in OVX rats

No death occurred in any of the HPC 03 groups to the end of study, and no obvious clinical sign, including loss of fur, changes in skin color, scabbing, eyes and mucous membranes and change in behavior patterns were observed in any of the HPC 03 groups throughout the experimental period (data not shown). In all groups, body weight increased over time, but the increase was significantly more in the OVX group compared with the SHAM group. A significant difference in body weight was observed between the E2-treated group and the OVX group at 1 week after the start of administration (Fig. 5A). As expected, the body weight of

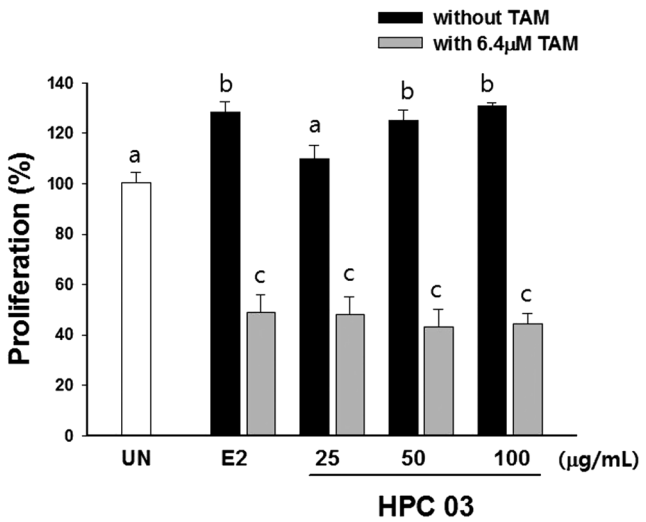

Figure 2 Effects of HPC 03 on MCF-7 cell proliferation. Tamoxifen was used as an estrogen antagonist. The results are presented as the mean ± S.D. Values with different letters (a, b, c) are significantly different one from another (one-way ANOVA followed by Newman–Keuls multiple range test, $P<0.05$).

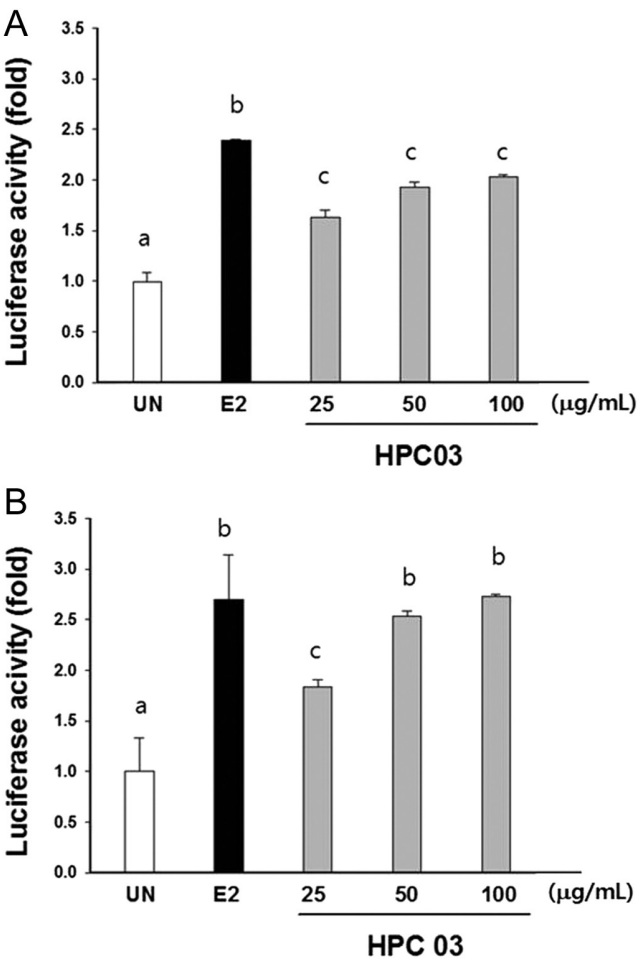

Figure 3 Effects of HPC 03-induced $ER_{\alpha/\beta}$-mediated luciferase activity. (A) ER_{α} and (B) ER_{β} reporter assays of cells treated with the indicated concentrations of HPC 03 for 24 h. The results are presented as the mean ± S.D. Values with different letters (a, b, c) are significantly different one from another (one-way ANOVA followed by Newman–Keuls multiple range test, $P<0.05$).

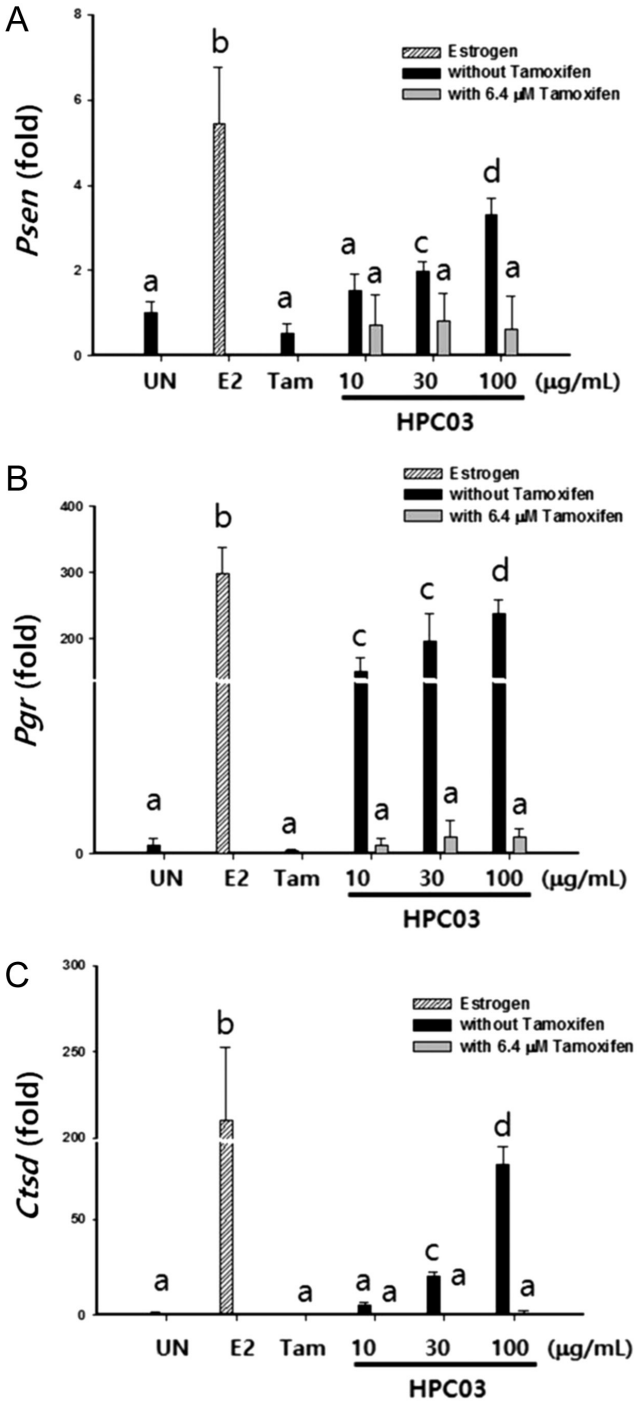

Figure 4 Effects of HPC 03 on the mRNA levels of estrogen-dependent genes. HPC 03 was added to the medium at 10–100 μg/mL where they measured estrogenic activity, either with or without the ER-agonist tamoxifen. mRNA levels of (A) *Psen2*, (B) *Pgr* and (C) *Ctsd* were quantified using real-time PCR. The results are presented as the mean ± S.D. Values with different letters (a, b, c, d) are significantly different from one another (one-way ANOVA followed by Newman–Keuls multiple range test, $P<0.05$).

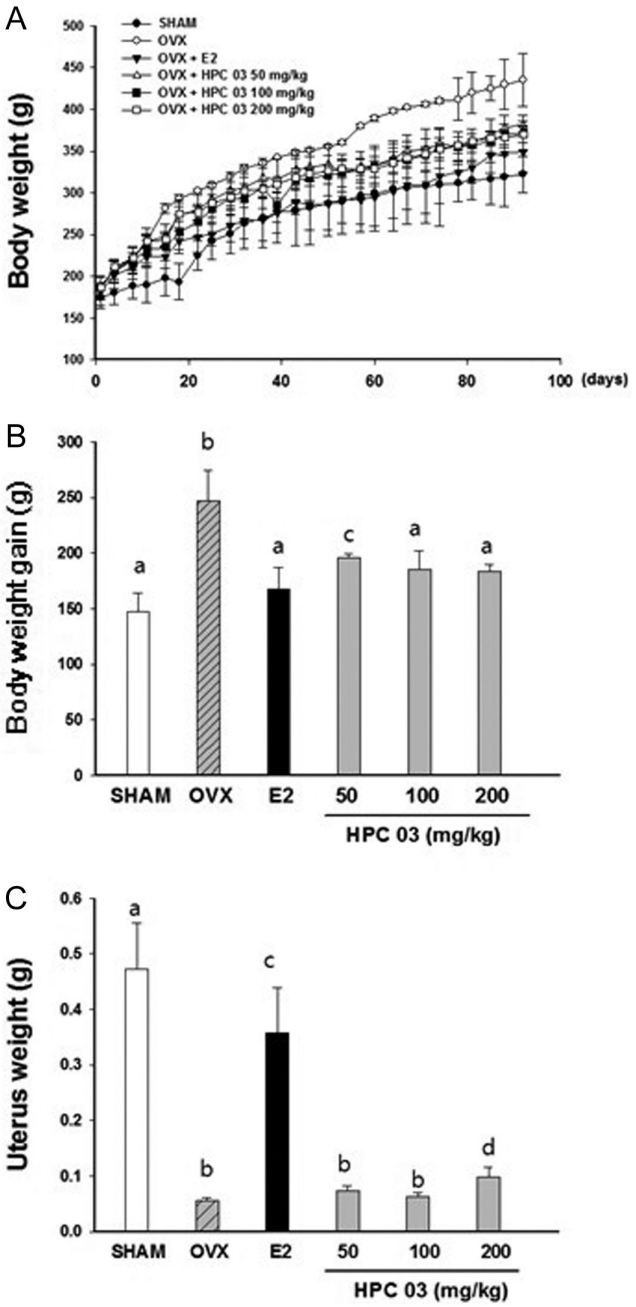

Figure 5 Effects of HPC 03 on the (A) body weight change, (B) body weight gain and (C) uterine weight in OVX rats. During the experimental period, body weight was measured every 3 days. Body weight gain was calculated by the equation: final body weight – initial body weight. The results are presented as the mean ± S.D. Values with different letters (a, b, c, d) are significantly different from one another (one-way ANOVA followed by Newman–Keuls multiple range test, $P<0.05$).

animals in the E2 group was significantly less than that of the OVX group. Furthermore, the body weight gain of animals in the HPC 03-treated group was significantly less than that of the OVX group (Fig. 5B). OVX caused atrophy of uterine tissue, which indicated the success of the surgical procedure in the E2 group, the uterine weight was significantly increased compared with the

OVX group. In the group treated with 200 mg/kg HPC 03, a significant amelioration of the uterine weight decrease caused by the OVX operation was observed (Fig. 5C).

Effects of HPC 03 on BMD in OVX rats

At 3 weeks after the OVX operation, the OVX group showed a significant decrease in the femur BMD compared to the SHAM group (Fig. 6A). After 12 weeks of treatment, the final femur BMD of the 200 mg/kg HPC 03-treated group (0.3203 ± 0.0031 g/cm^2) was significantly higher than that of the OVX group (0.2832 ± 0.0210 g/cm^2). HPC 03 (200 mg/kg) induced a 13% increase in the low femur BMD induced by OVX (Fig. 6B).

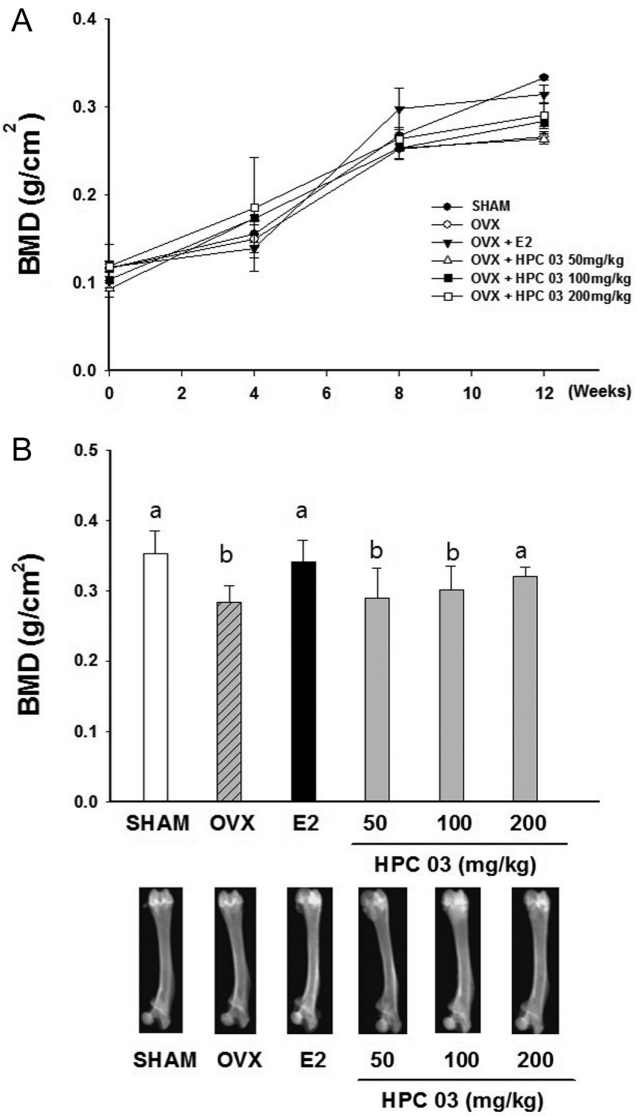

Figure 6 Effects of HPC 03 on (A) femur BMD change and (B) final femur BMD in OVX rats. During the experimental period, BMD was determined after anesthetization every 4 weeks. At the end of the treatment period, the femur was separated and the BMD was determined. The results are presented as the mean ± S.D. Values with different letters (a, b) are significantly different from one another (one-way ANOVA followed by Newman–Keuls multiple range test, $P<0.05$).

Effects of HPC 03 on bone parameter in OVX rats

Levels of ALP, BALP, CTx and OC in the OVX were significantly higher than the SHAM. After 12 weeks of treatment, the HPC 03 displayed significantly lower levels of ALP, BALP, CTx and OC compared with the OVX group. The observed decrease was stronger at higher doses of HPC 03: the highest dose (200 mg/kg) resulted in 29.4, 74.2, 12.1 and 17.9% decreases in ALP, BALP, CTx and OC levels (all $P<0.01$) compared with the OVX group, respectively (Table 1).

Effects of HPC 03 on serum estrogen, LH and FSH in OVX rats

The OVX group was lower levels of serum E2 and higher levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) compared with the SHAM group. HPC 03 treatment at 50, 100 or 200 mg/kg significantly raised the levels of circulating estrogen. HPC 03 (200 mg/kg) induced a 34% increase in the estrogen level compared with that of OVX group, which was comparable to the SHAM group. HPC 03-treated group also had significant inhibitory effects on LH and FSH. The observed decrease was stronger at higher doses: the highest dose (200 mg/kg) resulted in a decrease of 12.4% and 26.2% in LH and FSH, respectively, (both $P<0.01$) compared with the untreated OVX group (Table 2).

Effects of HPC 03 on the mRNA levels of estrogen-dependent genes in OVX rats

The uterus of OVX group was lower expression of *Psen2*, *Pgr* and *Ctsd* compared with the uterus of SHAM group. HPC 03 treatment at 200 mg/kg significantly raised the expression of *Psen2* and *Pgr* compared with the uterus of OVX group (Fig. 7A and B). However, no change in the *Ctsd* was observed with HPC 03-treated group in uterus of OVX group (Fig. 7C).

Discussion

Herbal formulas are widely used as complementary medicines. There is some dispute regarding the use of herbal formula because complex mixtures of medicinal herbs are employed in contrast to isolated single natural products. It is often suggested that herbal formula extracts work synergistically to increase the therapeutic effect while reducing the number of adverse side effects to healthy tissues (Huntley & Ernst 2003, Hiruma *et al.* 2013, Satoh 2013). The present study has shown that

Table 2 Effects of HPC 03 on serum estrogen, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in OVX rats.

Factor	SHAM	OVX	OVX + E2	OVX + HPC 03		
				50 mg/kg	100 mg/kg	200 mg/kg
Estrogen (pg/mL)	62.8 ± 4.0[a]	32.0 ± 2.0[b]	70.0 ± 4.0[a]	42.5 ± 4.5[c]	42.1 ± 5.2[c]	55.0 ± 3.0[c]
LH (pg/mL)	210.0 ± 4.3[a]	295.1 ± 10.4[b]	245.2 ± 4.2[c]	290.4 ± 5.0[b]	279.4 ± 12.6[b]	258.3 ± 10.8[d]
FSH (ng/mL)	65.2 ± 3.4[a]	109.8 ± 7.0[b]	72.4 ± 5.9[c]	102.5 ± 6.2[b]	98.4 ± 6.2[b]	80.4 ± 1.8[c]

Serum levels of estrogen, LH and FSH were measured at the end of the treatment period. Values with different letters (a, b, c, d) are significantly different from one another (one-way ANOVA followed by Newman–Keuls multiple range test, $P < 0.05$).

the herbal formula HPC 03 is a candidate for novel menopause therapies. HPC 03 is an herbal formula containing extracts of *A. gigas*, *C. officinale* Makino and *C. cassia* Presl. as the main ingredients. Although the effects of the individual medicinal herbs have been previously reported (Choi *et al.* 2012, de la Cruz *et al.* 2014, Sun *et al.* 2016), it is the first to report the effects of this herbal formula HPC 03. This study aimed to clarify the effects of HPC 03 in the estrogenic potential *in vitro* and *in vivo*.

Estrogens are known stimulants of cellular proliferation (Soto *et al.* 1995, Lee *et al.* 2012, Resende *et al.* 2013). The proliferation induced by HPC 03 was blocked by the addition of tamoxifen, an estrogen antagonist. These results indicated that HPC 03 was the estrogen-like physiological action. Estrogen and ERs are involved in the physiological function and regulation of the female reproductive system. Under physiological conditions, the biological effects of estrogen depend on not only the level of estrogen, but also the distribution and expression levels of the corresponding ERs in the target cell, ERα and ERβ (Nagaraj & Ma 2015, Warner *et al.* 2017). As the first step in the activation of ERs involves the binding of a ligand, the measurement of ligand binding is important in the characterization of the potential estrogenicity of test materials. Based on data, HPC 03 was thought to bind to ERs by the displacement of E2 binding. The binding of estrogens or selective estrogen-receptor modulators to ERs initiates a molecular signaling cascade that results in the transcriptional regulation of specific genes and protein synthesis (Lee *et al.* 2012, Nagaraj & Ma 2015). Finally, the expression of estrogen-related genes may indicate the presence of a functional estrogen signaling pathway (Mestries *et al.* 1997, Jorgensen *et al.* 2000, Mueller 2002).

Upregulation of the expression of endogenous genes *Psen2, Pgr* and PTGES in MCF-7 cells is indicative of estrogenic effects via ER. In addition to *Psen2*, mRNA expression of the *Pgr*, *Ctsd* and PTGES genes in MCF-7 cells are also regulated in a characteristic manner by estrogenic compounds (Stossi *et al.* 2004, Chang *et al.* 2006). HPC 03 induced mRNA production of all three genes, suggesting that in MCF-7 cells, this herbal formula may act as estrogen agonists.

Ovariectomy is a widely used standard surgical procedure for the study of menopausal symptoms and postmenopausal osteoporosis, because OVX animals are characterized by reduced ovarian function, bone mass and strength (Xu *et al.* 2016, Garcia *et al.* 2017). The success of ovariectomy was also confirmed by examining the serum estradiol level. The serum estradiol levels were markedly decreased in all OVX rats and provided evidence of the success of the ovariectomy. OVX showed a significant 2-fold decrease on serum of estrogen compared with SHAM. This is similar to that observed in previous researches (Sharkey *et al.* 1998, Kang *et al.* 2016, Lee *et al.* 2016*a*). Estrogen, a reproductive hormone, is important in the maintenance of body weight, and estrogen deficiency following menopause is a serious cause of obesity in females. Thus, it is important to inhibit body weight gain to maintain the health of postmenopausal women (Roberts & Hickey 2016). In our research, OVX showed a significant increase in body weight, which was also observed in other studies in comparison with the SHAM. However, HPC 03 resulted in a significant decrease in body weight. Furthermore, HPC 03 also restored the uterine weight decrease observed following OVX. The present study showed that HPC 03 increased serum estrogen levels. This result suggested that HPC 03 may stimulate

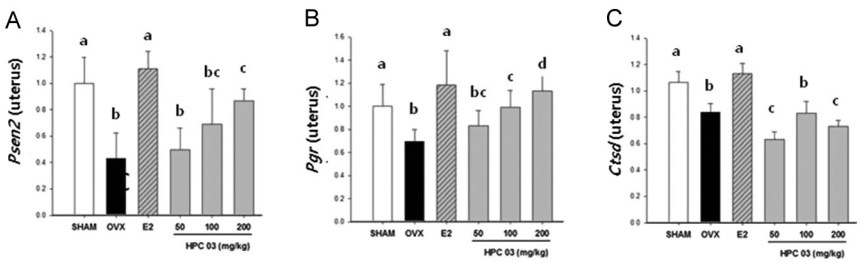

Figure 7 The effect of treatment with HPC 03 on mRNA expression of estrogen-dependent genes in the uterus of OVX rats. At the end of the treatment period, the uterus was separated and then the estrogen-dependent genes were determined. mRNA levels of (A) pS2, (B) PR and (C) cathepsin D were quantified using real-time PCR in uterus. The results are presented as the mean ± S.D. Values with different letters (a, b, c, d) are significantly different one from another (one-way ANOVA followed by Newman–Keuls multiple range test, $P < 0.05$).

the biosynthesis of estrogen. The serum levels of LH and FSH are a marker of the estrogenic effect (Cheng & Tian 2012, Santoro *et al.* 2016). Also, HPC 03-treated group produced estrogen-mediated changes in pS2 and PR mRNA levels in uterus tissue of OVX group.

Bone loss caused by estrogen deficiency in both humans and experimental animals occurs primarily through an increase in osteoclastic bone resorption (Nishizawa *et al.* 2005, Varela *et al.* 2017). OVX rats, which exhibit most of the characteristics of human postmenopausal osteoporosis, have been widely used as a model for the evaluation of potential osteoporosis treatments (Ardawi *et al.* 2016, Xiao *et al.* 2016). OVX rats experienced a significant decrease in the femur BMD after 8 weeks. This BMD loss was accompanied by a significant increase in bone remodeling, which was evidenced by an enhanced bone turnover markers. An increase in ALP and BALP serum levels, the most widely used biochemical bone turnover markers, was observed in OVX rats (Hou *et al.* 2012). Furthermore, an increase in the serum levels of telopeptides of CTx, which correlates with high levels of bone resorption that indicates excessive osteoclastic activity and bone formation marker OC, were observed in OVX rats, and these results were supported by Lim and coworkers (Lim *et al.* 2014). A 12-week treatment with HPC 03 significantly decreased the BMD loss in the femur, which was reflected by the decrease in ALP, BALP, CTx and OC serum levels compared with the OVX: this effect may have resulted from decreased bone resorption (Abuohashish *et al.* 2015). Therefore, HPC 03 appears to be very effective for the inhibition of bone resorption and improvement in bone formation.

In conclusion, HPC 03 enhanced the cell growth and the estrogen-dependent genes such as *Psen2*, *Pgr* and *Ctsd* and stimulated proliferation of ER-positive MCF-7 cells.. HPC 03 attenuates bone loss, uterine atrophy and estrogen depletion in ovariectomized rats. It is thus expected that the HPC 03 may work as a new potent protective agent for relieving especially in terms of hormone imbalance and osteoporosis in menopausal women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors read and approved the final version of the manuscript. S Y K conceived and designed the experiments. B Y C and D E K performed the experiments. B Y C, D E K and H S K analyzed the data. S Y K and B Y C drafted the manuscript. B Y C, D E K, H S K and S Y K revised the manuscript.

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