



## Evaluation of Estrogenic Activity of Extract from the Herbal Mixture *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turczaninow, and *Angelica gigas* Nakai

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Hormone replacement therapy (HRT) consists of highly effective prescription medications for treating menopausal symptoms; however, these agents have exhibited side effects including the risk of estrogen-induced carcinogenesis. Therefore, interest in phytotherapy-based materials as a natural source of alternatives to estrogen therapy has increased. However, some of these herbal medicines have been reported to increase the risk of estrogen-induced cancer. Herbal formulations composed of a combination of *Cynanchum wilfordii* Hemsley (CW), *Phlomis umbrosa* Turczaninow (PU), and *Angelica gigas* Nakai (AG) extracts (CPAE) have been used for treating menopausal symptoms. Therefore, in this study, we aimed to examine the safety of CPAE by determining its potential adverse estrogenic activity using the Organization for Economic Cooperation and Development (OECD) test guideline 455 (TG455) in a stably transfected transcriptionally activated human estrogen receptor  $\alpha$  (hER $\alpha$ )-HeLa9903 cell model. We found that CPAE did not show any estrogenic activity or stimulate promoters containing estrogen response elements in MCF-7 cells. In addition, CPAE showed no significant selective activity against hER $\alpha$  and hER $\beta$ , non-selective activity against the ER, or effects on ER target gene expression. Furthermore, CPAE did not significantly induce MCF-7 cell proliferation and uterine weight increase in ovariectomized rats. These results demonstrate that CPAE can be used as beneficial herbal drug for prevention and therapeutic intervention of estrogen carcinogenesis in menopausal women.

**Key words:** *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turczaninow, *Angelica gigas* Nakai, Estrogenic activity, Estrogen receptor

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Abbreviations: CPAE, *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turczaninow, and *Angelica gigas* Nakai extract; HRT, Hormone replacement therapy; OECD, Organization for Economic Cooperation and Development; hER, human estrogen receptor; CU, *Cynanchum wilfordii* Hemsley; PU, *Phlomis umbrosa* Turczaninow; AG, *Angelica gigas* Nakai; AMA, American Medical Association; MFDS, Ministry of Food and Administration Safety; BMI, body mass index; FSH, follicle-stimulating hormone; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; STTA, stably transfected transcriptional activation; CERL, Chemicals Evaluation and Research Institute; DTT, dithiothreitol; BSA, bovine serum albumin; OVX, ovariectomized; ERE, estrogen response element; PGR, progesterone receptor; TFF1, transcription factor 1.

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## INTRODUCTION

In 1992, the American Medical Association (AMA) published a recommendation that menopausal women should consider the use of hormone replacement therapy (HRT) for treating menopausal symptoms caused by low estrogen (specifically estradiol, E2) levels (1). Since then, HRT has been considered the best alternative for improving menopausal women's health, and its clinical use has drastically increased. However, the Women's Health Initiative performed a multi-center, randomized, double-blind, placebo-controlled study with approximately 16,000 post-menopausal women for 5 years and reported that HRT increased the number of cases of breast cancer, heart disease, and stroke by 26, 29, and 41%, respectively (2).

There has been an increasing interest in developing natural compounds with high efficacy and low risk of adverse effects, such as isoflavones, black cohosh, and pomegranate, as alternative phytotherapies. Unfortunately, the National Institutes of Health reported that genistein, an aglycone of

isoflavone from soybean, shows carcinogenic potential through its binding with estrogen receptors (ER)  $\alpha$  and  $\beta$  (3,4). Although the genistein contents in isoflavone-related ingredients may not be high enough to cause cancer, further investigations are needed to determine the possible side effects of phytoestrogens.

*Cynanchum wilfordii* Hemsley (CW), *Phlomis umbrosa* Turczaninow (PU), and *Angelica gigas* Nakai (AG) have been used for centuries as traditional herbal treatments and are classified as raw materials for food ingredients by the Ministry of Food and Administration Safety (MFDS). The use of these traditional herbs was described in the Dong-Eui-Bo-Gam, the most famous book on Korean traditional medicine, which is an encyclopedic guideline of medical knowledge and treatment techniques first compiled by Joon Heo in 1613. These herbal treatments have shown various beneficial activities including anti-inflammation, antioxidant, anticancer, and anti-atherosclerosis. Recently, the extracts of CW, PU, and AG were reported to have beneficial effects as an alternative HRT (5-8).

The effectiveness of an herbal formulation composed of a combination of CW, PU, and AG extracts (CPAE; brand name, EstroG-100™) has been proven in several animal studies and two randomized, double-blind, placebo-controlled clinical studies. The positive data obtained from the 1-year clinical study with 48 patients revealed that the formulation significantly ameliorated various menopausal symptoms in South Korea. At the same time, it did not show any serious side effects or increase body weight, body mass index (BMI), and serum levels of E2 and follicle stimulating hormone (FSH) (9). In addition, a randomized, double-blinded, placebo-controlled clinical study was performed in non-Asian American women at Friends Medical Group and Shady Canyon Medical Group in CA, USA. In this study, the CPAE-treated group showed a more significant improvement in various menopausal symptoms such as hot flashes and night sweats, paresthesia, insomnia, nervousness, melancholia, vertigo, fatigue, rheumatic pain, and vaginal dryness than the placebo group did. Furthermore, no adverse effects or significant changes were observed in body weight, BMI, serum E2 and FSH levels, and liver enzymes that showed changes following treatment with HRT and other phytoestrogens (10).

However, to further confirm the safety of CPAE in terms of its estrogenic activity, it has been tested in estrogen-mimicking activity assays according to the Organization for Economic Cooperation and Development (OECD) guideline 455 (11).

## MATERIALS AND METHODS

**Plant material and preparation of CPAE.** To prepare the CPAE, CW and PU were purchased from Jirisan Farming Association (Sancheong, Gyeongsangnam-do, Korea)

and Baocheng Chinese Herbal Med Co., Ltd. (Hunan, China), in July 2015, respectively, while AG was provided by Yeongdong Herb Medicine Farming Association (Jecheon, Chungcheongbuk-do, Korea) in October 2015. CW, PU, and AG were mixed at a ratio of 1 : 1 : 1.08, extracted with distilled water (1 : 8 w/v) by boiling under reflux for 8 hr, cooled to room temperature for 30 min, and filtered using Whatman # 4 filter paper (Whatman, Maidstone, MA, USA). Then, the filtrate was subsequently evaporated using a rotary evaporator (R-215, Buchi, Switzerland) under reduced pressure until 40°Brix, lyophilized using a freeze drier (DW-86L728, Haier, Qingdao, China), and then the dry residues were dissolved in 40% dimethyl sulfoxide (DMSO) to a concentration of 40 mg/mL for the *in vitro* assays.

**Cells and culture medium.** The human estrogen receptor  $\alpha$  (hER $\alpha$ )-expressing HeLa-9903 cell line (HeLa9903), which is stably transfected with a human *ER $\alpha$*  gene containing a luciferase reporter gene, was provided by the Chemicals Evaluation and Research Institute (CERI), Japan. The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in a humidified 5% CO<sub>2</sub> incubator at 37°C with 10% dextran-coated charcoal-treated fetal bovine serum (FBS) and phenol-free Dulbecco's modified Eagle's medium (DMEM).

**Stably transfected transcriptional activation (STTA) assay.** The luciferase activities were determined in accordance with the OECD TG 455 method (11). HeLa9903 cells were exposed to reference chemicals and the serially diluted test extract, followed by incubation for 20–24 hr (at 37°C in an atmosphere of 5% CO<sub>2</sub>). The cell preparations were then lysed, centrifuged (12,000 rpm, 10 min), and the supernatant was assayed for luciferase activity. Each compound was tested in three independent experiments in triplicate and all the plates contained E2 as a positive control.

**Cell-based ER-mediated luciferase activity.** MCF-7 cells were transfected with 1  $\mu$ g human estrogen response element (ERE)-Luc reporter plasmid (Addgene plasmid no. 11354), 0.2  $\mu$ g pCMV- $\beta$ -gal (Addgene plasmid no. 155), or both per well using Lipofectamine™ 2000 (Invitrogen, Paisley, UK). Then, 6 hr after transfection, the cells were maintained for at least 24 hr in DMEM without phenol red (supplemented with 10% FBS previously treated with charcoal dextran), followed by incubation with CPAE, E2, ICI 182,780 (ICI, ER antagonist), or a combination of these compounds for 24 hr. Luciferase and  $\beta$ -galactosidase activities were subsequently measured in the cellular extracts as described previously (12).

**Estrogen ligand-binding affinity assay.** The binding ability of the test compound to the control or recombinant

full-length hER $\alpha$  was assessed as described previously (13). Briefly, hER $\alpha$  (2088 pmol/mg estrogen binding activity; Invitrogen Corp., Carlsbad, CA, USA) as diluted to a concentration of 5 nM with a binding buffer containing 10 mM Tris (pH 7.5), 10% glycerol, 1 mM dithiothreitol (DTT), and 1 mg/mL bovine serum albumin (BSA). hER $\alpha$  was labeled with [ $^3$ H]-E2 (3 nM) in the presence or absence of  $10^{-6}$  M unlabeled E2 or CPAE (10 or 100  $\mu$ g/mL). For the hER $\beta$  binding assay, hER $\beta$  (4500 pmol/mg estrogen binding activity; Invitrogen Corp.) was diluted to a concentration of 10 nM with the binding buffer and labeled with [ $^3$ H]-E2 (10 nM) in the presence or absence of  $10^{-6}$  M unlabeled E2 or CPAE. After incubation for 2 hr at 27°C, the reactions were terminated by rapid filtration through glass fiber filters (Packard Instrument B.V.-Chemical Operations, Groningen, The Netherlands) presoaked with ice-cold 0.05% polyethylenimine using a filter mate harvester (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). The filters were washed five times with ice-cold buffer, dried, and then placed in scintillation vials containing 3 mL Packard Ultima Gold scintillation cocktail (Canberra Packard; Frankfurt, Germany). After shaking the vials and allowing them to equilibrate overnight, the radioactivity trapped in each filter was measured using a Packard 2000CA liquid scintillation counter. Non-specific [ $^3$ H]-E2 binding was determined in the presence of  $10^{-6}$  M unlabeled E2. The specific binding percentage of each ER was determined as follows: [(d.p.m. sample) – (d.p.m. non-specific binding)]/[(d.p.m. control) – (d.p.m. non-specific binding)]  $\times$  100%.

**Estrogen-related gene expression.** One-Step RT-PCR System (Invitrogen) as described previously (14). The product formation was monitored continuously during the polymerase chain reaction (PCR) using the Sequence Detection System software (ver. 1.7, Applied Biosystems, Foster City, CA, USA). The accumulated PCR products were detected directly by monitoring the increase in SYBR $^{\circledR}$  reporter dye fluorescence. The mRNA expression levels of E2-inducible genes (progesterone receptor [PGR] and trefoil factor 1 [TFF1]) in the MCF-7 cells treated with CPAE, E2, ICI, or a combination of these compounds were compared to those in the control cells at each time point using the comparative cycle threshold (C) method. The following primer sequences were used: *hPGR*, forward, 5'-GAC GTG GAG GGC GCA TAT-3' and reverse, 5'-GCA GTC CGC TGT CCT TTT CT3'; *hTFF1*, forward, 5'-CCC TCC CAG TGT GCA AAT A-3' and reverse, 5'-CTG GAG GGA CGT CGA TGG TA-3'; and *h $\beta$ -ACTIN*, forward, 5'-TGG CAC CCA GCA CAA TGA A-3' and reverse, 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of  *$\beta$ -ACTIN*, which was the housekeeping gene.

**Cell proliferation assay (E-screen assay).** The viability of MCF-7 cells was assessed using the water-soluble tetrazolium (WST)-1 (Roche Molecular Biochemicals, Mannheim, Germany). The cells (10,000 cells/well) were seeded in 96-well plates for 24 hr, followed by treatment with CPAE, E2, ICI, or a combination of these compounds and then they were incubated for 3 days. Based on the manufacturer's instructions, we added 10  $\mu$ L of the WST-1 cell proliferation reagent to each well, incubated the plates for 2 hr at 37°C, and then the cell viability was quantified by measuring the absorbance at 440 nm using a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, USA).

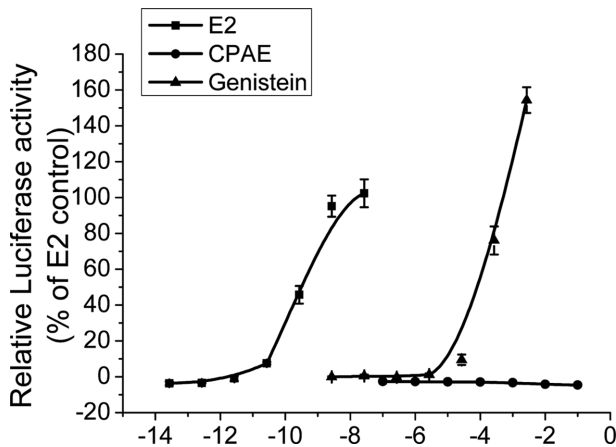
**Animals and experimental design.** All the animal experiments were performed in accordance with the guidelines of the MFDS. Specific-pathogen-free (SPF), 10-week-old female Sprague-Dawley rats were obtained from KOATECH Korea. The rats were acclimatized for at least 1 week prior to use, and were housed in an air-conditioned room with a 12-hr light/dark cycle at a temperature of  $22 \pm 2^\circ\text{C}$  and  $50 \pm 15.0\%$  humidity. The rats were anesthetized with tribromoethanol (Avertin), and either sham-operated (SHAM, controls,  $n = 7$ ) or bilaterally ovariectomized (OVX,  $n = 28$ ) surgically. One week after surgery, the OVX rats were divided into the following four groups of seven rats each and treated as indicated: OVX control group, E2 (10  $\mu$ g/kg), and low- and high-dose CPAE (125 and 250 mg/kg, respectively) groups. The CPAE was dissolved in saline prior to administration for 12 weeks, and the SHAM and OVX control rats were orally administered the same volume of saline alone. After the treatment period, the rat uteri were quickly removed, the connective tissue was excised, and then they were nicked, blotted, and weighed. The effects of CPAE on the uterus wet weight were determined by normalizing the uterine absolute and wet weights to the respective body weights of each rat. The animal experiments were conducted after approval (WJIACUC20160120-1-01) by WOJUNG Life Science Research Center (WOJUNGBSC, Suwon, Korea).

**Statistical analysis.** All experiments were repeated at least thrice, and a one-way analysis of variance (ANOVA) was used to determine the significance of differences between the treatment groups. The Newman-Keuls test was used for multi-group comparisons and a  $p$ -value  $< 0.05$  was considered statistically significant.

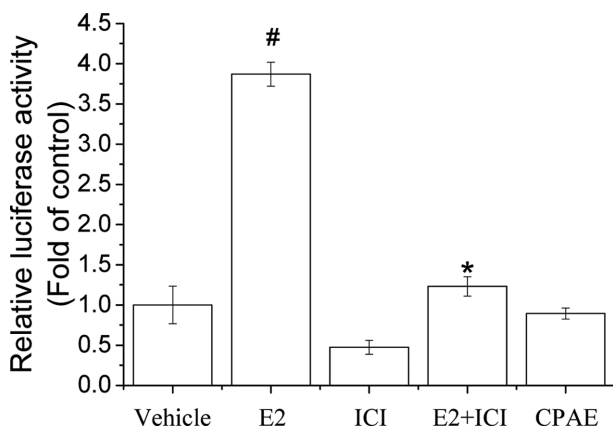
## RESULTS AND DISCUSSION

**Estrogenic activity of CPAE in STTA assay.** The STTA assay developed by the CER1 was accepted as the official TG 455 in 2009 (11). This assay is used to evaluate the ability of chemicals to function as ER $\alpha$  ligands and induce ago-

nistic responses in HeLa9903 cells stably transfected with the *hERα-Luc* gene. In the STTA assay performed in this study, CPAE did not exhibit any estrogenic activity at any tested concentrations while E2 and genistein, well-known phytoestrogens used as reference chemicals, showed significant estrogenic activity in a concentration-dependent manner (Fig. 1).



**Fig. 1.** Concentration-response curve of transcriptional activation by *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turczaninow, and *Angelica gigas* Nakai extracts (CPAE) using HeLa9903 cells. Estradiol (E2,  $10^{-13.6}$ - $10^{-7.6}$  g/L,  $10^{-4}$ - $10^2$  pM), genistein ( $10^{-8.6}$ - $10^{-2.6}$  g/L,  $10^{-6}$ - $10$   $\mu$ M), and CPAE ( $10^{-7}$ - $10^{-1}$  g/L). Cells were harvested and assayed for luciferase activity. All experiments were performed in triplicate. Bars represent mean  $\pm$  SD.



**Fig. 2.** Effect of estrogen response element (ERE) luciferase promoter activity induced by *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turczaninow, and *Angelica gigas* Nakai extract (CPAE) in MCF-7 cells. MCF-7 cells were transfected with pERE-Luc, treated with CPAE (100  $\mu$ g/mL), estradiol (E2; 10 nM), ICI 182,780 (ICI, 10  $\mu$ M) or a combination of these compounds for 24 hr. Cells were harvested and assayed for luciferase activity, which was normalized to  $\beta$ -galactosidase activity. All experiments were performed in triplicate. Bars represent mean  $\pm$  SD. # $P$  < 0.05 vs. control and \* $P$  < 0.05 vs. E2.

### Estrogenic activity of CPAE in pERE-Luc transfected MCF-7 cells.

MCF-7 human breast cancer cells were also used to examine the estrogenic activity of CPAE. To determine if CPAE has classical ER agonist activity, we transiently transfected MCF-7 cells with an ERE-luciferase reporter gene construct and used this system to indirectly measure the transcription induced by ERs activated by CPAE. We found that 100  $\mu$ g/mL CPAE did not induce luciferase activity while E2 caused a robust response (Fig. 2). To confirm the reliability of the transient transfection assays, the ER antagonist ICI was used and it strongly inhibited E2-induced luciferase activity. This observation suggests that CPAE did not show ERE-mediated estrogenic activity in MCF-7 cells. The extract of *Larrea nitida* is used to treat menstrual pain in South America and was reported to have no significant effect on *ERE-Luc* gene expression (15), which is similar to our present result.

### Competitive binding affinities of CPAE to ERs.

The comparative ER binding assay is also generally used to detect ligand-receptor binding and measure the affinity of chemicals to ER receptor proteins (16). The binding affinities of CPAE to the ERs as an hER ligand were determined based on its ability to displace [ $^3$ H]-E2 bound either the hER $\alpha$  or recombinant purified ERs, and the binding of the E2 standard was set to 100. As expected, E2 showed strong affinities in the ER binding assay while CPAE showed low binding affinities for hER $\alpha$ , hER $\beta$ , and non-selective ER (Table 1). A previous study also reported that none of the compounds contained in the extracts of *Cimicifuga racemosa*, one of the most promising available alternative therapies to HRT, was bound to the recombinant ER $\alpha$  or ER $\beta$  (17). These results suggest that CPAE does not have significant effects on ER signaling and the associated adverse effects.

### CPAE stimulates estrogen-related genes in MCF-7 cells.

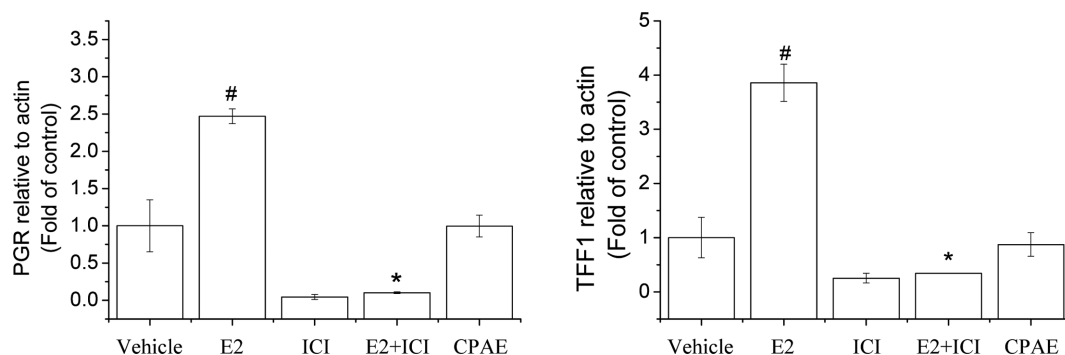
To elucidate the specificity of the estrogenic response, we assessed the effects of CPAE on the expression of estrogen-regulated genes, *PGR* and *TFPI*, which are known E2-inducible genes in MCF-7 cells. American Society of Clini-

**Table 1.** Competitive binding affinities of CPAE to ERs

Type	CPAE concentration ( $\mu$ g/mL)	Inhibition rate (%)*
ER $\alpha$	100	21 $\pm$ 5.3
	10	3 $\pm$ 6.2
ER $\beta$	100	20 $\pm$ 2.3
	10	8 $\pm$ 1.8
Non-selective ER	100	11 $\pm$ 6.8
	10	-2 $\pm$ 6.3

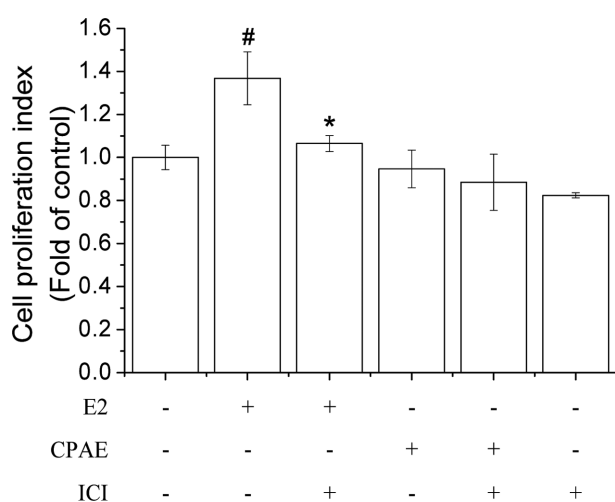
No significant results noted.

\*Inhibition rate of  $\geq$  50% is considered to be significant.



**Fig. 3.** Gene expression of progesterone receptor (*PGR*) and trefoil factor 1 (*TFF1*) in MCF-7 cells. Effect of CPAE (100  $\mu$ g/mL) on *PGR* and *TFF1* mRNA levels determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). All experiments were performed in triplicate. Bars represent mean  $\pm$  SD. # $P$  < 0.05 vs. control and \* $P$  < 0.05 vs. E2.

cal Oncology (ASCO) regards *PGR* as a standard marker in all invasive breast cancers (18). Activation of *TFF1* gene is one of the earliest responses to E2 treatment (19), and while CPAE did not significantly change the expression of *TFF1* and *PGR*, E2 increased the expression of these estrogen-regulated genes (Fig. 3). To confirm the reliability of the observed ER-mediated change in gene expression, we used an ER antagonist, ICI, which strongly inhibited the E2-induced gene expression of *TFF1* and *PGR*. A diarylheptanoid isolated from *Curcuma comosa* Roxb. recently identified as a phytoestrogen did not significantly change the gene expression of *TFF1* and *PGR* (20). This observation indicates that potent oncogenes induced by E2 may not be associated with the use of CPAE treatment in menopausal women.

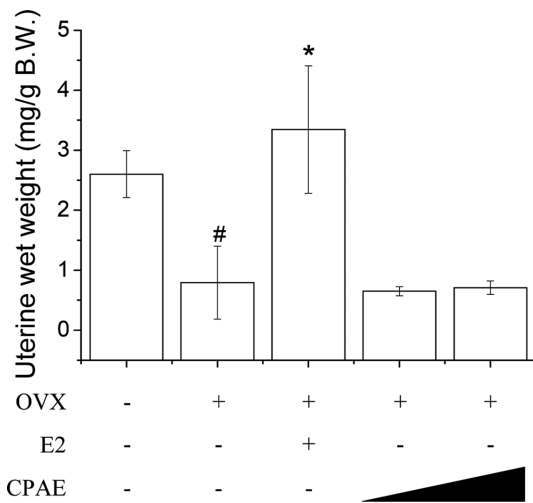


**Fig. 4.** Effects of *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turczaninow, and *Angelica gigas* Nakai extract (CPAE) on MCF-7 cell growth. MCF-7 cells were exposed to CPAE (100  $\mu$ g/mL), estradiol (E2; 10 nM), ICI 182,780 (ICI, 10  $\mu$ M) or a combination of these compounds for 24 hr. Cell proliferation was measured using water-soluble tetrazolium (WST)-1 assay. Bars represent mean  $\pm$  SD. # $P$  < 0.05 vs. control and \* $P$  < 0.05 vs. E2.

**Effect of CPAE on growth of MCF-7 cells.** The E-screen assay is an MCF-7 cell proliferation assay that measures the number of target cells during the exponential phase of proliferation (21). The MCF-7 cell line is known to be a hormone-dependent breast cancer cell line that expresses both ER $\alpha$  and ER $\beta$ . We attempted to determine if CPAE exposure affects the proliferation of MCF-7 cells. The result showed that the CPAE did not affect the proliferation of MCF-7 cells compared with the effects of the positive control E2 (Fig. 4), indicating that CPAE did not affect the development of E2-mediated breast cancer. Wild yam extract, which is known as “natural progesterone” and popularly used to alleviate menopausal symptoms, was reported not to induce the growth of MCF-7 cells (22), which is similar to our present result.

**In vivo effect of CPAE in uterotrophic assay.** An *in vivo* rodent uterotrophic assay was developed in which the estrogen-induced uterotrophic response was estimated based on an increase in the uterine tissue mass in OVX rats. Several studies have reported that some phytoestrogen-rich herbs inhibit bone loss in OVX rats without inducing any or only mild increases in uterine weight (23-25). CPAE was tested *in vivo* to determine if it induces uterine weight gain in rats and the results show that it did not increase uterine wet weight; however, E2 significantly increased both the absolute and relative wet weight compared to that of animals receiving saline only. The result demonstrated that CPAE was not estrogenic in adult female Sprague-Dawley rats *in vivo* (Fig. 5). A previous study reported that the mixture of CW and PU extracts decreased the uterus wall thickness and increased the body weight of OVX rats (26), which is consistent with our *in vivo* data.

In conclusion, our present study provided evidence that CPAE does not show any estrogenic effects in the STTA and ERE reporter assays on HeLa9903 and MCF-7 cells, respectively. CPAE showed no significant activity against hER $\alpha$ , hER $\beta$ , and non-selective ER, as well as the expres-



**Fig. 5.** Effects of *Cynanchum wilfordii* Hemsley, *Phlomis umbrosa* Turczaninow, and *Angelica gigas* Nakai extract (CPAE) in ovariectomized (OVX) rat uterotrophic assay. Each compound was administered subcutaneously at indicated concentration (125 and 250 mg/kg per day) for 12 weeks. # $P < 0.05$  vs. sham group and \* $P < 0.05$  vs. OVX group.

sion of ER target genes. Furthermore, CPAE showed no significant effects in the E-screening and uterotrophic assays. These results suggest that CPAE may be a beneficial herbal medicine for the treatment of menopausal symptoms in women without adverse estrogenic activity.

### CONFLICT OF INTEREST

None declared by all authors.

### ACKNOWLEDGMENTS

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