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Inhibition of estrogen signaling through depletion of estrogen receptor alpha by ursolic acid and betulinic acid from *Prunella vulgaris* var. *lilacina*

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ABSTRACT

Extracts of *Prunella vulgaris* have been shown to exert antiestrogenic effects. To identify the compounds responsible for these actions, we isolated the constituents of *P. vulgaris* and tested their individual antiestrogenic effects. Rosmarinic acid, caffeic acid, ursolic acid (UA), oleanolic acid, hyperoside, rutin and betulinic acid (BA) were isolated from the flower stalks of *P. vulgaris* var. *lilacina* Nakai (Labiatae). Among these constituents, UA and BA showed significant antiestrogenic effects, measured as a decrease in the mRNA level of GREB1, an estrogen-responsive protein; the effects of BA were stronger than those of UA. UA and BA were capable of suppressing estrogen response element (ERE)-dependent luciferase activity and expression of estrogen-responsive genes in response to exposure to estradiol, further supporting the suppressive role of these compounds in estrogen-induced signaling. However, neither UA nor BA was capable of suppressing estrogen signaling in cells ectopically overexpressing estrogen receptor α (ERα). Furthermore, both mRNA and protein levels of ERα were reduced by treatment with UA or BA, suggesting that UA and BA inhibit estrogen signaling by suppressing the expression of ERα. Interestingly, both compounds enhanced prostate-specific antigen promoter activity. Collectively, these findings demonstrate that UA and BA are responsible for the antiestrogenic effects of *P. vulgaris* and suggest their potential use as therapeutic agents against estrogen-dependent tumors.

1. Introduction

Breast cancer is the most prevalent cancer and the reading cause of cancer-related death in woman [1]. Because estrogen signaling is crucial for breast cancer cell growth, strategies designed to suppress estrogen signaling have been used to treat breast cancer patients. For instance, nonsteroidal selective estrogen receptor modulators (SERMs) such as tamoxifen and toremifene, which directly interact with estrogen receptors and suppress their function, have been widely used as adjuvant hormone therapeutic agents to treat breast cancer patients [2,3]. However, 30–40% of patients with estrogen receptor (ER)-positive tumors eventually develop resistance to tamoxifen and experience metastases [4,5]. In addition, tamoxifen is known to increase the risk of uterine cancer and bone loss. Raloxifene, a representative of a different class of SERM that exerts estrogenic effects on bones and antiestrogenic effects on the uterus and breast, has been used clinically to prevent osteoporosis in postmenopausal women [6]. In addition to SERMs, aromatase inhibitors such as exemestane, letrozole, and anastrozole have been developed to treat post-menopausal breast cancer patients by suppressing the production of estrogen [7]. Efforts have been made to identify natural compounds from medicinal herbs that are capable of regulating ER-mediated signaling [8,9]. Although several phytoestrogens, including ER-β-specific antagonists, have been identified, few compounds were found to exert antiestrogenic effects [10,11].

Prunella vulgaris, a perennial plant that is widely distributed in East Asia and Europe, has been commonly used as a traditional herbal medicine to treat a variety of symptoms, including hypertension, inflammation, sore throat, and fever. Several studies have demonstrated diverse biological activities of *P. vulgaris* extracts, including anti-microbial, anti-cancer, and anti-inflammatory

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actions [12–16]. *P. vulgaris*, a perennial plant that is widely distributed in East Asia and Europe, has been commonly used as a traditional herbal medicine to treat a variety of symptoms, including hypertension, inflammation, sore throat, and fever. Several studies have demonstrated diverse biological activities of *P. vulgaris* extracts, including anti-microbial, anti-cancer, and anti-inflammatory actions [17–19].

A recent study demonstrated *in vitro* and *in vivo* antiestrogenic effects of *P. vulgaris* extracts [20]. In the current study, we sought to identify biologically active compounds that are responsible for these antiestrogenic effects and establish the mechanism by which they suppress ER-mediated signaling.

2. Materials and methods

2.1. Plant materials

Spikes of *P. vulgaris* var. *lilacina* Nakai (Labiatae) used in this study were purchased from an herbal supplier (Inno-Pharm, Seoul, Korea). A voucher specimen (KHUOPS-08-33) has been deposited at the herbarium of the College of Pharmacy, Kyung Hee University (Seoul, Korea).

2.2. Plasmids and reagents

A 2× ERE (estrogen response element)-luciferase reporter and ERα expression plasmids were kindly provided by Prof. M.R. Stallcup (Univ. of Southern California). The ERβ expression plasmid was a kind gift from Prof. J.H. Choi (Kyung Hee Univ). The anti-ER antibody was purchased from Santa Cruz Biotechnology (sc-8002).

2.3. Cell culture and transfection

The MCF7 human breast cancer cell line and LNCaP (CRL-1740) human prostate cancer cell line were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). For luciferase assays and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) assays using 17β-estradiol (E2), MCF7 cells were maintained in phenol red-free RPMI containing 10% charcoal-stripped FBS for 1 day before E2 treatment. For transfection experiments, plasmids were introduced into MCF7 and LNCaP cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Extraction and isolation of compounds

Dried spikes of *P. vulgaris* var. *lilacina* (1 kg) were extracted twice with 1 L of 70% ethanol (EtOH) at 60 °C in an ultrasonic bath for 2 h. The extract was filtered, concentrated *in vacuo*, frozen, and lyophilized to give a 70% EtOH extract (100 g). The 70% EtOH extract was subjected to column chromatography on Diaion HP20 (Φ 6.9 × 70 cm) and eluted with an MeOH–H_2O gradient from 0:1 to 1:0, generating 15 fractions (F01−F15). Fraction 3 (F03; 10.8 g) was chromatographed over silica gel (6.5 × 32.5 cm, 70–230 mesh) and eluted with an EtOAc–MeOH–H_2O mixture (7.5:2:0.5) to give 6 subfractions (F03-1 to F03-6). Caffeic acid (54 mg) was isolated from fraction F03-2 by a reversed-phase column chromatography (Φ 2.8 × 21.5 cm, ODS 12 nm S-150 mm), eluting with acetonitrile:H_2O (2:8 v/v). A portion of fraction F07 (3 g/15.93 g) was chromatographed over reversed-phase silica gel (Φ 4.8 × 45 cm, 12 nm S-150 mm) and eluted with an MeOH–H_2O gradient from 3:7 to 6:4 (v/v) to give rosmarinic acid (450 mg). Rutin (240 mg) and hyperoside (36 mg) were obtained from fraction F10 (9.07 g) by silica gel column chromatography (Φ 6 × 48 cm, 230–400 mesh), eluting with CH_2Cl_2–MeOH–H_2O (7:2.7:0.3–6:3.5:0.5). Finally, ursolic acid (1.2 g), oleanolic acid (1.0 g), and betulinic acid (21 mg) were purified from fraction F14 (10.91) by silica gel column chromatography (Φ 6 × 42 cm, 230–400 mesh) using CH_2Cl_2–EtOAc–MeOH (13:6:1, v/v) as the mobile phase. The purity of isolates (>95%) was determined by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy. The structures of rosmarinic acid [21], caffeic acid [22], ursolic acid [23], betulinic acid [24], hyperoside [13], and rutin [12] were identified based on physical and spectroscopic data measurement and by comparison with published values. Analysis of purified compounds by Nuclear magnetic resonance was performed by the Korea Basic Science Institute (KBSI).

2.5. RNA preparation and RT-qPCR

Expression of GREB1 (growth regulation by estrogen in breast cancer 1), an estrogen- responsive gene, was analyzed by RT-qPCR using CFX-9000 (Bio-Rad) real-time PCR. Total RNA was extracted using an RNeasy RNA extraction kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen) with oligo$_{20}$(dT) primers. Two microliters of synthesized cDNA was used as a template for quantitative PCR. The following primer pairs were used for qPCR: GREB1, 5′-gtggtagccgagtggacaat-3′ (sense) and 5′-aaacccgtctgtggtacagc-3′ (antisense); ERα, 5′-tgtccagccaccaaccagt-3′ (sense) and 5′-tttcaacattctccctcctctt-3′ (antisense); and actin, 5′-gggaaatcgtgcgtgacatt-3′ (sense) and 5′-ggagttgaaggtagtttcgt-3′ (antisense).

2.6. Luciferase assay

Estrogen receptor activation was assessed by luciferase assays using an ERE-Luc (firefly luciferase) reporter plasmid. After seeding MCF7 cells on 24-well plates and incubating for 1 day, cells were co-transfected with ERE-Luc (250 ng) and thymidine kinase (TR)-Renilla expression plasmid (pRL-TK, 100 ng) and treated as described in the text and figure legends. Activation of the androgen receptor (AR) was assessed by transfecting LNCaP cells with a prostate specific antigen (PSA)-enhancer/promoter-luciferase reporter construct or mouse mammary tumor virus (MMTV) enhancer/promoter together with TK-Renilla expression plasmid. Luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions.

2.7. Immunoblotting

MCF7 cells were treated with different concentrations of UA and BA for 24 h. After lysing cells with RIPA buffer, proteins were resolved by SDS–PAGE and immunoblotted using anti-ERα and anti-β-actin antibodies. The densities of protein bands were analyzed using FusionCapt Advance (Vilber Lourmat) software.

2.8. Tumor-cell cytotoxicity assay

Cytotoxic activities of UA and BA were determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay. MCF7 cells in a 96-well plate were cultured in DMEM containing increasing concentrations of the indicated compounds. After incubation for 24- or 48 h, the culture medium was replaced with fresh medium containing 20 μl of MTT (5 mg/ml) and cells were incubated for an additional 4 h. Thereafter, the MTT-containing medium was removed by aspiration, and 200 μl of DMSO was added to lyse the cells and solubilize the resulting water-insoluble formazan crystals. The absorbance of lysates was determined at 570 nm using a microplate reader.

3. Results and discussion

3.1. Screening of antiestrogenic compounds of P. vulgaris

Since *P. vulgaris* has been shown to exhibit antiestrogenic effects, we sought to identify the compounds responsible for the activity. Among the compounds isolated from *P. vulgaris* extract were hyperoside, oleanolic acid, UA, caffeic acid, rutin, rosmarinic acid and BA (Fig. 1A). To analyze the effect of the compounds on ER-mediated transcriptional activity, we first treated human MCF7 breast cancer cells with each purified compound (20 μg/ml) and examined the mRNA expression of the ER target GREB1. As shown in (Fig. 1B), UA and BA significant inhibited the synthesis of GREB1 mRNA, suggesting a role for these compounds in mediating the suppressive effects of *P. vulgaris* extract on estrogen-mediated signaling. In addition, both UA and BA decreased ERα mRNA expression (Fig. 1C). The pentacyclic triterpenoid compounds, UA and BA, have been reported to possess numerous biological and pharmacological activities. For example, previous studies have demonstrated *in vitro* and *in vivo* anti-cancer effects of BA and UA on various cancer cell types, including breast cancer, prostate cancer, colon cancer, and pancreatic cancer [25–28]. A number of mechanisms have been suggested to account for the anti-tumor effects of these compounds, including induction of mitochondria-dependent apoptosis, transcriptional regulation, and anti-angiogenic activity [16,28–33]. However, no previous studies have reported a direct effect of UA or BA on estrogen signaling.

3.2. Inhibition of ER-mediated signaling by ursolic acid and betulinic acid

The effects of UA and BA on the estrogen signaling pathway were confirmed by luciferase assays using luciferase reporter plasmids containing estrogen-response elements (ERE). Both UA (40 μM) and BA (40 μM) significantly suppressed ERE-dependent luciferase activity (Fig. 2A); similar effects were observed for *P. vulgaris* extract, consistent with a previous report. In stark contrast, both UA and BA significantly increased PSA promoter- and MMTV enhancer/promoter-reporter activity in LNCaP cells, suggesting that the transcription-inhibiting effects of UA and BA are specific for estrogen-mediated signaling (Fig. 2B and C). Although the mechanism responsible for activation of AR-mediated signaling is not clear, a previous study showed that UA efficiently inhibits aromatase enzyme activity [34], which catalyzes the conversion of androstenedione to estrone and testosterone to estradiol. Accordingly, inhibition of aromatase activity by UA could result in depletion of estradiol and accumulation of androgen [35], leading to enhanced androgen-mediated transcriptional activity. Whether this mechanism is actually responsible for the androgen-related effects of UA and whether a similar mechanism is responsible for the actions of BA remain to be determined.

To test whether BA and UA suppress ER-mediated signaling by inhibiting the biosynthesis of estradiol, we analyzed the effects of BA and UA on GREB1 transcription in cells treated with exogenous β-estradiol (E2; 0.5 nM). Treatment with E2 increased GREB1 mRNA levels by approximately 10-fold. In this experimental

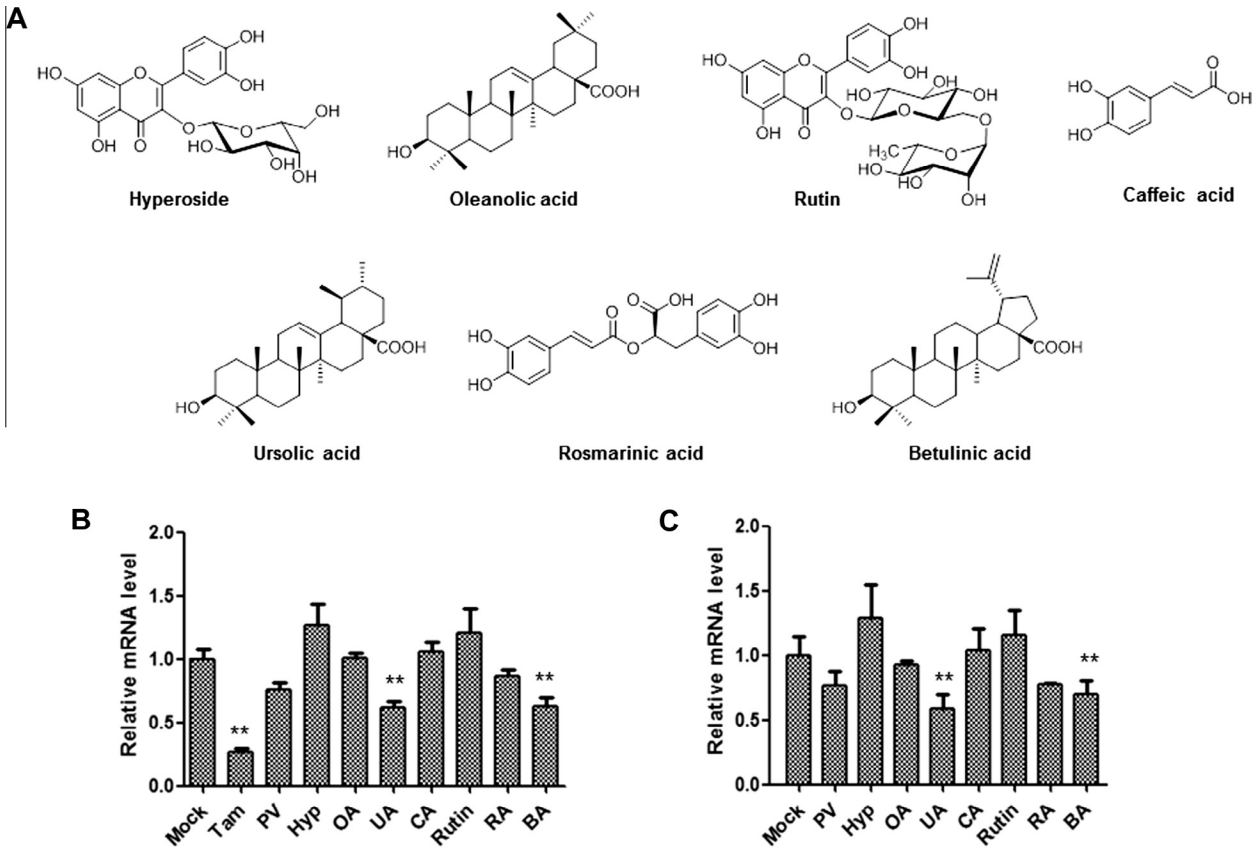

Fig. 1. Screening for antiestrogenic compounds in *P. vulgaris* extract. (A) Chemical structures of constituents isolated from *P. vulgaris* extract. (B, C) Antiestrogenic effects of rosmarinic acid (RA), caffeic acid (CA), UA, oleanolic acid (OA), hyperoside (Hyp), rutin, and BA. Tamoxifen (TAM, 10 μM) was used as a positive control. MCF7 cells were treated with the indicated compounds (20 μg/ml) for 12 h, and GREB1 (B) and ERα (C) mRNA levels were examined by RT-qPCR. Data are presented as means ± SD (**$p < 0.01$ versus mock treated).

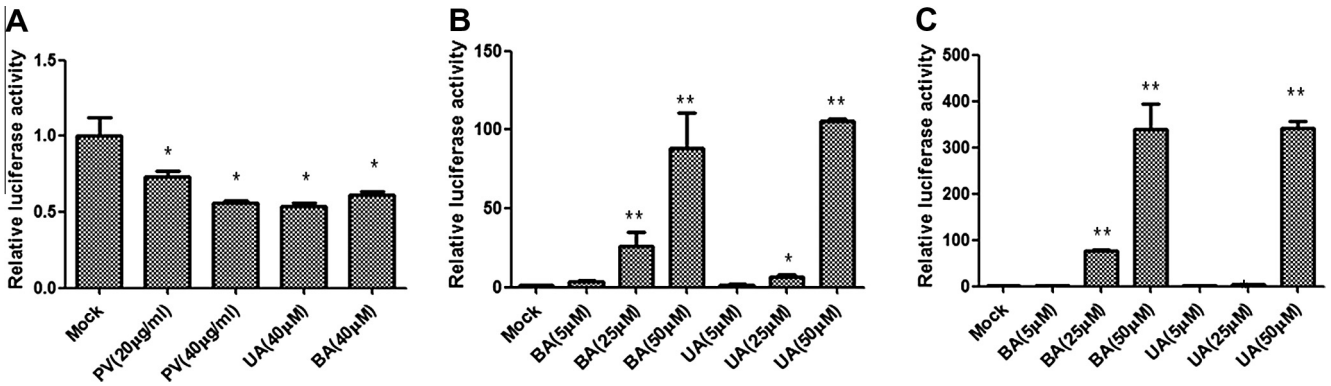

Fig. 2. ER-specific inhibition by UA and BA. (A) MCF7 cells were transfected with a 2X ERE-luciferase reporter plasmid and TK-Renilla luciferase expression plasmid. Twenty-four hours after transfection, cells were treated with total extract of *P. vulgaris* (PV), UA or BA for 12 h, and then assayed for luciferase activity. (B, C) LNCaP cells were transfected with a PSA-enhancer/promoter-luciferase reporter (B) or an MMTV enhancer/promoter (C) together with TK-Renilla reporter plasmid. Cells were treated with BA or UA as in (A) and assayed for luciferase activity. Data are presented as means ± SD ($^{*}p < 0.05$ and $^{**}p < 0.01$ versus mock treated).

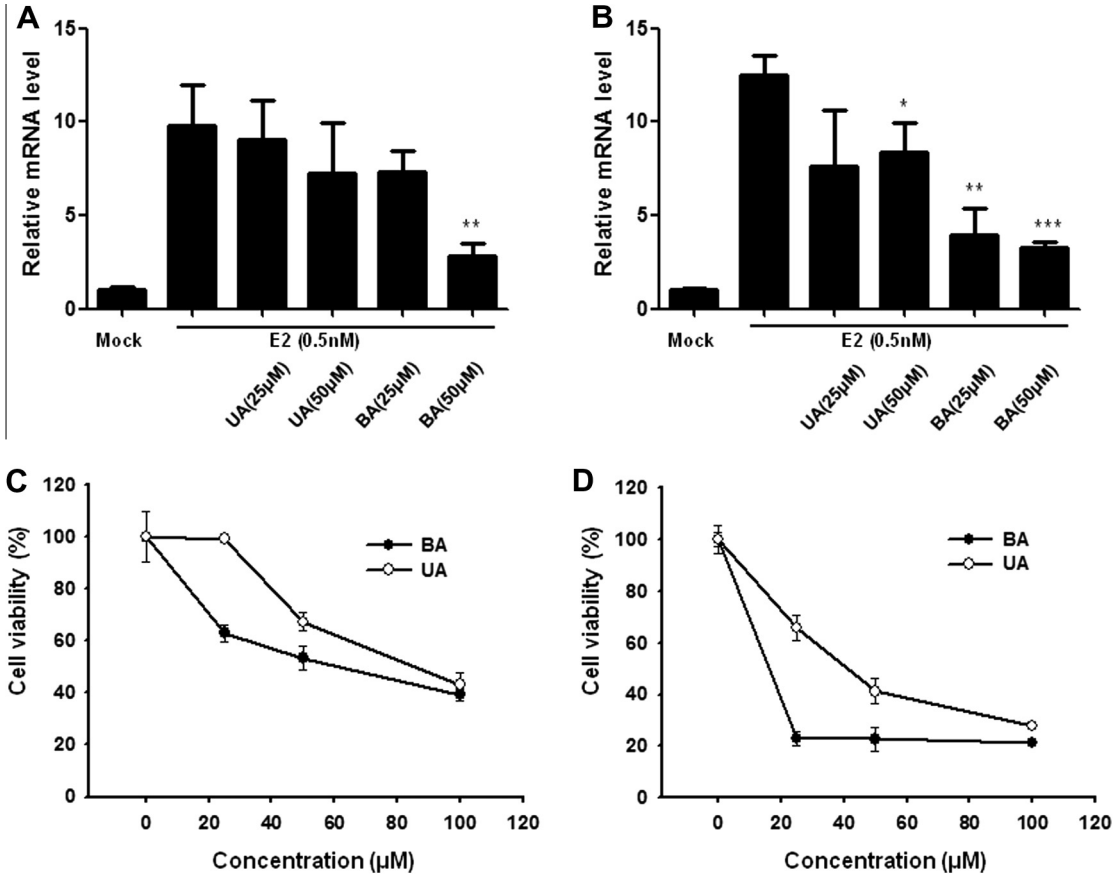

Fig. 3. Inhibition of ER-mediated transcriptional activity by UA and BA. (A, B) MCF7 cells were serum-starved for 18 h and treated with E2 with or without UA or BA for 12 h (A) or 24 h (B). GREB1 mRNA levels were quantified by RT-qPCR. Data are presented as means ± SD ($^{*}p < 0.05$ and $^{**}p < 0.01$ versus mock treated). (C, D) Effects of BA and UA on MCF7 cell viability. MCF7 cells were treated with different concentrations of UA and BA for 24 h (C) and 48 h (D). The viability of cells was determined by MTT assay. Data are presented as means ± SD.

setting, co-treatment with 50 μM BA significantly inhibited the induction of GREB1 mRNA 12 h after treatment (Fig. 3A). Twenty-four hours after treatment, significant reductions in E2-induced GREB1 mRNA expression was observed in both UA- and BA-treated cells, indicating that both compounds are capable of suppressing the transcriptional activity of ER regardless of the aromatase inhibitory activity (Fig. 3B).

We next assessed the ability of UA and BA to induce cell death in ER-positive MCF7 breast cancer cells using MTT assays. Treatment with BA or UA decreased cell viability in a concentration- and time-dependent manner, with BA exerting a stronger cytotoxic effect than UA, in line with their respective suppressive effects on ER signaling (Fig. 3C and D).

3.3. Inhibition of ER synthesis by betulinic acid and ursolic acid

Since ERα mRNA levels were reduced in cells treated with BA or UA (Fig. 1C), we tested the hypothesis that BA and UA suppress ER-mediated signaling by decreasing the amount of ERα. Addition of 50 μM BA or UA to MCF7 cells in charcoal-stripped serum containing E2 led to a dramatic decrease in ERα mRNA levels (Fig. 4A). Similarly, both BA and UA decreased the amount of ERα protein

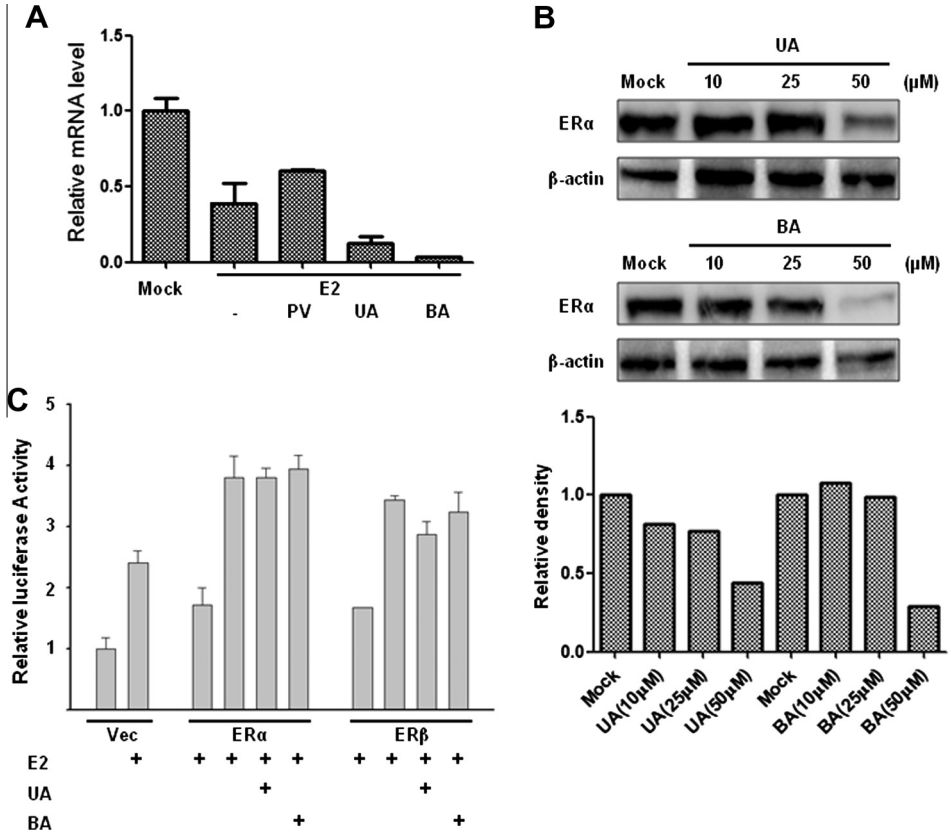

Fig. 4. Suppression of ERα expression by UA and BA. (A) MCF7 cells in charcoal-stripped serum were treated with UA or BA together with E2 (1 μM) for 12 h. mRNA levels of ERα were analyzed by RT-qPCR as described in Section 2. (B) MCF7 cells were treated with increasing amounts of UA or BA for 24 h. Cells were lysed, and proteins in lysates were resolved by SDS–PAGE and immunoblotted using anti-ERα and anti-β-actin antibodies. The graph (below) represents a densitometric analysis of ERα bands normalized to those of β-actin. (C) MCF7 cells were transfected with ERα- or ERβ-encoding plasmids or empty vector (control) together with an ERE-luciferase reporter construct and TK-Renilla expression plasmid. After a 24-h incubation, cells were treated with UA or BA (50 μM) for 12 h and assayed for luciferase activity. Data are presented as means ± SD.

(Fig. 4B). Collectively, these data suggest that BA and UA act by inhibiting ERα mRNA synthesis, thereby reducing ERα protein levels and suppressing ERα-mediated signaling. In line with this hypothesis, UA and BA failed to suppress E2-induced signaling in MCF7 cells exogenously overexpressing ERα or ERβ. These results suggest that BA and UA are unable to directly interact with the ER and instead suppress ER function by decreasing ER expression at the transcriptional level.

Collectively, our data identify UA and BA as antiestrogenic compounds responsible for the antiestrogenic effects of *P. vulgaris*. Both UA and BA suppressed ER-mediated signaling through inhibition of ER synthesis at the transcriptional level. Since the mechanism by which BA and UA suppress ER signaling is different from that of ER-binding SERMs and aromatase inhibitors, these compounds or their derivatives could be potential anti-cancer therapeutic agents against ER-dependent breast cancer.

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