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RESEARCH ARTICLE

Estrogenic effects of phytoestrogens derived from *Flemingia strobilifera* in MCF-7 cells and immature rats

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Abstract Phytoestrogen (PE) has received considerable attention due to the physiological significance of its estrogenicity. *Flemingia strobilifera* (FS) has been used as a folk medicine in Asia for the treatment of inflammation, cancer, and infection; however, the estrogenic effects and chemical components of FS have not yet been reported. We aimed to uncover the estrogenic properties and PEs derived from FS using phytochemical and pharmacological evaluation. PEs from FS extract (FSE) were analyzed by NMR, HPLC, and MS. To evaluate estrogenic activity, FSE and its compounds were evaluated by in vitro and in vivo assays, including human estrogen receptor alpha (hERα) binding, estrogen response element (ERE)-luciferase reporter assays, and uterotrophic assays. FSE and its compounds 1–5 showed binding affinities for hERα and activated ERE transcription in MCF-7 cells. Additionally, FSE and compounds 1–5 induced MCF-7 cell proliferation and trefoil factor 1 (pS2) expression. In immature female rats, significant increases in uterine weight and *pS2* gene were observed in FSE-treated groups. We identified estrogenic activities of FSE and its bioactive compounds, suggesting their possible roles as PEs via ERs. PEs derived from FSE are promising candidates for ER-targeted therapy for post-menopausal symptoms.

Keywords *Flemingia strobilifera* · Phytoestrogen · Estrogen receptor · ERE transcription · Uterotrophic effect · Immature rat

Introduction

The genus *Flemingia* belongs to the Fabaceae family. It is used in Asia and southern India as traditional medicine for the treatment of inflammation, cancer, and infections. Eighty species of *Flemingia* have been reported, including *Flemingia bhutanica, Flemingia brevipes*, *Flemingia chappar*, *Flemingia macrophylla, Flemingia fruticulosa, Flemingia rhodocarpa,* and *Flemingia strobilifera* (FS; The Plant List, 2010). The bark, leaves, flowers, and roots of these plants have been used in traditional medicine to treat a variety of illnesses, including epilepsy, dysentery, and diabetes mellitus (Cheyl 2006; Lans 2006; Gahlot et al. 2013). Among the *Flemingia* species, the effects of FS were reported in several studies; FS ethanol extract has shown anticonvulsant effects (Gahlot et al. 2013), analgesic activity (Kumar et al. 2011), and cytotoxicity against human leukemia cell lines (Ramcharan et al. 2010). Although previous studies have shown that flavonoids, flavonoid glycosides, chalcones, epoxy chromenes, and pterocarpans were the main constituents found in FS (Madan et al. 2008), it is still unknown whether FS includes compounds that can exert estrogenic effects.

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Estrogen, a major female hormone, regulates various physiological responses in many target tissues including the breast, uterus, brain, and bone (Paganini-Hill and Henderson 1994; Riggs et al. 1998). Estrogen exerts its biological effects by binding to estrogen receptors (ERs). ERs are mainly present in the nucleus and bind to ligand-forming complexes. These complexes bind to estrogen response elements (EREs) that are located in the promoter regions of target genes and act as a transcription factors for target genes (Nilsson et al. 2001). Estrogen plays an important role in the development and progression of breast and endometrial cancers (Hayashi et al. 2003; Bryant et al. 2005). Recruitment of co-activators to the promoter regions of target genes, such as cathepsin D, *pS2*, and c-myc, stimulates the growth of breast cancer cells (Shang et al. 2000). Many tissues express cofactors and ER subtypes that possess various functions throughout the body. ER activity is differentially modulated by a range of ligands. Some of these ligands show agonistic or antagonistic effects depending on ER subtype and are described as selective estrogen receptor modulators (SERMs) (Shang and Brown 2002). A typical SERM used to treat estrogen-positive breast cancer is tamoxifen (Fisher et al. 1998; Jordan 2003). Tamoxifen acts as an antagonist in breast cancer, an agonist in the uterus, and a partial agonist in bones (Martinkovich et al. 2014). In the uterus, however, long-term treatment of tamoxifen is associated with an increased risk of endometrial cancer due to its differential effects (Kedar et al. 1994; Wysowski et al. 2002; Shang 2006). Moreover, raloxifene is a second-generation SERM that has been approved for osteoporosis and breast cancer treatment; however, raloxifene is associated with an increased risk of thromboembolism (Palacios 2010). Therefore, development of SERMs with low estrogenic activity, anti-estrogenic activity, or tissue-specific activity would offer pharmacological advantages for hormonal substitutes or as complements in breast cancer treatments.

Phytoestrogens (PEs) refer to natural products that possess SERM properties. PEs can be derived from the seeds, roots, stems, leaves, and flowers of plants. Isoflavones and lignans from soybean and wheat are well-known PEs. These PEs are relevant in the treatment of hormone-related female diseases and cardiovascular diseases (Tham et al. 1998). PEs may be prospective candidates for the treatment of women's diseases due to their therapeutic efficacy and low side effect risks (Bhat and Pezzuto 2001). The PEs not only inhibit tumor growth, but also prevent heart disease, menopausal symptoms, and osteoporosis (Murkies et al. 1998; Mense et al. 2008). Additionally, PEs can function as SERMs whether or not they possess a steroidal structure (Moutsatsou 2007).

The present study aimed to determine the estrogenic properties of FS extract (FSE) and its compounds on MCF-7 human breast cancer cell line and immature female rat uteri. Five compounds were identified in FS fractions using NMR, HPLC, and MS. Binding affinity to hERα and ERE transcriptional activities of FSE was measured. Furthermore, cell proliferation of MCF-7 and *pS2* gene expression was examined. The uterotrophic effects of FSE, as well as protein expression of ERα, progesterone receptor A/B (PRA/B), and *pS2* mRNA levels were analyzed.

Materials and methods

Plant materials

Stems and leaves of *Flemingia strobilifera* were collected from the Institute of Ecology and Biological Resources in Binh Thuan province, Vietnam in 2012 and identified by Dr. Sei-Ryang Oh at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). A voucher specimen (access number FBM109-070) was deposited in the herbarium of the KRIBB.

Chemical analysis

Air-dried stems and leaves of *Flemingia strobilifera* (300 g) were pulverized and extracted with methanol to yield crude FSE (14.2 g). To identify bioactive constituents responsible for SERM efficacy, we fractionated FSE using reverse-phase silica column chromatography by eluting with a gradient mixture of H_2O-MeOH (80:20–0:100). We collected 20 fractions (FS1–20) from FSE. Fractionation of FS5 by reverse-phase medium-pressure column chromatography (MPLC) with a mixture of MeOH/H_2O (30:70) yielded compound **2** (83.5 mg, t_R = 3.36 min). Compound **1** (46.1 mg, t_R = 5.71 min) and compound **5** (55.3 mg, t_R = 4.74 min) were purified from FS6 by reverse-phase MPLC with a mixture of MeOH/H_2O (40:60). Chromatography of FS7 was performed on an HPLC column (YMC C18; 5 µm, 250 × 20 mm, 45% acetonitrile/H_2O, 7 mL/min, 210 nm) to yield compound **3** (16.4 mg, t_R = 31.57 min). Fractionation of FS8 by HPLC (YMC C18; 5 µm, 250 × 20 mm, acetonitrile/H_2O 45% 20 min, acetonitrile/H_2O 55% 35 min, 7 mL/min, 210 nm) yielded compound **4** (4.7 mg, t_R = 41.05 min).

Cajanin (**1**): wite amorphous powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ_H: 12.99 (1H, s, 5–OH), 9.38 (1H, br s, –OH), 9.30 (1H, br s, –OH), 8.23 (1H, s, H-2), 6.99 (1H, d, J = 8.4 Hz, H-6′), 6.63 (1H, d, J = 2.4 Hz, H-3′), 6.40 (1H, d, J = 2.0 Hz, H-6), 6.37 (1H, d, J = 2.0 Hz, H-8), 6.28 (1H, dd, J = 8.4, 2.4 Hz, H-5′), 3.86 (3H, s, 7–OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 156.2 (C-2), 121.1 (C-3), 181.0 (C-4), 162.1 (C-5), 98.3 (C-6), 165.5 (C-7), 92.8 (C-8), 158.0 (C-9), 105.9 (C-10), 108.2 (C-1′),

156.9 (C-2′), 103.0 (C-3′), 159.1 (C-4′), 106.7 (C-5′), 132.6 (C-6′), 56.5 (7–OCH_3); HRTOFESIMS *m/z* 299.0567 $[M-H]^-$ (calcd for $C_{16}H_{11}O_6^-$: 299.0561).

2′-Hydroxygenistein (**2**): white amorphous powder; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.99 (1H, s, H-2), 7.03 (1H, d, *J* = 8.4 Hz, H-6′), 6.39 (1H, d, *J* = 2.4 Hz, H-3′), 6.36 (1H, d, *J* = 8.4, 2.4 Hz, H-5′), 6.34 (1H, d, *J* = 2.4 Hz, H-8), 6.21 (1H, d, *J* = 2.4 Hz, H-6); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 156.9 (C-2), 122.7 (C-3), 182.8 (C-4), 163.8 (C-5), 100.3 (C-6), 166.1 (C-7), 94.9 (C-8), 159.9 (C-9), 106.3 (C-10), 110.9 (C-1′), 158.0 (C-2′), 104.4 (C-3′), 160.4 (C-4′), 108.2 (C-5′), 133.3 (C-6′); HRTOFESIMS *m/z*: 285.0402 $[M-H]^-$ (calcd for $C_{15}H_9O_6^-$: 285.0405).

Pisatin (**3**): colorless oil; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.33 (1H, d, *J* = 8.8 Hz, H-5), 6.82 (1H, s, H-6′), 6.62 (1H, dd, *J* = 8.4, 2.4 Hz, H-6), 6.42 (1H, d, *J* = 2.4 Hz, H-8), 6.35 (1H, s, H-3′), 5.90 (1H, d, *J* = 1.0 Hz, 1″a), 5.87 (1H, d, *J* = 1.0 Hz, 1″b), 5.20 (1H, s, H-4), 4.10 (1H, d, *J* = 11.6 Hz, H-2b), 3.95 (1H, d, *J* = 11.6 Hz, H-2a), 3.75 (3H, s, 7–OCH_3); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 70.8 (C-2), 77.8 (C-3), 86.1 (C-4), 133.2 (C-5), 110.3 (C-6), 162.6 (C-7), 102.5 (C-8), 157.5 (C-9), 114.3 (C-10), 121.6 (C-1′), 155.9 (C-2′), 94.7 (C-3′), 151.0 (C-4′), 143.7 (C-5′), 104.5 (C-6′), 55.9 (7-OCH_3), 102.9 (C-1″); HRTOFESIMS *m/z*: 313.0716 $[M-H]^-$ (calcd for $C_{17}H_{13}O_6^-$: 313.0718).

2′,3′,4′,6′-Tetramethoxychalcone (**4**): colorless oil; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.58 (2H, m, H-2, 6), 7.39 (3H, m, H-3, 4, 5), 7.31 (1H, d, *J* = 16.0 Hz, H-*β*), 6.97 (1H, d, *J* = 16.0 Hz, H-α), 6.53 (1H, s, H-5′), 3.92 (3H, s, -OMe), 3.81 (3H, s, -OMe), 3.783 (3H, s, -OMe), 3.777 (3H, s, -OMe);^{13}C NMR (100 MHz, CD_3OD) δ_C: 136.1 (C-1), 129.7 (C-2, 6), 130.2 (C-3, 5), 131.9 (C-4), 129.9 (C-α), 147.3 (C-*β*), 196.0 (C = O), 117.1 (C-1′), 152.9 (C-2′), 137.3 (C-3′), 157.0 (C-4′ or C-6′), 94.1 (C-5′), 154.9 (C-4′ or C-6′), 62.4 (2′–OCH_3), 61.6 (3′–OCH_3), 56.9 (4′ or 6′–OCH_3), 56.8 (4′ or 6′-OCH_3); HRTOFESIMS *m/z*: 373.1310 $[M+COOH]^-$ (calcd for$C_{20}H_{21}O_7^-$: 373.1293).

Genistein (**5**): white amorphous powder; ^1H NMR (400 MHz, CD_3OD) δ_H: 8.02 (1H, s, H-2), 7.36 (2H, d, *J* = 8.8 Hz, H-2′, 6′), 6.83 (2H, d, *J* = 8.8 Hz, H-3′, 5′), 6.31 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 154.9 (C-2), 124.9 (C-3), 182.4 (C-4), 164.0 (C-5), 100.2 (C-6), 166.1 (C-7), 94.9 (C-8), 159.8 (C-9), 106.4 (C-10), 123.4 (C-1′), 131.5 (C-2′, 6′), 116.4 (C-3′, 5′), 159.0 (C-4′); HRTOFESIMS *m/z*: 269.0457 $[M-H]^-$ (calcd for$C_{15}H_{10}O_5^-$: 269.0455).

Animals

All animal studies were performed in accordance with the guidelines stated by the Korean Food and Drug Administration. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC, SMWU-IACUC-1703-004) of Sookmyung Women's University. Immature female Sprague–Dawley rats (20–21 days old) with body weights ranging from 38–48 g were obtained from the Samtaco Animal Farm (Osan, Korea). All animals were housed in a purpose-built facility with a controlled environment and maintained in an isolator in which a control was set to keep temperature and relative humidity at 23 ± 2 °C and 50 ± 10%, respectively. Artificial lighting provided a 24 h cycle of 12:12 h light and dark.

Uterotrophic assay

Flemingia strobilifera was tested in vivo to determine its ability to induce uterine weight gain in immature rats. Immature female Sprague–Dawley rats (20–21 days old) with body weights ranging from 38–48 g were housed under standard animal laboratory conditions with ad libitum access to food and water. Animals were randomly divided into three groups, with group body weights within 10% of the population mean. Animals were subcutaneously injected daily for a period of 3 days with (i) corn oil (5 mL/kg; vehicle control), (ii) 17β-Estradiol (E2, 3 μg/5 mL/kg; positive control), or (iii) *Flemingia strobilifera* (300 mg/5 mL/kg). Each treatment group consisted of 10 rats. On day 4, the animals were weighed and euthanized. The uteri were quickly removed and the connective tissues were excised. Then, uteri were nicked, blotted, and weighed. The effects of *Flemingia strobilifera* treatment on uterus wet weight were determined by reporting absolute uterine weights normalized to rat respective body weights.

Cell culture and materials

MCF-7 cells were kindly provided by Dr. Anna Soto (Tufts University, Boston, MA, USA). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution. Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. All cell culture reagents were purchased from Gibco (Carlsbad, CA, USA). [2,4,6,7-^3H]-17β-Estradiol ([^3H]-E2, 88.0 Ci/mmol) was purchased from Perkin Elmer Inc. (Boston, MA, USA). Reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and unless stated otherwise, were of research grade suiratio for cell culture or of the highest grade available. All chemicals were dissolved in

DMSO before being tested in the various assay systems. Stock solutions were stored at − 30 °C. The final solvent concentration in the culture medium did not exceed 0.5%.

ER ligand-binding assay

Binding abilities to recombinant full-length human estrogen receptor α (hERα) was assessed as described previously (Obourn et al. 1993). Recombinant hERα (2010 pmol/mg, Invitrogen Corp, Carlsbad, CA, USA) was diluted to a concentration of 3 nM with a binding buffer containing 10 mM Tris (pH 7.5), 10% glycerol, 1 mM DTT, and 1 mg/mL bovine serum albumin (BSA). hERα was incubated with [^{3}H]-E2 (5 nM) in the presence or absence of 10^{-6}–10^{-13} M unlabeled E2 and various concentrations of FSE and bioactive compounds. After incubation for 2 h at 28 °C, the reactions were terminated by rapid filtration through glass-fiber filters (Packard Instrument B.V. Chemical Operations, The Netherlands) presoaked in ice-cold 0.05% polyethylenimine solution using a PerkinElmer Filter Mate Harvester. The filters were washed five times with ice-cold washing buffer containing 40 mM Tris (pH 7.5) and 100 mM KCl. Then, the filters were dried and placed in scintillation vials containing 3 mL of Packard Ultima Gold scintillation cocktail. After shaking and overnight equilibration of the vials, the radioactivity trapped on 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}–10^{-13} M unlabeled E2. The specific binding percentage for hERα was determined as follows: $[(dpm_{sample} - dpm_{non\ specific})/(dpm_{control} - dpm_{non\ specific})] \times 100$ (%). RBA was calculated as follows: $RBA = [IC_{50(E2)}/IC_{50(sample)}] \times 100$ (%). The results for each treatment condition were expressed as the mean ± standard error (SE).

ERE-luciferase reporter gene assay

ERE transcription activity was assessed as described previously (Obourn et al. 1993). MCF-7 cells were seeded in 24-well plates (3×10^5 cells/well) and cultured in phenol red-free DMEM containing 10% charcoal–dextran-treated FBS (CD-FBS) in triplicate. MCF-7 cells were then transiently transfected with recombinant plasmids that contain ERE-luciferase (luc) sequences (0.5 μg/well) and with 0.2 μg of an inactive control plasmid encoding a β-galactosidase gene using Lipofectamine 2000 Reagent (Invitrogen). The pERE-Luc plasmid contains three copies of the *Xenopus laevis* vitellogenin A2 ERE upstream of firefly luciferase (a gift from Dr. V. C. Jordan, Lombardi Comprehensive Cancer Center, Georgetown University). One day after transfection, cells were exposed to vehicle (0.1% DMSO), E2 (10^{-9} M), ICI182,780 (ICI; pure ER antagonist, 10^{-6} M), E2 + ICI, genistein (Gen; 10^{-6} M), FSE (1–10 μg/mL), FSE (10 μg/mL) + ICI, or compound 1–5 (0.5–20 μM) for 24 h. Cells were lysed for luciferase activity analysis after incubation for 24 h. Luciferase activity was measured using a luciferase reporter assay system (Promega, Madison, WI, USA). Luminescence was detected with a TD-20/20 luminometer (Turner Design, Sunnyvale, CA, USA). Finally, luciferase activities were normalized to β-galactosidase activity.

Quantitative polymerase chain reaction (qPCR)

To confirm if FSE and its bioactive compounds activated transcription of target genes, we examined expression of the *pS2* gene. Total mRNA was isolated from uteri obtained from the uterotrophic assay and MCF-7 cells. After chopping and homogenizing, uteri or cells were incubated with TRIzol reagent (Life technologies, Carlsbad, CA, USA) for 5 min at 25 °C. Then, 300 μL of chloroform was added, followed by incubation for 3 min at 25 °C. After centrifugation at ≥ 10,000×*g* (13,000 rpm) and 4 °C for 30 min, the supernatant was collected. Isopropanol was added to the collected supernatant and then centrifugation was conducted at ≥ 10,000×*g* (13,000 rpm) and 4 °C for 15 min. The supernatant was discarded and the pellets were dissolved in DEPC water. Using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), cDNA was synthesized from 1 μg of total mRNA. cDNA, a pair of primers capable of recognizing cDNA target genes, and the PCR SYBR Green kit (Bio-Rad) reagent were mixed. Quantitative gene expression of target genes was analyzed by the Applied Biosystem 7500 Realtime PCR system (Life technologies). To normalize experimental errors of several steps, gene expression of GAPDH (housekeeping gene) was analyzed at the same time. The ratio of target gene expression and housekeeping gene expression was used for determining quantitative gene expression. The primers used were, rGAPDH forward 5′-CTCTCTGCTCCTCCTGTTCGAC and reverse 5′-TGAGCGATGTGGCTCGGCT; r*pS2* forward 5′-GGAAAAGGGTTGCTGTTTTG and reverse 5′-ACAGGTGTGTATGAAGCAGGTG; hGAPDH forward 5′-GGCTGAGAACGGGAAGCTTGTCAT and reverse 5′-CAGCCTTCTCCATGGTGGTGAAGA; h*pS2* forward 5′-CGTGAAAGAC AGAATTGTGGTTTT and reverse 5′-CGTCGAAACAGCAGCCCTTA; mGAPDH forward 5′-TGCCAAGTATGATGACATCAAGAA and reverse 5′-GCCCAAGATGCCCTTCAGT. qPCR consisted of 40 cycles (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s for *pS2*). Each gene expression curve was presented as a logarithm and the threshold cycles (C_T) were calculated mathematically. The

values were substituted in $-2^{\Delta\Delta C_T}$ and relative gene expression was calculated.

Western blotting

After behavioral tests, animals were sacrificed and uteri tissues were collected for western blot analysis. Whole protein was extracted from uteri and brain tissues. Uteri and brains were separately digested with lysis buffer containing protease inhibitor and phosphatase inhibitor. Protein concentration was determined by the BCA assay, then 20 μg of the protein was loaded onto 7.5% SDS-PAGE gels. Electrophoresis was performed for 2 h at 80 V. Next, the protein was transferred onto a PVDF membrane for 3 h at 25 V. After blocking the membrane with BSA solution, the primary antibody was incubated with the membrane at 4 °C overnight. After washing the membrane with TPBS (phosphate buffered saline (PBS) with 0.1% Tween 20), the secondary antibody was added and the membrane was incubated for 1 h at 25 °C. Enhanced Chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA) was then used to detect target proteins (Amersham™ Imager 600 (GE Healthcare Biosciences; Pittsburg PA). For primary antibodies, 1:500 dilutions of ERα (sc-7207, Santa Cruz Biotechnology, CA, USA) and PRA/B (sc-538, Santa Cruz Biotechnology) were used. β-actin (Sigma-Aldrich) was used to normalize target protein expression.

MCF-7 cell proliferation assay

Cell proliferation was assessed as described previously (Soto and Sonnenschein 1985). MCF-7 cells were plated in 96-well plates at an initial density of 1×10^4 cells/well. After 24 h, vehicle, E2, genistein, ICI, E2 + ICI, FSE + ICI, or bioactive compounds were added to the experimental medium (phenol red-free DMEM containing 10% CD-FBS and the cells were incubated for 2 days (late exponential phase). Cell growth was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Statistical analysis

Data are expressed as the mean ± standard error (SE). Comparisons between groups were performed by one-way analysis of variance (ANOVA) with appropriate Newman-Keuls methods using the GraphPad Prism Software (San Diego, CA, USA). A p value of less than 0.05 was considered statistically significant.

Results

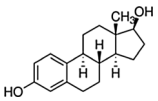
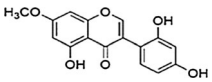
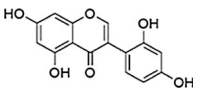
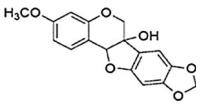
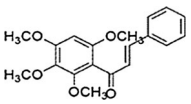
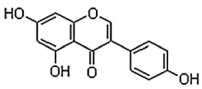
Isoflavones, pterocarpans, and chalcones were identified in FSEs through phytochemical analysis

Twenty fractions were separated from FSE by liquid chromatography and tested for their hERα binding abilities and ERE transcription activation. Among the 20 fractions, FS5, FS6, FS7, FS8, and FS20 showed over 60% binding affinity for hERα (data not shown). FS5, FS6, FS7, and FS8 were selected for further studies due to their binding affinity for hERα and their ERE transcriptional activity. Fractionation of FS5 by reverse-phase MPLC with a mixture of MeOH-H_2O (30:70) yielded compound 2 (83.5 mg, t_R = 3.36 min). Compound 1 (46.1 mg, t_R = 5.71 min) and compound 5 (55.3 mg, t_R = 4.74 min) were purified by reverse-phase MPLC with a mixture of MeOH-H_2O (40:60) from FS6. Chromatography of FS7 was performed on an HPLC column (YMC C18; 5 μm, 250 × 20 mm, 45% ACN, 7 mL/min, 210 nm) to yield compound 3 (16.4 mg t_R = 31.57 min). Fractionation of FS8 by HPLC (YMC C18; 5 μm, 250 × 20 mm, ACN 45% 20 min, ACN 55% 35 min, 7 mL/min, 210 nm) yielded compound 4 (4.7 mg, t_R = 41.05 min). The structures of the compounds were identified as cajanin **(1)** (Waffo et al. 2000), 2′-hydroxygenistein **(2)** (Uchida et al. 2015), pisatin **(3)** (Hegazy et al. 2011), 2′,3′,4′,6′-tetramethoxychalcone **(4)** (Panichpol and Waterman 1978), and genistein **(5)** (Coward et al. 1993) on the basis of MS, ^{1}H and ^{13}C NMR spectroscopic data, and literature comparison. Chemical characteristics of the five compounds are summarized in Table 1.

FSE and its compounds bind to recombinant hERα and activate ERE transcription in MCF-7 cells

To examine the estrogenic properties of FSE and its five bioactive compounds, we evaluated their hERα binding affinity and ERE transcriptional activity. Binding affinity of FSE for hERα was determined by a competitive binding assay using [^{3}H]-E2 and recombinant purified hERα. FSE exhibited a binding affinity for hERα that increased in a dose-dependent manner with an IC_{50} value of 1.6×10^{-5} g/mL (Table 1). At concentrations of 10^{-5} g/mL, FSE competitively inhibited 83% of [^{3}H]-E2 binding to hERα. The binding affinities were expressed as relative bioavailability (RBA) values, with an RBA value of the E2 standard set to 100. E2 showed binding affinity for hERα with an IC_{50} value of 7.68×10^{-9} M (Table 1). The RBA value of FSE for hERα was 0.048. Compounds 1, 2, 4, and 5 exhibited binding affinities for hERα with IC_{50} values ranging from 10^{-7} to 10^{-5} M (Table 1). Compound 2 has

Table 1 Chemical structure, molecular weight and ligand-binding affinity and ERE transcriptional activity of *Flemingia strobilia* extract (FSE) and its bioactive compounds

	Structure	Chemical name	Molecular formula (molecular weight)	hERα binding affinity IC_{50} (M), RBA (%)	ERE promoter activity EC50 (M)
E2		17β-Estradiol	$C_{18}H_{24}O_2$ (272.38)	7.68×10^{-9} M, (100)	1.25×10^{-11}
FSE				1.60×10^{-5} g/mL (0.048)	2.96×10^{-8}
1		Cajanin	$C_{16}H_{12}O_6$ (300.26)	1.86×10^{-5} M, (0.014)	3.35×10^{-6}
2		2-Hydroxy genistein	$C_{15}H_{10}O_6$ (286.24)	4.63×10^{-7} M, (0.931)	1.45×10^{-5}
3		Pisatin	$C_{17}H_{14}O_6$ (314.29)	N.D.	1.37×10^{-5}
4		2′,3′,4′,6′-tetramethoxychalcone	$C_{19}H_{20}O_5$ (328.36)	8.37×10^{-7} M, (0.515)	N.D.
5		Genistein	$C_{15}H_{10}O_5$ (270.24)	1.99×10^{-7} M, (1.325)	1.25×10^{-5}

[^{3}H]-E2 at a dose of 5 nM was incubated with hERα in the presence of compounds at concentrations of 10^{-4}–10^{-9} M. As a positive control group, [^{3}H]-E2 at a dose of 5 nM was incubated with the hERα in the presence of E2 at concentration of 10^{-6}–10^{-13} M. The IC_{50} was calculated using Prism software. RBA (Relative bioavailability) was calculated by dividing the IC_{50} of 17β-estradiol (E2) by that of the *Flemingia strobilia* extract (FSE) or its compounds, and multiplying by 100. Relative binding affinity (%); RBA = [$IC_{50(E2)}/IC_{50(FSE\ or\ compound)}$] × 100

potent binding affinities with an IC_{50} value of 0.46 μM for hERα. The RBA values of compounds 1, 2, and 4 for hERα were 0.014, 0.931, and 0.515, respectively.

The estrogenic activities of FSE and its compounds on ERE transcription were evaluated by ERE-luciferase reporter gene assays in MCF-7 cells. Cells were treated with FSE, ER agonist E2 (10^{-9}–10^{-12} M; positive control), ER antagonist ICI (10^{-6} M; negative control), or DMSO (0.1% v/v; vehicle control) and incubated for 24 h. Then, ERE transcriptional activity was evaluated by the luciferase assay. As shown in Fig. 1, E2, FSE, and FSE compounds 1–5 activated ERE transcription in a dose-dependent manner. Compounds 1, 2, 3, and 5 dose-dependently increased ERE transcriptional activities and E2 and genistein activated ERE transcription as well, indicating functional agonistic properties (Fig. 1a, b). Our results indicate that FSE and its compounds activate ERE transcriptional activity via ER binding.

FSE and its bioactive compounds induced cell proliferation and *pS2* mRNA expression in MCF-7 cells

To evaluate the effects of FSE and its bioactive compounds on MCF-7 cell proliferation, an MTT assay was performed. FSE induced cell proliferation in a dose-dependent manner in a concentration range of 10^{-8}–10^{-4} g/mL. E2 and genistein increased cell proliferation twofold compared with vehicle control. Compound 1 strongly induced MCF-7 cell proliferation by threefold, while compounds 2, 3, and 5 significantly increased cell proliferation by approximately 2.5-fold in a concentration range of 10^{-5}–10^{-6} M (Fig. 2a). To determine if FSE regulates nuclear ERα activity in MCF-7 cells, we examined mRNA levels of the ERα target gene, *pS2*. E2 and genistein increased *pS2* mRNA expression by approximately 1.5-fold compared with the vehicle. *pS2* mRNA expression was upregulated by FSE at concentrations of 10^{-7}–10^{-5} g/mL (Fig. 2b). At a concentration of 10^{-5} g/mL, compounds 1, 2, 3, and 5 increased *pS2* mRNA levels by more than 1.2-fold compared with the vehicle (Fig. 2b).

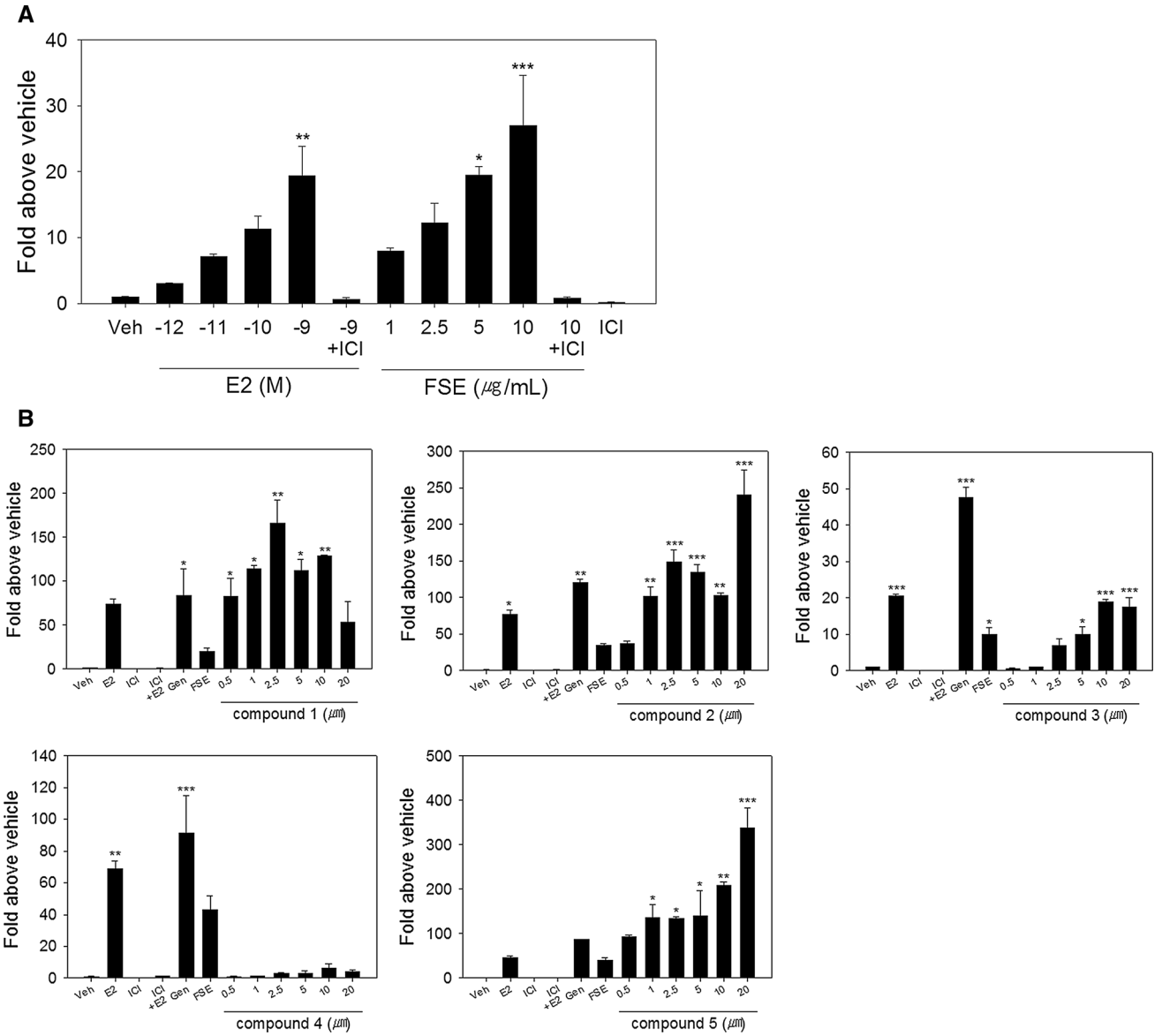


Fig. 1 Effect of FSE and its compounds on ERE transcriptional activity in MCF-7 cells. **a** Estrogen response element (ERE) transcriptional activity of FSE on ERE-luciferase plasmid transfected MCF-7 cells. MCF7 cells were transfected with recombinant plasmids that contain ERE-luc sequences. The next day, cells were exposed to vehicle (0.1% DMSO), E2 (10^{-12}–10^{-9} M), ICI182,780 (ICI; pure ER antagonist, 10^{-6} M), E2 + ICI, FSE (1–10 μg/mL), and FSE (10 μg/mL) + ICI for 24 h. E2 activated ERE transcription at concentrations of 10^{-9}–10^{-12} M, while FSE activated ERE transcription at concentrations of 1–10 μg/mL. *p< 0.05, **p< 0.01, and ***p< 0.001; significantly different from the vehicle group. Values are presented as the mean ± standard error (SE; $n = 3$). **b** ERE transcriptional activity of bioactive compounds in MCF-7 cells. E2 and genistein activated ERE transcription. FSE, compound 1, 2, 3, and 5 activated ERE transcription in a concentration-dependent manner, while compound 4 did not activate ERE transcription. Relative activities were expressed as fold above the vehicle control. *p< 0.05, **p< 0.01, and ***p< 0.001; significantly different from the vehicle group. Results (mean ± SE) are representative of three experiments

FSE induces a uterotrophic effect in association with ERα, PRA/B protein expression, and *pS2* mRNA levels in immature rats

The uterotrophic response to FSE was measured based on the increase in uterine tissue mass in immature rats. Since uteri express ERs, E2 induces cell proliferation of uterine tissue, leading to uterine weight gain. E2 increased uterine weight when administered subcutaneously to rats. Blotted uterine weights normalized to body weights were 1.97-fold higher in rats treated with 0.003 mg/kg E2 than in rats of the vehicle group (Table 2). FSE (300 mg/kg) increased uterine weight significantly compared with the vehicle group (Table 2). The impact of FSE treatment on ERα and PRA/B expression in the rat uterus was examined by western blotting (Fig. 3a). In the uterus of immature rats

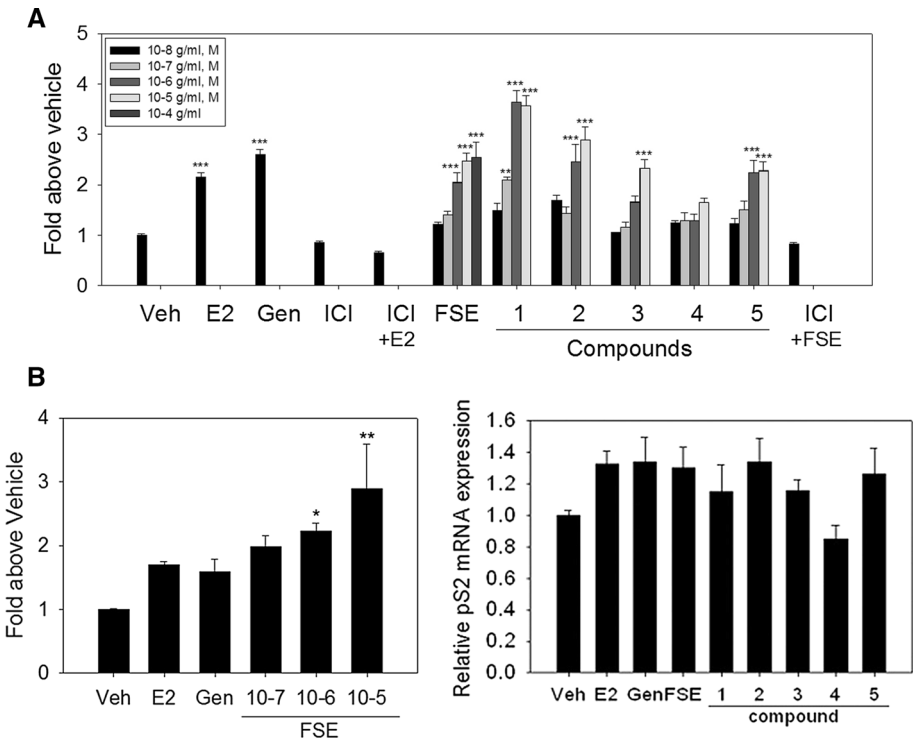


Fig. 2 Effect of FSE and its bioactive compounds on MCF-7 cell proliferation and *pS2* mRNA expression. **a** MCF-7 cell proliferation after treatment with FSE and bioactive compounds. The medium was replaced with vehicle (0.1% DMSO), E2 (10^{-9} M), genistein (10^{-6} M), ICI, E2 + ICI, FSE (10^{-8}–10^{-4} g/mL), bioactive compounds (10^{-8}–10^{-5} M), and FSE (10^{-5} g/mL) + ICI. Cells were incubated for 2 days (late exponential phase). Cell growth was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. *p< 0.05, **p< 0.01, and ***p< 0.001; significantly different from the vehicle group (n = 3). **b** The effects of FSE and bioactive compounds on estrogen-responsive *pS2* mRNA levels in MCF-7 cells. FSE and its compounds increased *pS2* mRNA expression in a dose-dependent manner. The ratio of target gene expression to housekeeping gene expression was used for quantitative gene expression. The results (mean ± SE) are representative of three experiments. *p< 0.05 and **p< 0.01; significantly different from the vehicle group

Table 2 Uterotrophic effect of the *Flemingia strobilia* extract (FSE) in immature rat uterus

Treatment	Dose (mg/kg)	Body weight (g)	Blotted Uterine weight (mg)	Blotted Uterine weight (mg)/body weight (g) ratio
Veh		69 ± 1.4	43.6 ± 5.4	0.63 ± 0.08
E2	0.003	62 ± 1.0	122.5 ± 5.4	1.97 ± 0.08***
FSE	300	63 ± 0.9	118.2 ± 4.6	1.88 ± 0.06***

Animals were injected subcutaneously every day with vehicle (corn oil), E2 at 0.003 mg/kg body weight, or FSE at a dose of 300 mg/kg body weight. Uterine weight data are shown as mean ± standard error (SE; n = 10 per group). Values significantly different from the vehicle treated group are indicated by asterisks (***p < 0.001)

treated with 0.003 mg/kg E2, ERα protein expression decreased by approximately 44% (Fig. 3b); this is in accordance with the decrease in ERα protein expression by E2 in MCF-7 cells (Gierthy et al. 1996). ERα protein expression decreased by approximately 38% after FSE treatment (Fig. 3b). Interestingly, FSE increased protein expression of PRA and PRB in the uterus compared with the vehicle group. We further examined if FSE affects mRNA levels of the ERα target gene, *pS2*. mRNA expression of *pS2* significantly increased after FSE treatment, compared with the vehicle group (Fig. 3c). These results show that FSE regulates ERα, PRA/B protein expression, and *pS2* mRNA expression, exerting a uterotrophic effect in immature rats.

Discussion

Although previous studies reported on the pharmacological activities of FS, the estrogenic property of FS remained unknown. In the present study, we analyzed phytochemical components and evaluated the estrogenic effects of FSE

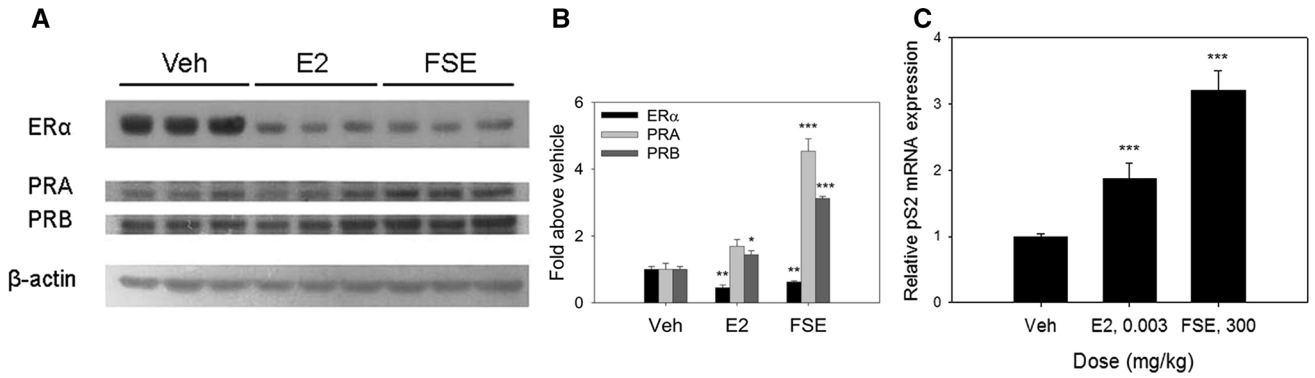


Fig. 3 Protein expression of ERα, PRA, and PRB, and *pS2* mRNA levels in FSE-treated immature rat uterus. FSE was administered subcutaneously at a dose of 300 mg/kg for 3 days. Uterine proteins were extracted and analyzed for expression levels of ERα, PRA, and PRB by western blotting. **a** Detection of ERα protein (66 kDa), PRA protein (81 kDa), and PRB protein (116 kDa) by western blotting. Results shown are representative of three independent experiments. Equal amounts of protein in each lane were confirmed using β-actin as loading control. **b** Quantitative graph of western blotting results. Intensity was calibrated by using β-actin as a loading control. ERα protein levels significantly decreased in FSE-treated uteri. PRA and PRB protein levels significantly increased in FSE-treated uteri. **c** Total RNA was extracted then *pS2* and GAPDH mRNA levels were determined using quantitative PCR and normalization to GAPDH expression. Significance was determined versus vehicle group (*p< 0.05, **p< 0.01, and ***p< 0.001). Values represent the mean ± SE of three independent experiments

and its compounds. Our bioassay-guided isolation of FSE led to the purification of cajanin (1), 2-hydroxygenistein (2), pisatin (3), 2′,3′,4′,6′-tetramethoxychalcone (4), and genistein (5).

To elucidate the estrogenic properties of FS, we examined the effect of FSE and bioactive compounds on hERα binding affinity, ERE transcriptional activity, MCF-7 cell proliferation, and *pS2* mRNA expression. FSE and its compounds 1, 2, 3, and 5 showed binding affinities to hERs and dose dependently increased cell proliferation as well as *pS2* mRNA expression in MCF-7 cells. Furthermore, we examined in vivo estrogenic properties of FSE in immature female rats. FSE treatment increased uterine weight and regulated ERα, PRA, and PRB protein expression. Expression of *pS2* mRNA in rat uteri was significantly increased in the FSE-treated group as well. Our results indicate that FSE and its bioactive compounds have estrogenic properties in vitro and in vivo and possibly act as PEs via ERs.

Using bioassay-guided analysis, we identified five PEs (compounds 1–5) that have binding affinities for hERα and ERE transcriptional activity. Compounds 1, 2, and 5 belong to the isoflavone family. Compounds 3 and 4 belong to the pterocarpan and chalcone family, respectively. Identification of isoflavones and flavonoids, such as genistein, daidzein, and naringin, was reported for the root and stems of FS (Madan et al. 2009; Yang et al. 2016). In the present study, our chemical analysis of FSE included isoflavones such as cajanin, 2-hydrogygenistein, and genistein. Moreover, we further discovered the presence of pterocarpan and chalcone family compounds, pisatin and 2′,3′,4′,6′-tetramethoxychalcone, respectively. Recently, chemical compositions of *Flemingia philippinensis* were reported to show aromatase inhibitory activities (Sun et al. 2017). Consistent with our results, phyto-SERM constituents and activities of *Flemingia macrophylla* were also reported (Lai et al. 2013), as these are common properties of *Flemingia* genus plants. To our knowledge, however, this study is the first to report on the estrogenic properties of FS.

Our results on hERα ligand binding and ERE transcription assays imply that bioactive constituents of FS may act as agonists or partial agonists of ERα in a tissue-specific manner. There are two subtypes of ERs, ERα and ERβ, that form homodimers and heterodimers following ligand binding (Björnström and Sjöberg 2005). The tissue distribution of ERα is different from that of ERβ. For example, Taylor and Al-Azzawi found that ERα is mainly expressed in endometrium glandular epithelia (Taylor and Al-Azzawi 2000), while both ERα and ERβ are present in rat uterine glands (Saunders et al. 1997). PE interactions with ERs have different efficacies and safety profiles in the breast, bone, and uterus depending on the tissue and its environment (Patisaul and Jefferson 2010; Komm and Mirkin 2014). To assess PE properties of FS in breast tissue, the MCF-7 cell line was used due to its high expression of ERs. Estrogenic properties of FSE and its PEs in MCF-7 cells were examined by measuring cell proliferation and *pS2* mRNA expression. Compounds 1, 2, 3, and 5 increased MCF-7 cell proliferation and *pS2* mRNA expression; therefore, FSE and bioactive compounds possibly act as PEs via ERs in breast cells. Additionally, we uncovered a uterotrophic effect of FSE in immature female rats. FSE PEs may bind to ERs in the uterus and activate transcription of target genes, resulting in increased uterine cell proliferation. Genistein, a soy PE, strongly binds to

ERs (Morito et al. 2001) and induces fluid accumulation in ovariectomized rat uteri (Chinigarzadeh et al. 2014). Hertrampf et al. reported that subcutaneous injections of genistein increased uterine wet weight in ovariectomized rats (Hertrampf et al. 2009). In our study, we also observed fluid accumulation in FSE-treated rat uteri. This uterotrophic response induced by FSE may be related to the action of its PEs. ERα protein expression in the uterus was downregulated after treatment with FSE or E2, similar to the decreased ERα protein expression by E2 in MCF-7 cells (Gierthy et al. 1996). On the other hand, it is unclear if FSE increased PRA and PRB protein expression directly or indirectly in the uterus. Saegusa and Okayasu suggested that alteration of ERα expression is related to the regulation of PRs during endometrial tumorigenesis (Saegusa and Okayasu 2000), and PRA reportedly regulates the activity of a number of ERs (Wen et al. 1994). It is possible that decreased ERα protein expression induced by FSE regulates the expression or activity of PRA/B. Further experiments are needed to elucidate the progestogenic mechanisms and functions of FSE in the uterus.

Our results demonstrate that FSE and its bioactive compounds (compounds 1–5) exhibit estrogenic properties. They showed hERα binding affinity and ERE transcriptional activity, as well as increased proliferation and *pS2* mRNA expression in MCF-7 cells. In addition, FSE led to uterotrophic effects in immature rats. These results reveal the estrogenic effects of FSE in breast and uterine cells.

In the present study, we examined estrogenic properties of FSE in vitro and in vivo for the first time, and provided insights into the estrogenic effects of PEs—derived from FSE—via estrogen receptors. An expanded understanding of tissue specificity and the molecular mechanisms of FSE and its compounds will prompt the development of FSE as a novel agent or promising candidate for the treatment of menopausal symptoms.

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Compliance with Ethical standards

Conflicts of interest The authors declare no conflict of interest.

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