College of Life Science[1], Institute of Biotechnology, Department of Bioscience and Biotechnology, Sejong University, Seoul; Food Function Research Center[2], Korea Food Research Institute, Gyeonggi-Do, Korea

Estrogen receptor mediated effects of *Cimicifuga* extracts on human breast cancer cells

JOONWOO PARK[1], MYEONGKUK SHIM[1], MEE-RA RHYU[2], YOUNGJOO LEE[1]

Received March 1, 2012, accepted March 30, 2012

YoungJoo Lee, Ph. D., Department of Bioscience and Biotechnology, Sejong University, Kwang-Jin-Gu, Seoul 143-747, Korea
yjlee@sejong.ac.kr

Pharmazie 67: 947–950 (2012) *doi: 10.1691/ph.2012.2571*

Cimicifuga racemosa extracts have long been used to treat female reproductive disorders both in Asia and Europe. Here in this study, we examined the possible estrogen receptor (ER)α effects of *Cimicifuga heracleifolia* var. *bifida* ethanol extract (C-Ex), which has been used traditionally in Asia, in MCF-7 cells. The activity of C-Ex was characterized in a transient transfection system, using ERα and estrogen-responsive luciferase plasmids in HEK 293 cells and endogenous target genes were studied in MCF-7 cells. C-Ex failed to activate ERα and at a concentration of 0.005–0.5 mg/ml as examined by reporter activity. In addition, no statistically significant antiestrogenic activity was observed. However, to our interest, C-Ex enhanced expression of VEGF at 0.5 mg/ml concentration and repressed ERα both at the mRNA and protein levels in MCF-7 cells. These results suggested that C-Ex does not activate or inactivate ERα in a direct manner, but the extracts may affect factors in ER signal transduction pathway.

1. Introduction

Women between the age of 45 and 54 experience a decrease in ovarian function and the number of women who are experiencing menopausal symptoms is increasing. Menopausal symptoms include vasomotor symptoms, including hot flushes and night sweats; central nervous system (CNS)-related symptoms such as insomnia and changes in memory, concentration, and mood; urogenital symptoms including vaginal dryness, urinary tract infections, and urinary urgency; and long-term disease processes including osteoporosis (Barber et al. 2004). Estrogen or combined estrogen and progestin therapy (hormone replacement therapy) has been used to alleviate various symptoms associated with diminished ovarian hormone secretion such as heart disease, osteoporosis, hot flushes, mood swings, night sweats, vaginal dryness and postmenopausal symptoms (Cos et al. 2003; Dennerstein et al. 2000). However, the relief from the menopausal symptoms using female steroid hormones is challenged by the increased risk of cardiovascular disease and breast cancer during and after menopause (Dennerstein et al. 2000; Hulley et al. 1998). These concerns have heightened public interest in alternative medicines as a substitute for pharmacological hormone replacement therapy.

Phytoestrogens are a heterogeneous group of nonsteroidal plant-derived compounds that share structural similarities with naturally occurring estrogenic substances, i.e. 17-β-estradiol (Setchell et al. 1998). Although they are less potent than genuine estrogens, it is surmised that increased concentration will overcome their comparatively low affinity for the estrogen receptor (ER) in exerting their effects (Cos et al. 2003). The most commonly used alternative herbal medicines for estrogen replacement are soy, black cohosh, ginseng, pomegranate, evening primrose oil and flax seed (Kronenberg et al. 2002; Newton et al. 2005; Kwon et al. 2008). Intensive research must be conducted to provide scientific rationales for traditional uses and to search for new phytoestrogen sources from natural products. Black cohosh is an herbal extract that is often used for the treatment of climacteric complaints such as hot flushes that frequently accompany the transition to menopause (Davis et al. 2008). Several studies have shown that black cohosh is safe and effective for reducing climacteric symptoms, primarily hot flushes, osteoprotective effects, and possibly mood disorders (Osmers et al. 2005). In a small randomized controlled trial, black cohosh reduced menopausal symptoms without affecting endometrial thickness of the uterus
(Kronenberg et al. 2002). However, intriguingly, studies have showed that black cohosh does not contain estrogenic components (Ruhlen et al. 2007). Some studies showed an uterotrophic effect of black cohosh whereas others showed contrasting results (Bolle et al. 2007). The Sumpter group showed estrogenic effects of black cohosh using a yeast based system (Routledge et al. 1996). In contrast, an antiestrogenic effect of black cohosh was reported (Zierau et al. 2002). Other reports showed that black cohosh is capable of enhancing the production of osteoprotegerin by human osteoblasts and increases osteoblastic differentiation markers osteocalcin and bone-specific alkaline phosphatase indicating estrogenic function (Viereck et al. 2005). It is also reported that black cohosh has no effect on serum levels of luteinizing hormone, follicle stimulating hormone, prolactin, sex hormone binding globulin, and estradiol (Liske et al. 2002).

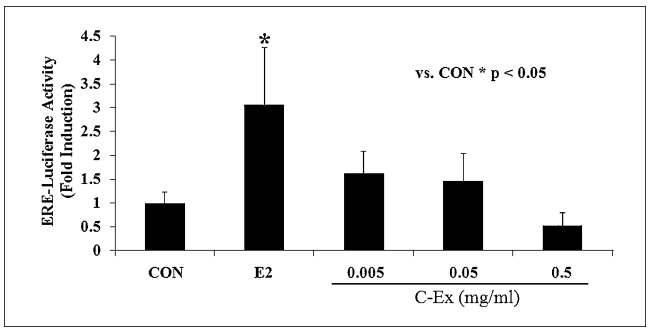

Fig. 1: MCF-7 cells were transiently transfected with ERE-luc plasmid. The cells were treated for 24 h with either E2 or C-Ex at designated concentrations as indicated in the figure. An untreated group served as a control. The data are representative of at least three independent experiments, performed in triplicate, with similar results, expressed as relative luciferase units and the SD of the triplicate samples

Here in this study, we have examined ER mediated effects of *C. heracleifolia* var. *bifida* (C-Ex), which has long been used in Asia.

2. Investigations and results

2.1. C-Ex fails to activate estrogen-responsive luciferase genes in the presence of ERα

Proper ligand binding to the ER initiates transcriptional activation through the specific estrogen response element (ERE) in certain target genes (Gehm et al. 2000). We have examined whether C-Ex activates the transcription of an ERE containing reporter plasmid in the presence of ERα in ER-negative HEK 293 cells. C-Ex failed to activate luciferase expression driven by the ERE in accordance with the results of the Vollmer group (Zierau et al. 2002) on black cohosh (Fig. 1).

2.2. C-Ex down-regulates ERα in MCF-7 cells

To characterize the effects of C-Ex on ERα, ERα protein levels were examined because it is well known that 17-β-estradiol (E2) induces downregulation of ERα as early as 30 min of treatment in E2-responsive cells. ERα protein levels were down-regulated at 24 h of either E2 or C-Ex as compared with control (Fig. 2).

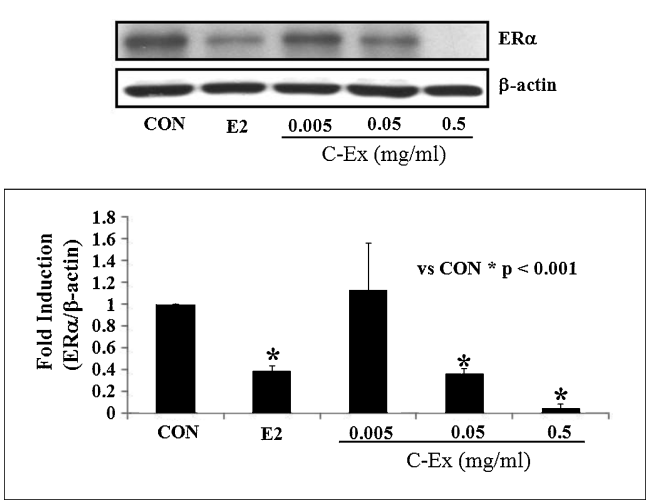

Fig. 2: MCF-7 cell were treated for 24 h with E2 or increasing concentrations of C-Ex, respectively. An untreated group served as a control. After the incubation, the cells were lysed and total protein extracts were resolved by SDS-PAGE and immunoblotted using an anti-ER□antibody or an anti-□-actin antibody. * Represent $p < 0.001$

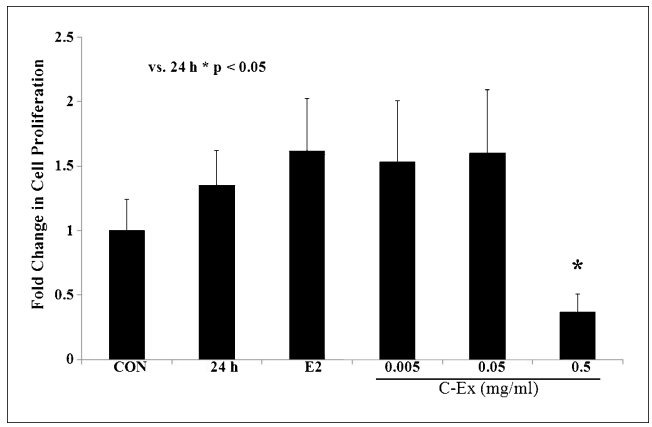

Fig. 3: MCF-7 cells were treated with C-Ex 0.5, 0.05, or 0.005 mg/ml or E2 (10 nM) for 24 h. The percentage cell survival was measured by WST-1 assay

2.3. Effects of C-Ex on MCF-7 cell proliferation

To examine the toxicity of C-Ex, its effects on MCF-7 cell proliferation were determined by WST-1 assay. The treatment of C-Ex had no effect on cell proliferation at concentrations upto 0.05 mg/ml (Fig. 3). However, we observed cell toxicity at 0.5 mg/ml concentration.

2.4. Effects of C-EX on endogenous ER and VEGF genes

Although we were unable to observe any estrogenic activity by reporter gene assays, the effects of C-EX on ERα protein downregulation prompted us to characterize the effects of C-Ex at the cellular gene expression level of ER gene. After treatment of MCF-7 cells with the extract for 24 h, steady-state mRNA levels were measured by qPCR. Constitutively expressed human β-actin mRNA was used as an internal control. C-EX downregulated ER mRNA levels at 24 h of treatment at 0.5 mg/ml concentration even at the concentration exhibiting cell toxicity (Fig. 4A) . In addition, C-Ex enhanced expression of VEGF at 0.5 mg/ml concentration in MCF-7 cells (Fig. 4B). However, these effects were not affected by 1 μM antiestrogen

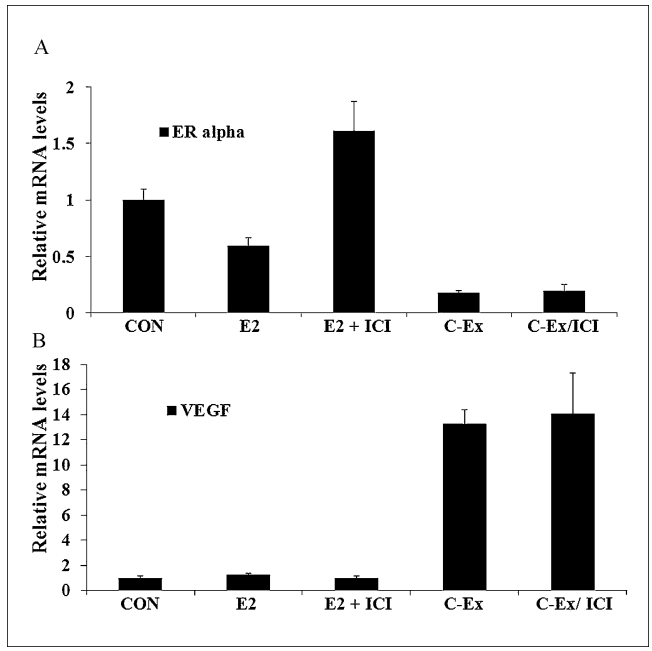

Fig. 4: MCF-7 cells were exposed to E2, C-Ex0.5 mg/ml, or in combination with 1 □M ICI for 24 h. RT-PCR results for ER(A) and VEGF (B) mRNA are shown

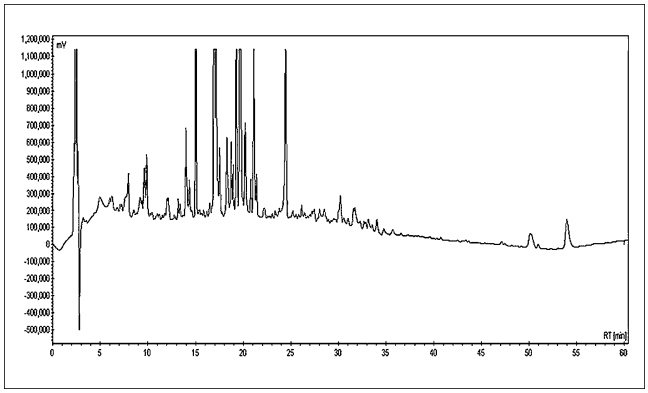

Fig. 5: HPLC profile of *Cimicifuga heracleifolia* var. *bifida* extracts at 203 nm. HPLC condition: linear gradient from 0 % to 80 % A and 100 % to 20 % B in 50 min (A: 1 % Formic acid in ACN, B: 1 % Formic acid in Water). Flow rate: 1 mL/min. Column: Thermo BDS hypersil C18 (250 × 4 mm)

ICI182,780(ICI) cotreatment indicating that the response is not mediated by ER.

3. Discussion

Phytoestrogens are produced in a wide variety of plants and mimic the actions of endogenous estrogens, usually by binding to ER (Setchell et al. 1998; Harris et al. 2005). Phytoestrogens can act as partial ER agonists or antagonists (Kuiper et al. 1998). However, despite the clinical efficacy of black cohosh on climacteric symptoms, *in vitro* data are still controversial with growth inhibitory effects on human breast cancer cells (Einbond et al. 2008; Gaube et al. 2007; Rice et al. 2007). The discrepancy may be due to the differential experimental systems that estrogenic effect of black cohosh is tissue specific. It has been shown that black cohosh acts as a selective estrogen receptor modulator with estrogenic actions in the bone, fat and hypothalamus/pituitary axis (Seidlova-Wuttke et al. 2003). Black cohosh has been reported to have dopamine and serotonin receptor-mediated activities as well (Powell et al. 2008). Black cohosh in 3 out of 7 clinical trials showed some improvements on hot-flush measures in postmenopausal women (Oktem et al. 2007). The efficacy of black cohosh is quite ambiguous at current stage. Further, some results report association of liver damage and increases of metastatic mammary cancer (Davis et al. 2008). Considering the popularity of black cohosh more intensive research is necessary to validate such use on postmenopausal women to ensure safety and efficacy.

We studied the estrogenic effects of extract of *C. heracleifolia* var. *bifida in vitro*, an herbal material related with black cohosh and used to treat female reproductive vascular symptoms related to menopause in Korea. Intriguingly, C-Ex enhanced downregulated ERα expression in the absence of estrogen responsive reporter activity. It is possible that Cimicifuga extract contains components that indirectly activate a small pool of ER target genes without directly activating ERα. Further *in vitro* mechanistic studies and identification of the active components responsible for the cellular effects will improve our understanding of the clinical applications of *C. heracleifolia* var. *bifida*.

4. Experimental

4.1. Materials

E2 was purchased from Sigma (St. Louis, MO, USA) and dissolved in 100% ethanol. ICI was obtained from ZENECA Pharmaceuticals. All of the compounds were added to the medium such that the total solvent concentration was never higher than 0.1%. An untreated group served as a control.

4.2. Preparation of plant extracts

Cimicifuga heracleifolia var. *bifida* Nakai rhizomes/roots were provided by Korea Botanic Garden (405–2 Byungnae-ri, Pyeongchang-gun, Gangwon-do, Korea) and were botanically verified. The rhizomes/roots were freeze-dried and milled with a commercial food mixer. Milled rhizomes/roots of *C. heracleifolia* were separately extracted by 80% ethanol using a homogenizer and evaporated under reduced pressure at low temperatures (37- 40 °C) and then lyophilized to a powder (C-Ex). The solids were stored at -20 °C until use. Crude extracts were dissolved in 80% ethanol. A voucher specimen (CH001) has been deposited at Korea Food Research Institute, Gyeonggi-do, Korea. A HPLC fingerprint of C-Ex was developed by dissolving in 75% MeOH. After centrifugation at 12,000 g for 30 min, the supernatant was filtered through a 0.22-μm filter prior to analysis. HPLC was carried out on a JASCO HPLC System 2000 Series (Jasco Co., Tokyo, Japan) equipped with an UV detector (203 nm), an on-line degasser and an autosampler. HPLC separation was achieved on a Thermo BDS hypersil C18 column (250 × 4 mm) with a linear solvent gradient comprised of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B). Gradient profile: 0–50 min: linear gradient from 0% B in A to 80% B in A. Column temperature: ambient; flow rate: 1 ml/min; injection volume: 20 μl (Fig. 5).

4.3. Cell culture and treatment

MCF-7 cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2 in phenol red-free RPMI supplemented with 10% fetal bovine serum (FBS) (WelGENE, Korea). HEK 293 cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS.

Before treatment, the cells were washed with phosphate-buffered saline and cultured in RPMI or DMEM/5% charcoal-dextran stripped FBS for 1 day to eliminate any estrogenic source before treatment. We used 10 nM E2 and 0.005, 0.05, 0.5 mg/ml for C-Ex as indicated in the Figure.

4.4. Transient transfection and luciferase assay

MCF-7 cells were transiently transfected with plasmids by polyethylenimine (Polysciences, Warrington, PA, USA). Luciferase activity was determined 24 or 48 h after treatment with an AutoLumat LB953 luminometer using the luciferase assay system (Promega Corp., Madison, WI, USA) and expressed as relative light units. The means and standard deviations (SD) of three replicates are shown for the representative experiments. All transfection experiments were repeated three or more times with similar results.

4.5. Quantitative real-time PCR

RNA from MCF-7 cells was extracted using TRIzol reagent (Invitrogen) according to the manufacture's instruction. The yield of RNA was quantified by spectroscopy at 260 nm. To synthesize first strand cDNA, 3 μg total RNA was incubated at 70 °C for 5 min with 0.5 μg of random primer (Promega) and diethylpyrocarbonate-treated water. The reverse transcription reaction was performed using 40 unit of MMLV reverse transcriptase (Promega) in 5X reaction buffer, and 2.5 mM dNTP mixtures at 37 °C for 60 min. The reaction was terminated by heating at 70 °C for 8 min, followed by cooling at 4 C. qPCR was perfomed using iQ™ SYBR Green Supermix (Bio-Rad). The β-actin primers were sense (5'-CAAATGCTTCTAGGCGGACTATG-3') and antisense (5'-TGCGCAAGTTAGGTTTTGTCA-3'). The ERα primers were sense (5'-CTACTGTGCAGTGTGCAATGACTA-3') and antisense (5'-ATGTCCTTGAATACTCTCTTGAAG-3'). The VEGF primers were sense (5'-CTGCTGTCTTGGGTGCATTGG-3') and antisense (5'-GTTTGATCCGCATAATCTGCAT-3'). A final volume was 25 μl, and an iCycler iQ Real-time PCR Detection System (Bio-Rad) was used for qPCR. The amplification data were analyzed by iQ™5 optical system software version 2.1 and calculated using the $\Delta\Delta C_T$ method. The $\Delta\Delta C_T$ method was used to calculate relative mRNA expression. The relative target gene expression was calculated using $2^{-\Delta\Delta CT}$, where ΔC_T = target C_T - control C_T, $\Delta\Delta C_T = \Delta C_T$ target - ΔC_T calibrator.

4.6. Western blot analysis

Protein was separated using a radioimmune precipitation buffer (including 150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS, with a protease inhibitor cocktail) on ice for 1 h and then centrifuged for 25 min at 13000 rpm. Protein concentrations were determined using the Bradford method (Bio-Rad). For immunoblotting, same amounts of proteins were separated by SDS-PAGE. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween (TBST), and incubated with rabbit polyclonal antibody to ERα (0.4 mg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or mouse polyclonal anti-

body to β-actin (Sigma). After washing with TBST, blots were incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence ECL kits (Amersham Bioscience, Little Chalfont, UK).

4.7. Cell proliferation measurements

MCF-7 cells were treated at a density of 3000 cells/well in a final volume of 100 µl and incubated in 96 well plates for 24 h followed by 1 h with a tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate). 10 µl of WST-1/100 µl medium was added to each well. After 1 h at 37 °C, absorption was measured at 450 nm. The absorbance observed at 630 nm reference wavelength was used as background control. Three independent set of experiments were performed in triplicate and evaluated. The growth inhibition rate was calculated as percentage of parallel untreated controls.

4.8. Statistical analysis

Values shown represent the mean ± SD. Statistical analysis was performed by Student's *t*-test, and a p value < 0.05 was considered significant.

Acknowledgements: This work was supported by grants from the Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea (109127-03-1-HD110).

References

Barber CA, Margolis K, Luepker RV, Arnett DK (2004) The impact of the Women's Health Initiative on discontinuation of postmenopausal hormone therapy: the Minnesota Heart Survey (2000–2002). J Womens Health (Larchmt) 13: 975–985.

Bolle P, Mastrangelo S, Perrone F, Evandri MG (2007) Estrogen-like effect of a Cimicifuga racemosa extract sub-fraction as assessed by *in vivo*, ex vivo and *in vitro* assays. J Steroid Biochem Mol Biol 107: 262–269.

Cos P, De Bruyne T, Apers S, Vanden Berghe D, Pieters L, Vlietinck AJ (2003) Phytoestrogens: recent developments. Planta Med 69: 589–599.

Davis VL, Jayo MJ, Ho A, Kotlarczyk MP, Hardy ML, Foster WG, Hughes CL (2008) Black cohosh increases metastatic mammary cancer in transgenic mice expressing c-erbB2. Cancer Res 68: 8377–8383.

Dennerstein L, Dudley EC, Hopper JL, Guthrie JR, Burger HG (2000) A prospective population-based study of menopausal symptoms. Obstet Gynecol 96: 351–358.

Einbond LS, Wen-Cai Y, He K, Wu HA, Cruz E, Roller M, Kronenberg F (2008) Growth inhibitory activity of extracts and compounds from Cimicifuga species on human breast cancer cells. Phytomedicine 15: 504–511.

Gaube F, Wolfl S, Pusch L, Kroll TC, Hamburger M (2007) Gene expression profiling reveals effects of Cimicifuga racemosa (L.) NUTT. (black cohosh) on the estrogen receptor positive human breast cancer cell line MCF-7. BMC Pharmacol 7: 11.

Gehm BD, McAndrews JM, Jordan VC, Jameson JL (2000) EGF activates highly selective estrogen-responsive reporter plasmids by an ER-independent pathway. Mol Cell Endocrinol 159: 53–62.

Harris DM, Besselink E, Henning SM, Go VL, Heber D (2005) Phytoestrogens induce differential estrogen receptor alpha- or beta-mediated responses in transfected breast cancer cells. Exp Biol Med (Maywood) 230: 558–568.

Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E (1998) Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and estrogen/progestin replacement study (HERS) research group. JAMA 280: 605–613.

Kronenberg F, Fugh-Berman A (2002) Complementary and alternative medicine for menopausal symptoms: a review of randomized, controlled trials. Ann Intern Med 137: 805–813.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139: 4252–4263.

Kwon HY, Rhyu MR, Lee YJ (2008) The effects of Cirsium japonicum on lipid profile in ovariectomized rats. Biomol Therap 16: 293–298.

Liske E, Hänggi W, Henneicke-von Zepelin HH, Boblitz N, Wüstenberg P, Rahlfs VW (2002) Physiological investigation of a unique extract of black cohosh (Cimicifugae racemosae rhizoma): a 6-month clinical study demonstrates no systemic estrogenic effect. J Womens Health Gend Based Med. 11: 163–174.

Newton KM, Reed SD, Grothaus L, Ehrlich K, Guiltinan J, Ludman E, Lacroix AZ (2005) The herbal alternatives for menopause (HALT) Study: background and study design. Maturitas 52: 134–146.

Oktem M, Eroglu D, Karahan HB, Taskintuna N, Kuscu E, Zeyneloglu HB (2007) Black cohosh and fluoxetine in the treatment of postmenopausal symptoms: a prospective, randomized trial. Adv Ther 24: 448–461.

Osmers R, Friede M, Liske E, Schnitker J, Freudenstein J, Henneicke-von Zepelin HH (2005) Efficacy and safety of isopropanolic black cohosh extract for elimacterie symptoms. Obstet Gynecol 105: 1074–1083.

Powell SL, Gödecke T, Nikolic D, Chen SN, Ahn S, Dietz B, Farnsworth NR, van Breemen RB, Lankin DC, Pauli GF, Bolton JL (2008) *In vitro* serotonergic activity of black cohosh and identification of N(omega)-methylserotonin as a potential active constituent. J Agric Food Chem 56: 11718–11726.

Rice S, Amon A, Whitehead SA (2007) Ethanolic extracts of black cohosh (Actaea racemosa) inhibit growth and oestradiol synthesis from oestrone sulphate in breast cancer cells. Maturitas 56: 359–367.

Routledge EJ, Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15: 241–248.

Ruhlen RL, Haubner J, Tracy JK, Zhu W, Ehya H, Lamberson WR, Rottinghaus GE, Sauter ER (2007) Black cohosh does not exert an estrogenic effect on the breast. Nutr Cancer 59: 269–277.

Seidlova-Wuttke D, Hesse O, Jarry H, Christoffel V, Spengler B, Becker T, Wuttke W (2003) Evidence for selective estrogen receptor modulator activity in a black cohosh (Cimicifuga racemosa) extract: comparison with estradiol-17beta. Eur J Endocrinol 149: 351–362.

Setchell DR, Zimmer-Nechemias L, Cai J, Heubi JE (1998) Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life. Am J Clin Nutr 68: 1453–1261.

Viereck V, Gründker C, Friess SC, Frosch KH, Raddatz D, Schoppet M, Nisslein T, Emons G, Hofbauer LC (2005) Isopropanolic extract of black cohosh stimulates osteoprotegerin production by human osteoblasts. J Bone Miner Res. 20(11): 2036–2043.

Zierau O, Bodinet C, Kolba S, Wulf M, Vollmer G (2002) Antiestrogenic activities of Cimicifuga racemosa extracts. Steroid Biochem Mol Biol 80: 125–130.