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# Establishment of *In Vitro* Test System for the Evaluation of the Estrogenic Activities of Natural Products

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In order to evaluate estrogenic compounds in natural products, an *in vitro* detection system was established. For this system, the human breast cancer cell line MCF7 was stably transfected using an estrogen responsive chloramphenicol acetyltransferase (CAT) reporter plasmid yielding MCF7/pDsCAT-ERE119-Ad2MLP cells. To test the estrogenic responsiveness of this *in vitro* assay system, MCF7/pDsCAT-ERE119-Ad2MLP cells were treated with various concentrations of 17β-estradiol. Treatments of $10^{-8}$ to $10^{-12}$ M 17β-estradiol revealed significant concentration dependent estrogenic activities compared with ethanol. We used *in vitro* assay system to detect estrogenic effects in *Puerariae radix* and *Ginseng radix Rubra* extracts. Treatment of 500 and 50 μg/ml of *Puerariae radix* extracts increased the transcriptional activity approximately 4- and 1.5-fold, respectively, compared with the ethanol treatment. Treatment of 500, 50, and 5 μg/ml of *Ginseng radix Rubra* extracts increased the transcriptional activity approximately 3.2-, 2.7, and 1.4-fold, respectively, compared with the ethanol treatment. These observations suggest that *Puerariae radix* and *Ginseng radix Rubra* extracts have effective estrogenic actions and that they could be developed as estrogenic supplements.

**Key words:** Chloramphenicol acetyltransferase (CAT), Estrogen, Isoflavone, *Ginseng radix Rubra*, Korea red ginseng, MCF7 cells, Phytoestrogen, *Puerariae radix*

## INTRODUCTION

In recent decades, many compounds of plant origin that have an ability to bind to the estrogen receptor have been identified. There is evidence that the consumption of some of these plant-derived estrogens has beneficial effects. For this reason, there is a requirement in characterizing the estrogenic potencies of these substances. *In vitro* test systems offer the possibility to efficiently screened compounds. Tests routinely used for the determination of estrogenicity include: (i) receptor binding assays, (ii) E-screen, (iii) reporter gene assays, and (iv) the analysis of the regulation of endogenous estrogen sensitive genes in cell lines (Diel *et al.*, 1999). Of these tests, the reporter gene assays have proven to be useful and powerful tools for identifying substances that are capable of activating estrogen dependent transcription and for determining their estrogenic potency (Diel *et al.*, 1999). Reporter gene assays are suitable for characterizing the agonistic and antagonistic properties of a substance and for analyzing a large number of substances within a short period of time (Diel *et al.*, 1999). Because of these advantages, we chose a reporter gene assay to establish an *in vitro* test system and to evaluate the estrogenic activities of natural products. Bronstein *et al.* (1994) used a reporter gene assay to determine the abilities of substances to activate the transcription of an estrogen-sensitive promoter. In the present study, human breast cancer MCF cells were transfected with the estrogen responsive reporter plasmid. For the reporter plasmid, we chose minimal chimeric promoters that were comprised of the TATA region of the adenovirus-2 major late promoter (Ad2MLP) (Miyamoto *et al.*, 1985) and the two perfect estrogen responsive elements

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(ERE119) of the *Xenopus* vitellogenin A2 gene that are linked to a reporter gene (Ponglikitmongkol *et al.*, 1990).

To date, more than 300 plants have been found that possess compounds with estrogenic activity (Fransworth *et al.*, 1975; Price and Fenwich, 1985). These compounds are referred as phytoestrogens. A number of classes of phytoestrogens have been identified, established, and studied: the hormone-like bisphenolic phytoestrogens, the isoflavonoids daidzein and genistein, the coumestans (coumestrol), and the lignans secoisolariciresinol (SECO), and matairesinol (MAT) are of great interest because of their estrogenic, antiestrogenic, anticarcinogenic, antiviral, antifungal, and antioxidant activities (Adlercreutz, 1984; Adlercreutz *et al.*, 1982; Setchell *et al.*, 1980, 1981; Whitten and Naftolin, 1991). Moreover, these phytoestrogens are structurally and functionally similar to estrogen (Knight and Eden, 1996).

*Puerariae radix* is an oriental medicinal plant that has been used as an antipyretic, antispasmodic, and as a migraine agent. Many isoflavones, such as daidzin (Keung and Vallee, 1998a), puerarin (Guerra *et al.*, 2000), and sapogenols (Arao *et al.*, 1998), have been reported to be extracted from this plant. Also, a number of investigations have been recently carried out internationally to identify the compounds responsible for the biological activity of *Puerariae radix* (Keung and Vallee, 1998b; Lin *et al.*, 1996; Lin and Li, 1998; Xuan *et al.*, 1999). The isolated constituents in the ethanol extract of *Puerariae radix* contains flavonoids, coumarines, and especially isoflavones, such as daidzein, daidzin, puerarin, and daidzin-4,7-diglucoside (Cao *et al.*, 1999). Moreover, six phenolic glucosides were isolated from the methanol extract of *Puerariae radix* (Hirakura *et al.*, 1997).

Panax ginseng has been used for more than 2000 years as a general tonic in oriental medicine. Among the various types of Panax ginseng, Korea red ginseng (*Ginseng radix Rubra*) has several pharmacological and physiological effects. In particular, the saponin fraction of Korea red ginseng shows a variety of efficacies such as anticancer, antihypertension, antidiabetes, antinociception, and improving weak body conditions (Jung and Jin, 1996). A number of components have been isolated and characterized from ginseng including ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids (Liu *et al.*, 2000). Among these ingredients, a group of saponin glycosides, known as ginsenosides, comprises a four trans-ring rigid steroid skeleton with a modified side chain (Nah *et al.*, 1995). Ginseng has been recommended for use in the alleviation of the symptoms of menopause, indicating that some components of ginseng can possibly act as phytoestrogens (Amato *et al.*, 2002).

In this investigation, an *in vitro* detection system was established and used to evaluate the estrogenic activities of the ethanol extracts of *Puerariae radix* and *Ginseng radix Rubra* as model natural products.

## MATERIALS AND METHODS

### Plasmid Construction

Complementary oligonucleotides spanning a minimal promoter composed of the TATA region of the adenovirus-2 major later promoter (Ad2MLP, -33 to +34; Table I) (Miyamoto *et al.*, 1985) were synthesized. After annealing, the double-stranded oligonucleotide was subcloned between the *Pst*I and *Xba*I sites in pCAT-Basic (Promega, USA) to yield pCAT-Ad2MLP. Complementary oligonucleotides spanning two perfectly palindromic *Xenopus* vitellogenin A2 gene EREs (ERE119; Table I) (Ponglikitmongkol *et al.*, 1990) were also synthesized. After annealing, the double-stranded oligonucleotide was subcloned between the *Hin*dIII and *Pst*I sites in pCAT-Ad2MLP to yield pCAT-ERE119-Ad2MLP. In order to achieve stable transfection, pDsCAT-ERE119-Ad2MLP was constructed by inserting the 1,800-bp *Hin*dIII-*Bam*HI fragment of pCAT-ERE119-Ad2MLP into the corresponding sites of pDSRed1-1 (Clontech, USA).

The integrities of all constructs were verified by restriction analysis and sequencing.

### Cell culture and stable transfection

The human breast cancer cells line, MCF7, was obtain-

**Table I.** Oligonucleotides for plasmid construction

| | |
|---|---|
| Ad2MLP | |
| Sense | 5'-GCTATAAAAGGGGGTGGGGGCGCGTTCGTCCTCAC<br>*Pst*I<br>TCTCTTCCGCATCGCTCTCTGCGAGGGCCAGCT3'<br>*Xba*I |
| Antisense | 5'-CTAGAGCTGGCCCTCGCAGACAGCGATCCGGAAG<br>*Xba*I<br>AGAGTGAGGACGAACGCGCCCCCACCCCCTTTTAT<br>AGCTGCA 3'<br>*Pst*I |
| ERE119 | |
| Sense | 5'-AGCTTCGAGATCAGGTCACAGTGACCTGACTCGAC<br>*Hin*dIII<br>ATCAGGTCACAGTGACCTGACTCTGCA-3'<br>*Pst*I |
| Antisense | 5'-GAGTCAGGTCACTGTGACCTGATCTCGAGTCAGGT<br>*Pst*I<br>CACTGTGACCTGATCTCGA-3'<br>*Hin*dIII |

The ERE sequences are underlined

ed from ATCC (Manassas, VA, USA), and was cultured at 37°C in humidified 10% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker, USA), which was supplemented with 10% fetal bovine serum (BioWhittaker). Transfection was carried out in serum free medium in 60 mm dishes using the Lipofectamine plus reagent (Invitrogen BV, Netherlands) and 5 μg of pDsCAT-ERE119-Ad2MLP plasmid. Plasmid that was used for the transfection was purified using a Plasmid Midi Kit (Qiagen, USA). For stable transfection, DMEM containing 10% fetal bovine serum and G418 (final concentration: 0.8 mg/ml) (both from BioWhittaker) was placed on the cells one day after the transfection, and the cells were fed every 3 days. Stably transfected MCF7 cells possessing the estrogen responsive CAT reporter plasmid in their chromosomal DNA were termed MCF7/pDsCAT-ERE119-Ad2MLP and stored in liquid nitrogen.

### Preparation of *Puerariae radix* and *Ginseng radix rubra* extracts, and estrogenic activity testing

*Puerariae radix* and *Ginseng radix Rubra* were dried and extracted with 95% ethanol for 3 hours at boiling point (about 65°C). Ethanol extracts were filtered, concentrated, and lyophilized to give dried extracts. Five hundred mg of dried extracts were then dissolved in 99% ethanol and stored at 4°C.

MCF7/pDsCAT-ERE119-Ad2MLP cells were cultured at 37°C in a humidified 10% $CO_2$ atmosphere in DMEM (without phenol-red, BioWhittaker, USA), which was supplemented with 10% dextran-coated charcoal stripped fetal bovine serum (Ponglikitmongkol *et al.*, 1990). At 90% confluency, extracts of the natural product, 17β-estradiol (RBI, USA), or vehicle (ethanol, 5 μL) were added to the corresponding dishes. The cells were harvested 24 h after sample treatment, lysed by freeze/thawing (four cycles), and then centrifuged.

CAT assays were carried out using a CAT-enzyme linked immunosorbent assay (ELISA) kit (Boehringer Mannheim). All CAT assay results were normalized to the protein concentration of lysates as determined by a BCA Protein Assay Reagent kit (Pierce, Rockford, IL).

## RESULTS

### Construction of an estrogen responsive reporter plasmid

In order to verify the estrogenic activities of natural products, an estrogen responsive CAT reporter plasmid was constructed using a minimal promoter that was composed of the TATA region of the adenovirus-2 major later promoter (Ad2MLP, -33 to +34; Table I) (Miyamoto *et al.*, 1985) and two perfectly palindromic *Xenopus* vitellogenin A2 gene EREs (ERE119; Table I) (Ponglikitmongkol *et al.*, 1990). The double-stranded Ad2MLP fragment was inserted between the *Pst*I and *Xba*I sites of pCAT-Basic to yield pCAT-Ad2MLP (Fig. 1). The double-stranded ERE119 fragment was then inserted between the *Hin*dIII and *Pst*I sites in pCAT-Ad2MLP to yield pCAT-ERE119-Ad2MLP (Fig. 1), and to yield the pDsCAT-ERE119-Ad2MLP, the 1,800-bp *Hin*dIII-*Bam*HI fragment of pCAT-ERE119-Ad2MLP was inserted into the corresponding sites of pDSRed1-1 (Fig. 1).

### Establishment of estrogen responsive reporter cells

To simplify the estrogenic activity analysis procedure, stably transfected MCF7 cells were established. Because MCF7 cells are known to express an endogenous estrogen receptor protein, cotransfection with an estrogen receptor expression plasmid was not necessary to perform a trans-activation assay (Diel *et al.*, 1999). MCF7 cells were transfected with an estrogen responsive CAT reporter plasmid (pDsCAT-ERE119-Ad2MLP), and stably transfected MCF7 cells possessing an estrogen responsive CAT reporter plasmid in their chromosome were termed MCF7/pDsCAT-ERE 119-Ad2MLP cells.

To test the estrogenic responsiveness of this *in vitro* assay system, MCF7/pDsCAT-ERE119-Ad2MLP cells were treated with various concentrations of 17β-estradiol (Fig. 2). Treatment with $10^{-8}$ to $10^{-12}$ M 17β-estradiol revealed significant estrogenic activities compared with ethanol treatment, in a concentration dependant manner (Fig. 2). Treatments of $10^{-8}$ M and $10^{-9}$ M 17β-estradiol increased the transcriptional activity 2.5-fold and 2-fold, respectively, relative to the ethanol treatment (Fig. 2). Treatment with $10^{-12}$ M 17β-estradiol also resulted in a significantly greater amount of transcriptional activity compared with ethanol treatment (Fig. 2).

### Estrogenic activities were detected in *Puerariae radix* and *Ginseng radix Rubra* extracts

Four hundred grams of *Puerariae radix* was extracted in 2 L of ethanol, and 100 g of *Ginseng radix Rubra* was extracted in 1 L of ethanol. The extracts were then filtered, concentrated, and lyophilized to give 13 g of dried *Puerariae radix* extract and 2 g of dried *Ginseng radix Rubra* extract, respectively.

The MCF7/pDsCAT-ERE119-Ad2MLP cell based *in vitro* assay system was then used to detect the estrogenic effects of *Puerariae radix* and *Ginseng radix Rubra* extracts. After treating MCF7/pDsCAT-ERE119-Ad2MLP cells with *Puerariae radix* extract, *Ginseng radix Rubra* extract (final concentrations; 500, 50, or 5 μg/mL), or 17β-estradiol (final concentrations; $10^{-8}$, $10^{-9}$, or $10^{-10}$ M) as the standard solution, cell extracts were prepared and assayed for the CAT protein. Treatment with 500 μg/mL of

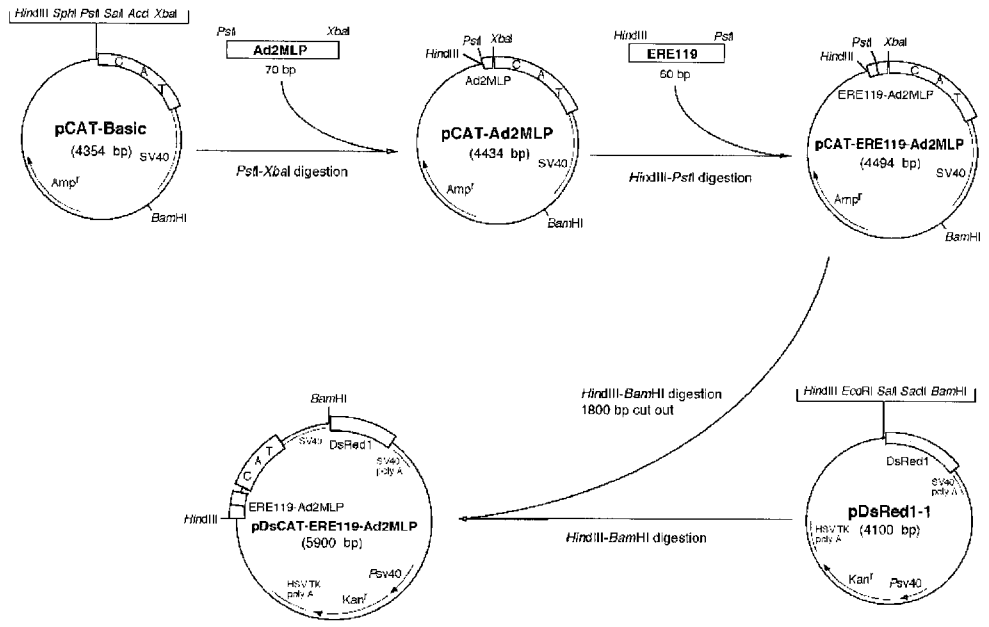


**Fig. 1.** Construction of an estrogen responsive reporter plasmid. An Ad2MLP fragment was inserted into the *Pst*I-*Xba*I sites of pCAT-Basic, which yielded pCAT-Ad2MLP. And then, an ERE119 fragment was inserted into the *Hin*dIII-*Pst*I sites of pCAT-Ad2MLP yielding pCAT-ERE119-Ad2MLP. A 1,800 bp *Hin*dIII-*Bam*HI fragment of pCAT-ERE119-Ad2MLP was then inserted into the corresponding sites of pDsRed1-1 yielding pDsCAT-ERE-119-Ad2MLP. CAT; chloramphenicol acetyltransferase gene, Amp$^r$; ampicillin registance gene, SV40; simian virus 40 small T antigen region, Ds xRed1; *Discosoma* sp. Human codon-optimized red fluorescent protein gene, SV40 poly A; SV40 polyadenylation signal, PSV40; SV40 early promoter, Kan$^r$; kanamycin resistance gene, HSV TK poly A; herpes simplex virus thymidin kinase polyadenylation signal.

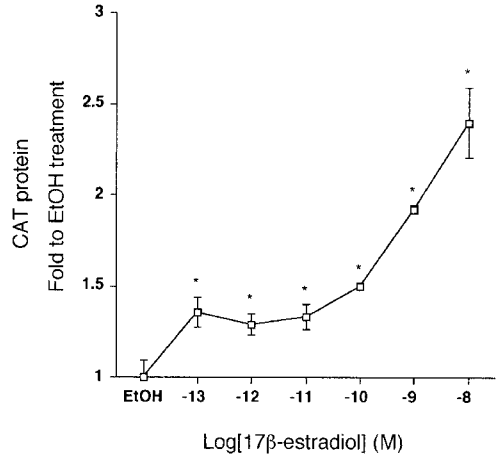


**Fig. 2.** Estrogenic responsiveness of the developed *in vitro* assay system. MCF7/pDsCAT-ERE119-Ad2MLP cells were treated with the indicated concentrations of 17β-estradiol or ethanol. CAT activity was measured using a CAT-ELISA kit and was normalized to the protein concentrations of cell lysates. Means±SEM for three plates are shown in reference with the ethanol treatment. *ANOVA $p<0.0001$ compared with the ethanol treatment. This experiment was repeated at least twice and has yielded reproducible results.

*Puerariae radix* extract increased transcriptional activity approximately 4-fold relative to the ethanol treatment (Fig. 3). Furthermore, treatment with 50 μg/mL of *Puerariae radix* extract also showed a 1.5-fold increase in transcriptional activity versus ethanol (Fig. 3). On the other hand, treatment with 5 μg/mL of *Puerariae radix* extract did not increase transcriptional activity (Fig. 3). Treatment with 500 μg/mL of *Ginseng radix Rubra* extract allowed for approximately a 3.2-fold increase of transcriptional activity compared to the ethanol treat-ment (Fig. 3). Furthermore, treatment with 50 μg/mL of *Ginseng radix Rubra* extract also showed a 2.7-fold increase in transcriptional activity versus ethanol (Fig. 3), and treatment with 5 μg/mL of *Ginseng radix Rubra* extract also showed a 2.7-fold increase in transcriptional activity versus ethanol (Fig. 3).

Transcriptional activities induced by 50 μg/mL of *Puerariae radix* extract and 5 μg/mL of *Ginseng radix Rubra* extract were statistically significant compared with the transcriptional activities induced by ethanol treatment, and they were the same as that induced by $10^{-10}$ M 17β-estradiol (Fig. 3). Moreover, the transcriptional activities induced by 500 μg/mL of *Puerariae radix* and *Ginseng radix Rubra* extracts were higher than that induced by $10^{-8}$ M of 17β-estradiol. In addition, transcriptional activity induced by 50 mg/mL of *Ginseng radix Rubra* extract was higher than that induced by $10^{-9}$ M of 17β-estradiol (Fig. 3).

## DISCUSSIONS

Phytoestrogens, in particular isoflavonoids, are believed

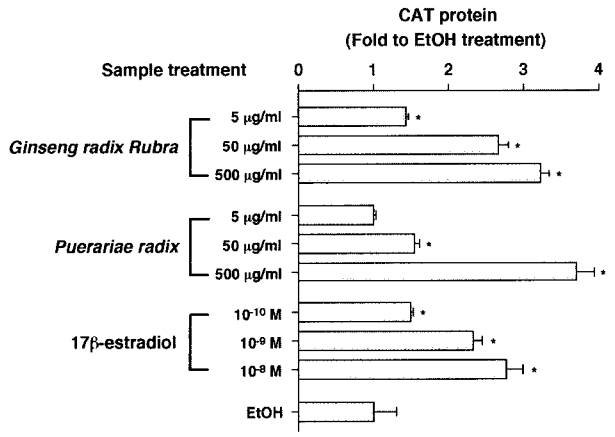


**Fig. 3.** Estrogenic activities of *Puerariae radix* and *Ginseng radix Rubra* extracts. MCF7/pDsCAT-ERE119-Ad2MLP cells were treated with the indicated concentrations of *Puerariae radix* or *Ginseng radix Rubra* extract, 17β-estradiol, or ethanol. CAT activity was measured using a CAT-ELISA kit and was normalized with respect to the protein concentrations of cell lysates. Mean±SEM for three plates are shown as multiples of the ethanol treatment results. *ANOVA $p<0.0001$ compared with the ethanol treatment. This experiment was repeated at least twice and has yielded reproducible results.

to have a range of beneficial effects that are largely related to their mild estrogenic activities (Lee *et al.*, 1991; Whitten and Patisaul, 2001). Since they are weak estrogens, in principle, they could be used to attenuate estrogenic activity in situations where estrogens have devastating effects, e.g. hormone dependent cancer growth (Auborn *et al.*, 2003; Barnes, 1995), and stimulate it when estrogenic activity appears to be deficient, e.g. in case of osteoporosis (Melis *et al.*, 1992). Isoflavonoids are also receiving much attention as food supplements for the purposes of enhancing health and preventing several common diseases, such as cardiovascular diseases, cancers of reproductive tissues, and osteoporosis (Anderson and Garner, 1998). Moreover, the isoflavones of soy, mainly genistein and daidzein, have been shown by at least three different laboratories to conserve bone in ovariectomized rodent models, and they may have similar conservatory effects in higher mammalian species (Anderson and Garner, 1998; Anderson *et al.*, 1998). Phytoestrogens can also improve memory and offer protection against Alzheimer's disease (Howes and Perry, 2003; Lephart *et al.*, 2002; Zhao *et al.*, 2002).

Using our *in vitro* assay system, we successfully detected estrogenic activities in *Puerariae radix* and *Ginseng radix Rubra* extracts as model natural products. These observations suggest that our system can adequately detect estrogenic activity in natural products. Furthermore, the active components in *Puerariae radix* and *Ginseng radix Rubra* extracts could be developed as an estrogenic supple-ment to relieve the symptoms of menopausal patients suffering from defective estrogen secretion.

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