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Screening of Korean Medicinal Herbs for Hormonal Activities using Recombinant Yeast Assay and MCF-7 Human Breast Cancer Cells

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재조합효모와 MCF-7 사람유방암세포주를 이용한 한국산 약용식물의 호르몬 활성 스크리닝

양세란 · 홍희도[1] · 조성대 · 안남식 · 정지원 · 박준석 · 조은혜 · 황재웅 · 순 보 · 박정란 · 이성훈 · 정지윤 · 최창순[2] · 강경선[†] · 이영순[†]

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ABSTRACT – To investigate whether there are estrogenic and anti-estrogenic activities in various medicinal herbs and discover prominent chemo-preventive agents, we screened and compared the ethanol extracts of 9 plants through the recombinant yeast assay and MCF-7 human breast cancer cell assay. In recombinant yeast assay, seven medicinal herbs showed estrogenicity, and four extracts showed androgenecity. In MCF-7 proliferation assay, the growth of MCF-7 cells was inhibited by eight extracts before and even after co-treatment with bisphenol A. It is interesting that the extracts of *Glycyrrhiza uralensis*, *Cassia tora*, *Syringa velutina*, *Zingiber officinale*, *Malva verticillata*, and *Panax ginseng* C.A. Meyer exhibited inhibitory effects as phytoestrogens in estrogen-responsive human breast cancer cells. This study suggests that some Korean medicinal herbs might be considered as phytoestrogens and be useful to further analyze those plants which contain the estrogenic effect in order to identify the active principles.

Key words: Breast cancer, MCF-7 human breast cancer cell, Phytoestrogen, Recombinant yeast assay, Screening

Herbal medicines have been used in medical practice for thousands of years and recognized especially as a valuable and readily available resource for healthcare in East Asian nations. In addition, it may well be that phytoestrogen can be found in many medicinal herbs since they are composed of various plant materials.[1)] Phytoestrogens are estrogen-like substances that have a diphenolic and nonsteroidal structure and are found in many plants including fruits, vegetables, legumes, whole grains, and especially in soybean products.[2)] Phytoestrogen-rich diets have been suggested to prevent or alleviate estrogen-related diseases or conditions including cardiovascular diseases, menopausal symptoms and post-menopausal osteoporosis.[3)] In addition, phytoestrogen research has brought about a great impact in plant consumption by humans, and most the results concluded that routine consumption of phytoestrogen-rich plant products would benefit the body for all ages and sex, mostly in terms of cancer protection.[4)]

As the other issues, endocrine disruptors may cause the dysfunction of reproductive organs (cryptochidism, hypospadias, decline in sperm concentration etc.) and steroid hormone-dependent human cancers (breast, prostate, colon, etc.), which are a leading cause of morbidity and mortality in industrialized countries.[5,6)] Among them, bisphenol A (BPA) is a well-known estrogenic

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molecule[7] used extensively as a monomer in the plastics industry.[8] BPA has been reported to have estrogenic actions such as uterotrophic effects,[9] decreased sperm production[10] and promotion of cell proliferation in a breast cancer cell line.[11]

In the present study, we screened medical herbs in Korea regarding their effects as phytoestrogens by recombinant yeast assay and MCF-7 proliferation assay. In addition, we discussed whether these plants have inhibitory effects of BPA. These results may provide useful information on further discovering chemopreventive phytoestrogens.

MATERIALS AND METHODS

Plant materials

All plants from Kyungsan Province, Korea, were purchased in the Kyungdong oriental medicine market, Seoul, Korea. The fresh plants were washed, disintegrated and extracted with ethanol for 24 h. The crude extracts were obtained by subjecting them into silica gel chromatography and were evaporated to dryness with a rotary evaporator. The names of these medicinal herbs, plant parts, and their traditional use are listed in Table 1.

Estrogen and androgen-receptor assay

Recombinant yeast strains (*Saccharomyces cerevisiae* ER + LYS 8127(YER) and *S. cerevisiae* AR + 8320 (YAR)) were kindly provided by Dr. Donald P. Mcdonnell (Duke University Medicinal Center, USA). The yeast cells were allowed to grow the $OD_{600\ nm}$ value of 1~2, then treated with natural products in diluted yeast. As positive controls, 17-β estradiol (E2; 10^{-9} M) and nortestosterone (NT; 10^{-9} M) were used in the estrogen- and androgen-receptor assay, respectively. After 18 h in a shaking incubator, β-galactosidase activity was measured with OD_{420nm} and OD_{590nm} values from each well using Titertek multiskan MCC/344 plate reader after allowing the tube to stand for 20 min. The OD_{420nm} value of each well was corrected by subtracting the OD_{590nm} value. Values represented the mean (ratio) of 3 independent experiments for each samples.

MCF-7 cell proliferation assay

MCF-7 cell proliferation assay was referred to our previous procedure.[12] The cells were placed in an incubator maintained under 5% CO_2, 95% air condition at 37°C. All compounds were diluted with the phenol red-free D-media supplemented with 5% dextran-coated charcoal-stripped FBS (DCC-FBS; Hyclone, USA) and 3ml/ Penicillin-Streptomycin-Neomycin (PSN) antibiotic mixture (test media). The cells (5×10^4/ml) were seeded in a 6-well culture plate (2 ml/well) in triplicate, and allowed to attach for 24 h. The cells were incubated at 37 °C for 3 days, and the test media were changed once. DNA content in the clear lysate was measured at OD_{260nm} with a spectrophotometer (Du 650, Beckman, Fullerton, USA). Values represented the mean (ratio) of 3 independent experiments for each samples.

Statistical Analysis

Data were analyzed using SAS program (SAS institute, USA) for one-way ANOVA. If the overall F-test was significant, the two-tailed Dunnett's t-test was performed to determine significant differences at the level of $P<0.05$ between the means of the treatment and control groups. Fold induction of natural product relative to control was determined by OD_{420nm} and OD_{590nm} values.

Table 1. Korean medicinal herbs collected and their traditional use

Herbal medicines	Family	Plant parts	Uses in Korea
Glycyrrhiza uralensis FISCH	Leguminosae	Root	Allergic-inflammation
Angelica gigas NAKAI	Umbelliferae	Root	Anemia, circulatory disorders
Cassia tora L.	Leguminosae	Seed	Various ailments
Syringa velutina var. Kamibayashii. T Lee	Oleaceae	Root	Abdominal distension
Zingiber officinale Rosc.	Zingiberaceae	Root	Inflammation, osteoarthritis
Carthamus tinctorius L.	Compositae	Seed	Inflammation, osteophoresis
Astragalus membranaceus BUNGE	Leguminosae	Root	Cardiotonic, diuretic
Malva verticillata L.	Malvaceae	Root	Diuretic
Panax ginseng C.A. Meyer	Araliaceae	Root	Anticancer

RESULTS

Estrogenic or Androgenic effects of plant extracts

A total of 9 ethanolic extracts were investigated. Table 2 show that extracts from *Syringa velutina*, *Astragalus membranaceus* and *Panax ginseng* C.A. Meyer exhibited estrogenic activities at the high concentrations (1000 μg/ml). However, *Glycyrrhiza uralensis*, *Cassia tora*, *Zingiber officinale*, and *Malva verticillata* showed estrogenic activities at the high and medium concentrations (1000 μg/ml and 100 μg/ml). By the yeast-based androgen receptor assay, *Glycyrrhiza uralensis*, *Cassia tora*, and *Syringa velutina* are shown to possess androgenic activities at the high concentrations. However, *Zingiber officinale* showed androgenecity in high and medium concentrations.

Anti-proliferation effects of the plant extracts and antagonic effects treated with bisphenol A (BPA)

Anti-proliferation effects of the same set of plant extracts were evaluated by measuring the abilities to inhibit proliferation of estrogen-dependent MCF-7 breast cancer cells in culture. Table 3 shows that *Glycyrrhiza uralensis*, *Angelica gigas*, *Cassia tora*, *Zingiber officinale*, *Carthamus tinctorius* and *Malva verticillata* possessed anti-proliferation effects at the high concentrations. On the other hand, *Syringa velutina* and *Panax ginseng* C.A. Meyer showed anti-proliferation activities at the medium concentrations. *Zingiber officinale* showed anti-proliferation activity in high and medium concentrations.

We next examined the combination of 8 μg/ml BPA

Table 2. Hormonal activities of Korean medicinal herbs in the yeast screen

Herbal medicine	Estrogen Receptor		Androgen Receptor	
	1000 (μg/ml)	100 (μg/ml)	1000 (μg/ml)	100 (μg/ml)
Glycyrrhiza uralensis FISCH	2.5539*	4.3844*	2.0470*	1.0490
Angelica gigas NAKAI	0.5055	0.7972	1.1681	1.0559
Cassia tora L.	2.7423*	2.1689*	1.3390*	1.1125
Syringa velutina var. Kambibayashii. T Lee	1.6587*	0.7778	2.1261*	0.9734
Zingiber officinale Rosc.	1.7896*	3.7354*	1.2308*	1.2303*
Carthamus tinctorius L.	1.0699	0.9408	1.1490	1.0869
Astragalus membranaceus BUNGE	2.1271*	0.9681	0.8788	0.8683
Malva verticillata L.	2.2366*	1.3205*	0.6458	1.1316
Panax ginseng C.A. Meyer	1.6206*	0.8944	0.7769	0.7547

Fold induction of natural product relative to control was determined by OD_{420nm} and OD_{590nm} values. As positive controls, 17β estradiol and nor-testosterone were used in recombinant yeast assay. *, Significantly different from the non-treated control group by Dunnett's t-test ($P<0.05$). Each experiment was performed in triplicate.

Table 3. Inhibitory effects of Korean medicinal herbs in MCF-7 cells

Herbal medicine	Treated alone		with bisphenol A
	1000 (μg/ml)	100 (μg/ml)	100 (μg/ml)
Glycyrrhiza uralensis FISCH	0.2646*	0.9663	0.6047*
Angelica gigas NAKAI	0.1706*	0.9140	0.1553*
Cassia tora L.	0.5434*	1.7540	2.6472
Syringa velutina var. Kambibayashii. T Lee	1.8656	0.4615*	0.6239*
Zingiber officinale Rosc.	0.4512*	0.2916*	0.2853*
Carthamus tinctorius L.	0.1254*	1.1423	1.1615*
Astragalus membranaceus BUNGE	0.9754	1.0001	1.4258*
Malva verticillata L.	0.4096*	1.2410	1.3082*
Panax ginseng C.A. Meyer	1.1317	0.7777*	1.2465*

Fold induction of natural product relative to control was determined by OD_{420nm} and OD_{590nm} values. As a positive control, bisphenol A was used in MCF-7 cell proliferation assay. *, Significantly different from the non-treated control group by Dunnett's t-test ($P < 0.05$). Each experiment was performed in triplicate.

with 100 μg/ml plant extracts. Eight extracts except *Cassia tora* were found to inhibit cell growth of the estrogen-dependent cell line at 100 μg/ml, with corresponding treatment of BPA (Table 3).

DISCUSSION

The recombinant yeast assay in the present study was adapted to 96-well microtiter plate, which requires only a single treatment of the indicator cells with the estrogenic substance to induce an estrogenic response. This system makes the yeast estrogen screen ideal for large-scale screening purposes, in addition to being one of highly sensitive estrogen or androgen screens available. In the present study, the recombinant yeast screen was capable of detecting a number of korean medicinal herbs. In the recombinant yeast assay, *Glycyrrhiza uralensis* showed estrogenic potency 1.10~1.90 times higher than positive control (17β-estradiol). In addition, some of the medicinal herbs, such as *Cassia tora*, *Syringa velutina*, *Zingiber officinale*, *Astragalus membranaceus*, *Malva verticillata* and *Panax ginseng* C.A. Meyer were found to have estrogenic activity related to vehicle control (DMSO 0.1%). *Glycyrrhiza uralensis*, *Cassia tora*, *Syringa velutina* and *Zingiber officinale* exhibited potency of estrogenicity and androgenecity. Messina and co-workers have reported the potential clinical benefits of dietary phytoestrogens in their study showing that Asian populations, who share a high consumption of soy-based foodstuffs, have markedly lower incidences of breast and prostate cancers.[13] In addition, the estrogenic activity of some of the flavo-estrogens, such as naringenin, apigenin and kaempferol have been suggested as cancer protective substances in foodstuffs.[14] These results are in agreement with that of previous study comparing estrogenicity of foodstuffs.[15]

The medicinal herbs were evaluated in the mammalian MCF7 cell proliferation assay. The MCF-7 human breast cancer cell line is well-established *in vitro* model characterized by its estrogen responsiveness.[16] It has been frequently used to test the effects of E2 mimics on the pathway(s) linking estrogen receptor binding to gene expression and cell proliferation.[17] In this assay, eight herbs such as *Glycyrrhiza uralensis*, *Angelica gigas*, *Cassia tora*, *Syringa velutina*, *Zingiber officinale*, *Carthamus tinctorius*, *Malva verticillata*, and *Panax ginseng* C.A. Meyer exhibited inhibition of cell growth. Several studies have shown that some of flavo-estrogen were active in the MCF-7 cell proliferation assay in accord with results from the yeast screen.[14,18,19] However, genistein as representative flavonoid showed a much lower activity in MCF7 assay.[20] Recently a biphasic effect of genistein was seen with a low dose stimulating intestinal cell proliferation through the estrogen receptor, whereas a high dose of genistein inhibited intestinal cell proliferation and altered cell cycle dynamics.[21] *In vivo* study, no comprehensive effects on the reproductive organs have been reported with genistein during the perinatal period.[22] In our results by recombinant yeast and MCF-7 proliferation assay, the medicinal herbs exhibited estrogenicity or androgenicity in recombinant yeast assay, and some of them inhibited growth of MCF7 cells. In our previous study, *Glycyrrhiza uralensis* induced chemopreventive effect through the Bcl-2/Bax family of apoptotic regulatory factors in MCF-7 cell.[12] There were some of the discrepancies between the two systems, at least partly, due to differences in biotransformation capacity between the yeast mammalian cells.

Bisphenol-based polymers have been used in thousands of products. Bisphenol induce a higher luciferase activity in the MCF-7 transfected cells than that reported for E2.[23] This phenomenon has been described in different reporter gene assays[24,25] but its mechanism remains unknown. Our investigations revealed that *Glycyrrhiza uralensis*, *Angelica gigas*, *Syringa velutina*, *Zingiber officinale*, *Carthamus tinctorius*, *Astragalus membranaceus*, *Malva verticillata* and *Panax ginseng* C.A. Meyer inhibited the growth of MCF 7 cells even after co-treatment with BPA. It has been reported that chronic exposure to environmental estrogens has major impacts on reproductive impairments in humans and animals.[22] On the other hand, phytoestrogens-natural compounds were reported to decrease the risk of breast cancer and heart disease.[26] Both environmental estrogens and phytoestrogens have been reported to exert estrogenic activity mediated through estrogen receptors.[27] We conclude that some of the medicinal herbs might exert a higher estrogenic activity than BPA. Ramanathan *et al* isolated two phytoestrogen (PE) specific genes (PE-13.1 and pRDA-D) from MCF-7 cells, and reported that PE-13.1 transcript was up-regulated by phytoestrogens (genistein and zearalenone) and was

nonresponsive to estradiol. Conversely, the pRDA-D transcript was down-regulated by both phytoestrogens and estradiol.[28] This suggests that PE specific genes might be used as molecular markers in understanding the chemopreventive mechanism between phytoestrogen and endocrine disruptors in MCF-7 cells.

We showed the inhibitory effects of the Korean medicinal herbs as phytoestrogens using recombinant yeast and MCF-7 cell proliferation assay. Although the active principles responsible for the antioxidant activity of the tested extracts have not been identified in this study, it will be useful to further analyze those herbal medicines.

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국문요약

약용식물내 에스트로겐성과 항-에스트로겐성을 조사하고 항암인자를 발견하기 위하여, 본연구는 에탄올추출로 제조된 9종류의 한국산 약용식물에 대하여 재조합효모와 MCF-7 사람유방암세포주를 이용하여 스크리닝하고 비교하였다. 재조합효모를 이용한 실험결과, 7종류의 약용식물에서 에스트로겐성이 나타났고, 4종류에서 안드로겐성이 나타났다. 또한 MCF-7 사람유방암세포주를 이용한 실험결과, 8종류의 추출물이 MCF-7 세포의 성장을 억제하는 것으로 확인되었으며 비스페놀 A와 동시 처치한 경우에도 유의적으로 억제하는 것으로 나타났다. 또한 *Glycyrrhiza uralensis*, *Cassia tora*, *Syringa velutina*, *Zingiber officinale*, *Malva verticillata*, *Panax ginseng* C.A. Meyer는 식물성 에스트로겐으로서 에스트로겐에 양성인 사람유방암세포의 증식을 유의적으로 억제시키는 흥미로운 결과가 제시되었다. 따라서 이번 연구는 한국산 약용식물이 식물성 에스트로겐과 항암인자로서 이용될 수 있으며, 에스트로겐의 활성을 조사하는데 유용하게 이용될 수 있을 것으로 사료된다.

REFERENCES

1. Hayakawa, J., Noda, N., Yamada, S. & Uno, K. [Studies on physical and chemical quality evaluation of crude drug preparations. I. Analysis of Pueraria Radix and species Puerariae]. *Yakugaku Zasshi* **104**, 50-6 (1984).
2. Setchell, K. D. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clin Nutr* **68**, 1333S-1346S (1998).
3. Lephart, E. D., Thompson, J. M., Setchell, K. D., Adlercreutz, H. & Weber, K. S. Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats. *Brain Res* **859**, 123-31 (2000).
4. Murkies, A. L., Wilcox, G. & Davis, S. R. Clinical review 92: Phytoestrogens. *J Clin Endocrinol Metab* **83**, 297-303 (1998).
5. Ekbom, A. & Akre, O. Increasing incidence of testicular cancer--birth cohort effects. *Apmis* **106**, 225-9; discussion 229-31 (1998).
6. Facemire, C. F., Gross, T. S. & Guillette, L. J., Jr. Reproductive impairment in the Florida panther: nature or nurture? *Environ Health Perspect* **103 Suppl 4**, 79-86 (1995).
7. Staples, C. A., Dorn, P. B., Klecka, G. M., O'Block, S. T. & Harris, L. R. A review of the environmental fate, effects, and exposures of bisphenol A. *Chemosphere* **36**, 2149-73 (1998).
8. Upmeier, A., Degen, G. H., Diel, P., Michna, H. & Bolt, H. M. Toxicokinetics of bisphenol A in female DA/Han rats after a single i.v. and oral administration. *Arch Toxicol* **74**, 431-6 (2000).
9. Ashby, J. & Tinwell, H. Uterotrophic activity of bisphenol A in the immature rat. *Environ Health Perspect* **106**, 719-20 (1998).
10. Nikula, H., Talonpoika, T., Kaleva, M. & Toppari, J. Inhibition of hCG-stimulated steroidogenesis in cultured mouse Leydig tumor cells by bisphenol A and octylphenols. *Toxicol Appl Pharmacol* **157**, 166-73 (1999).
11. Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L. & Feldman, D. Bisphenol-A: an estrogenic substance is

released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279-86 (1993).
12. Jo, E. H. et al. Modulations of the Bcl-2/Bax family were involved in the chemopreventive effects of licorice root (Glycyrrhiza uralensis Fisch) in MCF-7 human breast cancer cell. *J Agric Food Chem* **52**, 1715-9 (2004).
13. Messina, M. & Barnes, S. The role of soy products in reducing risk of cancer. *J Natl Cancer Inst* **83**, 541-6 (1991).
14. Breinholt, V. & Larsen, J. C. Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. *Chem Res Toxicol* **11**, 622-9 (1998).
15. Hanna, K., O'Neill, S., Patterson, C. & Lyons-Wall, P. Intake of phytoestrogen-rich foods and associated lifestyle and sociodemographic characteristics in Australian women. *Asia Pac J Clin Nutr* **13**, S73 (2004).
16. Soto, A. M. & Sonnenschein, C. The role of estrogens on the proliferation of human breast tumor cells (MCF-7). *J Steroid Biochem* **23**, 87-94 (1985).
17. Soto, A. M. et al. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* **103 Suppl 7**, 113-22 (1995).
18. Lee, Y. S. et al. Requirement of metabolic activation for estrogenic activity of Pueraria mirifica. *J Vet Sci* **3**, 273-7 (2002).
19. Stroheker, T., Picard, K., Lhuguenot, J. C., Canivenc-Lavier, M. C. & Chagnon, M. C. Steroid activities comparison of natural and food wrap compounds in human breast cancer cell lines. *Food Chem Toxicol* **42**, 887-97 (2004).
20. Gutendorf, B. & Westendorf, J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **166**, 79-89 (2001).
21. Chen, A. C. & Donovan, S. M. Genistein at a concentration present in soy infant formula inhibits Caco-2BBe cell proliferation by causing G2/M cell cycle arrest. *J Nutr* **134**, 1303-8 (2004).
22. Kang, K. S., Che, J. H. & Lee, Y. S. Lack of adverse effects in the F1 offspring maternally exposed to genistein at human intake dose level. *Food Chem Toxicol* **40**, 43-51 (2002).
23. Rivas, A. et al. Estrogenic effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast cancer cells. *J Steroid Biochem Mol Biol* **82**, 45-53 (2002).
24. Meerts, I. A. et al. In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. *Environ Health Perspect* **109**, 399-407 (2001).
25. Legler, J. et al. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. *Toxicol Sci* **48**, 55-66 (1999).
26. Knight, D. C. & Eden, J. A. A review of the clinical effects of phytoestrogens. *Obstet Gynecol* **87**, 897-904 (1996).
27. Soto, A. M., Justicia, H., Wray, J. W. & Sonnenschein, C. p-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environ Health Perspect* **92**, 167-73 (1991).
28. Ramanathan, L. & Gray, W. G. Identification and characterization of a phytoestrogen-specific gene from the MCF-7 human breast cancer cell. *Toxicol Appl Pharmacol* **191**, 107-17 (2003).