Evaluation of the Estrogenic Activity of Leguminosae Plants

Hye Hyun Yoo, Taehyeong Kim, Soyun Ahn, Yoon Jung Kim, Hyun Young Kim, Xiang Lan Piao, and Jeong Hill Park*

College of Pharmacy, Seoul National University; Seoul 151–742, Korea.
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The plant extracts of the Leguminosae family were screened for their estrogenic activity with the Ishikawa cell system. Of the tested plants, *Desmodium oxyphyllum*, *Dunbaria villosa*, *Kummerowia striata*, *Lespedeza bicolor*, *Maackia amurensis*, *Maackia fauriei*, *Pueraria thunbergiana*, and *Sophora flavescens* were highly estrogenic with EC_{50} values of less than 10 μg/ml.

Key words Leguminosae; phytoestrogen; Ishikawa cell

During the period of menopause and postmenopause, many women experience one or more symptoms such as hot flashes, depression, mood swings, sleeping disorders, vaginal dryness, and joint pain, largely due to a lack of estrogens.[1] Hormone replacement therapy has helped to relieve these menopausal symptoms. In addition, the risk of osteoporosis, cardiovascular disease, dementia from Alzheimer's disease, and certain types of cancer can be reduced by hormone replacement therapy.[2—5] However, the existing estrogen replacement therapy using synthetic estrogens has some side effects that include a slight but significant increase in the risk of developing breast and endometrial cancer due to unselective estrogenic action.[3,6—9] For this reason, women are increasingly using herbal remedies as alternative therapy for menopausal symptoms.[10—12] Generally, herbal remedies are not as potent in their estrogenic action as the synthetic estrogens but safer than them in terms of side effects. Epidemiologic data show that a diet containing soy products, which are known to be rich in natural estrogenic ingredients, reduces the number of hot flashes and the incidence of cancer in Oriental women.[13]

More than 300 plants have been found to contain compounds with estrogenic activity.[14,15] These compounds have been defined by the general name of phytoestrogens. It has been known that the plant family most abundant in phytoestrogens is the Leguminosae. In particular, isoflavones of the soybean have attracted the most attention in the Leguminosae family.[16] Other leguminous plants such as *Phaseolus vulgaris*, *Pueraria lobata*, and *Trifolium pretense* have been known to be rich in such phytoestrogens as genistein and daidzein.[17,18] However, it can be assumed that other leguminous plants in addition to those well-known plants also contain phyoestrogens. Therefore, as a part of our search for novel natural estrogens for the improvement of menopausal symptoms, we evaluated the estrogenic property of various plant extracts belonging to the legume family using the Ishikawa cell system. Ishikawa cells are estrogen-responsive human endometrial adenocarcinoma cells, and alkaline phosphatase (AP) activity in these cells is markedly stimulated when estrogens bind to the estrogen receptors in the cell membranes.[19,20] Therefore estrogenic activity can be measured easily using the enzyme-substrate reaction in the Ishikawa cell system. In the present study, we report the estrogenic potential of plant extracts of the legume family using the Ishikawa cell system.

MATERIALS AND METHODS

Plant Material The MeOH extracts of the selected leguminous plants were provided by the Korea Plant Extract Bank (Daejeon, Korea).

Induction of AP with Cultured Ishikawa Cells The procedure of Pisha and Pezzuto[21] was used. Briefly, Ishikawa cells (5×10^4/well) were incubated for 24 h with estrogen-free media in 96-well plates. Test samples dissolved in DMSO were added, and the cells in a total volume of 200 μl media/well were incubated at 37 °C for 4 d. Enzyme activity was measured by reading the liberation of *p*-nitrophenol at 405 nm every 15 s for 16—20 readings with a microplate reader. The maximum slope of the lines generated by the kinetic readings was calculated using a computer program. The percent induction for determination of estrogenic activity was calculated as follows:

$$\%\text{AP induction} = [(\text{slope}_{\text{sample}} - \text{slope}_{\text{control cells}})/(\text{slope}_{\text{estrogen}} - \text{slope}_{\text{control cells}})]\times 100$$

Cytotoxicity Assay Ishikawa cells (5×10^4/well) were preincubated overnight in estrogen-free media in a 96-well culture plates. The Ishikawa cells were incubated with test samples for 4 d. The cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) colorimetric assay.[22] Twenty microliters of MTT solution (5 mg/ml) was added to each well of a 96-well culture plate, incubated at 37 °C for 4 h and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with DMSO 100 μl and the absorbance of each well was measured at 570 nm.

RESULTS AND DISCUSSION

As summarized in Table 1, all the plants extracts tested showed distinctive induction of AP in Ishikawa cells. Estradiol as a positive control induced alkaline phosphate activity to saturate the enzyme activity completely at the concentration of 10 nM (data not shown). When assuming the AP activity to be 100% activity with estradiol 10 nM, extracts of eight plants including *Desmodium oxyphyllum*, *Dunbaria villosa*, *Kummerowia striata*, *Lespedeza bicolor*, *Maackia amurensis*, *Maackia fauriei*, *Pueraria thunbergiana*, and *Sophora flavescens*, effectively induced AP activity to more than 50% when their concentrations were 10 μg/ml. All of these active extracts showed estrogenic activity in a concentration-depen-

* To whom correspondence should be addressed. e-mail: hillpark@snu.ac.kr

Table 1. AP Inductive Activity of Leguminous Plant Extracts at 10 μg/ml in Ishikawa Cells

Plant	Plant part used	% AP induction[a)]
Aeschynomene indica	Leaves, stem	4.4
Amorpha fruticosa	Leaves	0
Amorpha fruticosa	Stem	13
Amphicarpaea edgeworthii var. *trisperma*	Whole plants	8.1
Caesalpinia japonica	Leaves, stem	1.5
Canavalia lincata	Peel	32
Caragana sinica	Stem	3.1
Caragana sinica	Leaves	0.2
Cassia mimosoides var. *nomame*	Whole plants	3.8
Cercis chinensis	Roots	7.3
Desmodium caudatum	Leaves, stem	12
Desmodium caudatum	Whole plants	31
Desmodium oxyphyllum	Whole plants	82
Dunbaria villosa	Whole plants	69
Gleditsia japonica var. *koraiensis*	Leaves	−1.9
Gleditsia japonica var. *koraiensis*	Stem-heart wood	−1.0
Gleditsia japonica var. *koraiensis*	Stem-bark	0.7
Gleditsia japonica var. *koraiensis*	Peel	3.8
Indigofera kirilowii var. *coreana*	Leaves, stem	3.8
Indigofera pseudotinctoria	Whole plants	4.6
Kummerowia striata	Whole plants	59
Lathyrus japonica	Whole plants	6.1
Lathyrus japonica var. *aleuticus*	Whole plants	3.4
Lespedeza bicolor	Flowers	11
Lespedeza bicolor	Leaves	−2.4
Lespedeza bicolor	Stem	60
Lespedeza cuneata	Whole plants	19
Lespedeza cyrtobotrya	Leaves	4.2
Lespedeza cyrtobotrya	Stem	18
Lespedeza maximowiczii	Leaves, stem	30
Lespedeza thunbergii var. *intermedia*	Leaves, stem	14
Lespedeza X robusta	Leaves	11
Lespedeza X robusta	Stem	30
Lotus corniculatus var. *japonicus*	Whole plants	4.8
Maackia amurensis	Leaves	76
Maackia amurensis	Stem	87
Maackia amurensis	Fruits	67
Maackia fauriei	Leaves	23
Maackia fauriei	Stem-heart wood	83
Maackia fauriei	Stem-bark	65
Pueraria thunbergiana	Branches, leaves	16
Pueraria thunbergiana	Flowers	56
Pueraria thunbergiana	Stem	99
Rhynchosia volubilis	Leaves, stem	34
Robinia pseudo-accacia	Flowers	−2.7
Robinia pseudo-accacia	Leaves	0.3
Robinia pseudo-accacia	Stem-bark	3.3
Sophora flavescens	Whole plants	75
Trifolium pratense	Whole plants	30
Trifolium repens	Whole plants	0
Vicia angustifolia var. *segetalis*	Whole plants	1.5
Vicia villosa	Whole plants	−1.4
Vigna sinensis	Aerial parts	−1.1
Wisteria floribunda	Leaves	3.5
Wisteria floribunda	Stem-bark	27

a) % AP induction at the concentration of 10 μg/ml. The data represent the average of triplicate determinations.

dent manner, and subsequently the EC_{50} value of each of the extracts was calculated. Additionally cytotoxicity testing using MTT assay was performed in parallel. As a result, all of the retested extracts had EC_{50} values of less than 10 μg/ml and exhibited almost no cytotoxicity upto the concentration of 100 μg/ml (Table 2). The extract of the stem of *P. thunbergiana* showed the strongest estrogenic activity with an EC_{50} value of 1.0 μg/ml and it did not show any cytotoxicity. *D. oxyphyllum*, *M. amurensis* leaves, and *M. fauriei* stem extracts were active considering their cytotoxic potential and their EC_{50} values were 2.6, 2.7, and 1.8 μg/ml, respectively. The *M. amurensis* stem extract had a fairly low EC_{50} value of 1.6 μg/ml but exhibited some cytotoxic activity with a GI_{50} of 18 μg/ml, and thus it was not considered an effective estrogenic source. Generally, it is considered that the estrogenic constituents of leguminous plants are isoflavonoids.[23)]

The results of this study show that many plants in the Leguminosae family have estrogenic properties and thus

Table 2. Estrogenic Potency and Cytotoxicity of Leguminous Plant Extracts

Specific name	Plant part used	Estrogenic activity EC_{50} (μg/ml)[a)]	Cytotoxicity GI_{50} (μg/ml)[b)]
Desmodium oxyphyllum	Whole plants	2.6	>100
Dunbaria villosa	Whole plants	4.4	>100
Kummerowia striata	Whole plants	7.7	>100
Lespedeza bicolor	Stem	8.6	>100
Maackia amurensis	Leaves	2.7	>100
Maackia amurensis	Stem	1.6	18
Maackia amurensis	Fruits	3.9	>100
Maackia fauriei	Stem-bark	2.8	76
Maackia fauriei	Stem-heart wood	1.8	81
Pueraria thunbergiana	Flowers	8.6	>100
Pueraria thunbergiana	Stem	1.0	>100
Sophora flavescens	Whole plants	3.2	>100

a) 50% effective concentration, *b*) 50% growth inhibitory concentration. EC_{50} and GI_{50} were calculated using the probit method.[24)]

leguminous plants could be good source of natural estrogen. Although the results obtained in this study were limited to those of *in vitro* cellular reactions, they suggest that these active plants could be used as effective herbal medicines for the treatment of menopausal dysfunctions in the future. Further investigations using *in vivo* models and for the identification of their active principles should be conducted.

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