Estrogenic and Antiestrogenic Activities of the Roots of Moghania philippinensis and Their Constituents

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In the course of our search for natural estrogenic compounds from medicinal plants, we found that the methanolic extract from the roots of Moghania philippinensis (Fabaceae) showed significant effects on the proliferation of MCF-7 cells (human breast cancer) and induction of β-galactosidase activity in a yeast two-hybrid assay. Through estrogenic activity-guided fractionation, we isolated several active flavonoids including prenylated ones. The CHCl3 fraction and its new constituent, 8-(1,1-dimethylallyl)genistein (9), appreciably increased the uterine weight in ovariectomized rats when administered orally for 14 consecutive days, in which compound 9 showed stronger estrogenic activity than genistein. Antiestrogenic activities were also examined based on the inhibition of MCF-7 cell proliferation and β-galactosidase activity in the yeast two-hybrid assay, mediated by 17β-estradiol. 5,7,3′,4′-Tetrahydroxy-6,8-diprenylisoflavone (6) showed the strongest antiestrogenic activity.

Key words Moghania philippinensis; MCF-7; yeast two-hybrid assay; 8-(1,1-dimethylallyl)genistein; phytoestrogen

In recent years there has been increasing interest in hormone replacement therapy (HRT), which seems to protect women against hot flashes, reduced bone density, cardiovascular problems, and osteoporosis.1 On the other hand, synthetic estrogen replacement therapy was reported to increase the risk of certain types of cancer, such as endometrial and breast cancer.2) Since a variety of plants and vegetables were shown to exert beneficial effects on the above-mentioned estrogen mediated diseases,3) many phytoestrogens, such as isoflavones, coumestans, resorcyclic acid lactones, and dihydroxychalcones have been isolated.4) Ichikawa et al.5) have recently reported that 4,4′-dihydroxy-2,6-dimethoxydihydrochalcone (retrodihydrochalcone) and 5,7-dihydroxy-3-(4-hydroxybenzyl)-4-chromanone (homoisoflavone) from the stem wood of Dracaena loureiri exhibited significant estrogenic activity.

In a search for new phytoestrogens that are effective in preventing and treating estrogen-mediated diseases, we examined many medicinal plants for their estrogenic activity. Of these, a methanolic extract of the roots of Moghania philippinensis was demonstrated to have potent estrogenic activity. M. philippinensis (Fabaceae) is a shrubby herb mainly growing in southwestern China. Its roots have traditionally been used for the treatment of menopausal syndrome, leukorrhea, rheumatism, arthropathy, and improving bone mineral density.5,7) In the present paper, we report estrogenic and antiestrogenic activities of the constituents of M. philippinensis, as monitored in two independent in vitro estrogen assay systems: the estrogen-dependent proliferation of MCF-7 (human breast cancer) cells and a yeast two-hybrid assay developed by Nishikawa et al.8) Furthermore, we investigated the estrogenic effect induced by oral administration of the extract of M. philippinensis, 8-(1,1-dimethylallyl)genistein (9) and genistein (1) in ovariectomized (OVX) rats.

MATERIALS AND METHODS

Materials Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Gibco BRL. (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was purchased from ICN Biomedicals, Inc. (Aurora, OH, U.S.A.). Streptomycin, 0.25% trypsin, and o-nitrophenyl β-d-galactopyranoside (ONPG) were purchased from Nacalai Tesque Co. (Kyoto, Japan). 17β-Estradiol was obtained from Calbiochem Co. (Darmstadt, Germany). Human serum was obtained from Bio-Whittaker Co. (Walkersville, MD, U.S.A.). 3,4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), penicillin, and norit SX-II charcoal were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Genistein was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 20T-zymolyase was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Dextran 70T was obtained from Amersham Pharmacia Biotech AB (Upsalsa, Sweden).

Plant Material The roots of M. philippinensis were obtained from Nanning, Guangxi Province, China, in March 2002 and the botanical source was identified by Dr. Katsuko Komatsu, Toyama Medical and Pharmaceutical University (TMPU). A voucher specimen (TMPU 19843) was deposited at the Institute of Natural Medicine, TMPU, Toyama, Japan.

Extraction and Fractionation Procedures The pulverized roots of M. philippinensis (2.5 kg) were extracted with MeOH under reflux (5×3) for 3 h. The combined solutions were evaporated to dryness in vacuo to give a MeOH-soluble fraction. The MeOH-soluble fraction (127.1 g) was suspended in 1 l of water and then extracted with CHCl3 (800 ml), which was suspended in 1 l of water and then extracted with CHCl3 (800 ml). The combined solutions were evaporated to dryness to give a debris (107.2 g), which was suspended in 11 of water and then extracted with CHCl3 (800 ml×3). The combined solutions were evaporated to dryness to give a ChCl2-soluble fraction (MC, 56.2 g). The H2O phase was concentrated and applied to a Diaion HP-20 column (9×40 cm) and the column was eluted stepwise with H2O, MeOH–H2O (1:1) and MeOH. The respective eluates were evaporated to dryness in vacuo to yield three fractions [H2O (MA, 31.4 g), 50% MeOH (MM2, 16.1 g), and MeOH (MM1, 3.5 g) eluted fractions].

Test Compounds Compounds 1—16 (Fig. 1) were iso-
lated from a CHCl₃-soluble fraction (MC) of *M. philippinensis* through bioassay-guided fractionation, as reported in the previous paper.⁹) Compound 9 was synthesized from genistein by a method described in previous papers⁹,¹⁰) and used for the *in vivo* experiment. The purity of the compound was more than 98%, as determined based on the ¹H-NMR spectrum and HPLC.

**MCF-7 Cell Proliferation Assay** Estrogen-sensitive human MCF-7 breast cells were grown in DMEM supplemented with 5% FBS, penicillin, and streptomycin. The cells were harvested by trypsinization (0.25% trypsin) and plated at a concentration of 5 × 10³ cells/well in DMEM supplemented with 5% FBS in 96-well tissue culture plates (Iwaki, Co., Chiba, Japan) and allowed to attach for 24 h. Then the culture medium was replaced with phenol red-free DMEM containing 10% heat-inactivated dextran/charcoal-stripped (DC) human serum prior to the addition of compounds.¹¹) Stock solutions of test compounds in DMSO were diluted with DC medium. The final DMSO concentration in culture medium did not exceed 0.1%, and this concentration did not affect cell viability. After 4 d in a humidified incubator at 37 °C, the growth of the cells was measured using the MTT method.¹²)

**Yeast Two-Hybrid Assay** The yeast two-hybrid assay was carried out according to the method of Nishikawa et al.⁸) Briefly, yeast cells expressing estrogen receptor α (ERα) was grown overnight at 30 °C with shaking in synthetic defined (SD) medium lacking tryptophan and leucine. Yeast cells were treated with a test compound for 4 h at 30 °C, and β-galactosidase activity was determined as follows. The growth of the yeast cells was monitored by measuring the turbidity at 600 nm. The treated yeast cells were collected by centrifugation (8000 × g, 5 min) and resuspended in 200 μl of Z-buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, and 1 mM MgSO₄) containing 1 mg/ml of zymolyase at 37 °C for 15 min. The reaction was started by the addition of 40 μl of 4 mg/ml ONPG as a substrate. When the yellow color developed (incubation time: t), 100 μl of 1 M Na₂CO₃ was added to stop the reaction. The absorbance of solution (150 μl) was measured at 420 and 550 nm. β-Galactosidase activity (U) was determined using the following formula:

\[
U = 1000 \times \frac{(A_{420} - 1.75 \times A_{550}) \times (t \times 0.05 \times A_{600})}{t}
\]

**Antiestrogen Assay** To examine antagonistic activity of compounds, the inhibition of β-galactosidase activity and the growth of MCF-7 cells, which had been induced by 10⁻⁷ and 10⁻¹⁰ M 17β-estradiol, respectively, were measured at various concentrations of test compounds.

**Uterotrophic Assay** Female Sprague-Dawley rats (10—11 weeks old, 210—220 g) rats were purchased from Nippon SLC Co. (Shizuoka, Japan). All rats were allowed free access to water and pelleted commercial diet (MM-3, Funabashi Farm) during the experimental period. The light/dark cycle was 12 h light and 12 h dark. The room temperature and humidity were controlled automatically. All rats were OVX or sham-operated by the supplier (Nippon SLC) 1 week before treatment, and randomly divided into groups of 5 rats each: (1) sham, sham-operated (treated with 10% DMSO), (2) bilaterally OVX (treated with 10% DMSO), (3) OVX+Gen (treated with 8 mg/kg of genistein), (4) OVX+compound 9 (treated with 2 mg/kg or 10 mg/kg of 9), and (5) OVX+MC (treated with 200 mg/kg of a CHCl₃-soluble fraction). All treatments were given by oral gavage for 14 d. On days 15, urine samples were collected using metabolic cages for the measurement of the bone resorption marker deoxypyridinoline (DPD). The animals were weighed and killed 24 h after the last treatment. The uterus, spleen, and thymus were quickly removed and weighed.

![Fig. 1. Chemical Structures of Isolated Compounds from the Roots of *Moghania philippinensis*](image-url)
Table 1. Effects of the Fractions of *M. philippinensis* on the Proliferation of MCF-7 Cells and Induction of β-Galactosidase Activity in an Yeast Two-Hybrid Assay

<table>
<thead>
<tr>
<th>Fraction</th>
<th>MCF-7 cell proliferation (% of control)</th>
<th>β-Galactosidase activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 µg/ml</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>MH</td>
<td>119.7±6.40</td>
<td>117.1±4.54</td>
</tr>
<tr>
<td>MC</td>
<td>163.5±4.68**</td>
<td>177.9±1.54**</td>
</tr>
<tr>
<td>MM₁</td>
<td>162.3±5.33**</td>
<td>199.5±4.34**</td>
</tr>
<tr>
<td>MM₂</td>
<td>117.1±4.94</td>
<td>139.8±6.56**</td>
</tr>
<tr>
<td>MA</td>
<td>107.6±2.65</td>
<td>108.9±3.07</td>
</tr>
</tbody>
</table>

β-Galactosidase activity (U) was 53.47±2.94 in a control experiment (DMSO). 17β-Estradiol was 963.56±75.7 (U) at a concentration of 10⁻⁷ M. Asterisks indicate significant difference from the control at p<0.05 (**), p<0.01 (**), and p<0.001 (***). MH, hexane-soluble fraction; MC, CHCl₃-soluble fraction; MM₁, MeOH-eluted fraction; MM₂, 50% MeOH-eluted fraction; MA, H₂O-eluted fraction.

**Bone Turnover Assays** DPD (a marker of bone metabolism) is released and excreted in the urine during the process of bone resorption, and the amount was measured using an enzyme immunoassay provided by BML Co. (Tokyo, Japan).

**Statistical Analysis** Each set of experiments was repeated at least three times. Values are expressed as mean±S.E.M. One-way analysis of variance followed by Dunnett’s test was used for statistical analysis.

**RESULTS**

**Estrogenic Activity of *M. philippinensis* Extracts** The MeOH extract of the roots of *M. philippinensis* was subjected to fractionation by solvent extraction followed by column chromatography on Diaion HP-20 to give five fractions. These fractions were evaluated for their estrogenic activity at various concentrations (Table 1) depending on the assay system. The CHCl₃-soluble (MC) and MeOH-eluted (MM₂) fractions were active in an MCF-7 cell proliferation assay (ca. 160—200% of control) at concentrations of 1 and 10 µg/ml and the yeast two-hybrid assay (ca. 170—450 U at 10 and 100 µg/ml). The 50% MeOH-eluted (MM₁) fraction including a flavonoid glycoside genistin (data not shown) increased the growth of MCF-7 cells at the high concentration of 10 µg/ml, but was inactive in the yeast two-hybrid assay. The other extracts showed no or weak estrogenic activity.

Although the weights of uteri in the OVX rats were significantly decreased 14 d after ovariectomy when compared with those of control rats (sham) (Table 2), the weights were increased by consecutive oral administration of the MC fraction at a dose of 200 mg/kg/d (p<0.05).

**Estrogenic and Antiestrogenic Activities of Isolated Compounds** Using an MCF-7 cell-proliferating assay method, all the compounds isolated from a chloroform fraction of *M. philippinensis* were tested for their estrogenic activities at various concentrations. Under the conditions where 17β-estradiol (positive control) showed the maximum proliferation of cells at a concentration of 10⁻¹⁰ M (data not shown), genistein (1), biochanin A (2), 2'-hydroxygenistein (3), lupinalbin A (4), and 3'-O-methylgenistein (5) stimulated cell proliferation in a concentration-dependent manner (Fig. 2A), suggesting that these isoflavonoids are agonistic for estrogen receptors. Similarly, 8-(1,1-dimethylallyl)genistein (9) and 5,7,3'-trihydroxy-2'-(-3-methylbut-2-enyl)-4',5'-3,3-dimethylpyranol)isoflavone (11) showed significant cell-proliferating activity at a concentration of 10⁻⁵ M, at which the potency of (cell-proliferating activity 186% of control) is comparable to that of 1 (196%) (Fig. 2B). On the other hand, auricularin (7), flemiphilippinin A (8), and 5,7,3',4'-tetrahydroxy-2',5'-di(3-methylbut-2-enyl)isoflavone (10) did not show any substantial cell-proliferating activity at concentrations of less than 10⁻⁵ M. The proliferating activity of a flavanone [(2S)-liquiritigenin (12)], and isoflavone isoferrerin (16) was also observed at concentrations of more than 10⁻⁶ M (Fig. 2C).

In the yeast two-hybrid assay (Fig. 3), appreciable induction of β-galactosidase activity was observed for genistein (1), 2'-hydroxygenistein (3), lupinalbin A (4), and (2S)-liquiritigenin (12) at a concentration of 10⁻⁴ M. The other isoflavones 2, 5, and 16 showed weak estrogenic activity, but the prenylated isoflavones (6—11, 14, 15) did not show induction of β-galactosidase activity.

As shown in Fig. 4, under the conditions when tamoxifen (an antagonist of estradiol) inhibited the 17β-estradiol-mediated proliferation of MCF-7 cells by 16%, 2'-hydroxygenistein (3) inhibited the proliferation by 17% at a concentration of 10⁻⁶ M. Prenylated flavonoids 6—9 and 11 inhibited the proliferation of the MCF-7 cells stimulated by 17β-estradiol (15—35% inhibition at concentrations of 10⁻⁷—10⁻⁵ M). Of these, flemiphilippinin A (8) showed greater antagonistic activity (35% inhibition compared with control) than tamoxifen (16% inhibition at the same concentration). These findings show that prenylated flavonoids inhibit the proliferation of MCF-7 cells mediated by 17β-estradiol.

Similarly, using the yeast two-hybrid assay, antagonistic activities of the isolated compounds were determined under the conditions at which these compounds did not inhibit the growth of cells due to cytotoxic effects. Inhibition of the induced β-galactosidase activity was observed for 2 (43% inhibition) and 4 (39% inhibition) (Fig. 5A). Most of the prenylated flavonoids 6—11 appreciably inhibited the induction of β-galactosidase (Fig. 5B). At a concentration of 10⁻⁴ M, 6
showed 88% inhibition, which was greater than that of tamoxifen (73% inhibition). Compounds 12—15 slightly inhibited the induction of β-galactosidase. It is of interest that genistein (1) did not show any inhibitory activity but rather stimulated the estradiol mediated proliferation of the cells.

**Influence on the Weights of the Uterus, Spleen and Thymus, and the Excretion of Urinary DPD**

Table 3 shows the effects of 1 and 9, which showed the most potent proliferative activity against MCF-7 cells, on the uterine weight in OVX rats. The uterine weights were markedly reduced in OVX rats compared with those of sham-operated rats. However, following oral administration of 1 and 9 for 14 d, the uterine weights were significantly increased at doses of 8 mg/kg and 10 mg/kg (equivalent on a molar basis) by 121% and 130%, respectively. Furthermore, an increasing effect was also observed at the lower dose of 2 mg/kg of 9, when the uterine weight increased by 124% in comparison with that of controls (OVX).

The size of the thymus gland and spleen are influenced by sex hormones in male mice.15) Ovariectomy leads to an increase in thymus weight and cellularity. Exposure to endogenous estrogen during pregnancy,16) or treatment with exogenous sex hormone causes massive atrophy of the thymus.17,18) In the present experiment, oral administration of compounds 1 and 9 showed no effects on thymus and spleen weights.

Ovariectomy caused a significant increase in urinary DPD (Table 4), but oral administration of 1 and 9 tended to attenuate the decrease in urinary DPD levels in OVX rats.

**DISCUSSION**

In the present study, we examined the extract and its fractions of *M. philippinensis* together with isolated compounds for their estrogenic/antiestrogenic activities. The isolation of active constituents was achieved by monitoring the proliferation of MCF-7 cells and the yeast two-hybrid assay. In the MCF-7 cell proliferation assay, the cells show wide variation in regard to their sensitivity to estradiol.19) Furthermore, since proliferation of MCF-7 cells can be influenced not only by estrogens but also by cytokinins, growth factors, mito-
gens, and nutrients, it is sometimes difficult to conclude that the proliferation is due to the presence of phytoestrogens in the plant extract. On the other hand, the yeast two-hybrid assay is associated with steroid hormone receptors and its gene expression including a coactivator, so that the system closely resembles the mammalian hormone system. However, the disadvantage is the potential for false-negative results due to some action via receptors other than ERα, some involve a pathway other than via receptor-mediated gene expression, and some cannot be transported into the cell. Therefore it

![Fig. 4. Assay of Antiestrogenic Activities of Isolated Compounds](image)

The inhibition of MCF-7 cell proliferation mediated by 10^{-10} M 17β-estradiol was measured by adding isolated compounds at various concentrations. The relative cell inhibition was expressed as a percentage of a control (100%), observed in the presence of 10^{-10} M 17β-estradiol alone. The bar at each point is the standard error of three independent experiments (n=3). Significantly different from the control at p<0.01 (**). (A) Isoflavonoids, (B) prenylated isoflavonoids, (C) other compounds.

![Fig. 5. Coincubation of a Yeast Strain ERα with 17β-Estradiol and Isolated Compounds](image)

The yeast strain was incubated with 10^{-7} M 17β-estradiol in the presence or absence of isolated compounds. The Y-axis indicates relative β-galactosidase activity induced by 10^{-7} M 17β-estradiol in comparison with a control (100%) (17β-estradiol alone). The bar at each point is the standard error of three independent experiments (n=3). Significantly different from the control at p<0.05 (*), p<0.01 (**), and p<0.001 (***)). Compound 9 was cytotoxic at a concentration of 10^{-7} M. (A) Isoflavonoids, (B) prenylated isoflavonoids, (C) other compounds.

| Table 3. Change in Body Weight (BW), Uterine Weight, and Spleen and Thymus Weight in Sham-Operated Rats and Ovariectomized Rats (OVX) Treated with or without Genistein (1) and 8-(1,1-Dimethylallyl)genistein (9) |
|---------------------------------|--------|--------|--------|--------|--------|
|                                 | Initial B/W (g) | Final B/W (g) | Uterus (g) | Spleen (g) | Thymus (g) |
| Sham (10% DMSO)                | 213.2±3.84 | 235.8±3.80 | 0.424±0.0587** | 0.600±0.049 | 0.310±0.019** |
| OVX (10% DMSO)                | 215.2±3.06 | 245.8±4.78 | 0.092±0.0037 | 0.722±0.055 | 0.430±0.017 |
| OVX (1, 8 mg/kg)              | 218.0±2.66 | 242.6±6.35 | 0.112±0.0034** | 0.654±0.048 | 0.434±0.072 |
| OVX (9, 2 mg/kg)              | 217.8±2.15 | 249.8±4.51 | 0.114±0.0036** | 0.662±0.047 | 0.424±0.038 |
| OVX (9, 10 mg/kg)             | 212.0±3.54 | 236.8±7.55 | 0.120±0.0044** | 0.684±0.050 | 0.360±0.046 |

The rats were orally administered test compounds for 2 weeks. Values are mean±S.E. Asterisks indicate groups significantly different from the OXV control at p<0.01 (**).
flat structure, giving a structural similarity to 17\alpha\text{estradiol} in the gastrointestinal tract. However, compound 9, with less potent estrogenic activity by human intestinal microflora, was more resistant to degradation by intestinal bacteria due to the presence of a bulky 1,1-dimethylallyl group at C-8 in the A ring of the isoflavone skeleton, which may account for the more potent estrogenic activity of 9 in the in vivo experiment.

In conclusion, we demonstrated that genistein (1) and 8-(1,1-dimethylallyl)genistein (9) were major estrogen substances in the extract of _M. philippinensis_, with potent proliferating activity on MCF-7 cells and resulting in a significant increase in uterine weight in OVX rats. Oral administration of 9 showed estrogenic effects stronger than those of 1. In addition, 9 was more antiestrogenic than 1, as shown by the MCF-7 proliferation and \(\beta\)-galactosidase induction in yeast cells, which were stimulated by estradiol. This result shows that 9 may serve as a lead compound for developing preventive and therapeutic agents for various estrogen-mediated diseases.

### REFERENCES