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Evaluation of the Estrogenic Activity of Isoflavones from the Rhizome of *Belamcanda chinensis*

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Abstract Four compounds were isolated from the rhizome of *Belamcanda chinensis* (Iridaceae) by repeated column chromatography and were elucidated as iristectorigenin A, irigenin, tectorigenin and tectoridin by spectral analysis. The estrogenic activity of these isoflavones was tested using Ishikawa cells. Irigenin, tectorigenin and tectoridin were highly estrogenic (EC_{50} = 0.75, 0.42 and 0.81 μg/ml, respectively), while iristectorigenin A exhibited weak estrogenic activity ($EC_{50}\geq$ 4 μg/ml).

Keywords: *Belamcanda chinensis*, Iridaceae, isoflavone, estrogenic activity

Introduction

Belamcanda chinensis (Iridaceae) is a perennial shrub growing on hillsides in East Asia, including the Korean peninsula, and has been used as a Chinese traditional medicine for the treatment of throat ailments such as asthma and tonsillitis (1).

In our studies on the bioactive constituents of the family Iridaceae, four compounds were isolated from the rhizome of *B. chinensis*. This plant has been known to have a number of isoflavonoids (2-8). As some of these isoflavonoids are known as phytoestrogens (9-17), the isolated isoflavones were tested for their estrogenic potential. Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens and therefore are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms and osteoporosis (18-20). For these reasons, phytoestrogens can be a good alternative to conventional hormone replacement therapy with synthetic estrogens that carries the risks of breast or endometrial cancers. Isoflavones are the most well-known compounds of phytoestrogens (21).

In this paper, we examined the estrogenic activity of isoflavones from the rhizome of *B. chinensis*.

Materials and Methods

Plant material The rhizome of *Belamcanda chinensis* DC. was collected in vicinity of Seoul, Korea in Oct. 2001, and a voucher specimen (NPRI-97-037) was deposited at the Herbarium of Natural Products Research Institute, Seoul National University, Korea.

Instruments and reagents Electron impact (EI)- and fast atom bombardment (FAB)-mass spectra (MS) were measured with a Jeol JMS-AX505WA mass spectrometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 400 NMR spectrometer in dimethylsulfoxide (DMSO) using tetramethylsilane (TMS) as an internal standard. Ultraviolet (UV) absorbance for specimens in a 96-well plate was measured with a Molecular Devices Spectra MAX 340pc. Other reagents were commercially available, first-grade material.

Extraction and isolation The air-dried rhizome was extracted four times with methanol (MeOH) by refluxing for 5 hr. After removal of the solvent *in vacuo*, the residue was suspended in water and then successively extracted with *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and butanol (*n*-BuOH). A portion of the CH_2Cl_2 fraction (20 g) was chromatographed on a silica gel column (7 × 60 cm) while eluting with a gradient of chloroform ($CHCl_3$) and MeOH to afford compounds 1 (57 mg, 80:20), **2** (56 mg, 79:21) and **3** (142 mg, 78:22). A portion of the *n*-BuOH fraction (20 g) was chromatographed on a silica gel column while eluting with a gradient of $CHCl_3$ and MeOH to afford compound **4** (157 mg, 70:30).

Alkaline phosphatase (AP) induction in Ishikawa cells The estrogenic/antiestrogenic activity employing Ishikawa cells was evaluated as described previously (22). Ishikawa cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 medium supplemented with 2 mM glutaMAX-1, antibiotic-antifungal reagent (10 units/ml penicillin G sodium, 10 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B), 1 mM sodium pyruvate and 10% fetal bovine serum (FBS). One day before plating the cells, the medium was changed to a phenol red-free formulation of DMEM/F-12 containing charcoal/dextran-stripped FBS to remove estrogens (estrogen-free medium). Cell suspensions (190 μl containing 5×10^4 cells) were plated into 96-well culture plates and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Test compounds (10 μl dissolved in DMSO, diluted 10-fold in ethanol and then diluted an additional 10-fold in phenol red-free medium), either alone or with 10 nM estradiol and

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the relevant controls (DMSO as a vehicle, estradiol as a positive control and tamoxifen as a negative control), were added to the plated cells and incubated for 4 days. The plates were processed by removing the test medium, washing twice with PBS, adding 50 µl of 0.1% Triton X-100 (v/v) in 0.1 M Tris-HCl buffer (pH 9.8) and freezing at -80°C. For analysis, the plates were rapidly thawed at 37 °C, and 150 µl of 0.1 M Tris-HCl buffer (pH 9.8) containing 1 mg/ml of *p*-nitrophenyl phosphate was added to each well. The plates were monitored at 405 nm with a microplate reader every 15 s, with a 10 s shake between each reading, for the first 8 min. The slopes of the obtained curves were calculated and those obtained with cell preparations treated with test samples were compared with standards. The percentage of induction for determination of estrogenic activity was calculated as follows:

$$\% \text{ Induction} = (\text{Slope}_{\text{sample+cells}} - \text{Slope}_{\text{cells}})/(\text{Slope}_{\text{DMSO}} - \text{Slope}_{\text{cells}}) \times 100$$

For determination of antiestrogenic activity, induction was calculated as follows:

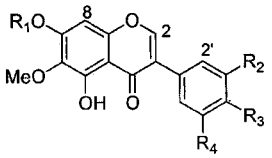
$$\% \text{ Induction} = (\text{Slope}_{\text{sample+cells}} - \text{Slope}_{\text{DMSO}})/(\text{Slope}_{\text{estradiol}} - \text{Slope}_{\text{DMSO}}) \times 100$$

Dose-response curves were plotted and the data represent the average of triplicate determinations.

Results and Discussion

Four compounds, isolated with repeated chromatographic purification of the CH_2Cl_2 and *n*-BuOH fractions, were elucidated as iristectorigenin A (**1**), irigenin (**2**), tectorigenin (**3**) and tectoridin (**4**), by spectral analysis (see appendix) and comparison of their spectral data in the literature (2-5, 7, 8). Their structures are shown in Fig. 1.

The estrogenic activity of the isolated isoflavones was estimated by AP inductive effect in Ishikawa cells. The Ishikawa cell is an estrogen receptor positive endometrial adenocarcinoma cell line derived from a glandular epithelial



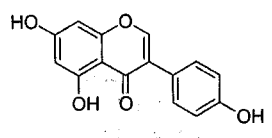
1 R_1: H, R_2: OH, R_3: OMe, R_4: H

2 R_1: H, R_2: OMe, R_3: OMe, R_4: OH

3 R_1: H, R_2: H, R_3: OH, R_4: H

4 R_1: Glc, R_2: H, R_3: OH, R_4: H

A



B

Fig. 1. Structures of compounds 1-4 from *B. chinensis* (A) and genistein (B).

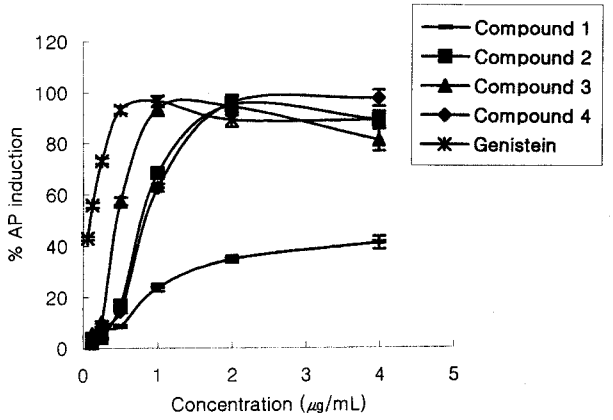


Fig. 2. AP-inductive activity of compounds 1-4 from *B. chinensis* in Ishikawa cells. (Compound **1**: iristectorigenin A, Compound **2**: irigenin, Compound **3**: tectorigenin, Compound **4**: tectoridin).

cell line. This cell responds to estrogens at concentrations approximating physiological levels (13). Induction of AP activity in Ishikawa cells indicates an estrogenic response, whereas inhibition represents an antiestrogenic effect (23). AP activity was supposed to be induced to about 100% by addition of 10 nM estradiol. The Ishikawa cell system allows easy and rapid measurement of the estrogenic potentials of various compounds.

In Ishikawa cells, irigenin, tectorigenin and tectoridin were highly estrogenic with EC_{50} values of 0.75, 0.42 and 0.81 µg/ml, respectively, while iristectorigenin A exhibited weak estrogenic activity ($EC_{50} \geq 4$ µg/ml). The relative activity is shown in Fig. 2. None of the isoflavones exhibited antiestrogenic activity (data not shown).

All of the tested isoflavones were less estrogenic than genistein, a reference compound (EC_{50} = 0.09 µg/ml), which is known to be one of the most estrogenic isoflavones of the known phytoestrogens. These results suggest that the presence of a methoxy group in ring (A) of isoflavones decreases the estrogenic potential (Fig. 1). Nevertheless, tectorigenin, while is structurally the most similar to genistein, was the most estrogenic of all the compounds tested. Moreover, there was little difference between the activities of tectorigenin and tectoridin. It has been suggested that the presence of glucose in tectoridin does not affect its estrogenic potential. It is supposed that tectoridin, an isoflavone glucoside, is metabolized to cleave the glycosidic bond and that, therefore, tectoridin exerts its estrogenic action in the form of tectorigenin, its aglycone. Research on the metabolism of tectoridin in the Ishikawa cells is in progress.

Mechanistically, phytoestrogens have been shown to bind to two types of estrogen receptors: α (ERα) and β (Erβ). Phytoestrogens show a lower binding affinity than estradiol and some show a higher binding affinity for ERβ than for ERα, which may suggest different pathways for their actions and explain the tissue-specific variability of phytoestrogenic action. Therefore, further research on the effect of these isoflavones on ERα and ERβ is needed.

The effects of phytoestrogens on the development of breast or endometrial cancers *in vivo* have not yet been established clearly. Although further studies are needed to confirm the characterization of the estrogenic potential of

these isoflavones, it appears that a rapid and simple screening of estrogenic activity using the Ishikawa cells of these isoflavones identified from *B. chinensis* may elucidate the clinical benefit of *B. chinensis*.

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Appendix

Compound **1**; EI-MS (70 eV, rel. int., %): *m/z* 330 $[M]^+$ (61.1), 315 (24.0), 312 (34.1), 301 (3.0), 287 (40.1), 272 (8.1), 182 (1.0), 149 (13.0), 118 (3.0), 69.1 (100); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.07 (s, 5-OH), 8.38 (s, H-2), 7.13 (d, J = 1.8 Hz, H-2'), 6.99 (dd, J = 1.8, 8.1 Hz, H-6'), 6.82 (d, J = 8.1 Hz, H-5'), 6.51 (s, H-8), 3.80 (s, -OMe), 3.75 (s, -OMe); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 154.4 (C-2), 121.6 (C-3), 180.6 (C-4), 152.7 (C-5), 131.4 (C-6), 153.3 (C-7), 93.9 (C-8), 157.5 (C-9), 104.8 (C-10), 121.8 (C-1'), 115.3 (C-2'), 146.7 (C-3'), 147.3 (C-4'), 113.2 (C-5'), 121.7 (C-6'), 59.9 (-OMe), 55.7 (-OMe).
Compound **2**; EI-MS (70 eV, rel. int., %): *m/z* 360 $[M]^+$ (20.1), 345 (10.1), 330 (13.0), 317 (13.0), 312 (10.1), 287 (12.1), 182 (3.1), 149 (7.0), 105 (21.2), 69.1 (100); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.07 (s, 5-OH), 8.37 (s, H-2), 6.71 (d, J = 1.9 Hz, H-2'), 6.66 (d, J = 1.9 Hz, H-6'), 6.50 (s, H-8), 3.78 (s, -OMe), 3.75 (s, -OMe), 3.69 (s, -OMe); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 154.8 (C-2), 121.7 (C-3), 180.3 (C-4), 152.9 (C-5), 131.5 (C-6), 153.3 (C-7), 94.0 (C-8), 157.7 (C-9), 104.8 (C-10), 126.1 (C-1'), 110.4 (C-2'), 150.3 (C-3'), 136.4 (C-4'), 152.7 (C-5'), 104.5 (C-6'), 60.1 (-OMe), 59.9 (-OMe), 55.8 (-OMe).
Compound **3**; EI-MS (70 eV, rel. int., %): *m/z* 300 $[M]^+$ (44.2), 282 (26.5), 257 (30.1), 182 (1.0), 149 (0.9), 118 (9.0); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.06 (s, 5-OH), 8.32 (s, H-2), 7.37 (dd, J = 1.8, 8.7 Hz, H-2',6'), 6.83 (dd, J = 1.8, 8.7 Hz, H-3',5'), 6.50 (s, H-8), 3.75 (s, -OMe); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 154.1 (C-2), 121.8 (C-3), 180.6 (C-4), 152.8 (C-5), 131.4 (C-6), 153.3 (C-7), 93.9 (C-8), 157.6 (C-9), 104.9 (C-10), 121.3 (C-1'), 130.2 (C-2',6'), 115.1 (C-3',5'), 157.4 (C-4'), 59.9 (-OMe).
Compound **4**; FAB-MS: *m/z* 463 $[M + 1]^+$; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 12.92 (s, 5-OH), 8.44 (s, H-2), 7.39 (dd, J = 1.8, 8.7 Hz, H-2',6'), 6.82 (dd, J = 1.8, 8.7 Hz, H-3',5'), 6.50 (s, H-8), 5.11 (anomeric H-1), 3.77 (s, -OMe); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 154.9 (C-2), 122.2 (C-3), 180.9 (C-4), 152.6 (C-5), 132.6 (C-6), 153.1 (C-7), 94.2 (C-8), 156.8 (C-9), 106.6 (C-10), 121.2 (C-1'), 130.3 (C-2',6'), 115.3 (C-3',5'), 157.6 (C-4'), 100.3 (Glc C-1), 73.3 (Glc C-2), 76.9 (Glc C-3), 69.8 (Glc C-4), 77.5, (Glc C-5), 61.0 (Glc C-6), 59.9 (-OMe).