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Estrogenic Activity of Furanocoumarins Isolated from *Angelica dahurica*

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In our efforts to discover novel phytoestrogens to treat menopausal symptoms, eleven furanocoumarins were isolated from *Angelica dahurica* and tested for their estrogenic activity on the Ishikawa cell line. Among the compounds tested, 9-hydroxy-4-methoxypsoralen and alloisoimperatorin showed strong abilities to induce alkaline phosphatase (AP) with EC_{50} values of 1.1 and 0.8 μg/mL, respectively, whereas the other nine furanocoumarins were weakly or only slightly active.

Key words: *Angelica dahurica,* Umbelliferae, Furanocoumarin, Estrogenic activity

INTRODUCTION

During menopause and postmenopause, many women experience one or more undesirable symptoms, including hot flashes, depression, mood swings, sleeping disorders, vaginal dryness, and joint pain, largely due to a lack of estrogens (Brosage, 1995). Hormone replacement therapy helps to relieve menopausal symptoms, as well as reduces the risk of osteoporosis, cardiovascular disease, dementia from Alzheimer's disease, and certain types of cancer (Harris *et al.*, 1990; Colditz *et al.*, 1995; Grodstein *et al.*, 1997; Wickelgren, 1997). Epidemiological data show that a diet rich in phytoestrogens, such as those found in soy, also reduces the number of hot flashes and the incidence of cancer in Asian women (Kurzer *et al.*, 1997). However, traditional estrogen replacement therapy induces a slight, but significant, increase in the risk of developing breast and endometrial cancer (Colditz *et al.*, 1995; Henderson *et al.*, 1988; Liehr, 1990; Stampfer *et al.*, 1992; Bolton *et al.*, 1998). Women are therefore increasingly turning to herbal remedies as alternative therapies for menopausal symptoms (Setchell, 1998, 1999; Murkies *et al.*, 1998).

In the present investigation, we studied the estrogenic constituents of the root of *Angelica dahurica* Bentham et Hooker (Umbelliferae). This root is an important herbal medicine (Perry, 1980; Hsu *et al.*, 1986) listed in the Chinese Pharmacopoeia and has been used as an antipyretic and analgesic for colds, headaches, and toothaches (Tang *et al.*, 1992). Furthermore, *A. dahurica* is one of the components in traditional restoratives for women in Asia. These restoratives commonly have some estrogenic effects, so it was expected that *A. dahurica* would also have some estrogenic activity. We evaluated the estrogenic activity of eleven furanocoumarins isolated from *A. dahurica* using the Ishikawa cell line, an estrogen-responsive human endometrial adenocarcinoma cell line. Estrogenic compounds markedly increase the activity of alkaline phosphatase (AP) in these cells (Nishida *et al.*, 1985; Holinka *et al.*, 1986).

MATERIALS AND METHODS

Materials

A. dahurica (Umbelliferae) was purchased at an herbal market in Seoul, Korea. All components and reagents for cell media were purchased from Life Technologies, Inc. (Grand Island, NY).

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Instruments

NMR spectra were measured on a Bruker Avance 500 spectrometer (Bruker, Ettlingen, Germany, 500 MHz for ^{1}H). EI-MS was recorded on a Jeol JMS-700 mass spectrometer (Jeol, Tokyo, Japan). The Hitachi 7100 HPLC-UV system was used for chromatographic analysis. The eluent was collected with a Gilson FC204 fraction collector (FC). Absorbance for Ishikawa assays were measured with the Molecular Devices Spectra$_{max}$ 340PC.

Preparation of extracts

The dried root of *A. dahurica* (1 kg) was refluxed with methanol for 3 h. The organic solvent was then removed *in vacuo*. The residue was suspended in 1000 mL of water and subsequently extracted with 800 mL of methylene chloride. The organic solvent was evaporated to yield 70 g of the methylene chloride-soluble fraction.

Bioassay-guided LC-UV-FC

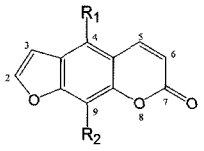
Fifty microliters of the CH_2Cl_2-fraction (20 mg/mL in MeOH) was injected into an ODS column (Kanto Mightysil, RP-18, 5 μm, 4.6 × 250 mm) and eluted with CH_3CN - H_2O (30:70, 0 min; 100:0, 15 min; 100:0, 30 min) at the rate of 1 mL/min. The eluent was analyzed by UV detection (254 nm), collected in 96-well plates, and dried for use in testing estrogenic and anti-estrogenic activities.

Isolation of Compounds

The major components responsible for the observed estrogenic/anti-estrogenic activity were detected by bioassay-linked HPLC-UV, which indicated the corresponding peaks on the HPLC chromatogram (Fig. 2). Based on this detection, the active components were isolated using the following procedure. The methylene chloride-soluble portion (70 g) was chromatographed over a silica gel column (400 g, 7×100 cm) using a stepwise gradient elution with a mixture of hexane/ethyl acetate (5:1 to 0:1). Six fractions were obtained. Repeated silica gel column chromatography of fraction-IV yielded the following 11 compounds (Fig. 1): oxypeucedanin hydrate (**1**), 9-hydroxy-4-methoxypsoralen (**2**), byakangelicin (**3**), pabulenol (**4**), alloisoimperatorin (**5**), neobyakangelicol (**6**), byakangelicol (**7**), oxypeucedanin (**8**), imperatorin (**9**), phellotorin (**10**), isoimperatorin (**11**) (Stanley and Vannier, 1967; Saiki *et al.*, 1971; Bennett and Bonner, 1953; Dreyer and Lee, 1969; Bergendorff *et al.*, 1997; Ishihara *et al.*, 2001).

AP Induction in ishikawa cells

Ishikawa cells were cultured in DMEM/F-12 medium supplemented with 2 μM glutaMAX-1, antibiotic-antifungal (10 units/mL penicillin G sodium, 10 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B), 1 mM sodium pyruvate, and 10% FBS. AP induction was evaluated as



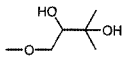
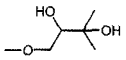
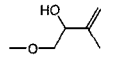
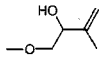
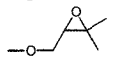
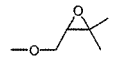
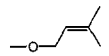
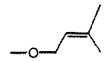
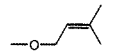
Compound	R_1	R_2
1		H
2	OCH_3	OH
3	OCH_3	
4		H
5	OH	
6	OCH_3	
7	OCH_3	
8		H
9	H	
10	OCH_3	
11		H

Fig. 1. Furanocoumarins isolated from *A. dahurica*

previously reported (Pisha and Pezzuto, 1997). Briefly, one day prior to plating the cells for the assay, the medium was changed to a phenol red-free formulation of DMEM/F-12 containing charcoal/dextran-stripped FBS to remove estrogen (estrogen-free medium). Cell suspensions (190 μL containing 5×10^4 cells) were subsequently plated into 96-well microtiter plates and incubated overnight at 37°C in a 5% CO_2 incubator. Each furanocoumarin solution, alone or with 2×10^{-6} M 17β-estradiol (E_2), and the relevant controls (DMSO, E_2, and tamoxifen) were added to the plated cells and incubated for 4 days. The microtiter plate was then washed twice with PBS; 0.1% Triton X-100 (v/v) in 0.1 M Tris-HCl buffer (pH 9.8) was added to the washed cells, which were then frozen at -80 °C. For the AP 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 were added to each well. For 8 min, the plates were monitored at 405 nm at 15 s intervals with 10 s of shaking between each reading. The measured absorbances were converted to kinetic readings, and the maximum slope of the lines generated by the kinetic readings was calculated using a computer program installed in microplate reader. Determination of the percent induction of estrogenic activity was calculated as follows:

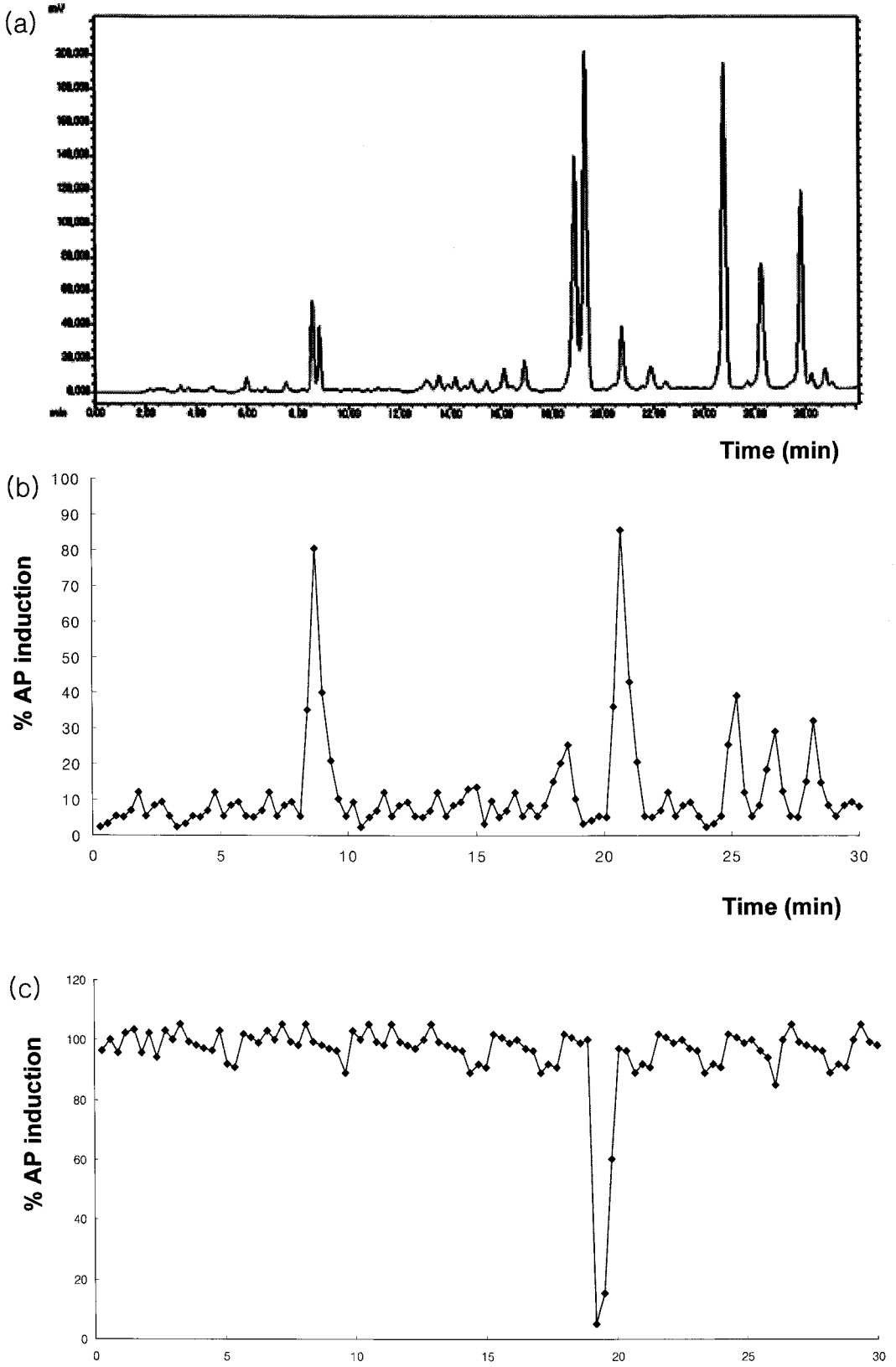


Fig. 2. HPLC-UV chromatogram and activity profiles. (a) HPLC-UV chromatogram, (b) Estrogenic activity of CH_2Cl_2-fraction of *A. dahurica*, (c) Anti-estrogenic activity of CH_2Cl_2-fraction of *A. dahurica*.

$$\%\text{Inhibition} = \frac{\text{Slope}_S - \text{Slope}_D}{\text{Slope}_{E_2} - \text{Slope}_D} \times 100$$

For determination of anti-estrogenic activity, inhibition was calculated as follows:

$$\%\text{Induction} = \frac{\text{Slope}_S - \text{Slope}_C}{\text{Slope}_D - \text{Slope}_C} \times 100$$

where $Slope_S$, $Slope_C$, $Slope_D$, and $Slope_{E2}$ are the slopes from sample solution, control cells, vehicle blank (DMSO), and E_2 solution, respectively. Dose-response curves were plotted, and EC_{50} or IC_{50} values were calculated.

RESULTS AND DISCUSSION

Using an adapted bioassay-linked HPLC-UV process, we isolated 11 furanocoumarins from a methylene chloride extract of *A. dahurica*. Their structures were elucidated as oxypeucedanin hydrate, 9-hydroxy-4-methoxypsoralen, pabulenol, alloisoimperatorin, neobyakangelicol, byakangelicin, byakangelicol, oxypeucedanin, imperatorin, phellotorin, and isoimperatorin using NMR and UV spectroscopy, mass spectrometry, and physico-chemical data (Stanley and Vannier, 1967; Saiki *et al.*, 1971; Bennett and Bonner, 1953; Dreyer and Lee, 1969; Bergendorff *et al.*, 1997; Ishihara *et al.*, 2001).

The Ishikawa cell line is an estrogen receptor (ER)-positive endometrial adenocarcinoma cell line derived from glandular epithelial cells. These cells respond to estrogens and anti-estrogens at approximately physiological concentrations (Holinka *et al.*, 1986). Induction of AP activity in Ishikawa cells indicates an estrogenic response, whereas inhibition of AP represents an anti-estrogenic effect (Pisha and Pezzuto, 1997); numerous studies have reported estrogenic or anti-estrogenic activity in Ishikawa cells (Lee *et al.*, 2005; Yoo *et al.*, 2005). Among the 11 compounds isolated in this study, 9-hydroxy-4-methoxypsoralen (**2**) and alloisoimperatorin (**5**) showed a strong ability to induce AP in Ishikawa cells. Their EC_{50}s were 1.1 and 0.87 μg/mL, respectively, whereas the other nine furanocoumarins were weakly or only slightly active (i.e., AP induction was less than 50% at 4 μg/mL) (Table I). In the AP inhibition tests, oxypeucedanin (**8**) exhibited anti-estrogenic activity with an IC_{50} of 3.2 μg/mL. None of the furanocoumarins exhibited cytotoxicity at the concentrations used in these assays (data not shown). Meanwhile, the total extract of *A. dahurica* showed neither estrogenic nor anti-estrogenic activity. This may be caused by the activities of the estrogenic and anti-estrogenic components contained within *A. dahurica* offsetting each other. Nevertheless, active components were effectively isolated from the apparently inactive extract through an adapted bioassay-linked HPLC-UV process.

Table I. Estrogenic and anti-estrogenic activities of furanocoumarins from *A. dahurica*

Compound	Molecular Weight	AP induction EC_{50} (μg/mL)	AP inhibition IC_{50} (μg/mL)
1	304	>20	>20
2	232	1.1±0.1	>20
3	334	>20	>20
4	286	>20	>20
5	270	0.87±0.06	>20
6	316	>20	>20
7	316	>20	>20
8	286	>20	3.2±0.17
9	270	>20	>20
10	300	>20	>20
11	270	>20	>20

The data represent the average of triplicate determinations.

The estrogenic activity of the individual furanocoumarins (i.e, the ability to induce AP) appears to be related to the positions of the hydroxyl groups. The nine furanocoumarins that do not have a hydroxyl group in the aromatic ring displayed little estrogenic activity. On the other hand, 9-Hydroxy-4-methoxypsoralen (**2**) and alloisoimperatorin (**5**), which have a hydroxyl group in the aromatic ring, were effective phytoestrogens. The hydroxyl group in the aromatic ring is therefore an important determinant for estrogenic potential. An epoxide substitution at position 4 of furanocoumarin is regarded as the determinant for anti-estrogenic potential, as observed with oxypeucedanin (**8**).

Numerous reports have demonstrated the multiplicity of effects that furanocoumarins have, including anti-mutagenic effects (Edenharder *et al.*, 1995), an affinity for brain benzodiazepine receptors (Bergendorff *et al.*, 1997), iNOS inhibitory activity (Wang *et al.*, 2000), and tyrosinase inhibitory activity (Piao *et al.*, 2004). However, to our knowledge, few have investigated the phytoestrogenic activities of furanocoumarins. In fact, this is the first report of the estrogenic effects of 9-hydroxy-4-methoxypsoralen (**2**) and alloisoimperatorin (**5**) and the anti-estrogenic effect of oxypeucedanin. Further investigations of their effects at the level of proteins and genes are necessary to fully evaluate their potential estrogenic or anti-estrogenic activity. However, the current results suggest that these furanocoumarins can be developed as natural hormone replacements or as novel chemopreventive agents for hormone-dependent cancers.

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