Arch Pharm Res Vol 28, No 2, 186-189, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Anti-Estrogenic Activity of Lignans from *Acanthopanax chiisanensis* Root

Sanghyun Lee, Hye Hyun Yoo, Xiang Lan Piao, Ju Sun Kim[1], Sam Sik Kang[1], and Kuk Hyun Shin[1]

College of Pharmacy, Seoul National University, Seoul 151-742, Korea, and [1]Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea

(*Received October 2, 2004*)

Anti-estrogenic activity of (-)-sesamin (**1**), helioxanthin (**2**), savinin (**3**), taiwanin C (**4**), and 3-(3,4-dimethoxybenzyl)-2-(3,4-methylenedioxybenzyl)butyrolactone (**5**) isolated from the root of *Acanthopanax chiisanensis* was tested using Ishikawa cells. Among them, compound **3** exhibited anti-estrogenic activity (IC_{50} = 4.86 μM).

Key words: *Acanthopanax chiisanensis*, Araliaceae, Lignan, Savinin, Anti-estrogenic activity

INTRODUCTION

The root of *Acanthopanax chiisanensis* has traditionally been used as a tonic, and sedative as well as in the treatments of rheumatoid arthritis and diabetes mellitus (Perry, 1980; Yook, 1990). In our studies on the bioactive constituents of the *Acanthopanax* species, five lignans were isolated from the root of *A. chiisanensis.* As some lignans are known as phytoestrogens (Ososki and Kennelly, 2003), the isolated lignans were tested for their estrogenic and anti-estrogenic potentials. The estrogenic/anti-estrogenic activities of the isolated lignans were estimated by their alkaline phosphatase (APase) inductive effect in Ishikawa cells. Ishikawa cells are an estrogen receptor positive endometrial adenocarcinoma cell line derived from a glandular epithelial cell line. They respond to estrogens at concentrations approximating physiological levels (Holinka *et al.*, 1986). Induction of APase activity in Ishikawa cells indicates an estrogenic response, whereas inhibition suggests an anti-estrogenic effect (Pisha and Pezzuto, 1997). The Ishikawa cell system makes it possible to simply and easily measure the estrogenic/anti-estrogenic potentials of various compounds. In this paper, we present the estrogenic and anti-estrogenic activities of lignans from the root of *A. chiisanensis.*

Correspondence to: Dr. Sanghyun Lee, College of Pharmacy, Seoul National University, Shillim-dong, Gwanak-gu, Seoul 151-742, Korea
Tel: 82-2-880-9178, Fax: 82-2-887-0246
E-mail: jnp@korea.com

MATERIALS AND METHODS

Plant material

The root of *Acanthopanax chiisanensis* Nakai (Araliaceae) was collected at Gongju, Korea, and authenticated by Prof. S. H. Cho, Gongju National University of Education, Korea. A voucher specimen (Shin 9910-2) was deposited at the Herbarium of Natural Products Research Institute, Seoul National University, Korea.

Extraction and isolation

The air-dried powdered root (1.5 kg) of *A. chiisanensis* was extracted with MeOH under reflux, as described previously (Lee *et al.*, 2003). After removal of the solvent *in vacuo*, the residue (159 g) was suspended in water and then successively extracted with *n*-hexane (42 g), $CHCl_3$ (26 g), EtOAc (10 g) and *n*-BuOH (24 g). A portion of the $CHCl_3$ fraction (26 g) was subjected to chromatography on a silica gel column (7 × 60 cm), and eluted with a gradient of *n*-hexane and EtOAc, to afford compounds **1** (26 mg, 95:5), **2** (45 mg, 90:10), **3** (33 mg, 87:23), **4** (27 mg, 80:20), and **5** (45 mg, 78:22).

Compound **1**: $C_{20}H_{18}O_6$; EIMS (70 eV, rel. int., %): *m/z* 354 $[M]^+$ (100), 323 (12.6), 219 (7.5), 203 (34.7), 161 (64.8), 149 (90.6), 135 (53.8), 103 (8.3); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.87 (2H, br s, H-2',2"), 6.82 (2H, dd, *J* = 8.0, 1.2 Hz, H-6',6"), δ 6.79 (2H, d, *J* = 8.0 Hz, H-5',5"), 5.96 (2× -OCH_2O-), 4.74 (2H, d, *J* = 4.2 Hz, H-2,6), 4.25 (2H, dd, *J* = 9.0, 6.8 Hz, H-4_e,8_e), 3.89 (2H, dd, *J* = 9.0, 3.5 Hz, H-4_a,8_a), 3.07 (2H, m, H-1,5); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 147.9 (C-3',3"), 147.1 (C-4',4"), 135.0 (C-1',1"),

119.3 (C-6',6"), 108.1 (C-5',5"), 106.5 (C-2',2"), 101.0 (2× -O*C*H$_2$O-), 85.7 (C-2,6), 71.7 (C-4,8), 54.3 (C-1,5).

Compound **2**: $C_{20}H_{12}O_6$; EIMS (70 eV, rel. int., %): *m/z* 348 $[M]^+$(100), 319 (16.2), 291 (7.7), 261 (5.1), 233 (6.4), 205 (4.2), 174 (6.1), 145 (4.5); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.45 (H, s, H-4), 7.73 (H, d, *J* = 8.7 Hz, H-5), 7.33 (H, d, *J* = 8.7 Hz, H-6), 6.91 (1H, d, *J* = 7.7 Hz, H-5'), 6.83 (1H, s, H-2'), 6.81 (1H, d, *J* = 7.7 Hz, H-6'), 6.08 (-O*C*H$_2$O-), 5.98 (-O*C*H$_2$O-), 5.23 (2H, q, lactone -C*H*$_2$-); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.1 (*C*=O), 147.4 (C-8), 146.9 (C-7), 141.7 (C-1'), 139.7 (C-1), 130.7 (C-3',4'), 130.4 (C-9), 129.0 (C-10), 127.4 (C-4), 125.4 (C-5), 122.3 (C-6'), 121.5 (C-2), 121.0 (C-3), 111.8 (C-6), 109.6 (C-2'), 107.9 (C-5'), 101.5 (-O*C*H$_2$O-), 101.2 (-O*C*H$_2$O-), 69.5 (lactone -*C*H$_2$-).

Compound **3**: $C_{20}H_{16}O_6$; EIMS (70 eV, rel. int., %): *m/z* 352 $[M]^+$ (62.4), 217 (61.2), 189 (6.4), 159 (18.6), 135 (100), 131 (16.3), 103 (10.9), 77 (23.1); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.51 (1H, d, *J* = 1.6 Hz, H-6), 7.09 (1H, dd, *J* = 8.1, 1.4 Hz, H-6'), 7.06 (1H, d, *J* = 1.4 Hz, H-2'), 6.89 (1H, d, *J* = 8.1 Hz, H-5'), 6.75 (1H, d, *J* = 7.9 Hz, H-5"), 6.68 (1H, d, *J* = 1.3 Hz, H-2"), 6.65 (1H, d, *J* = 7.9, 1.3 Hz, H-6"), 6.06 (-OC*H*$_2$O-), 5.95 (-OC*H*$_2$O-), 4.27 (2H, m, H-4), 3.76 (1H, m, H-3), 3.00 (1H, dd, *J* = 14.2, 4.5 Hz, H-5$_e$), 2.61 (1H, dd, *J* = 14.2, 10.0 Hz, H-5$_a$); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 172.5 (C=O), 149.1 (C-5'), 148.3 (C-4'), 147.9 (C-5"), 146.5 (C-4"), 137.2 (C-1), 131.4 (C-1"), 128.1 (C-2'), 126.0 (C-1'), 125.8 (C-2), 122.0 (C-2"), 109.1 (C-6'), 108.8 (C-6"), 108.6 (C-3'), 108.5 (C-3"), 101.7 (-O*C*H$_2$O-), 101.0 (-O*C*H$_2$O-), 69.5 (C-5), 39.9 (C-3), 37.5 (C-4).

Compound **4**: $C_{20}H_{16}O_6$; EIMS (70 eV, rel. int., %): *m/z* 348 $[M]^+$(100), 319 (9.4), 289 (12.4), 261 (18.5), 233 (8.2), 159 (7.9); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.71 (1H, s, H-4), 7.22 (1H, s, H-5), 7.14 (1H, s, H-8), 6.98 (1H, d, *J* = 7.8 Hz, H-5'), 6.83 (1H, d, *J* = 1.0 Hz, H-2'), 6.81 (1H, dd, *J* = 7.8, 1.0 Hz, H-6'), 6.10 (-OC*H*$_2$O-), 6.08 (-OC*H*$_2$O-), 5.39 (2H, s, lactone -C*H*$_2$-); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 169.7 (*C*=O), 149.9 (C-7), 148.6 (C-6), 147.5 (C-3',4'), 140.1 (C-1), 139.8 (C-1'), 134.6 (C-2,3), 130.5 (C-9), 128.3 (C-10), 123.4 (C-6'), 118.9 (C-4), 110.5 (C-2'), 108.2 (C-5'), 103.6 (C-5,8), 101.7 (-O*C*H$_2$O-), 101.2 (-O*C*H$_2$O-), 67.9 (lactone -*C*H$_2$-).

Compound **5**: $C_{21}H_{22}O_6$; EIMS (70 eV, rel. int., %): *m/z* 370 $[M]^+$ (92.7), 356 (22.1), 177 (34.9), 151 (94.6), 135(100), 105 (13.1), 77 (20.9); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.77 (2H, d, *J* = 8.1 Hz, H-5"), 6.71 (2H, d, *J* = 7.7 Hz, H-5'), 6.60 (2H, d, *J* = 1.5 Hz, H-2'), 6.58 (2H, br d, *J* = 7.7 Hz, H-6'), 6.57 (2H, br d, *J* = 8.1 Hz, H-6"), 6.49 (2H, d, *J* = 1.9 Hz, H-2"), 5.93 (-OC*H*$_2$O-), 4.15 (2H, dd, *J* = 9.1, 6.9 Hz, H-4$_e$), 3.88 (2H, dd, *J* = 7.3, 1.9 Hz, H-4$_a$), 3.86 (-OC*H*$_3$), 3.83 (-OC*H*$_3$), 2.90 (2H, m, H-5), 2.57 (2H, m, H-6); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 178.5 (C=O), 149.0 (C-3"), 147.8 (C-3',4), 146.4 (C-4'), 131.3 (C-1'), 130.4 (C-1"), 122.2 (C-6'), 120.6 (C-6"), 111.7 (C-2"), 111.3 (C-5"), 109.4 (C-2'), 108.1 (C-5'), 101.0 (-O*C*H$_2$O-), 71.2 (C-4), 55.9 (-O*C*H$_3$), 55.7 (-O*C*H$_3$), 46.4 (C-2), 41.2 (C-3), 38.2 (C-6), 34.7 (C-5).

APase Induction in Ishikawa cells

The estrogenic/anti-estrogenic activities were evaluated employing Ishikawa cells, as described previously (Pisha and Pezzuto, 1997). The Ishikawa cells were routinely cultured in Dulbeccos 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 the 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 5% CO_2 in air atmosphere. 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 2×10^{-6} M estradiol, and the relevant controls (DMSO, estradiol, and tamoxifen) 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 0.1% Triton X-100 (v/v) in 0.1 M Tris-HCl buffer (pH 9.8), and frozen at -80°C. For the analysis, the plates were rapidly thawed to 37°C, and 150 μl 0.1 M Tris-HCl buffer (pH 9.8), containing 1 mg/mL of *p*-nitrophenyl phosphate, added to each well. The plates were monitored at 405 nm with an ELISA 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 compounds compared with a standard. The percentage induction for the determination of estrogenic activity was calculated as follows:

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

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

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

Dose-response curves were plotted, and IC_{50} values calculated.

Cytotoxicity assay

Ishikawa cells (5×10^4/well) were preincubated overnight in estrogen-free media in a 96-well culture plate, and then further incubated with test compounds for 4 days. The cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983). Twenty micro liter of MTT solution (5 mg/mL) was added to each well of the 96-well culture plate, incubated for 4 h at 37°C and the medium containing MTT removed. The incorporated formazan crystals in the viable cells were solublized with 100 μL DMSO and the absorbance of each well measured at 570 nm.

RESULTS AND DISCUSSION

A portion of the $CHCl_3$ fraction from *A. chiisanensis* root was subjected to chromatography on a silica gel column, and eluted with a gradient of *n*-hexane and EtOAc, to afford compounds **1-5**. They were identified as (-)-sesamin (**1**), helioxanthin (**2**), savinin (**3**), taiwanin C (**4**), and 3-(3,4-dimethoxybenzyl)-2-(3,4-methylenedioxybenzyl)butyrolactone (**5**) by spectral analysis and comparison with their spectral data from the literature (Banerji *et al.*, 1984; Ina *et al.*, 1987; Shieh *et al.*, 1990; Stevenson and Weber, 1989). Their structures are shown in Fig. 1.

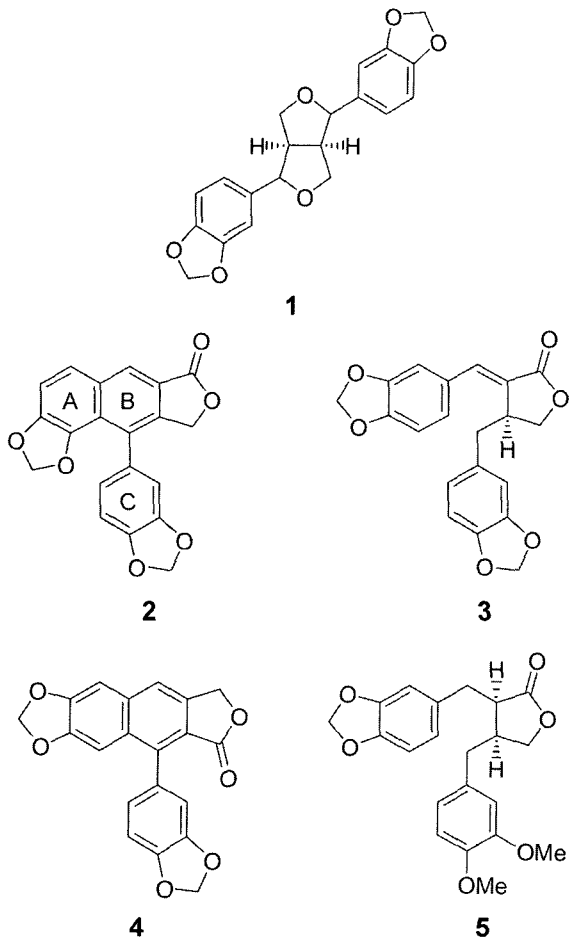

Fig. 1. Structures of compounds **1-5**

The five lignans isolated from *A. chiisanensis* were evaluated for their estrogenic/anti-estrogenic potentials using the Ishikawa cell system. As a result, without estradiol, none of the lignans significantly induce APase activity in the Ishikawa cells (data not shown). However, when cells were treated with 1 nM estradiol, the APase activity increased approximately 10-fold, with compounds **3** and **5** inhibiting the induction in dose-dependent manners, their IC_{50} being 4.86 and 3.62 μM, respectively. When the methylenedioxy group on the (C) ring was substituted with methoxyl groups, and the (B) ring opened, the anti-estrogenic inhibitory activity was appreciably increased. When compounds **3** and **5** were tested at the same concentration, compound **5**, which possesses methoxyl groups on the (C) ring, was revealed to be the most potent. To determine whether their inhibitory activities on APase induction was due to an estrogen-antagonistic or a cytotoxic mechanism, Ishikawa cells were treated with the lignans for 4 days, and the survival of the cells then measured using the MTT assay. All of the tested lignans exhibited partial cytotoxic activity. The active lignans, compounds **3** and **5**, were more or less cytotoxic, with Growth Inhibition $(GI)_{50}$ values of 30.23 and 4.38 μM, respectively. From these results, the APase inhibitory activity of compound **5** appears to be caused by its cytotoxic effect, so it can be inferred that compound **5** is not a real anti-estrogenic principle (Fig. 2 and Table I). Accordingly, compound **3**, which possesses a methylenedioxy group on the (C) ring, and an opened (B) ring, was a real anti-estrogenic principle.

Isoflavones, coumestans, and lignans are representative phytoestrogens. Among the phytoestrogenic lignans, secoisolariciresinol and matairesinol, which are converted by bacterial action in the gut into enterodiol and enterolactone, are the most well known mammalian lignans (Setchell and Adlercreutz, 1988). Recently, other enterolactone precursors have been identified: arctigenin,

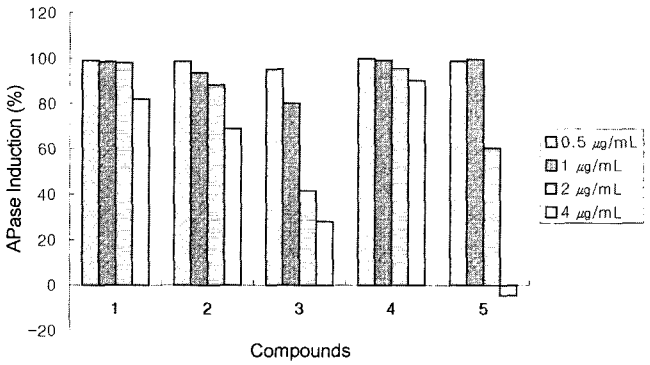

Fig. 2. APase inhibitory activity of compounds **1-5** in Ishikawa cells

Table I. APase inhibitory activity and cytotoxicity of compounds **1-5** in Ishikawa cells

Compounds	APase Inhibition (IC_{50}, μM)	Cytotoxicity (GI_{50}, μM)
Tamoxifen*	1.16	>100
1	>20	99.80
2	>20	71.29
3	4.86	30.23
4	>20	>100
5	3.62	4.38

*A positive control.

7-hydroxymatairesinol, lariciresinol, pinoresinol, and syringaresinol (Meagher *et al.*, 1999; Heinonen *et al.*, 2001; Xie *et al.*, 2003). However, their estrogenic or anti-estrogenic properties have not been studied as thoroughly as those of isoflavones and coumestans. In fact, up to now, there have been few reports on the anti-estrogenic properties of lignans, with some having been reported as inactive in anti-estrogenic tests (Chang *et al.*, 2000). Therefore, the anti-estrogenic activity of compound **3**, as first reported in Ishikawa cells, might be valuable. Further, compound **3** exhibited cytotoxic activity against Ishikawa cells at higher concentrations.

Therefore, it can be suggested that compound **3** might be a potential candidate for the development as a novel chemopreventive agent for hormone-dependent cancers. Further researches on the mechanism and relationship of its anti-estrogenic and cytotoxic actions are in progress.

ACKNOWLEDGMENT

This study was supported, in part, by a grant from the Korean Gasiogalpi Association.

REFERENCES

Banerji, J., Das, B., Chatterjee, A., and Shoolery, J. N., Gadain, a lignan from *Jatropha gossypifolia*. *Phytochemistry*, 23, 2323-2327 (1984).

Chang, L. C., Gills, J. J., Bhat, K. P., Luyengi, L., Farnsworth, N. R., Pezzuto, J. M., and Kinghorn, A. D., Activity-guided isolation of constituents of *Cerbera manghas* with antiproliferative and antiestrogenic activities. *Bioorg. Med. Chem. Lett.*, 10, 2431-2434 (2000).

Heinonen, S., Nurmi, T., Liukkonen, K., Poutanen, K., Wähälä, K., Deyama, T., Nishibe, S., and Adlercreutz, H., *In vitro* metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. *J. Agric. Food Chem.*, 49, 3178-3186 (2001).

Holinka, C. F., Hata, H., Kuramoto, H., and Gurpide, E., Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). *Cancer Res.*, 46, 2771-2774 (1986).

Ina, H., Asai, A., Iida, H., and Ushida, T., Chemical investigation of *Hydrocotyle sibthorpioides*. *Planta Med.*, 53, 228 (1987).

Lee, S., Shin, K. H., Lee, S. C., and Cho, S. H., Lignans from the root of *Acanthopanax chiisanensis* Nakai. *Kor. J. Med. Crop Sci.*, 11, 279-283 (2003).

Meagher, L. P., Beecher, G. R., Flanagan, V. P., and Li, B. W., Isolation and characterization of the lignans, isolariciresinol and pinoresinol, in flaxseed meal. *J. Agric. Food Chem.*, 47, 3173-3180 (1999).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55-63 (1983).

Ososki, A. L. and Kennelly, E. J., Phytoestrogens: a review of the present state of research. *Phytother. Res.*, 17, 845-869 (2003).

Perry, L. M., Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses, Cambridge, Massachusetts and London: MIT Press, p. 41 (1980).

Pisha, E. and Pezzuto, J. M., Cell-based assay for the determination of estrogenic and anti-estrogenic activities. *Methods Cell Sci.*, 19, 37-43 (1997).

Setchell, K. D. R. and Adlercreutz, H., In Role of the Gut Flora in Toxicity and Cancer, ed. by Rowland I. R., Academic Press, San Diego, pp. 315-345 (1988).

Shieh, H. L., Cordell, G. A., Lankin, D. C., and Lotter, H., The liquid, solid and molecular force field calculated conformations of savinin. *J. Org. Chem.*, 55, 5139-5145 (1990).

Stevenson, R. and Weber, J. V., Improved methods synthesis of lignan arylnaphthalene lactones *via* arylpropargyl arylpropiolat
e esters. *J. Nat. Prod.*, 52, 367-375 (1989).

Xie, L.-H., Akao, T., Hamasaki, K., Deyama, T., and Hattori, M., Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and isolation of *Enterococcus faecalis* strain PDG-1 responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol. *Chem. Pharm. Bull.*, 51, 508-515 (2003).

Yook, C. S., Coloured Medicinal Plants of Korea, Seoul: Academy Book Co., p. 370 (1990).