Isoguaiacins, Arylnaphthalene Types Identified as Novel Potent Estrogenic Signaling Molecules from *Larrea nitida*

Si-Yeon Jeong,[†] Hye-na Ahn,[†] Gyu-Un Bae,[†] Minsun Chang,[‡] Xiyuan Liu,[§] Hee-Kyung Rhee,[‡] Joongku Lee,[¶] Young-Won Chin,[‖] Sei-Ryang Oh,[††] and Yun Seon Song[†,*]

[†]*College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Republic of Korea.*
**E-mail: yssong@sookmyung.ac.kr*
[‡]*Department of Medical and Pharmaceutical Science, College of Science, Sookmyung Women's University, Seoul 140-742, Republic of Korea*
[§]*Graduate School of Life Systems Sciences, Sookmyung Women's University, Seoul 140-742, Republic of Korea*
[¶]*International Biological Material Research Center, KRIBB, Daejeon 305-806, Republic of Korea*
[‖]*College of Pharmacy, Dongguk University-Seoul, Goyang 410-820, Republic of Korea*
[††]*Natural Medicine Research Center, KRIBB, ChungBuk 363-883, Republic of Korea*
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In our study on the biological activities of isoguaiacins, we demonstrated lignans with a rigid structure from *Larrea nitida* as selective estrogen receptor modulators (SERMs). Estrogenic activity-guided isolation of the *L. nitida* extract led to the identification of four lignans compounds. All four lignans displayed high binding affinity for human estrogen receptor (hER) α and β as well as transcriptional activities for estrogen response element (ERE). Furthermore, arylnaphthalene type lignans **1** (5,6,7,8-tetrahydro-5-(3,4-dihydroxyphenyl)-6,7-dimethylnaphthalene-2,3-diol) and **2** (5,6,7,8-tetrahydro-5-(4-hydroxy-3-methoxyphenyl)-6,7-dimethylnaphthalene-2,3-diol) showed especially high potencies for ERβ and ERE with IC_{50} of 0.045 and 0.85 μM, respectively. Arylnaphthalene type lignans **1** and **2** and bibenzyl butane type lignans **3** (4-(4-(4-hydroxyphenyl)-2,3-dimethylbutyl) benzene-1,2-diol) increased the proliferation of MCF-7 cells and the ERα target gene *pS2*. Our results indicate that the four active lignans have selective affinities for hERβ and exhibit SERM properties. For the first time, we have discovered the estrogenic activities of isoguaiacins from *L. nitida*, which are promising candidates for the treatment of estrogen-related conditions and warrant further preclinical evaluation.

Keywords: *Larrea nitida*, Isoguaiacin, Phytoestrogen, Estrogen receptor

Introduction

Plants of the genus *Larrea* comprise six species: *Larrea ameghinoi*, *L. cuneifolia*, *L. divaricata*, *L. mexicana*, *L. nitida*, and *L. tridentate*. They have been used to treat a variety of illnesses, including cancer, inflammation, and menstrual pain.[1] The extract of *L. divaricata* has been shown to inhibit the proliferation of BW 5147T lymphoma and MCF-7 human breast cancer cells.[2,3] As a folk remedy, the genus *Larrea* is not recommended for pregnant and lactating women, implying that the *Larrea* genus regulates hormonal functions in them. Estrogen, a major female hormone, regulates various physiological responses in many target tissues and plays an important role in the development and progression of breast and endometrial cancers.[4,5] Women suffering from breast cancer have often been cured by the selective estrogen receptor modulator (SERM) tamoxifen, which inhibits the proliferation of mammary epithelial cells. Tamoxifen has been shown to decrease the incidence of breast cancer by ~50%, leading to a 25%–28% decline in cancer mortality rates.[6] However, long-term tamoxifen treatment is associated with an increased risk of thromboembolic events, stroke, and uterine cancer.[7] Because of these side effects, phytoestrogens (plant SERMs) are becoming important for the treatment of diseases in women due to their proven therapeutic efficacy and low risk of side effects.

Phytoestrogens can function as SERMs, with similar structures to 17β-estradiol (E2). The main classes of phytoestrogens in terms of chemical structure are isoflavones, prenylated flavonoids, coumestans, and lignans.[8] Phytochemical studies have shown that the genus *Larrea* contains lignans, flavonoids, condensed tannins, triterpene saponins, and naphthoquinone, with lignans being the major component.[9,10] It was reported that nordihydroguaiaretic acid (NDGA), as a member of the lignan family, exhibits estrogenicity.[11] Recently we reported the effects on the ER-mediated activities by the alcoholic extract of *L. nitida*.[12] Our results showed that *L. nitida* extract (LNE) and its fractions had binding affinities for both hERα and hERβ and that treatment with LNE reduced the proliferation of breast cancer MCF-7 cells in the presence of E2 and the uterotrophic effect in immature rats. These data indicated that LNE and its bioactive components may act as SERMs in female hormone-responsive organs.

Correction added on 01 October 2015, after first online publication: ISSN (Print) has been corrected.

In this paper, we report the isolation and identification of individual phytoestrogenic compounds from LNE via bioactivity-guided fractionation. Different types of ligans were identified as the novel estrogenic signaling molecules responsible for the SERM activities of the LNE. The lignans found in *L. nitida* differ from the typical lignans identified as phytoestrogens in other plants in terms of their structural rigidity. The SERM activities of these lignans were evaluated to determine their pharmacological importance as a source of phytoestrogens based on their hERα and hERβ binding affinities and ER-mediated gene transcription, as well as their effect on the proliferation on MCF-7 human breast cancer cells.

Experimental

Cell Culture and Materials. The MCF-7 cell line is a steroid-sensitive subline developed from MCF-7 human breast cancer cells (kindly provided by Dr. Anna Soto, Tufts University, Boston, MA, USA). MCF-7 cells were cultured in Dulbecco's Modified Eagle′s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. All cell culture reagents were purchased from Gibco (Carlsbad, CA, USA). [2,4,6,7-^{3}H]-17β-Estradiol (^{3}H-E2 88.0 Ci/mmol) was purchased from PerkinElmer Inc. (Boston, MA, USA). Other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and, unless stated otherwise, were of research grade suitable for cell culture or of the highest grade available. Each chemical was dissolved in either ethanol or DMSO before being tested in the various assay systems. The final solvent concentration in the culture medium did not exceed 0.5%.

Plant Materials. The stems and leaves of *L. nitida* were collected in Jarilla, Chile, in 2007 and identified by Dr. Joongku Lee, Korea Research Institute of Bioscience and Biotechnology (KRIBB). A voucher specimen (access number FBM026-052) has been deposited in the herbarium of the KRIBB.

Extraction and Isolation. Air-dried stems and leaves of *L. nitida* (52 g) were pulverized and extracted with methanol (MeOH) to yield the crude LNE (14 g). A portion of this LNE (12 g) was subjected to a reversed-phase silica column chromatography eluting with a gradient mixture of H_2O/MeOH (90:10–0:100) to give 16 subfractions (LN01–16). Separation of LN 08 by the aforementioned HPLC method yielded NDGA (6.8 mg, t_R = 38.04 min). This compound exhibited comparable spectroscopic data (^{1}H and ^{13}C NMR) and HRESIMS *m/z*: 303.1591 $[M+H]^+$ (calculated for $C_{18}H_{23}O_4$, 303.1596) with published values.[13] A bioactivity-guided fractionation and purification of LNE was performed. One of 16 fractions, LN08 (1.6 g), was further fractionated into 14 subfractions (LN08-01–14) using reversed-phase medium-pressure column chromatography (MPLC) eluted with a gradient mixture of methanol/water (25:75 to 100:0). From LN08-07, compound **1** (8.6 mg, t_R = 31.25 min) was purified by HPLC separation (Phenomenex Luna C_{18} 250 × 10 mm) and eluted with 40% acetonitrile at a flow rate of 2 mL/min. LN10 (830 mg) was chromatographed on an HPLC column (Phenomenex Luna C_{18} 250 × 10 mm), yielding compound **2** (1.5 mg t_R = 60.40 min). Fractionation of LN11 (1.2 g) by reversed-phase MPLC with a gradient of methanol/water (40:60 to 100:0) yielded 16 subfractions. Among these subfractions, LN11-06 was separated using HPLC, leading to the isolation of compound **3** (7.7 mg, t_R = 42.06 min). Using the same HPLC conditions, compound **4** (5.4 mg, t_R = 50.92 min) was purified from LN11-10.

ERE-luciferase Reporter Gene Assay. MCF-7 cells were seeded in 12-well plates (6×10^5 cells/well) and cultured in phenol red-free DMEM containing 5% charcoal dextran-treated FBS (CD) in triplicate. Cells were then transiently transfected with pERE-Luc plasmid (0.5 μg/well) and 0.2 μg of an inactive control plasmid encoding a β-galactosidase gene using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). The pERE-Luc plasmid contains three copies of the *Xenopus laevis* vitellogenin A2 ERE upstream of firefly luciferase (a gift from Dr. V. C. Jordan, Lombardi Comprehensive Cancer Center, Georgetown University).[14] One day after transfection, various concentrations of compounds and E2 were added. Cells were lysed for luciferase activity analysis after incubation for 24 h. Luciferase activity was measured using a luciferase reporter assay system (Promega, Madison, WI, USA). Luminescence was detected with a TD-20/20 luminometer (Turner Design, Sunnyvale, CA, USA). Finally, luciferase activities were normalized to β-galactosidase activity.

ER Ligand-binding Assay. The binding ability of the test compounds to control or treated recombinant full-length human estrogen receptor α (hERα) was assessed as described previously.[15] hERα (2088 pmol/mg estrogen binding activity, Invitrogen) was diluted to a concentration of 5 nM, and the hERα was labeled with [^{3}H]-E2 (3 nM) in the presence or absence of 10^{-6} M unlabeled E2 and various concentration of compounds derived from LNE. For the hERβ binding assay, hERβ (4500 pmol/mg estrogen binding activity, Invitrogen) was diluted to a concentration of 10 nM and labeled with [^{3}H]-E2 (10 nM) in the presence or absence of 10^{-6} M unlabeled E2 or compounds derived from LNE. After incubation for 2 h at 27 °C, the reactions were terminated with a Perkin Elmer FilterMate Harvester (Waltham, MA, USA). The radioactivity trapped on each filter was measured using a Packard 2000CA liquid scintillation counter. Nonspecific [^{3}H]-E2 binding was determined in the presence of 10^{-6} M unlabeled E2. The specific binding percent for each ER was determined as follows: $[(dpm_{sample} - dpm_{nonspecific})/(dpm_{control} - dpmnonspecific)] \times 100$. Relative binding affinity (RBA) was calculated as follows: $RBA = [IC_{50\ (E2)}/IC_{50\ (LNE\ or\ LNs)}] \times 100$.

Cell Proliferation Assay. MCF-7 cells were plated in 12-well plates at an initial density of 6×10^4 per well. Cells were allowed to attach for 24 h, and the medium was then replaced with the experimental medium (phenol red-free DMEM containing 5% CD-FBS. LNE, E2, ICI 182,780 (ICI; pure ER antagonist), or a combination of these compounds was added to the medium in a range of concentrations, and the cells were

incubated for 6 days (late exponential phase). Cell growth was determined using the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Quantitative Polymerase Chain Reaction (PCR). Total mRNA were isolated from MCF-7 cells using Trizol (Life Technologies) according to the user manual. Using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), the cDNA was synthesized from 1 μg of total mRNA. cDNA, a pair of primers, and PCR SYBR green kit (Bio-Rad) reagent were mixed. We examined the expression of Trefoil factor 2 (*pS2*) genes as the estrogen target gene. The quantitative gene expression of target gene was analyzed by an Applied Biosystem 7500 Realtime PCR system (Life Technologies). The gene expression of GAPDH, the housekeeping gene, was analyzed at the same time. The ratio of target gene expression and housekeeping gene expression was shown as a quantitative gene expression. The primer were *pS2*; forward 5'-CGTGAAAGAC AGAATTGTGGTTTT; reverse 5'-CGTCGAAACAGCAGCCCTTA. Real-time PCR involved 40 cycles (95 °C; 30 s, 60 °C; 30 s, 72 °C; 30 s). Each gene expression curve was shown by the logarithm, and the threshold cycle (C_T) was calculated mathematically. This value was substituted into $2-\Delta\Delta C_T$, and the relative gene expression was calculated.

Western Blot. The whole protein was extracted from MCF-7 cells. The concentration of the extracted protein was determined by BCA assay, and 20 μg of the protein was loaded on 7.5% SDS-PAGE gel. The electrophoresis was performed for 2 h at 80 V. Then the protein was transferred to a PVDF membrane, and the primary antibody was incubated with the membrane at 4 °C overnight. Secondary antibody was incubated for 1 h at RT. The ECL reagent was used to detect the target proteins. For primary antibody, ERα (sc-7207, Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:500. To normalize the experimental error, β-actin was used.

Statistical Analysis. Data are expressed as mean ± standard error (SE). Comparisons between groups were performed by one-way analysis of variance (ANOVA) with appropriate Bonferroni tests using the GraphPad Prism Software (San Diego, CA, USA). A *p*-value less than 0.05 was considered to indicate statistical significance.

Results and Discussion

The structures of the four compounds from LNE, 5,6,7,8-tetrahydro-5-(3,4-dihydroxyphenyl)-6,7-dimethylnaphthalene-2,3-diol (**1**), 5,6,7,8-tetrahydro-5-(4-hydroxy-3-methoxyphenyl)-6,7-dimethylnaphthalene-2,3-diol (**2**), 4-(4-(4-hydroxyphenyl)-2,3-dimethylbutyl) benzene-1,2-diol (**3**), and 4-(4-(4-hydroxy-3-methoxyphenyl)-2,3-dimethylbutyl) benzene-1,2-diol (**4**) were identified by analyzing their UV, MS, ^{1}H- and ^{13}C-NMR spectra and by comparing our data with those reported in the literature.[16–19] The chemical characteristics of the four compounds are summarized in Table 1.

To evaluate the SERM properties of compound **1–4**, we evaluated their binding affinities to both hERα and hERβ. The four compounds showed binding affinities for hERα and hERβ with IC_{50} values ranging from 10^{-8} to 10^{-5} M (Table 2). Compound **1** showed the most potent binding affinity, with IC_{50} values of 0.11 μM for hERα and 0.045 μM for hERβ (Table 2). The RBA values of compound **1** to hERα and hERβ were 1.09 and 10.89, respectively, indicating 10-fold higher selectivity for hERβ than hERα. Consequently, compound **1** showed the greatest preference for hERβ over hERα. Compound **2** also showed high binding affinities, with IC_{50} values of 0.14 μM for hERα and 0.36 μM for hERβ (Table 2). These four lignan compounds were also evaluated in terms of their effect on ER-mediated transcription using a transcription assay, with luciferase as the reporter gene. All four compounds increased transcriptional activity in dose-dependent manner (>80% intrinsic activity of E2), indicating that they posses functional agonistic properties. Compound **2**, the most potent compound, showed an IC_{50} of 0.85 μM for ERE activity. Therefore, compounds **1** and **2** are mainly responsible for the SERM activity of LNE. Our data suggest that compound **1** from LN08 and compound **2** from LN10 are the major contributors to the SERM activity of LNE.

To examine the biological activities of compounds **1–4** in MCF-7 breast cancer cells, we determined their effects on cell proliferation by MTT assay. Treatment with E2 increased cell proliferation, as did treatment with 10^{-9}–10^{-6} M of compounds **1–4** compared to the vehicle control (Figure 1). Compound **1** dose-dependently increased cell proliferation, and compound **3** considerably increased cell proliferation. To determine whether the isolated compounds affected nuclear ERα activities in MCF-7 cells, we assayed the mRNA of the ERα target gene *pS2*. *pS2* expression was significantly upregulated by compounds **1–4** at 10 μM; this effect was reversed by co-treatment with the ER inhibitor ICI (Figure 2), indicating that *pS2* mRNA was upregulated by compounds **1–4** via ER activation.

To further confirm the effects of these compounds on the expression of ERα, we examined ERα protein levels in MCF-7 breast cancer cells. E2 downregulates ERα mRNA and protein levels in MCF-7 cells.[20,21] ERα protein levels were downregulated by up to 20% compared to vehicle after treatment with E2 or compounds **1–3** (100 μM) (Figure 3). Compound **1** resulted in the greatest reduction in ERα protein levels. Therefore, compounds **1–3** from LNE may affect the ERα-mediated control of gene expression as functional ER ligands. These results indicated that compounds **1–3** increased ER-sensitive MCF-7 cell proliferation by acting on ERs.

Phytoestrogens are found in a variety of plants and represent a class of nonsteroidal compounds. Most of them belong to one of three classes: isoflavones, coumestans, or lignans. Reportedly, lignans, such as NDGA and triterpenes, are major components of the *Larrea* genus.[22,23] In this study, attempts were made to identify the arylnaphthalene lignans present in LNE acting as SERMs in addition to NDGA. Using bioassay-guided analysis of LNE, we identified that four different lignans had good binding affinities to both hERα and hERβ.

Table 1. Chemical structures and molecular formulas of compounds isolated from LNE.

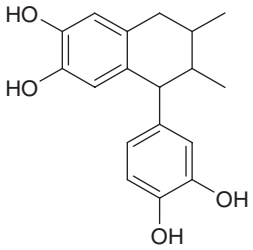
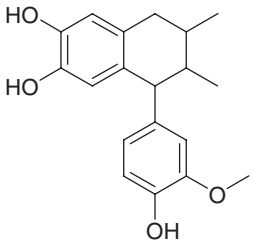
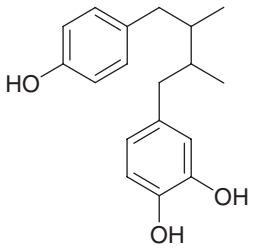
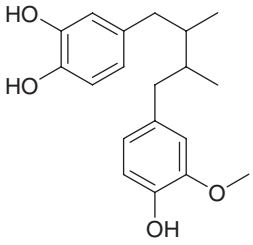
No.	Structures	Chemical names	Molecular formula (molecular weight)
1		5,6,7,8-Tetrahydro-5-(3,4-dihydroxyphenyl)-6,7-dimethyl naphthalene-2,3-diol	$C_{18}H_{20}O_4$ (300.35)
2		5,6,7,8-Tetrahydro-5-(4-hydroxy-3-methoxyphenyl)-6,7-dimethylnaphthalene-2,3-diol	$C_{19}H_{22}O_4$ (314.38)
3		4-(4-(4-Hydroxyphenyl)-2,3-dimethylbutyl) benzene-1,2-diol	$C_{18}H_{22}O_3$ (286.37)
4		4-(4-(4-Hydroxy-3-methoxyphenyl)-2,3-dimethylbutyl) benzene-1,2-diol	$C_{19}H_{24}O_4$ (316.39)

Table 2. Binding affinities for hERα and hERβ and ERE activities of compounds **1–4** isolated from LNE.

Compound	hERα		hERβ		ERE activity
	IC_{50} (μM) ± SE[a]	RBA[b] (%)	IC_{50} (μM) ± SE	RBA (%)	IC_{50} (μM) ± SE
E2	0.0012 ± 0.0003	100	0.0049 ± 0.0004	100	
1	0.11 ± 0.02	1.09	0.045 ± 0.01	10.89	13.80 ± 4.76
2	0.14 ± 0.02	0.86	0.36 ± 0.01	1.36	0.85 ± 0.12
3	8.28 ± 0.27	0.02	14.00 ± 0.38	0.04	3.90 ± 1.58
4	21.20 ± 6.11	0.01	44.00 ± 0.31	0.01	7.91 ± 4.73

[a] Values are means of three experiments; standard errors (SE) are shown in parentheses.
[b] Relative binding affinity; RBA = [$IC_{50(E2)}$/$IC_{50(compounds)}$] × 100.

Although NDGA was previously reported to possess estrogenic activity,[11] the presence of isoquaiacins in the LNE responsible for SERM properties was first demonstrated in this study. Competitive binding assays showed that compounds **1** and **2** bound to both hERα and hERβ with a high affinity. Although the RBA of compound **1** was 1% compared to E2, 0.045 μM IC_{50} of compound **1** for hERβ indicated high potency as a phytoestrogen. A lignan family nember, NDGA exhibited binding affinities for ERα and ERβ with an IC_{50} of 30 μM.[11] Our results showed that compounds **1** and **2** were 100-fold more potent than NDGA in terms of binding ERα and ERβ. Compound **1**, which comprises two catechol units

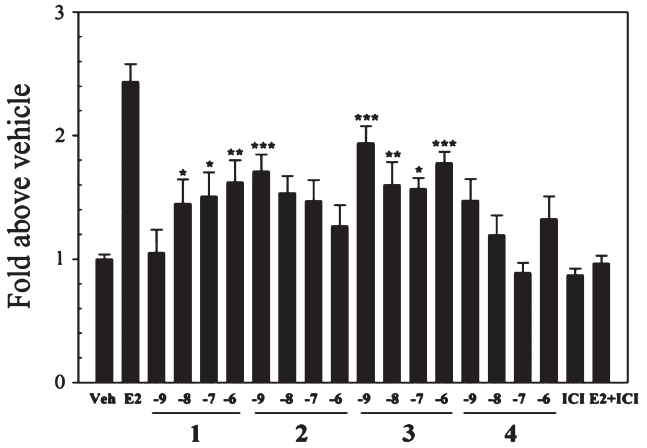


Figure 1. Effects of compounds **1–4** (10^{-9}–10^{-6} M) from LNE on MCF-7 cell proliferation. MCF-7 cells were cultured with various concentrations of compounds for 6 days. Results are expressed as fold increases above the vehicle control. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$: significantly different from the vehicle group. Values are means ± SE ($n = 3$).

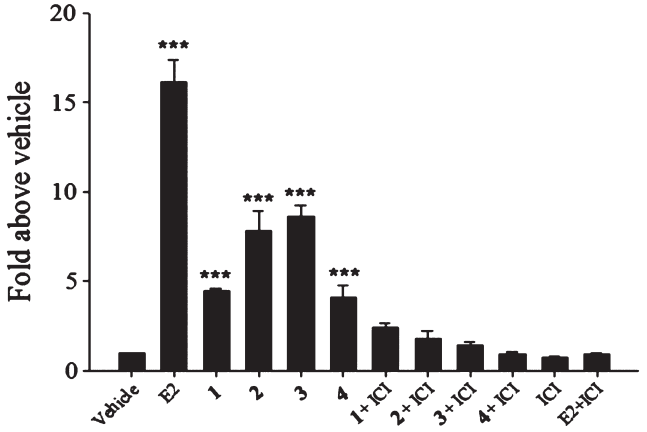


Figure 2. Effects of compounds **1–4** (10^{-5} M) on estrogen-responsive *pS2* mRNA in MCF-7 cells. Total RNA was extracted, and *pS2* and GAPDH mRNA levels were determined using real-time PCR and normalization to GAPDH expression. ***$p < 0.001$: significantly different from the vehicle group. The results are from values of means ± SE ($n = 3$).

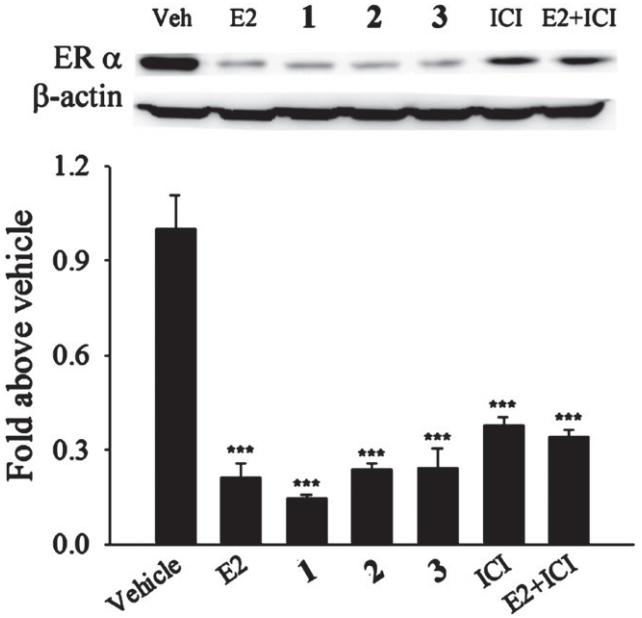


Figure 3. Changes of ER protein by compounds **1–3** (10^{-4} M) in MCF-7 cells. Detection of ERα protein (62 kDa) was done by western blotting. The results shown are representative of three independent experiments. Equal amounts of protein in each lane were confirmed by using β-actin as a loading control. ***$p < 0.001$: significantly different from the vehicle-treated group. Values are means ± SE ($n = 3$).

and a closed ring, showed the highest binding affinity to hERα and hERβ. Based on these results, the two catechols and the closed ring appear to be important structures for estrogenic activity. Compounds **1** and **2** are classified as arylnaphthalene type lignans, and include isoguaiacin or norguaiacin, whereas compounds **3** and **4** belong to bibenzyl butane type lignans. The rigidity of two phenyl groups is the most unique structural difference between the two types of lignans. Two phenyl groups in bibenzyl butane type lignans are characterized by the free rotation among the aliphatic bonds. The second structural feature among these four compounds is the presence of an *O*-methoxy group at the 3-position of the phenyl ring. These structural differences are clearly represented in competitive receptor binding assays. First, the arylnaphthalene type ligans (compounds **1** and **2**) displayed higher receptor binding affinities compared the less rigid lignans (compounds **3** and **4**) in both ERα and β binding assays, as shown in Table 2. Second, the 3-methoxy group found both in compounds **2** and **4** seems interfere the molecular interaction within the ligand-binding domain of the ERs since compounds **2** and **4** showed decreased relative binding affinities compared the compounds **1** and **3**. It has been reported that stimulatory activity on osteoblast differentiation by rigid isoguaiacin from *Machilus thunbergii* was involved with ER.[24] Our data suggest that the rigidity of the three rings or the ring-like alkane plays an important role in the receptor binding mode and that the 3-hydroxyl groups in the lignans are critical in the molecular interaction between the lignans and amino acids such as Glu, Arg, or His in the ligand-binding pocket of the ERs.[25] Furthermore, compounds **1** and **2** showed higher selectivity for hERβ compared to hERα. Preferential activities toward ERβ is suggested for alternative estrogen replacement therapy because the activation of ERα-mediated signaling pathways are attributed to increased risk of breast and endometrial cancers.[26,27] Preclinical and clinical data support a beneficial role in use of ERβ agonists to relieve hot flushes without increasing cancer risks.[28] Moreover, the estrogenic activities of these compounds were determined by measuring ERE-dependent reporter gene transcription in cells transiently transfected with a pERE-Luc plasmid. Compounds **1–4** induced ERE-dependent luciferase expression similar to E2 in MCF-7 cells. Although LNE compounds **1–4** increased ERE activity in MCF-7 cells, LNE exhibited antiproliferative effects on the

proliferation of MCF-7 cells in the presence of E2 and in the uterus.[12] Various SERM-like constituents can be disregarded because of their opposing effects, leading to both estrogenic action via classical ER signaling and a tissue-specific antiestrogenic effect. In this study, we focused on a naturally occurring SERM found in plants. These results provide insight into the medicinal properties of *L. nitida* via ERs in health and disease in women.

Conclusion

In summary, our results showed that active compounds had greater binding affinity for hERβ than hERα in terms of their effects on cell proliferation and ERE activity in MCF-7 breast cancer cells. Therefore, its active components may act as SERMs via ERs in estrogen-responsive organs. In conclusion, this study provides evidence for the phytoestrogenic properties of *L. nitida* and its active compounds as a hormonal therapeutic agents for menopausal diseases.

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Supporting Information. Additional supporting information is available in the online version of this article.

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