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Selective Estrogen Receptor Modulation by *Larrea nitida* on MCF-7 Cell Proliferation and Immature Rat Uterus

Hye-Na Ahn[1], Si-Yeon Jeong[1], Gyu-Un Bae[1], Minsun Chang[2], Dongwei Zhang[2], Xiyuan Liu[2], Yihua Pei[5], Young-Won Chin[5], Joongku Lee[3], Sei-Ryang Oh[4] and Yun Seon Song[1,*]

[1]College of Pharmacy, [2]Department of Medical and Pharmaceutical Science, College of Science, Sookmyung Women's University, Seoul 140-742, [3]College of Pharmacy, Dongguk University-Seoul, Goyang 410-820, [4]International Biological Material Research Center, Korea Research Institute of Bioscience & Biotechnology, Daejeon 305-806, [5]Natural Medicine Research Center, Korea Research Institute of Bioscience & Biotechnology, Cheongwon 363-883, Republic of Korea

Abstract

Larrea nitida is a plant that belongs to the Zygophyllaceae family and is widely used in South America to treat inflammatory diseases, tumors and menstrual pain. However, its pharmacological activity remains unclear. In this study we evaluated the property of selective estrogen receptor modulator (SERM) of *Larrea nitida* extracts (LNE) as a phytoestrogen that can mimic, modulate or disrupt the actions of endogenous estrogens, depending on the tissue and relative amount of other SERMs. To investigate the property of SERM of LNE, we performed MCF-7 cell proliferation assays, estrogen response element (ERE)-luciferase reporter gene assay, human estrogen receptor (hER) binding assays and *in vivo* uterotrophic assay. To gain insight into the active principles, we performed a bioassay-guided analysis of LNE employing solvents of various polarities and using classical column chromatography, which yielded 16 fractions (LNs). LNE showed high binding affinities for hERα and hERβ with IC_{50} values of 1.20 $\times 10^{-7}$ g/ml and 1.00$\times 10^{-7}$ g/ml, respectively. LNE induced 17β-estradiol (E2)-induced MCF-7 cell proliferation, however, it reduced the proliferation in the presence of E2. Furthermore, LNE had an atrophic effect in the uterus of immature rats through reducing the expression level of progesterone receptor (PR) proteins. LN08 and LN10 had more potent affinities for binding on hER α and β than other fractions. Our results indicate that LNE had higher binding affinities for hERβ than hERα, and showed SERM properties in MCF-7 breast cancer cells and the rat uterus. LNE may be useful for the treatment of estrogen-related conditions, such as female cancers and menopause.

Key Words: *Larrea nitida*, Lignan, Phytoestrogen, Estrogen receptor, MCF-7 cell, Uterus

INTRODUCTION

The genus *Larrea* (Zygophyllaceae) has a long history of ethnobotanical use by native people of south America. The genus comprises six species: *Larrea ameghinoi, Larrea cuneifolia, Larrea divaricata, Larrea mexicana, Larrea nitida* and *Larrea tridentate*. These plants have been used to treat a variety of illnesses, including cancer, inflammation and menstrual pain (Brent, 1999). The extract of *Larrea divaricata*, a species in the *Larrea* genus, has a biphasic effect on cell proliferation, exerting a stimulatory effect at low concentrations but inhibiting the proliferation of BW 5147 T lymphoma cells at high concentrations (Anesini *et al*., 1999). Methanolic extract of *Larrea divaricata* exhibited anti-tumor activity in the MCF-7 human breast adenocarcinoma cell line (Bongiovanni *et al*., 2008). As a folk remedy, the genus *Larrea* was not recommended for pregnant and lactating females, suggesting that the *Larrea* genus regulates hormonal functions in females. Estrogen, a major female hormone, regulates various physiological responses in many target tissues and plays important roles in the development and progression of breast and endometrial cancers (Hayashi *et al*., 2003; Bryant *et al*., 2005).

Estrogen exerts its biological effects by binding to estrogen receptors (ERs), which are present mainly in the nucleus and comprise ERα and ERβ (Hayashi and Yamaguchi, 2008). Selective ER modulators (SERMs) bind to ERs and exert agonistic actions in several target tissues, while acting as ER antagonists in others (Shang and Brown, 2002). Progesterone (P4)

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***Corresponding Author**
E-mail: yssong@sookmyung.ac.kr
Tel: +82-2-2077-7231, Fax: +82-2-710-9871

bound to the progesterone receptor (PR) is important in mammary gland and reproductive tract development, especially in the uterus and ovaries (Graham and Clarke, 1997; Mulac-Jericevic *et al*., 2003). PR has two predominant isoforms, PR-A and PR-B. PR-B mediates the proliferative effect of P4 in the mammary gland and PR-A is essential for the functional response of P4 in the uterus and ovaries (Mulac-Jericevic *et al*., 2003). Therefore, Kuhl and Stevenson suggested that the regulators of PR or ER are used for the prevention and treatment of breast cancer and postmenopausal osteoporosis (Kuhl and Stevenson, 2006). Phytoestrogens are compounds naturally found in plants that possess estrogenic properties associated with the prevention of cancer, heart disease, menopausal symptoms and osteoporosis (Bedell *et al*., 2014). The main classes of phytoestrogens in terms of chemical structure are isoflavones, prenylated flavonoids, coumestans, and lignans (Ward and Kuhnle, 2010).

Phytochemical studies have indicated that the genus *Larrea* contains lignans, flavonoids, condensed tannins, triterpene saponins and naphthoquinones with lignans being the major component (Gisvold and Thaker, 1974; Konno *et al*., 1990). Although various species of the genus *Larrea* have been studied to have therapeutic effects, pharmacological properties of *Larrea nitida* associated female hormones and their interaction with hormone receptors have not reported.

In this study, we evaluated the SERM activity of *Larrea nitida* to determine its pharmacological importance as a source of phytoestrogens. Extract of *Larrea nitida* (LNE) was investigated in terms of its ability to bind to purified hERα and hERβ, and to modulate ER-mediated gene transcription, proliferation in MCF-7 breast cancer cells, and its uterotrophic effect in rats.

MATERIALS AND METHODS

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) and 1% antibiotic-antimycotic solution. Cells were maintained in 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). 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. Stock solutions were stored at -20°C. The final solvent concentration in the culture medium did not exceed 0.5%.

Plant materials

The stems and leaves of *Larrea 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) was deposited in the herbarium of the KRIBB.

Preparation of LNE and its chromatography profile

Air-dried stems and leaves of *Larrea nitida* (52 g) were pulverized and extracted with methanol (MeOH) to yield the crude extract of *Larrea nitida* (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 sub-fractions (LN01-16). Separation of LN 08 by aforementioned HPLC method yielded nordihydroguaiaretic acid (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) to published values (Son *et al*., 2005).

Cell proliferation assay

MCF-7 cells were plated in 12-well plates at an initial density of 6×10^4 per well (Soto and Sonnenschein, 1984; Soto and Sonnenschein, 1985). Cells were allowed to attach for 24 h and the medium was then replaced with experimental medium (phenol red-free DMEM containing 5% charcoal dextran-treated FBS (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.

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% CD-FBS 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) (Catherino and Jordan, 1995). One day after transfection, various concentrations of LNE were added in the presence or absence of E2. 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 compound to control or treated recombinant full-length human estrogen receptor α (hERα) was assessed as described previously (Obourn *et al*., 1993). hERα (2088 pmol/mg estrogen binding activity, Invitrogen Corp.) was diluted to a concentration of 5 nM with a binding buffer containing 10 mM Tris (pH 7.5), 10% glycerol, 1 mM DTT and 1 mg/ml bovine serum albumin (BSA). The hERα was labeled with [^{3}H]-E2 (3 nM) in the presence or absence of 10^{-6} M unlabeled E2, various concentration of LNE or LNs fractions. For the hERβ binding assay, hERβ (4500 pmol/mg estrogen binding activity, Invitrogen Corp.) was diluted to a concentration of 10 nM with the binding buffer and labeled with [^{3}H]-E2 (10 nM) in the presence or absence of 10^{-6} M unlabeled E2, LNE or LNs. After incubation for 2 h at 27°C, the reactions were terminated by rapid filtration through glass-fiber filters (Packard

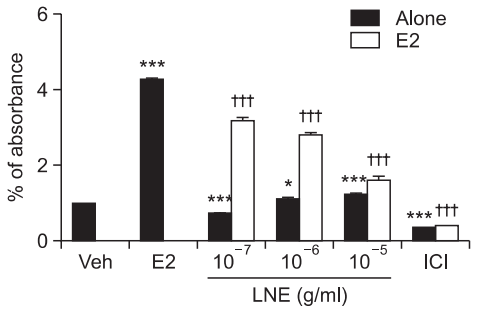

Fig. 1. Growth-stimulating effect of LNE were measured by MTT assay. MCF-7 cells (6×10^4 cells per well in 12-well plate) were incubated in phenol-red free DMEM supplemented with 10% CD-FBS. Cells were exposed to EtOH (Veh), E2 (10^{-10} M), ICI (10^{-6} M), LNE (10^{-7} to 10^{-5} g/ml), and LNE with E2 for 6 days. LNE with E2 reduced cell proliferation induced by E2. Values significantly different from vehicle group are indicated by asterisk: (***p<0.001) whereas different from the E2 group are indicated as follows: ($^{\dagger\dagger\dagger}p$<0.001). Values are mean ± SE from three separate experiments.

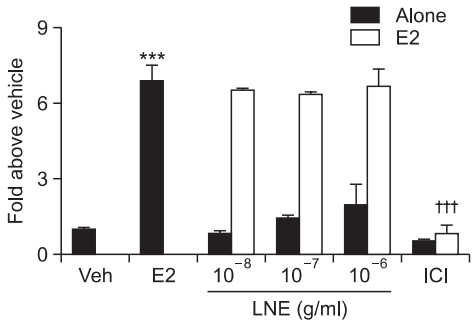

Fig. 2. ERE-regulated luciferase gene expression induced by E2 and LNE in MCF-7 cells. MCF-7 BOS cells were transiently co-transfected with plasmids containing either ERE-luciferase gene or β-galactosidase gene. Cells were treated with ethanol (EtOH) (Veh), E2 (10^{-10} M), ICI (10^{-6} M), LNE (10^{-8} to 10^{-6} g/ml), and LNE with E2 in phenol red-free DMEM with 10% cd-FBS. Luciferase activity was assayed after 24 hr treatment. LNE did not inhibit the E2-induced reporter gene activation in MCF-7 cells. Values significantly different from vehicle group are indicated by asterisk: (***p<0.001) whereas different from the E2 group are indicated as follows: ($^{\dagger\dagger\dagger}p$<0.001). Values are mean ± SE from three separate experiments.

Instrument B.V. Chemical Operations) presoaked with ice-cold 0.05% polyethylenimine using a PerkinElmer Filter Mate Harvester. The filters were washed five times with ice-cold buffer, dried, and then placed in scintillation vials containing 3 ml of Packard Ultima Gold scintillation cocktail. After shaking and overnight equilibration of the vials, the radioactivity trapped on each filter was measured using a Packard 2000CA liquid scintillation counter. Non-specific [^{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: $[(dpm_{sample}-dpm_{nonspecific})/(dpm_{control}-dpm_{nonspecific})]\times100$. Relative binding affinity (RBA) was calculated as follows: $RBA=[IC_{50\ (E2)}/IC_{50\ (LNE\ or\ LNs)}]\times100$. The final results for each treatment condition were expressed as means ± standard error (SE).

Uterotrophic assay

All animal studies were performed in accordance with the guidelines provided by the Korean Food and Drug Administration. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sookmyung Women's University. LNE was tested *in vivo* to determine its ability to induce uterine wet weight gain in rats. Immature female Sprague-Dawley rats (20-21 days old) with body weights ranging from 38-48 g were obtained from the Samtaco Animal Farm (Osan, Korea) and were housed under standard animal laboratory conditions with food and water available *ad libitum*. The light/dark cycle was 12-h light/12-h dark, with dark cycle starting at 21:00 h. Animals were randomly divided into four groups, with the group body weights within 10% of the population mean. Animals were subcutaneously injected once daily for a period of 3 days with (i) corn oil alone (vehicle control); (ii) E2 (0.003 mg/kg; positive control); or (iii) LNE (100 or 500 mg/kg). Each treatment group consisted of five animals. On day 4, the animals were weighed and euthanized (24 h following the last treatment). The uteri were quickly removed and the connective tissue was excised. The uteri were then nicked, blotted and weighed. The effects of LNE on uterus wet weight were determined by reporting absolute uterine weights and uterine wet weights normalized for the rats' respective body weights.

Statistical analysis

Data are expressed as means ± 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

Effect of LNE on MCF-7 cell proliferation

The MCF-7 cell proliferation assay evaluates the cellular response to either estrogenic or antiestrogenic compound in ER-mediated pathway. LNE was investigated for their ability to enhance the proliferation of estrogen-dependent MCF-7 breast cancer cell. E2 and ICI were used as a positive control (an ER agonist) and a negative control (an ER antagonist), respectively in estrogen-derived cell proliferation in MCF-7 cell. The cells were treated with the extracts of LNE (10^{-7}-10^{-5} g/ml), ICI (10^{-6} M), E2 (10^{-10} M), or the LNE in combination with E2 for 6 days and the proliferative effects were evaluated using MTT assay. As shown in Fig. 1, treatment of cells with LNE resulted in dose-dependent increases of cell growth in MCF-7 cells. Further investigation was conducted to test whether the cell proliferation was associated with the function of ER, the effects of co-treatment of the LNE with E2 on MCF-7 cell proliferation was examined. Interestingly, the LNE dose-dependently reduced cell growth caused by E2 (10^{-10} M) compared to E2 alone. Our investigation indicated that only LNE standalone treatment has a proliferative effect via ER, whereas LNE inhibited MCF-7 cell proliferation induced by E2. These data suggested that LNE has both estrogenic and anti-estrogenic effect in the absence or presence of E2 in MCF-7 cell.

Estrogenic effect in ERE mediated gene transcription

LNE was tested for the transcriptional activity via classical ER signaling pathway in MCF-7 cell. The cells were transfect-

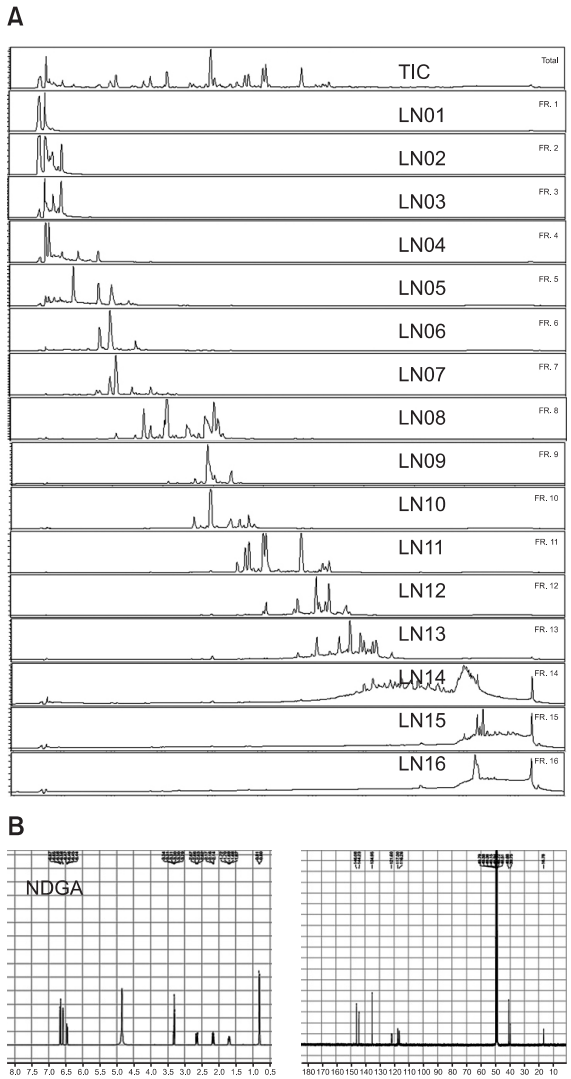

Fig. 3. Analysis of LNE, its fractions and nordihydroguaiaretic acid (NDGA). (A) HPLC chromatogram of extract of *Larrea nitida* (LNE) and its 16 fractions (LN01-LN16). (B) NMR spectra of NDGA.

ed with the plasmid containing the ERE in the promoter region of the luciferase reporter gene (pERE-Luc). Fig. 2 demonstrated the effect of LNE (10^{-8}-10^{-6} g/ml), E2 (10^{-10} M), and ICI (10^{-6} M) on luciferase activity in MCF-7 cell transfected with pERE-Luc. The results showed that E2 treatment induced 7-fold of luciferase gene expression compared to the vehicle control and LNE induced gene expression by 2-fold, which was not significant compared to E2 and much weaker than that of E2. Further investigation was conducted to test whether LNE was involved in the transcriptional activation of ERE-luciferase by E2. However, LNE did not inhibit the E2-induced luciferase in MCF-7 cell at any concentrations tested in this study. The known ER pure antagonist, ICI completely inhibited E2-induced luciferase activity, implying that luciferase induction was mediated via ER function. Our results showed that LNE possessed weak estrogenic activity in the absence of E2 in MCF-7 cells. However, a combination of LNE with E2 did not affect the E2-mediated transcriptional activity in the same cell line, which was different to what has been observed in MCF-7 cell proliferation experiment.

Fractions of LNE and chromatographic isolation of LNs fractions

To identify the bioactive constituents responsible for the SERM efficacy, first, we fractionated LNE using a reversed-phase silica column chromatography by eluting with a gradient mixture of H_2O-MeOH (90:10-0:100). Based on this separation, LNE constituents were resolved into 16 fractions (LN01-16) (Fig. 3A). Nordihydroguaiaretic acid (NDGA) was identified as one of active compounds by NMR in the LNE (Fig. 3B).

Competitive binding affinities of LNE and LNs to ERα and ERβ

LNE and LN 01-16 fractions were tested for their ability to bind ERs. The binding affinities of the LNE and LNs as hER ligands were determined its ability to displace [^{3}H]-E2 bound to the receptor from either hERα or hERβ using [^{3}H]-E2 and recombinant purified ERs. The binding affinities were expressed as RBA values with the relative binding of the E2 standard set to 100. As expected, the ER binding assay showed strong affinities for E2 with IC_{50} of 1.2×10^{-9} g/ml and 2.5×10^{-9} g/ml for the hERα and the hERβ, respectively (Table 1). LNE showed high binding affinities for the hERα and hERβ with the values of IC_{50} of 1.2×10^{-7} g/ml for hERα and 1.0×10^{-7} g/ml for hERβ, respectively. LN01-16, fractions 1–16 were also tested for their binding affinities to hERα and hERβ. Among the 16 fractions, LN08, LN09, LN10, LN11 and LN12 were active in the IC_{50} range of 0.38×10^{-6} g /ml and IC_{50} of 20.08×10^{-6} g /ml (Table 1). The IC_{50} values of LN08 for hERα and hERβ were 1.1×10^{-6} and 5.3×10^{-7} g/ml, respectively. LN10 also showed high binding affinity to hERα and hERβ, with IC_{50} values of 3.8×10^{-7} and 3.18×10^{-6} g/ml, respectively. It showed that LN08 and LN10 had more potent affinities for binding on both hER α and β than other fractions. RBA showed that the affinities of LNE and LN08 have selectivity for hERβ rather than hERα. Consequently, we suggest that LNE may have the potential to preferentially target hERβ over hERα in cells or in animals.

Atrophic effect on uterine of immature rats

An *in vivo* rodent uterine bioassay was developed in which an estrogen-induced uterotrophic response was estimated as an increase in uterine tissue mass in immature rodents. LNE was tested *in vivo* to determine its ability to induce uterine weight gain in rats. There was no significant difference of initial body weights among the rats in each group. The changes of body weight, calculated by subtraction of the body weight at 1 day before administration from that on the day after the last injection, are shown in Table 2. The positive agent E2 (0.003 mg/kg) increased uterine wet weights when rats were administered with E2 via the subcutaneous injection route. As shown in Table. 2, uterine wet weight was increased 1.8-fold following treatment with 0.003 mg/kg of E2 compared to vehicle group. However, the uteri of rats treated with 100 and 500 mg/kg of LNE were significantly atrophied compared to those of vehicle control rats (Table 2). In addition, rats administered with LNE (100 and 500 mg/kg) showed body weight gains of a few grams compared to control group and E2 treatment group.

Table 1. Ligand-binding affinities of LNE and LNs for hERα and hERβ

Treatment	hERα		hERβ	
	IC_{50} (μg/ml)	RBA (%)	IC_{50} (μg/ml)	RBA (%)
E2	0.0012 ± 0.0003	100	0.0025 ± 0.0004	100
Genistein	1.36 ± 0.10	0.09	0.055 ± 0.0083	4.55
LNE	0.12 ± 0.02	1.00	0.10 ± 0.01	2.50
LN08	1.10 ± 0.01	0.11	0.53 ± 0.01	0.47
LN09	9.11 ± 2.27	0.01	6.60 ± 0.38	0.04
LN10	0.38 ± 0.02	0.32	3.18 ± 0.31	0.08
LN11	20.08 ± 1.04	0.01	8.05 ± 0.69	0.03
LN12	9.52 ± 1.58	0.01	7.54 ± 1.60	0.03

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

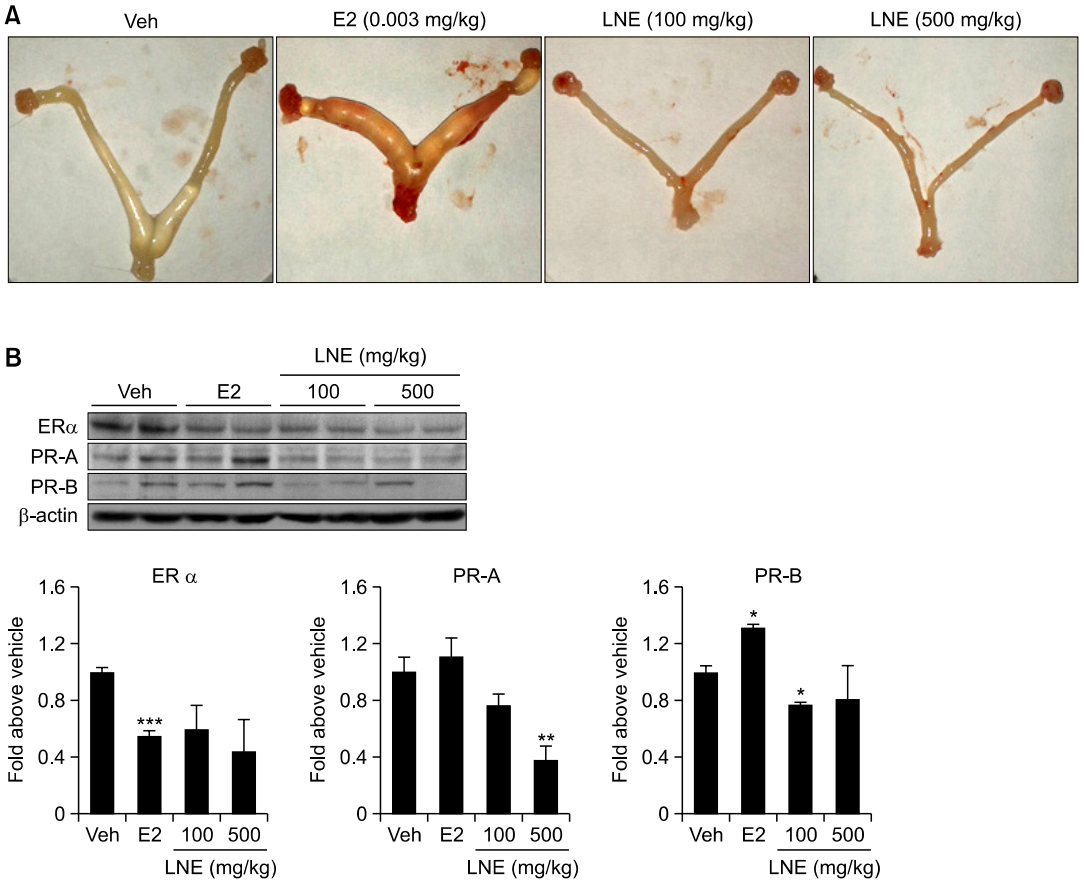

Fig. 4. LNE reduced wet weight and protein expression of estrogen responsive genes in immature rats. Each compound was administered subcutaneously at the indicated concentration (100 mg and 500 mg/kg/day) for 3 days. (A) Administration of LNE reduced the uterine wet weights in immature rat uterotrophic assay. (B) Whole cell proteins from uterus were extracted and analyzed for the expression of ERα, PR-A, and PR-B by Western blot analysis. ERα, PR-A and PR-B protein levels were significantly decreased in the LNE-treated uterus. Equal loading of protein in each lane was confirmed by β-actin as a loading control. Values significantly different from vehicle group are indicated by asterisk: (***p<0.001, **p<0.01, *p<0.05).

Anti-proliferative effect of LNE

LNE reduced uterine weights in immature uterotrophic assay and showed weight gains after treatment with the LNE for 3 days (Fig. 4A, Table 2). We hypothesized that this weight gain effect of LNE is attributed to its properties of progesterone. Therefore, we examined the effects of LNE on expression of PR-A, PR-B, and ERα proteins in rats. Steady-state protein levels were measured by Western blot analysis, as

Table 2. Effects of LNE in immature rat uterotrophic assay

Treatment	Dose (mg/kg)	Body weight (g)	Uterine wet weight (mg)	Uterine wet weight (mg)/Body weight (g) ratio
Veh		57 ± 1.2	256 ± 32	4.44 ± 0.64
E2	0.003	54 ± 1.1	446 ± 33	8.16 ± 0.38***
LNE	100	59 ± 1.0	97 ± 3	1.64 ± 0.05***
	500	61 ± 0.4	101 ± 2	1.64 ± 0.03***

Animals were treated subcutaneously every other day with LNE at doses of 100 and 500 mg/kg body weight, 17β-ES at 3 μg/kg body weight, or vehicle (corn oil) alone. The effect of uterine wet weight data are shown as the mean ± SE (n=5, each). Values significantly different from vehicle group are indicated by asterisk: (***p<0.001).

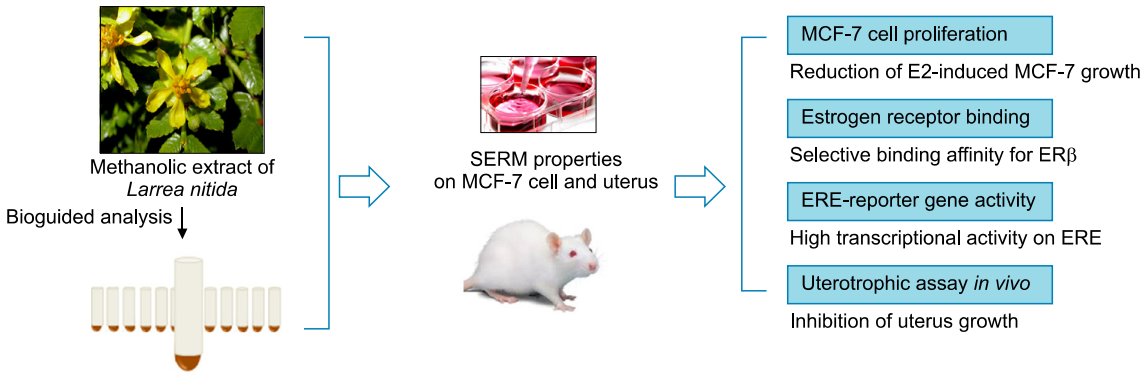

Fig. 5. LNE and its sub-fractions showed SERM activities on ER binding, ERE-reporter activity, and rat uterus.

indicated in Fig. 4B. ERα protein levels in the uterine tissues were down regulated after treatments with 0.003 mg/kg of E2. It is known that E2 downregulates ERα protein levels in MCF-7 cells, which is consistent with our result in uterus (Gierthy *et al*., 1996; Park *et al*., 2009). The 100 or 500 mg/kg of LNE significantly reduced the expression of ERα, PR-A and PR-B, compared with that of the E2-treated group in uterus (Fig. 4B). These data suggested that LNE may interact with the ER as well as modulate the expression levels of ER and PR.

DISCUSSION

The lives of women suffered from breast cancer have probably been saved because of the anti-proliferative effects of tamoxifen as a representative SERM on mammary epithelial cells. However use of SERM is limited because of undesirable tissue-specific effects such as thromboembolic events, strokes, and uterine cancer (Wysowski *et al*., 2002). Therefore phytoestrogens (plant SERMs) are gaining importance for the relief of acute women's diseases due to their therapeutic efficacy and low risk of developing potential side effects.

Larrea plant has a long history of use with the Native Americans and European settlers in the south-western deserts of the United States. It has been used for menstrual pain, however lactating women were not recommended to take products of *Larrea tridentate*, implying that the genus *Larrea* regulate women's hormonal actions. Although *Larrea nitida* has been used in South America as a traditional medicine for the women, therapeutic effects of *Larrea tridentate* on women's diseases and its mechanism of action have yet not been investigated.

The objective of our study was to investigate the potential estrogenic activity of LNE in breast cancer cell and uterine tissue, which may lead to development of SERM remedy. LNE induced cellular proliferation in MCF-7 cells in a dose-dependent fashion (Fig. 1). However, LNE inhibited E2-induced proliferation in the same cell line. Our data imply that LNE has properties of a partial agonist and mixed antagonist to ER depending on the level of endogenous estrogen. Estrogenic activity was also detected by measuring the activity of ERE-dependent reporter gene transcription in cells transiently transfected with pERE-Luc plasmid. LNE induced gene expression of ERE-dependent luciferase in MCF-7 cells, however, E2-induced gene activation was not affected by co-treatment of LNE (Fig. 2). The inconsistency in inhibitory effects of LNE on the E2-induced cell proliferation and ERE-dependent reporter gene expression implies that the molecular mechanisms by which MCF-7 cell proliferation may be mediated by alternative ER-mediated signaling pathways in addition to an event including classical ER-ERE pathway (Smith and O'Malley, 2004). Furthermore, it is possible that LNE contains various phytoestrogen chemicals, some of which are responsible for ER agonist actions and others for ER antagonistic function leading to both estrogenic action in classical ER signaling pathway and antiestrogenic action on E2-induced MCF-7 cell proliferation. Thus, it seems worth to isolate and identify individual phytoestrogenic compounds from LNE, which would serve as the direct ER agonist or the lead to develop a better SERM.

Phytochemical studies that were carried out on *Larrea tridentate* isolated and identified lignans such as NDGA and triterpenes (Abou-Gazar *et al*., 2004; Jitsuno and Mimaki, 2010).

NDGA found in the *Larrea tridentate* had binding affinities for ERα and β with IC_{50} of 30 μM (Fujimoto *et al*., 2004).

To trace the bioactive constituents responsible for the SERM efficacy of LNE, chromatographic analysis and NMR were performed (Fig. 3). LNE constituents were resolved into 16 fractions that were tested for their abilities to bind both ER α and β. Among sixteen fractions, LN08 and LN10 had more potent affinities for binding on hER α and β than other fractions, which would guide to next isolation of single compounds and their structural elucidation (Table 1). In the present study, we suggest that NDGA is partially responsible for SERM activity of LNE.

LNE and LN08 possessed higher binding affinity to hERβ than hERα. In human breast cancers, ERα is expressed as approximately 70% of breast tumor, but ERβ expression is lost during breast cancer progression, most likely due to promoter methylation (Skliris *et al*., 2008). Furthermore, clinical studies suggested that the signaling of ERβ in breast tumors increases the effectiveness of tamoxifen therapy (Honma *et al*., 2008). It is expected that higher binding affinity toward hERβ than hERα of LNE and LN8 is of clinical value in breast cancer therapy.

Furthermore, LNE had uterine atrophic effect, even at 100 or 500 mg/kg, suggesting that LNE possesses a SERM-like effect via action of ER antagonist in rat uterus (Table 2). The protein expression levels of PR-A and PR-B were down regulated after 3 days treatment with LNE compared with the vehicle (Fig. 4). PR-A has been shown to be a dominant inhibitor of the transcriptional activity of PR-B (Wen *et al*., 1994; Giangrande and McDonnell, 1999). It was reported that PR-A regulates the activity of a number of nuclear receptors, including the ERs and glucocorticoid receptor (Wen *et al*., 1994). We showed that the LNE reduced the PR expression in the uterus of rats, suggesting that the modulation of PR expression and thus function by LNE may be responsible for uterine atrophy and weight gain observed in immature rats treated with LNE.

Our data indicated that selectively targeting ERβ and PR by LNE may function as a potential suppressor of women's cancer that promotes differentiation and inhibits ERα-mediated proliferation. Since the first endocrine agent tamoxifen was described in 1986, tamoxifen as the standard endocrine therapy has been shown to reduce ER-positive breast cancer. However more recent studies are suggesting the positive roles of naturally occurring SERM such as a phytoestrogen as an alternative therapeutic option to circumvent increased risks of tamoxifen. Results from the studies of naturally-occurring SERM both in cell culture and in animals have provided insights into new dimension of the complex nature of ER action in health and disease.

In summary, our results showed that LNE and LN08 had selective binding affinity to hERβ rather than hERα and treatment of LNE reduced the cell proliferation in the presence of endogenous estrogen. LNE had uterine atrophic effects in immature rats through reducing the expression of ER/PR proteins (Fig. 5). It suggested that LNE and its bioactive principles may act as SERMs or SPRMs via ER and/or PR in female hormone-responsive organs.

In conclusion, our present study provided the first evidence for phytoestrogenic properties of LNE and its potential application as a hormonal therapy for women's disease. Further *in vitro* and *in vivo* studies and characterization of the active components of LNE will shed light on its health-promoting effects and suitability in hormone-dependent women's disease.

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