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Estrogenic activity of freeze-dried silkworm extracts through the activation of estrogen receptors in MCF-7 cells

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Abstract

Silkworm is an insect that feeds on mulberry leaves only, and silkworm powder has been reported to have antioxidant, hypoglycemic, and anticholesterol activities. In this study, we measured the content of 1-deoxynojirimycin (1-DNJ) in 24 different extracts from silkworm cocoons and also investigated the estrogenic activities of these extracts and their effects on the activation of intracellular signaling pathways in MCF-7 breast cancer cells. Among the 24 silkworm extracts, relatively high 1-DNJ content and estrogenic activity were shown by Extract 11 (30E3H20-1), which was prepared by a single extraction of silkworm powder with 20 times the volume of 30% ethanol against each powder weight for 3 h. The estrogen receptor activity of this extract was confirmed based on its promoting effects on the phosphorylation of the estrogen receptor α (ERα) and mRNA expression of the *ESR1* and *ESR2* genes. In addition, treatment with Extract 11 (30E3H20-1) increased the phosphorylation of AKT, p38, and JNK, which are downstream proteins of ERα. Based on our findings, a silkworm extract could be developed as a natural estrogen supplement in the future.

Keywords: Silkworm, 1-Deoxynojirimycin, MCF-7 breast cancer cells, Estrogen

Introduction

Silkworm (*Bombyx mori* L.) is an insect that undergoes complete metamorphosis through four stages: egg, silkworm, pupa, and moth; it overwinters as eggs. Silkworms are known to feed solely on mulberry (*Morus alba* L.) leaves [1]. Mulberry leaves contain large amounts of bioactive substances, such as flavones, plant sterols, and triterpenes [2, 3]. In particular, the γ-aminobutyric acid content in mulberry leaves is tenfold higher than that in green tea leaves, resulting in excellent blood pressure-lowering and antioxidant properties of mulberry leaves [2–5]. Recently, silkworms and their byproducts have been developed as edible, health, and medicinal materials in South Korea. Silkworm powder has been found to effectively lower blood sugar levels and has been approved since 2009 as a health supplement or functional ingredient to help control blood glucose level, since 2009 [4].

Recently, Ryu et al. and Yang et al. demonstrated that aqueous extracts of silkworm (*Bombyx mori*) cocoon had a binding affinity to estrogen receptors in cultured breast cancer cells and estrogenic activity using ovariectomized rats [6, 7]. Estrogen, a female hormone, is involved in important metabolic processes and regulates energy balance. Estrogen levels start to decrease by approximately 1% per year in women after the age of 30, which causes various menopausal symptoms, such as increased body fat, skin aging, decreased bone density, increased visceral fat, and depression [8–11]. The biological functions of estrogen are mediated in vivo via its binding to estrogen receptors (ERs), and thus, the expression and activity of these receptors are important for the function of female hormones [12]. Phytoestrogens are plant-derived compounds that are found in stems, roots, flowers, and seeds

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and are similar in function and structure to the female hormone estrogen. Phytoestrogens include isoflavones from beans, coumestan from clover, and lignans from flax seeds [13]. Phytoestrogens have been shown to be able to replace estrogen in postmenopausal women, playing an important role in preventing osteoporosis, which is caused by estrogen deficiency, preventing breast cancer, and alleviating menopausal symptoms [14, 15]. As mentioned above, substances derived from natural products with estrogenic activity can be candidate substances for treating various menopausal diseases. Therefore, we focused on the silkworm, which has been reported to have estrogen activity in water extracts, but the difference in estrogen activity between various extraction methods is unknown. In this study, we prepared 24 different extracts of silkworm powder and analyzed their estrogenic activities using MCF-7 cells. Subsequently, the mechanism of estrogenic activity was investigated using an extract with one of the highest activities.

Materials and methods

Preparation of silkworm extracts

Freeze-dried powder of 3rd-day 5th instar silkworm cocoons was purchased from a sericulture farmhouse in Geongbok Yechoen, Korea (May 2021) and stored at − 80 °C for subsequent use. Silkworm powder (15 g) was extracted with 10 or 20 volumes of water or ethanol (10% or 30% in water) using a reflux extractor. Extraction was performed once or twice for 3 or 5 h using each solvent. Consequently, we obtained 24 different silkworm extracts, which were dried using a freeze dryer.

Analysis of the 1-DNJ content

Quantitative analysis of 1-DNJ in the 24 different silkworm extracts was performed using high-performance liquid chromatography (HPLC). HPLC analysis was performed using Waters 1525 Binary HPLC and Waters 2424 ELSD, and a YMC Pack Pro C_{18} reverse-phase column with a gradient elution using acetonitrile (ACN) and water containing 0.5% acetic acid. An evaporative light scattering detector (ELSD) was set to 90 °C with N_2 gas at 40 psi. The detailed HPLC conditions are shown in Tables 1 and 2.

Cell culture

The ER-positive human breast cancer cell line (MCF-7) was purchased from American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Cellgro, Manassas, VA, USA) containing 10% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Table 1 HPLC analytical conditions for the determination of 1-DNJ in silkworm extracts

Parameter	Analytical condition
Equipment	Waters 1525 binary HPLC MA 01757 (USA) Waters 2424 ELSD MA 01757 (USA)
Column	YMC pack pro C_{18} column (25 cm × 4.6 mm, 5 μm)
Oven temperature	30 °C
Injection volume	10 μL
Mobile phase	Gradient (see Table 2)
Flow rate	1.1 mL/min
ELSD	90 °C; N_2 gas, 40 psi

Table 2 HPLC mobile phase gradient conditions

Time (min)	Mobile phase	
	A (0.5% acetic acid in water, %)	B (ACN, %)
0	97	3
10	97	3
15	60	40
20	0	100
25	0	100
30	97	3
40	97	3

Measurement of estrogenic activity

The E-screen assay was performed according to a method described by Soto et al. [13], with substantial modifications. Briefly, MCF-7 cells at a density of 1×10^4 cells/100 μL in RPMI-1640 medium supplemented with 10% FBS were seeded onto 48-well plates. After incubation for 24 h, the cells were treated with various concentrations (25, 50, and 100 μg/mL) of silkworm extracts in phenol red-free RPMI-1640 medium supplemented with 5% charcoal–dextran-stripped human serum. To test for antagonistic effect, the ER antagonist ICI 182,780 (ICI) was added to test samples. After incubation for 144 h, 10% EZ-Cytox assay reagent (Daeil Lab Service Co., Seoul, Korea) was added to the medium, followed by incubation for 1 h. The absorbance was measured at 450 nm using a microplate reader (PowerWave XS; Bio-Tek Instruments) to estimate cell proliferation. 17β-Estradiol was used as the positive control.

Immunoblotting

MCF-7 cells (4×10^5) were inoculated into a 6 cm cell culture dish, and on the next day, the medium was replaced with RPMI-1640 medium containing 10% dextran-coated charcoal-stripped FBS. Silkworm Extract 11 (30E3H20-1) was added at concentrations of 12, 25, and 50 μg/mL, and the cells were cultured for 96 h. After removing the culture supernatant and washing cells twice with phosphate-buffered saline, protein was extracted using RIPA buffer (Rockland, USA). The extracted proteins were mixed with sodium dodecyl sulfate (SDS) sample buffer and then separated by SDS–polyacrylamide gel electrophoresis (Mini-PROTEAN® TGX™ precast gels; Bio-Rad). After transfer onto a polyvinylidene difluoride membrane (Millipore, USA), relative expression of target proteins was detected using antibodies against phospho-ER alpha (p-ERα), ERα, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Real-time quantitative polymerase chain reaction (RT-qPCR)

MCF-7 cells (4×10^5) were inoculated into a 6 cm cell culture dish, and on the next day, the medium was replaced with RPMI-1640 medium containing 10% dextran-coated charcoal-stripped FBS. Silkworm Extract 11 (30E3H20-1) was added at various concentrations, and the cells were cultured for 96 h. RNA was extracted using an RNA extraction kit (Qiagen, Germany), and cDNA was synthesized from the RNA using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific). The mRNA expression levels of the ERα- and ERβ-encoding genes (*ESR1* and ESR2, respectively) were measured using an RT-PCR instrument (QuantStudio 3) and TaqMan gene expression primer kits (Hs01046816_m1, Hs01100353_m1, and Hs00607062_gH).

Statistical analysis

The results are expressed as the mean ± standard deviation of triplicate experiments. The results were statistically analyzed using the Mann–Whitney *U*-test using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), and $P < 0.05$ was considered statistically significant.

Results

Content of 1-DNJ in silkworm extracts

A total of 24 different extracts were prepared from the silkworm powder, and the 1-DNJ content was analyzed in each extract using HPLC-ELSD. As shown in Table 3, a relatively high 1-DNJ content (103.00 ± 5.88 μg/mg) was confirmed in Extract 11 (30E3H20-1), which was prepared using a one-time extraction with 20 volumes of 30% ethanol for 3 h, as well as in Extract 21 (30E5H10-1), which was prepared using a one-time extraction with 10 volumes of 30% ethanol for 5 h. The HPLC chromatograms of silkworm Extracts 11 and 21 are shown in Fig. 1.

Table 3 Content of 1-DNJ in the 24 silkworm extracts

Extract number	Extract code	Extraction conditions (solvent, volume, and time)	1-DNJ (μg/mg)
1	W3H10-1	Water (× 10), 3 h, 1 time	69.22 ± 8.86
2	W3H10-2	Water (× 10), 3 h, 2 times	90.35 ± 3.58
3	W3H20-1	Water (× 20), 3 h, 1 time	80.81 ± 7.98
4	W3H20-2	Water (× 20), 3 h, 2 times	86.35 ± 9.84
5	10E3H10-1	10% Ethanol (× 10), 3 h, 1 time	99.68 ± 8.11
6	10E3H10-2	10% Ethanol (× 10), 3 h, 2 times	94.14 ± 13.57
7	10E3H20-1	10% Ethanol (× 20), 3 h, 1 time	93.76 ± 8.17
8	10E3H20-2	10% Ethanol (× 20), 3 h, 2 times	91.96 ± 13.59
9	30E3H10-1	30% Ethanol (× 10), 3 h, 1 time	99.19 ± 13.40
10	30E3H10-2	30% Ethanol (× 10), 3 h, 2 times	104.46 ± 10.89
11	30E3H20-1	30% Ethanol (× 20), 3 h, 1 time	103.00 ± 5.88
12	30E3H20-2	30% Ethanol (× 20), 3 h, 2 times	94.87 ± 12.02
13	W5H10-1	Water (× 10), 5 h, 1 time	92.94 ± 12.55
14	W5H10-2	Water (× 10), 5 h, 2 times	81.40 ± 12.64
15	W5H20-1	Water (× 20), 5 h, 1 time	92.67 ± 11.91
16	W5H20-2	Water (× 20), 5 h, 2 times	65.53 ± 8.38
17	10E5H10-1	10% Ethanol (× 10), 5 h, 1 time	97.87 ± 7.68
18	10E5H10-2	10% Ethanol (× 10), 5 h, 2 times	71.35 ± 9.85
19	10E5H20-1	10% Ethanol (× 20), 5 h, 1 time	75.73 ± 6.84
20	10E5H20-2	10% Ethanol (× 20), 5 h, 2 times	86.33 ± 4.84
21	30E5H10-1	30% Ethanol (× 10), 5 h, 1 time	102.06 ± 5.86
22	30E5H10-2	30% Ethanol (× 10), 5 h, 2 times	95.01 ± 7.73
23	30E5H20-1	30% Ethanol (× 20), 5 h, 1 time	98.24 ± 7.68
24	30E5H20-2	30% Ethanol (× 20), 5 h, 2 times	100.36 ± 7.12

Estrogenic effects of silkworm extracts on MCF-7 cells

Estrogenic effects of the 24 silkworm extracts were evaluated using the ER-positive MCF-7 human breast cancer cell line and a modified E-screen assay [13]. MCF-7 cells were treated with each extract at concentrations of 25, 50, and 100 μg/mL for 144 h. As shown in Fig. 2, 17β-estradiol (positive control) strongly promoted

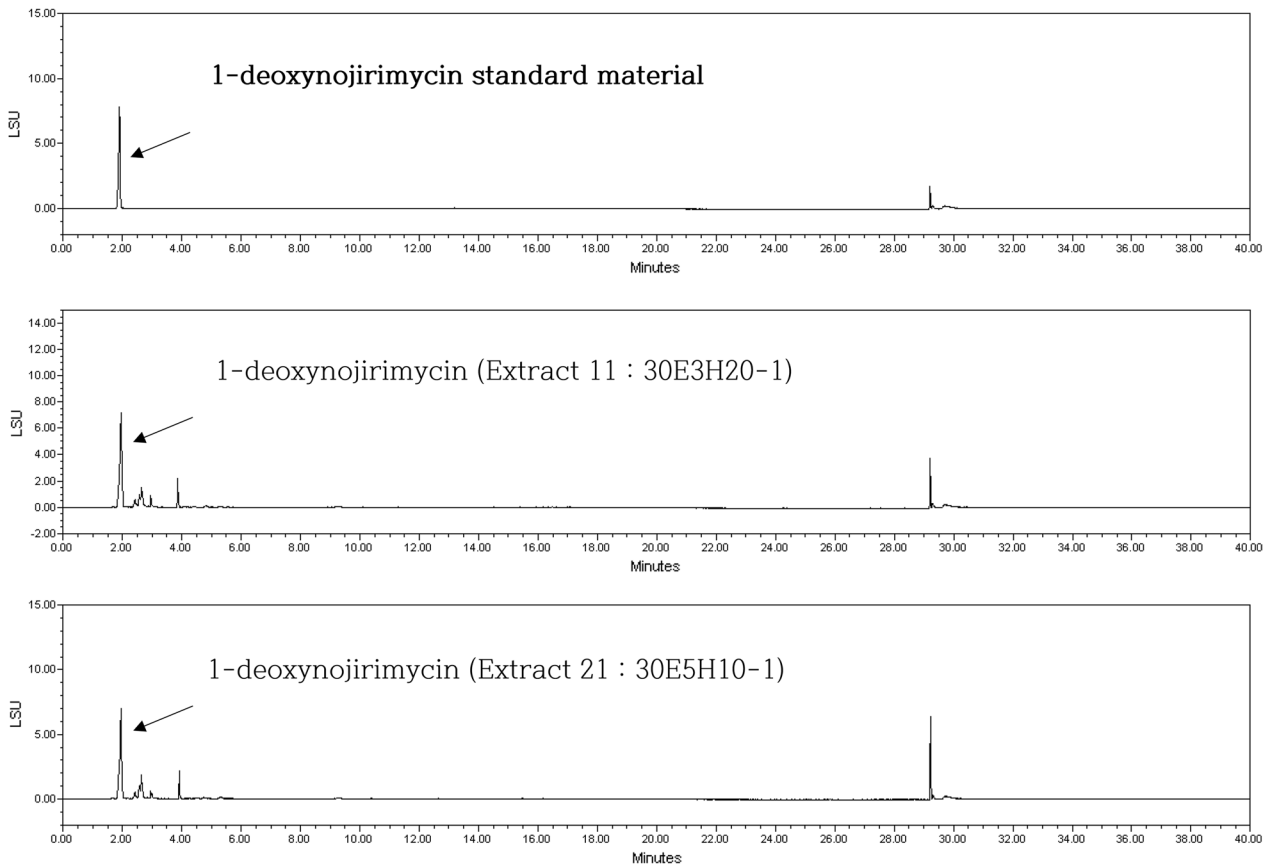

Fig. 1 HPLC chromatograms of 1-deoxynojirimycin standard material, silkworm Extract 11 and Extract 21

MCF-7 cell proliferation in a concentration-dependent manner at 0.1, 1, 10, and 100 μg/mL (120.2 ± 1.2%, 127.8 ± 1.9, 10%, 137.0 ± 2.5%, and 135.1 ± 1.7%, respectively). This effect was completely prevented by co-treatment with ICI, an estrogen receptor antagonist (Fig. 2), indicating that the significant 17β-estradiol-induced increase in cell proliferation was mediated via ER activation. Each of the 24 silkworm extracts significantly increased MCF-7 cell proliferation, in a concentration-dependent manner, compared with that in the control (Fig. 2). When cells were co-treated with ICI, the increases in cell proliferation with Extracts 10, 11, 12, 15, 19, 21, and 24 were attenuated, suggesting that these extracts had significant estrogenic effects. In particular, Extract 11 (30E3H20-1) greatly increased MCF-7 cell proliferation (50 μg/mL, 128.6 ± 2.4%; 100 μg/mL, 139.0 ± 1.4%); similar results were obtained with Extract 21 (30E5H10-1), and these effects were significantly suppressed by co-treatment with ICI. These results showed that Extracts 11 (30E3H20-1) and 21 (30E5H10-1) had some of the highest estrogenic activities among the 24 extracts; however, considering the 1-DNJ content and process of extraction, Extract 11 (30E3H20-1) was chosen for subsequent research.

ER activation in MCF-7 cells by Extract 11 (30E3H20-1)

ERα has been reported to be a ligand-dependent nuclear hormone receptor transcription factor. After ERα binds its ligand 17β-estradiol, the receptor binds with high-affinity estrogen response elements [16, 17]. Several ERα ligands, including 17β-estradiol, promote phosphorylation of serine 118 in ERα [18]. Thus, to investigate the activation of ERα by Extract 11 (30E3H20-1), we performed a western blotting analysis. As shown in Fig. 3A, Extract 11 (30E3H20-1) increased ERα phosphorylation in a concentration-dependent manner. The density of each phosphorylated band was normalized to that of the total protein, and the results are displayed in Fig. 3B. These results indicate that treatment with Extract 11 (30E3H20-1) triggers phosphorylation of ERα at the serine residue.

Phosphorylation of p38, JNK, AKT, and ERK in MCF-7 cells treated with Extract 11 (30E3H20-1)

The MCF-7 breast cancer cell line expresses high levels of estrogen receptors. It is known that estrogen and estrogenic substances induce the activation of downstream signaling proteins of ERs, such as AKT and mitogen-activated protein kinases (MAPKs). Phosphorylation of AKT increased upon treatment with Extract 11 (30E3H20-1) in a concentration-dependent manner (Fig. 4A). In particular, phosphorylation of AKT increased by 139% compared with that in the control group when MCF-7 cells were treated with Extract 11 (30E3H20-1) at a concentration of 50 μg/mL. In addition, phosphorylation of the MAPK proteins p38 and JNK increased in a concentration-dependent manner by Extract 11 (30E3H20-1) treatment. This extract also slightly affected ERK phosphorylation. The density of each phosphorylated band was normalized to that of the total protein, and the results are displayed in Fig. 4B. These results indicate that Extract 11 activates the intracellular signaling pathways and may be involved in the regulation of estrogenic activity in MCF-7 cells.

Expression of ESR1 and ESR2 in MCF-7 cells treated with Extract 11 (30E3H20-1)

An ER is a receptor protein that induces cell responses to estrogen signaling [17]. Currently, two ERs have been identified, ERα and ERβ. It is known that beta receptors

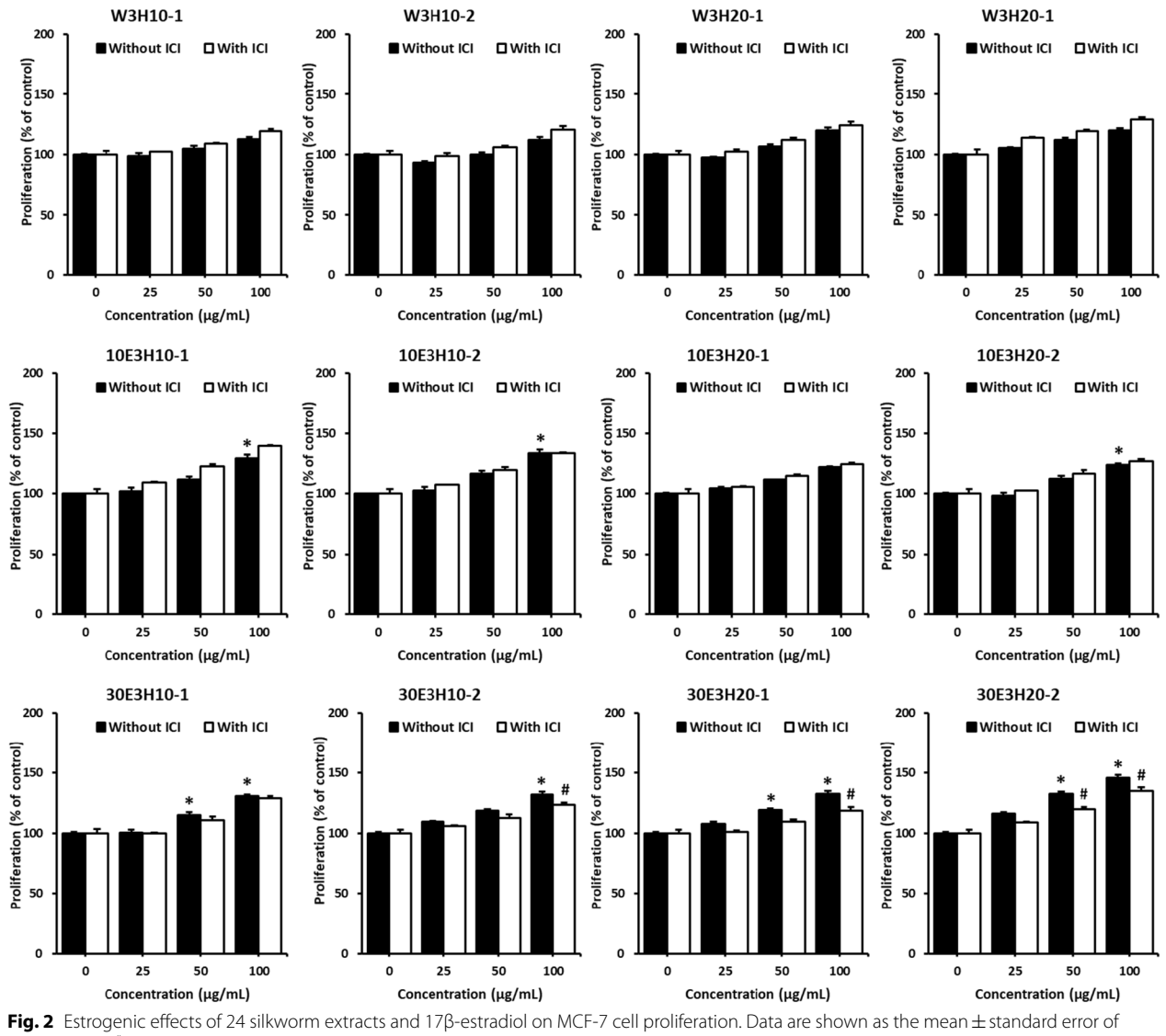

Fig. 2 Estrogenic effects of 24 silkworm extracts and 17β-estradiol on MCF-7 cell proliferation. Data are shown as the mean ± standard error of mean (n = 2). $^{\#}P < 0.05$ compared to the untreated group and $^{*}P < 0.05$ compared to each extract plus ICI (Mann–Whitney *U*-test)

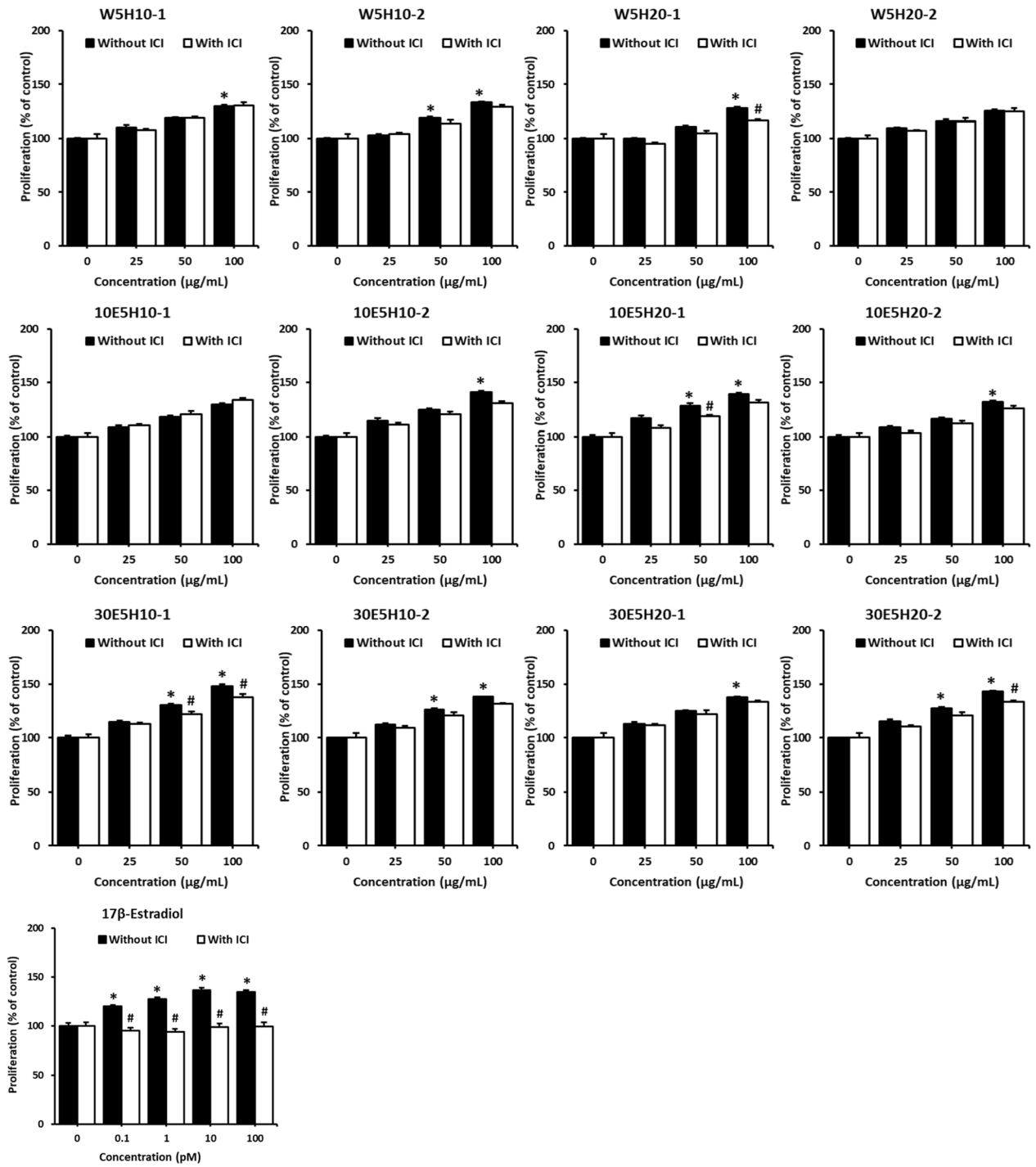

Fig. 2 continued

are present in the bone and blood vessel cells, which do not have alpha receptors, whereas organ cells have many alpha receptors but no beta receptors. Since the main symptoms of menopause are due to a decrease in estrogen concentration in the body, the reduced interaction between estrogen and ERs affects estrogen-responsive gene transcription. *ESR1* is an estrogen-responsive gene whose expression is increased by estrogen and is

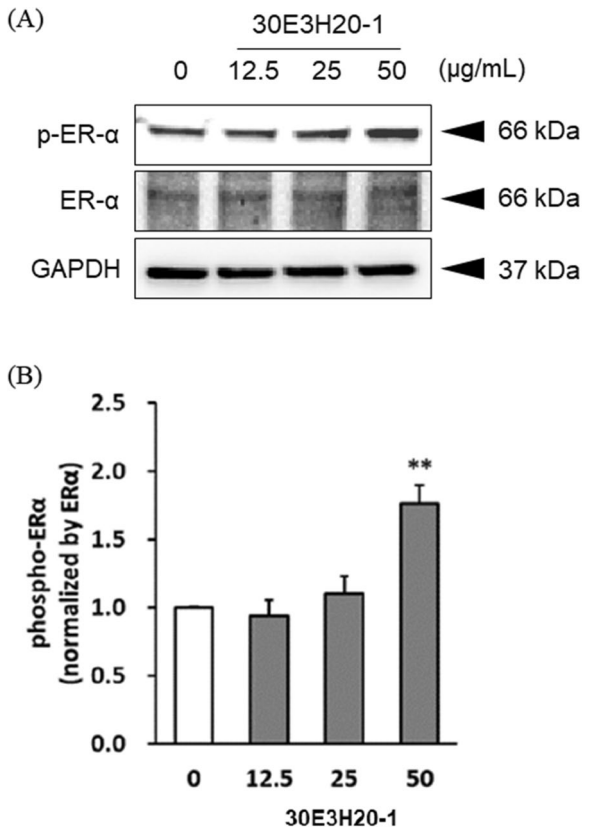

Fig. 3 Effect of Extract 11 (30E3H20-1) on the phosphorylation of ERα in MCF-7 cells. **A** Expression of p-ERα, ERα, and GAPDH was assessed by western blotting. **B** Protein band intensities were quantified using the ImageJ software and normalized to those of the indicated proteins. Data are presented as the mean ± standard deviation (n = 3). **P < 0.001 compared to the control

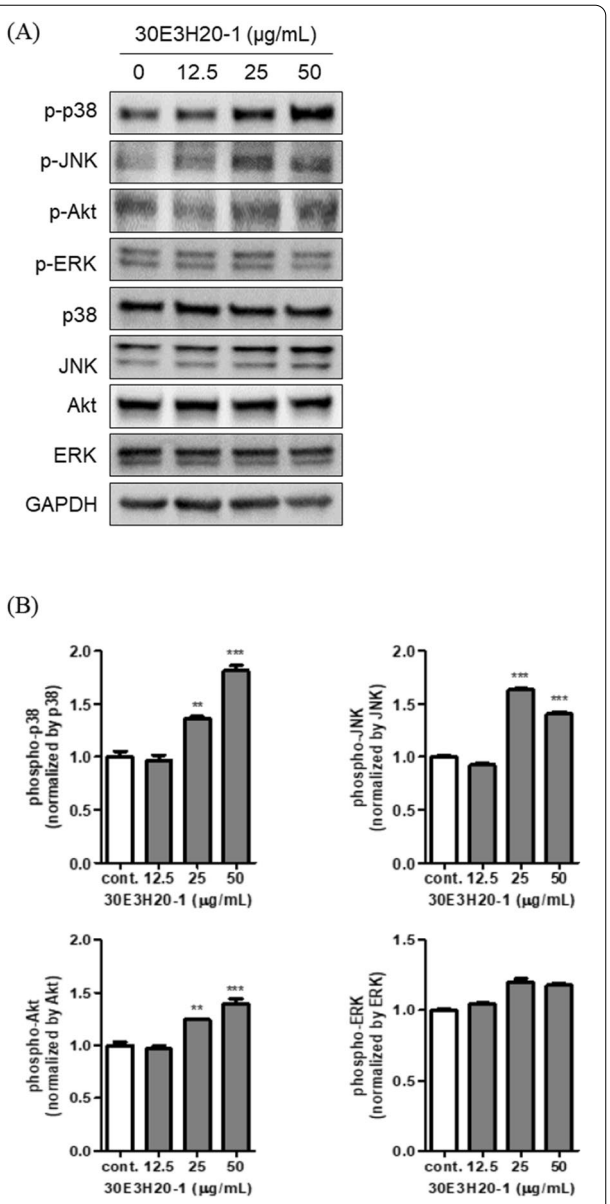

Fig. 4 Phosphorylation of p38, JNK, AKT, and ERK in MCF-7 cells treated with Extract 11 (30E3H20-1). **A** Levels of total and phosphorylated p38, JNK, AKT, and ERK. **B** Protein band intensities were quantified using the ImageJ software and normalized to those of the indicated proteins. Data are presented as the mean ± standard deviation (n = 3). **P < 0.001 and ***P < 0.0001 compared to the control

an indicator of the sensitivity to estrogen analogs. Treatment of MCF-7 cells with Extract 11 (30E3H20-1) for 96 h significantly increased the mRNA expression levels of *ESR1* and *ESR2* (Fig. 5).

Conclusions

Hormone replacement therapy (HRT), in which estrogen is artificially administered, is used to treat menopausal symptoms in women [19]. Although menopausal symptoms are effectively improved, studies have shown that estrogen administration increases the incidence of uterine or breast cancer [20]. Naturally derived phytoestrogens are therefore emerging as a possible safer alternative to estrogen in HRT [21, 22]. In this study, different silkworm cocoon extracts were prepared, and their chemical properties and estrogenic activities were evaluated. Among 24 type of silkworm extracts, Extract 11 (30E3H20-1) detected relatively high 1-DNJ contents and estrogenic activity in MCF-7 cells. Also, increases in mRNA expression and phosphorylation of ERα were induced by the treatment of MCF-7 cells with Extract 11. In addition, increased phosphorylation of downstream signaling pathway proteins, such as AKT, JNK, and p38, was confirmed. Moreover, it was reported that the contents of crude protein, crude fat, crude fiber, ash and moisture were detected as 57,79%,

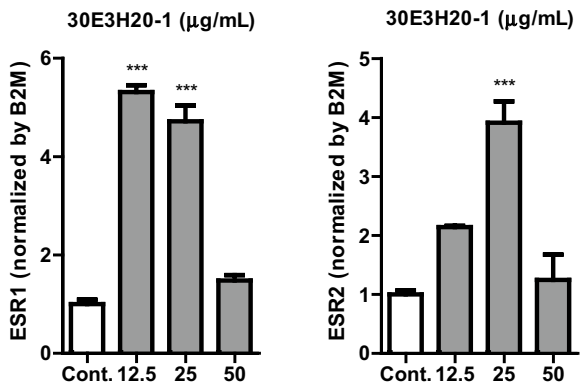

Fig. 5 Effects of treatment with Extract 11 (30E3H20-1) on the *ESR1* and *ESR2* gene expression in MCF-7 cells. *ESR1*, *ESR2*, and β-2-microglobulin mRNA expression was determined by RT-qPCR. Data are presented as the mean ± standard deviation. ***P < 0.0001 compared to the control

9.7%, 4.77%, 10.22% and 4.52%, respectively, in 3rd-day 5th instar silkworm cocoon [23]. This is different results from the contents of mulberry leaves (24.2%, 2.65%, 9.56%, 9.79% and 9.13%) [24, 25]. Therefore, in order to develop new functional material, it is considered that composition analysis studies for Extract 11 is also necessary.

Collectively all, it is suggested that silkworm extracts can be used as natural estrogen supplements in menopausal women with reduced estrogen levels.

Abbreviations

ACN: Acetonitrile; 1-DNJ: 1-Deoxynojirimycin; ELSD: Evaporative light scattering detector; ER: Estrogen receptor; FBS: Fetal bovine serum; HPLC: High-performance liquid chromatography; ICI: ICI 182780; p-: Phospho-; RPMI: Roswell park memorial institute; RT-qPCR: Real-time quantitative polymerase chain reaction; SDS: Sodium dodecyl sulfate.

Author contributions

SL, QNN, SJK, JL, and M-SS carried out the molecular genetic studies and drafted the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing Interests

The authors declare that they have no competing interests.

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