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Estrogen activity of Silkworm (*Bombyx mori*) Pupa water extract and its fractions

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SUMMARY

This study was conducted to evaluate the estrogen activity of silkworm (*Bombyx mori*) pupa extracts and their fractions. Powdered samples of freeze-dried silkworm pupa were extracted at room temperature (RT), 40 ºC, 60 ºC, 80 ºC, and 100 ºC in water (D.W), chloroform, ethyl acetate, and methanol for 6h and then filtered (0.45 um). The extracts were then freeze-dried. The estrogenic activity of these extracts was then investigated by competition binding assays using estrogen receptor α (ERα) and ERβ, and by evaluating their effects on the proliferation of the human breast cancer cell line, MCF-7. Among the extracts evaluated, water extracts prepared at RT showed the highest binding affinity to ERα (IC_{50}, 1.76 ug/ml) and ERβ (IC_{50}, 0.07 ug/ml). In addition, MCF-7 cells that were treated with 62.5 ug/ml of the RT extract showed the greatest increase in proliferation (2-fold; 1291.79%) when compared to control cells (659.82%). Next, the water extract that was prepared at RT (sample 1) was dissolved in D.W. and further fractionated using a Dowex 50W - 8X (H^+) column. The flow-through and wash were then pooled together and freeze-dried (sample 2). The bound materials were then eluted with 20 mM NaCl, after which they were applied to a Dowex 1X2 - 200 (Cl^-) column and washed with D.W. to remove the sodium ions. The eluants were then freeze-dried (sample 3). Of these fractions, sample 2 showed the highest binding affinity to ERα (IC_{50}, 1.44 ug/ml) and ERβ (IC_{50}, 1.18 ug/ml). In addition, MCF-7 cells that were treated with sample 2 (15.6 ug/ml) showed the largest increase in growth (1159.39%) when compared to control cells (525.26%). Taken together, these results suggest that the fraction of the RT water extract of silkworm pupa referred to as sample 2 may be useful as a phytoestrogen.

Key words: Silkworm pupa water extract; Estrogen; Estrogen receptor; MCF-7 cells

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INTRODUCTION

Estrogens, which are named for their importance in the estrous cycle, are a group of steroid hormones that are secreted from the ovaries and function as the primary female sex hormone. As such, estrogens play a prominent role in regulation of the maturation, proliferation, and differentiation of the mammary gland, and influence the growth and development of breast cancer (Yager, 2000). Estrogen is also a major hormone involved in the proliferation and differentiation of cells in various target organs. The biological activity of estrogen is primarily mediated through estrogen receptors (ER), which belong to the superfamily of steroid transcription factors (Farooqui *et al*., 2006). Estrogen receptor α (ERα) and ERβ are distinct gene products that can be expressed at different levels in the same tissue or in different tissues (Hall *et al*., 2001; Nilsson and Gustafsson, 2002). ERα is the dominant species expressed in the uterus, liver, adipose tissue, skeletal muscle, the pituitary and hypothalamus, whereas ERβ is the major form expressed in the ovaries, testis and the prostate, as well as in some regions, of the brain regions including the limbic system, cerebellum and cerebral cortex (Ruff *et al*., 2000). ERα and ERβ are co-expressed in breast tissue, the urogenital tract, bone and the cardiovascular system within the same cell-types, as well as in different cell populations (Nilsson and Gustafsson, 2002).

Previous studies have shown that dong quai and ginseng (Amato *et al*., 2002), soy and red clover (Bodinet and Freudenstein, 2004), and legumes (Boué *et al*., 2003) have estrogenic activity. Furthermore, Ruff *et al*. (2000) reported that phytoestrogen reduces cancer and hot flashes.

Therefore, in the present study, we evaluated the estrogen activity of silkworm pupa water extracts and their fractions using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the effects of the fractions on the proliferation of MCF-7 human breast cancer cells. In addition, we evaluated the binding activity of the extracts and their fractions using an ER binding competition assay.

MATERIALS AND METHODS

Preparation of extracts and of silkworm pupa and fractionation

Silkworm pupa was purchased from the Korean Society of Sericultural Science (Suwon, Republic of Korea). Silkworm pupa were then extracted at room temperature (RT), 40 °C, 60 °C, 80 °C, and 100 °C in distilled water (D.W.), chloroform, ethyl acetate, and methanol for 6 h, after which they were filtered (0.45 um). The extracts were then freeze-dried and evaluated for their effects on proliferation and ER binding as described below. The sample with the greatest effect was then dissolved in D.W. and further fractionated through a Dowex 50W - 8X (H^+) column. The flow-through and D.W. wash were pooled together and freeze-dried. The bound materials were then eluted with 20 mM NaCl. Next, the eluants were applied to a Dowex 1X2 - 200 (Cl^-) column and washed with D.W. to remove the sodium ions. The eluants were then freeze-dried and used for further analysis.

Cell culture

Human breast cancer MCF-7 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) supplemented with 1% antibiotic-antimycotic (10,000 unit/ml, Gibco BRL, Grand Island, NY) and 10% heat-inactivated charcoal/dextran treated fetal bovine serum (FBS; Hyclone, Logan, UT) at 37 °C under 5% CO_2 in a humidified cell incubator.

Immunofluorescence assay

MCF-7 cells were permeabilized with 0.25% Triton X-100 for 10mins at room temperature, after which they were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15min at room temperature. The fixed cells were then blocked for

20 min with PBS containing 5% bovine serum albumin (BSA). Next, the cells were then incubated for 16 h at 4 °C with mixtures containing primary antibodies specific to ERα and ERβ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS containing 1% BSA. Secondary antibody conjugated to fluorescent goat and anti-mouse IgG conjugated to FITC (Sigma, St Louis, MO) were applied at dilutions of 1: 500 and 1: 800, respectively. Next, the cells were washed with PBS, after which they were observed under a confocal microscope (Model FV300, Olympus, Japan).

MTT assay

Human breast cancer MCF-7 cells were seeded at a density of 5×10^3 cells/well in 24-well plates. The cells were then treated with the silkworm pupa water extracts and their fractions at concentrations of 250 ug/ml, 62.5 ug/ml, 15.6 ug/ml, and 3.9 ug/ml for 24 h, 48 h, 72 h, and 96 h, after which the cell proliferation was determined by a MTT assay. Briefly, each well was incubated in MTT (Sigma, St. Louis, MO) solution for 4 h, and the absorbance of each well at 490 nm was then determined using a spectrophotometer.

Competition ER binding assay

ER binding was performed using an ER (α, β) competitor assay red kit (Pan Vera, Madison, WI). Briefly, the silkworm pupa extract and their fractions were diluted to a concentration of 2X in ER Red assay buffer. Serial dilutions of the 2X solution were then made so the IC_{50} could be determined. Next, a 2X ER/Fluormone EL Red Complex containing ERα at a final concentration of 30 nM or ERβ at a final concentration of 60 nM and 2 nM Fluormone EL Red in ER red assay buffer was prepared. Twenty microliters of the diluted 2X test compounds were then added to each well, after which 20 ul of the 2X ER/Fluormone EL Red Complex was added. The solutions in the wells were then mixed, after which the plate was covered and incubated in the dark at room temperature for 1 - 18 h. The absorbance of each well at 535 nm excitation and 590 nm emission was then measured.

Statistical analysis

All data are presented as the mean ± S.D. The groups were compared using two-way ANOVA followed by Tukey's test for multiple comparisons. All statistical analyses were performed using Graphpad Prism version 5 (Graphpad software, San Diego, CA)

RESULTS

Immunofluorescence staining of MCF-7 cells

We first examined the expression of ERα and ERâ in the human breast cancer cell line, MCF-7. Briefly, the presence of ERα and ERβ was determined by immunofluorescence staining using monoclonal ERα and ERβ antibodies. As shown in Fig. 1, ERα and ERβ was localized to the MCF-7 cells.

Effect of ER binding in response to treatment with the water extract of silkworm pupa

To evaluate the affinity binding to ER, the effects of the 8 compounds on the binding affinities were evaluated. The resulting competitive binding curves were then analyzed using the PRISM software (Ver. 5.0; GraphPad Software Inc., USA) to assess the potency of the competitor molecule. The concentration that inhibited 50% (i.e. the IC_{50} value) of each test compound was then determined by nonlinear least-squares regression (Table 1). The binding affinity of RT water extract (ERα; 1.76 ± 0.13, ERβ; 0.07 ± 0.06) was found to be higher than that of 17β-estradiol (E2) (ERα; 8.37 ± 0.05, ERβ; 0.19 ± 0.05). These results suggest that water extract of silkworm pupa can bind to ER.

Effect of the extract of silkworm pupa on the proliferation of MCF-7 cells

Next, the proliferation effect of silkworm pupa extracts prepared at RT, 40 °C, 60 °C, 80 °C and 100 °C in water, chloroform, ethyl acetate, and methanol

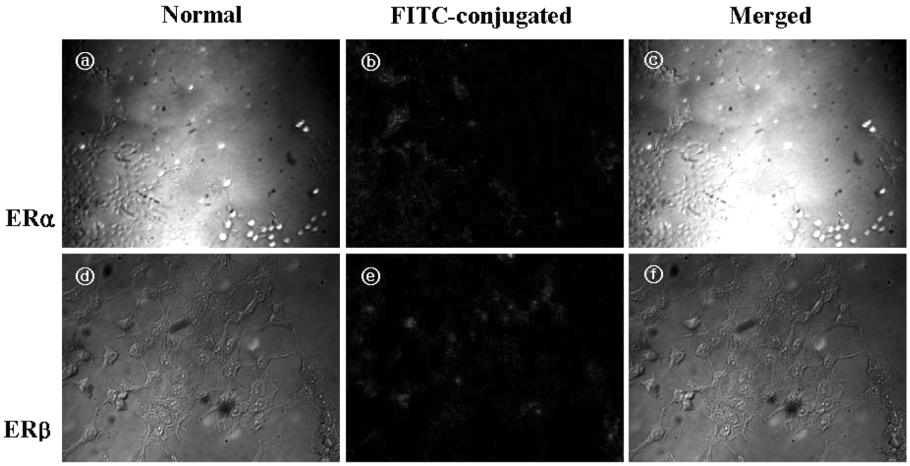

Fig. 1. Immunofluorescence staining of estrogen receptor α (ERα) and ERβ expressed in MCF-7 cells. Cells were immunostained with anti-ERα and ERβ Mab (primary), and FITC-conjugated goat anti-mouse IgG Mab (secondary) and then observed using laser scaning confocal microscopy. Panel: (a,b and c), ERα; (d, e and f), ERβ; (a and d), normal; (b and e), ERα and ERβ; (e and f) merged.

Table 1. IC_{50} value of ER binding of silkworm pupa water extracts

Sample Name	IC_{50} (ug/ml)[1)]	
	ERα	ERβ
E2	8.37 ± 0.05	0.19 ± 0.05
100°C	9.82 ± 0.12	0.14 ± 0.09
80°C	10.27 ± 0.06	0.26 ± 0.07
60°C	4.17 ± 0.05	0.09 ± 0.01
40°C	8.91 ± 0.06	0.26 ± 0.07
RT	1.76 ± 0.13	0.07 ± 0.06
Chloroform	6.92 ± 0.13	0.16 ± 0.14
Ethyl acetate	6.30 ± 0.07	0.19 ± 0.06
Methanol	7.82 ± 0.9	0.32 ± 0.06

ER competition data was generated from 384-well plates using an ELISA reader. The concentration of test compounds resulting in a half-maximal shift in polarization value equals its IC_{50}. Each value is expressed as the mean ± S.D. in triplicate experiments.
[1)]The IC_{50} value is the concentration of sample required for 50% inhibition.

on the proliferation of MCF-7 cells was evaluated. To accomplish this, the cells were cultured with the extracts of silkworm pupa at final concentrations of 250 ug/ml, 62.5 ug/ml, 15.6 ug/ml, and 3.9 ug/ml for 24 h, 48 h, 72 h, and 96 h, after which MTT assays were conducted (Fig. 2). Cells that were cultured in silkworm pupa-free media and cells that were treated with 17β-estradiol were used as negative and positive controls, respectively. The greatest increase in cell proliferation occurred in response to treatment with the RT extract for 96 h (1291.79 ± 37.1%, concentration 62.5 ug/ml) when compared to increase in the control cells (659.82 ± 48.7%) (Fig. 2B).

These results demonstrated that the water extract of silkworm pupa induced the greatest increase in cell proliferation. However, treatment with fat soluble (chloroform, ethyl acetate, and methanol) extracts of silkworm reduced cell proliferation. Based on these results, the RT water extract was used for the next experiment.

The ability of the RT water extract of silkworm pupa to bind to ER

In a previous study, we demonstrated that RT water extracts of silkworm pupa had the potential for use as potential phytoestrogens. Therefore, to assess the effects of the individual fractions of RT water extracts of silkworm pupa, the initial RT extract (sample 1) was dissolved in D.W. and further fractionated by passing it through a Dowex 50W - 8X (H^+) column. The flow-through and D.W. wash were pooled together and freeze-dried

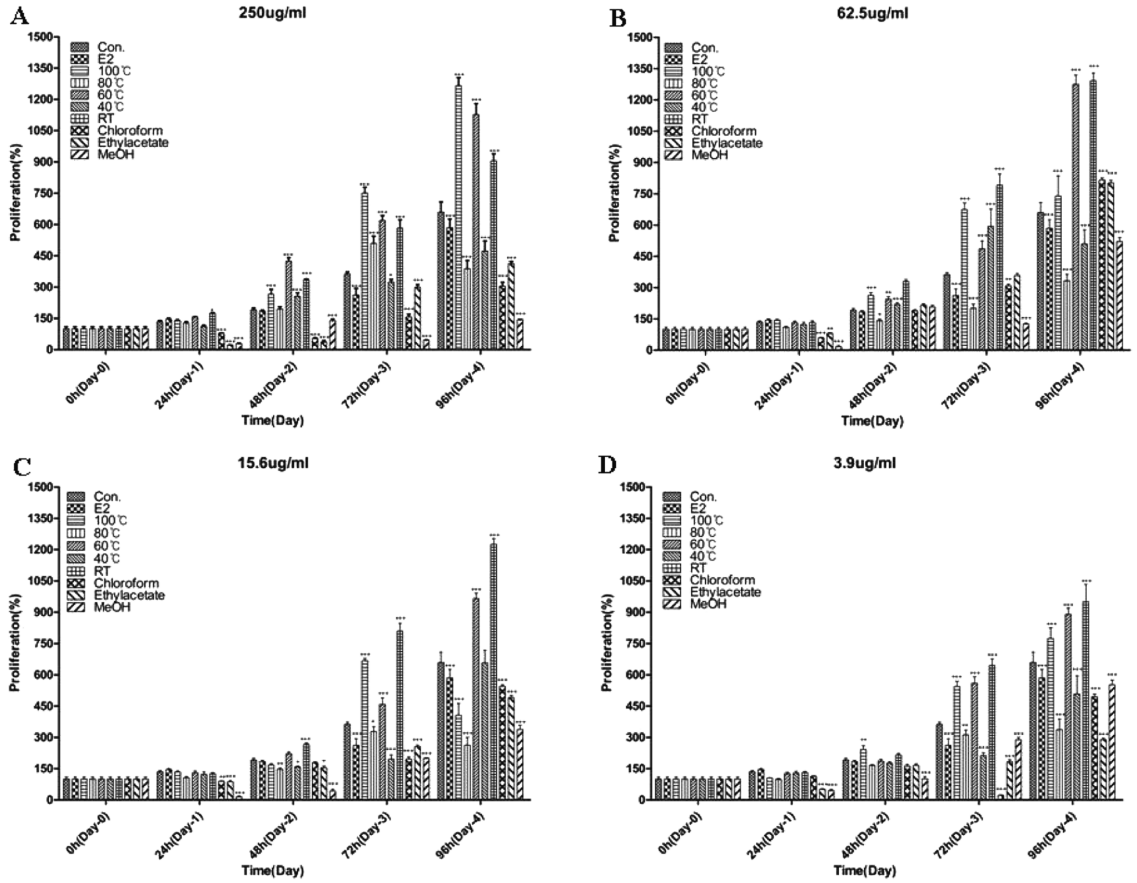

Fig. 2. Effect of silkworm (*bombyx mori*) pupa water extracts on the proliferation of MCF-7 human breast cancer cells, *in vitro*. MCF-7 cells (5×10^3 cells/well in 24-well plates) were treated with silkworm pupa extracted at room temperature (RT) (▦), 40°C (▧), 60°C (▨), 80°C (▥), and 100°C (▤) in water, chloroform (▩), ethyl acetate (▧), and methanol (▨) at concentrations of (A) 250 ug/ml, (B) 62.5 ug/ml, (C) 15.65 ug/ml and (D) 3.90 ug/ml for 24 h, 48 h, 72 h and 96 h. Data are presented as the mean ± S.D. of six separate experiments. Sample groups were compared using 2 way ANOVA. Con. *vs.* $^{*}P < 0.05$, Con. vs. $^{**}P < 0.01$, Con. *vs.* $^{***}P < 0.001$.

(sample 2). The bound materials were then eluted with 20 mM NaCl, after which they were applied to a Dowex 1X2 - 200 (Cl^-) column and washed with D.W. to remove the sodium ions. The eluants were then freeze-dried (sample 3).

Next, the binding affinities of the 3 compounds were determined. The binding affinity of sample 2 (ERα; 1.44 ± 0.05, ERβ; 1.18 ± 0.06) was found to be greater than that of E2 (ERα; 8.37 ± 0.05, ERβ; 1.66 ± 0.05) (Table 2). Taken together, these results suggest that the individual fractions of silkworm pupa water extract, specifically sample 2, could bind to ER, which indicates that it had estrogenic effects.

Table 2. IC_{50} value of ER binding of fractions of silkworm pupa water extracts

Sample Name	IC_{50} (ug/ml)[1)]	
	ERα	ERβ
E2	8.37 ± 0.05	1.66 ± 0.05
Sample 1	2.58 ± 0.05	1.21 ± 0.06
Sample 2	1.44 ± 0.05	1.18 ± 0.06
Sample 3	4.77 ± 0.05	1.35 ± 0.05

ER competition data was generated from 384-well plates using an ELISA reader. The concentration of test compound resulting in a half-maximal shift in polarization value equals its IC_{50}. Each value is expressed as the mean ± S.D. of triplicate experiments.
[1)] The IC_{50} value is the concentration of the sample required for 50% inhibition.

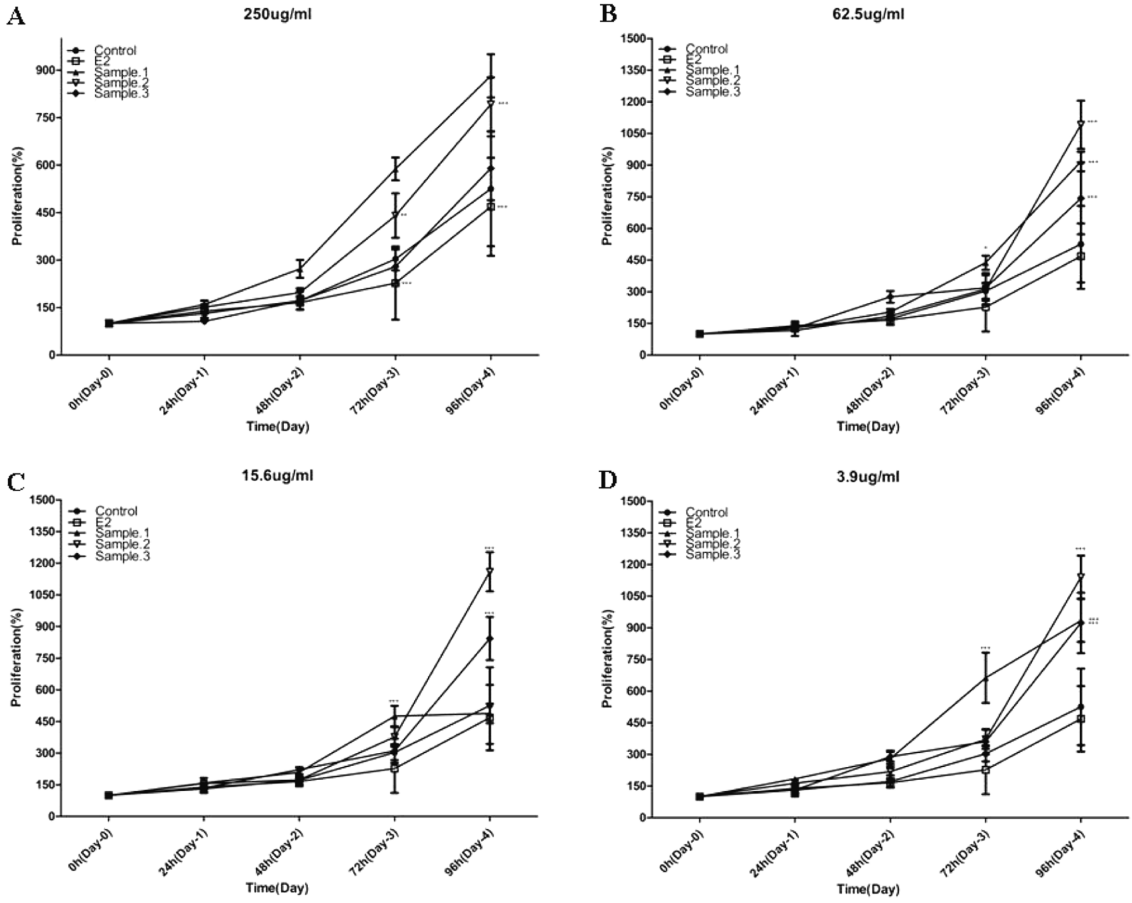

Fig. 3. Effect of fractions of silkworm (*bombyx mori*) pupa water extracts on the proliferation of MCF-7 human breast cancer cells *in vitro*. MCF-7 cells (5×10^3 cells/well in 24-well plates) were treated with silkworm pupa water extracted at RT, sample 1 (▲), sample 2 (▽), and sample 3 (◆) at concentrations of (A) 250 ug/ml, (B) 62.5 ug/ml, (C) 15.65 ug/ml and (D) 3.90 ug/ml for 24 h, 48 h, 72 h and 96 h. Data are presented as the mean ± S.D. of six separate experiments. Treatment groups were compared by 2way ANOVA. Con. vs. $^{*}P < 0.05$, Con. vs. $^{***}P < 0.001$.

Effect of the fractions of RT water extract of silkworm pupa on the proliferation of MCF-7 cells

MCF7 cells were cultured with the RT water extract of the silkworm pupa and its fractions at final concentrations of 250 ug/ml, 62.5 ug/ml, 15.6 ug/ml and 3.9 ug/ml for 24 h, 48 h, 72 h and 96 h, after which MTT assays were conducted (Fig. 3). The results revealed that sample 2 induced the greatest increase in growth (2-fold (1159.39%) at a concentration of 15.6 ug/ml), when compared to that of the control (525.26%) (Fig. 3B). Taken together, these results demonstrate that sample 2 of the silkworm pupa water extracts induced cell proliferation.

DISCUSSION

Silkworm pupa (*bombyx mori*) is a traditional Asian medicine that reportedly has antimycotic (Madana Mohana *et al.,* 2007), antijuvenoid (Saha *et al.,* 2007), and antioxidant effects, as well as the ability to improve lipid metabolism (Kwon *et al.,* 2006) prevent the proliferation of cancer (Lim *et al.,* 2007), and function as an osteoblast (Choi *et al.,* 2005). In the present study, we compared the effects of the water extract of silkworm pupa and its factions on estrogen activity in MCF-7 cells.

We found that, of the silkworm pupa extracts

prepared, water extracts prepared at RT and its fractions had the greatest ability to bind ER. It has been suggested that elevation of estrogen binding is associated with the functional relationship of DNA- and the ligand- binding domains of ER (Ruff *et al.,* 2000).

In addition, we found that the silkworm pupa water extract prepared at RT and its fractions induced a 2-fold increase in the growth of MCF-7 cells, while extracts prepared using solutions that are fat soluble did not. These findings are similar to the results of a study conducted by Yang *et al.* (2005). Taken together, these findings suggest that estrogen induces the proliferation of MCF-7 cells via an ER and proliferation signaling pathway. Interestingly, in a previous study, the proliferation of MCF-7 cells and human prostate stromal cells induced by E2 were primarily mediated by HRG/HER-2/PKC-δ/Ras/Raf/MEK/ERK (Venkateshar *et al.,* 2002; Zhang *et al.,* 2008).

In this study, we evaluated the binding affinities of silkworm pupa water extracts as well as their effects of MCF-7 cells. We found that water extracts prepared at RT and its fractions significantly bound to ERα and ERβ. In addition, these extracts induced the growth of MCF-7 cells. These results suggest that silkworm pupa water extracts that were prepared at RT, particularly sample 2, may be useful as a phytoestrogen. However, further studies using menopausal animal models and chemical experiments are needed to elucidate the mechanism by which the extract exerts its estrogen activity.

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REFERENCES

Amato P, Christophe S, Mellon PL. (2002) Estrogenic activity of herbs commonly used as remedies for menopausal symptoms. *Menopause* **9**, 145-150.

Atul Kumar Saha, Tapati Datta (Biswas), Salil Kumar Das, Niharendu Bikash Kar. (2007) Antijuvenoid action of terpenoid imidazole compound on larval - pupal - adult development of skilworm, *Bombyx mori* L. *Int. J. Indust. Entomol.* **14**, 127-135.

Bodinet C, Freudenstein J. (2004) Influence of marketed herbal menopause preparations on MCF-7 cell proliferation. *Menopause* **11**, 281-289.

Boué SM, Wiese TE, Nehls S, Burow ME, Elliott S, Carter-Wientjes CH, Shih BY, McLachlan JA, Cleveland TE. (2003) Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. *J. Agric. Food Chem.* **51**, 2193-2199.

Kwon EH, Jung MA, Rhee SJ, Choi SW, Cho SH. (2006) Antioxidant effects and improvement of lipid metabolism of mulberry fruit, mulberry leaves and silkworm powder with different mixing ratios in streptozotocin-induceed diabetic rats. *Korean J. Nut.* **39**, 91-99.

Choi EM, Lee KH, Koo SJ. (2005) Effect of silkworm (*bombyx mori*) pupa extract on the function of osteoblastic MC3T3-E1 cells. *Food Sci. Biotechnol*. **14**, 593-598.

Farooqui M, Geng ZH, Stephenson EJ, Zaveri N, Yee D, Gupta K. (2006) Naloxone acts as an antagonist of estrogen receptor activity in MCF-7 cells. *Mol. Cancer Ther*. **5**, 611-620.

Lim HY, Lim YE, Hong SW, Lee CH, Lim YH. (2007) Antiproliferative effect of silkworm extrat. *J. Appl. Biol. chem*. **50**, 293-295.

Hall JM, Couse JF, Korach KS. (2001) The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J. Biol. Chem*. **276**, 36869-36872.

Yang JW, Choi EM, Kwon MG, Koo SJ. (2005) *In vitro* estrogenic activity of silkworm (*Bombyx mori*) pupa and herbs. *J. East Asian Soc. Dietary Life.* **15**, 315-322.

Nilsson S, Gustafsson JA. (2002) Estrogen receptor action. *Crit. Rev. Eukaryot. Gene Expr*. **12**, 237-257.

Madana Mohana N, Gupta SK, Mitra P. (2007) Antimycotic activity of *Allum Sativum* Against *Beauveria Vassiana*, pathogenic fungus of white muscardine disease in silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). *Int. J. Indust. Entomol.* **14**, 81-85.

Ruff M, Gangloff M, Wurtz JM, Moras D. (2000) Estrogen receptor transcription and transactivation: Structure-function relationship in DNA- and ligand-

binding domains of estrogen receptor. *Breast Cancer Res.* **2**, 353-359.

Venkateshwar G, Keshamouni, Raymond R, Mattingly, Kaladhar B Reddy. (2002) Mechanism of 17-Estradiol-induced Erk1/2 Activation in Breast Cancer Cells. *J. Biol. Chem.* **277**, 22558-22565.

Yager JD. (2000) Endogenous estrogens as carcinogens through metabolic activation. *J. Natl. Cancer Inst. Monographs* **27**, 67-73.

Zhang Z, Duan L, Du X, Ma H, Park I, Lee C, Zhang J, Shi J. (2008) The proliferative effect of estradiol on human prostate stromal cells is mediated through activation of ERK. *Prostate* **68**, 508-516.