Master's Thesis of Science in Biomodulation

A seed coat extract of Yak-Kong as a potential alternative to selective aromatase modulator

선택적 아로마타제 조절제의 대체재로서
약콩껍질추출물에 대한 연구

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The Graduate School

Seoul National University

Biomodulation Major

Sumin Park

석사학위논문

ABSTRACT

Estrogen is a steroid hormone that plays an important role in female reproductive function and sexual development. As women get older, the secretion of estrogen decreases and estrogen supplementation is crucial to alleviate symptoms related to estrogen deficiency. CYP19 (also called aromatase) is the key enzyme responsible for estrogen production and it is expressed in a tissue-specific manner. Aromatase should be expressed in sites that require estrogen synthesis and inhibited overexpression in estrogen-dependent cancer tissues. Therefore, it is necessary to investigate natural products that can supplement estrogen and regulate aromatase expression tissue-specifically for menopausal women's health.

In this study, we found that Yak-Kong seed coat extract increases 17β-estradiol production in human adrenocortical carcinoma NCI-H295R cells and mouse antral follicles. It promotes 17β-estradiol biosynthesis by increasing the protein and gene expression of CYP19A1 and 3β-HSD in steroidogenesis pathway. Furthermore, it suppresses proliferation of breast and ovarian cancer cells known as estrogen-dependent cancer cells and regulates aromatase expression of adipocytes followed a different pattern from that of H295R cells. These results suggest that the extract of Yak-Kong seed coat could be a potential alternative to selective aromatase modulator for menopausal women.

Keyword: Yak-Kong; estrogen; steroidogenesis; aromatase; breast cancer;

Student Number: 2017-21102

CONTENTS

I. INTRODUCTION

Estrogen is a steroid hormone that plays an important role in female reproductive function and sexual development. There are three types of natural estrogen; estrone (E1), estradiol (E2), and estriol (E3). Estradiol (E2) is also known as 17β-estradiol, and it is the most potent form of estrogenic steroids [1]. The primary sites of estrogen synthesis are reproductive organs; ovary and testis [2], but estrogen is also regulated in many extra-gonadal sites including adrenal gland [3], body fat [4], bone [5], brain [6] and etc. In menopause, ovary which is primary site of estrogen production in women stops working gradually and the secretion of estrogen decreases [7]. Various symptoms related to estrogen deficiency such as hot flush [8], weight gain [9], insomnia [10] and etc. appear. Thus, for peri-menopausal women, it is important to promote estrogen

synthesis in dysfunctional ovary. There is a difference in their signaling that gonads secret estrogen into target tissues as an endocrine factor and extra-gonadal estrogen acts locally in paracrine, intracrine, and autocrine manners [11]. In post-menopausal women, ovarian estrogen no longer functions as an endocrine factor because the main sources of estrogen production are extra-gonadal sites [12]. Therefore, post-menopausal women should produce estrogen locally where it is needed.

In this study, NCI-H295R was used to observe the ability of estrogen synthesis because it is the most appropriate in vitro model for verifying steroid hormone synthesis and represents steroidogenesis in adrenal cortex [13-15]. Moreover, circulating androgens produced by adrenal cortex are sources of local estrogen production by aromatization and they might be more important than circulating

estrogens in post-menopausal women [16]. Mouse antral follicles were also used to observe estrogen production in gonadal site as functional unit of ovary [17].

Hormones can develop human cancer in certain organs [18], and estrogen especially acts as a causative factor of breast, endometrial, and ovarian cancers with reference to cellular proliferation [19, 20], decreased apoptosis, and genomic damage [21]. As mentioned above, there is a difference of hormone action between gonads and extra-gonadal sites. Thus, total circulating estrogen levels of post-menopausal women may not be high, but the levels of estrogen in certain tissue related to estrogen-dependent cancer may be high [16]. In aspect of breast cancer, locally produced estrogen in breast tumor and surrounding tissue influences breast cancer development [22].

Steroidogenesis is the biosynthetic pathway producing steroid hormones such as estrogen from cholesterol via steroidogenic enzymes. In steroidogenesis pathway,

steroidogenic enzymes are divided into two groups; the Hydroxysteroid Dehydrogenases and the Cytochrome P450 [23]. 3β-HSD is one of the hydroxysteroid dehydrogenases and it is responsible enzyme producing androgens that are direct precursors of estrogens: pregnenolone to progesterone, dehydroepiandrosterone to androstenedione [24]. CYP19 (also called aromatase) is involved in the cytochrome P450 heme-containing proteins and it is the key enzyme responsible for the conversion of androgens to estrogens: androstenedione to estrone, testosterone to estradiol [23].

CYP19 gene, encoding aromatase, is expressed with tissue-specificity because unique promoters are used for each tissue and they are regulated by distinct factors and signaling pathway [25]. In ovary, follicle stimulating hormone (FSH) binds to the receptor and cyclic AMP (cAMP) acts as second messenger. As a result, promoter II regulates aromatase expression [26]. In adipose tissue, promoter I.4 primarily controls

basal levels of aromatase expression under normal conditions. However, in breast cancer, tumorigenic breast epithelial cells increase adipose fibroblasts and the regulation of aromatase expression is switched to promoter I.3 and II under the control of prostaglandin E2 from breast tumor. [27]. Moreover, adipose tissue adjacent to malignant breast epithelial cells may affect aromatase overexpression of breast cancer tissue through paracrine interactions, which account for most of the aromatase expression of breast tumors [28, 29]. Thus, regulation of aromatase expression in adipose tissue can be therapeutic target for breast cancer.

Recently, aromatase inhibitors (AIs) such as letrozole and anastrozole are used to treat breast cancer. However, they inhibit aromatase expression in all tissues throughout the body and can cause osteoporosis and cognitive impairment as side effects [30]. Hence, selective aromatase modulator (SAM) can be used as an appropriate

therapeutic method in menopausal women [31]. SAM inhibits aromatase expression in tissues related to estrogen-dependent cancer, but not in sites that require estrogen synthesis [32]. For menopausal women, it is necessary to regulate local estrogen production and SAM can selectively regulate aromatase producing estrogen in each tissue.

The aim of this study is to identify the effects of Yak-Kong seed coat extract on the estrogen biosynthesis via steroidogenic enzymes and tissue-specific regulation of aromatase expression. It can be a potential alternative to SAM that stimulates aromatase in dysfunctional gonadal site in peri-menopausal women and extra-gonadal sites in post-menopausal women and inhibits aromatase overexpression in adipose tissues adjacent to estrogen-dependent breast cancer.

II. MATERIALS AND METHODS

1. Soybean materials

Two different soybeans (*Glycine max*) were used in this study. Standardized YK soybean (Registration number: 01-0003-2013-3) was provided by the Rural Development Administration, Republic of Korea. The yellow soybean was purchased from local suppliers in Danyang and Boeun, Republic of Korea in 2016. Peeled seed coats and embryos used were assessed using the different extraction conditions. The conditions selected were previously optimized for antioxidant extraction through multiple antioxidant assays (data not shown). Briefly, the peeled seed coats were extracted with 50% ethanol at 75 °C for 1.5 h. The 3 mm cut embryos were extracted with 70% ethanol at 75 °C for 3 h. YK seed coat and embryo extracts were freeze-dried as powders.

2. Animals

Animal study was performed in accordance with recommendations in *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. Animal handing was done in accordance with the protocols approved by the Seoul National University's Institutional Animal Care and Use Committee (IACUC). At 25-30 postnatal days of wild type C57BL/6 female mice were used.

3. Cell culture

The NCI-H295R human adrenocortical cell line (CRL-2128) was purchased from the American Type Culture Collection (ATCC, Virginia, USA). Cells were cultured in DMEM/F-12 medium; 1:1 mixture of Dulbecco's-modified Eagle's and Ham's F-12 media containing L-glutamine and 15mM HEPES (Welgene, Gyeongsan, South

Korea), supplemented with 2.5% Nu-Serum, 1% insulin/transferrin/selenium premix (Corning, New York, USA) and 0.1% Penicillin-Streptomycin (Corning, New Yok, USA). The serum-free media contained only 0.1% Penicillin-Streptomycin in DMEM/F-12. Cells were maintained in 75 cm^2 flasks at 37 °C in an atmosphere of 5% CO_2. For RNA, protein extraction and media collection, 2.5 x 10^6 cells were plated in a 6 cm^2 cell culture dish. After subculturing for 48 h, cells were treated with dimethyl sulfoxide (DMSO) (vehicle), Yak-Kong seed coat extract at the doses indicated, or 40 μg/ml of Yellow Soybean seed coat extract, Yak-Kong seed coat extract, Yellow Soybean embryo extract, and Yak-Kong embryo extract in serum-free media. RNA extraction was performed after 12 h of sample treatment. Protein extraction was done after 24 h of sample treatment. Media was collected after 48 h of sample treatment and immediately frozen in liquid nitrogen.

The MCF-7 human breast adenocarcinoma cell line (HTB-22) was purchased from the American Type Culture Collection (ATCC, Virginia, USA). The OVCAR-8 human ovarian carcinoma cell line was a kind gift from Laboratory of Prof. Zigang Dong (The Hormel Institute, University of Minnesota, MN 55912, USA). Cells were cultured in DMEM medium (Welgene, Gyeongsan, South Korea), supplemented with 10% Fetal Bovine Serum (VWR, PA, USA) and 0.1% Penicillin-Streptomycin (Corning, New Yok, USA). Cells were maintained in 75 cm^2 flasks at 37 °C in an atmosphere of 5% CO_2. For cell viability assay, cells were treated with dimethyl sulfoxide (DMSO) (vehicle), Yak-Kong seed coat extract at the doses indicated.

4. Antral follicle culture

Female mice were euthanized on postnatal days (PND) 25-30 and their ovaries were collected. Antral follicles about 250-400 μm were isolated mechanically from the ovaries using fine watchmaker forceps. Isolated antral follicles placed individually in wells of a 96-well culture plate, and covered with supplemented α-minimum essential media (α-MEM). Supplemented α-MEM was prepared with 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 IU/ml human recombinant follicle stimulating hormone (FSH; Sigma, Missouri, USA), 5% fetal bovine serum (VWR, PA, USA). 2 – 4 mice were used per experiment, approximately 20 - 30 antral follicles were isolated from each mouse. Each experiment contained a minimum of 3 - 5 follicles per group. Antral follicles were treated with dimethyl sulfoxide (DMSO) (vehicle), Yak-Kong seed coat extract at the doses indicated in supplemented

media and cultured for 96 hours in an incubator with 5% CO_2 at 37 °C. After culture, media was collected and frozen immediately in liquid nitrogen.

5. Hormone measurement

The concentrations of 17β-estradiol in the media were measured by enzyme-linked immunosorbent assays ELISA (DRG International, Germany). The samples were run in triplicates and had intra- and inter-assay coefficients of variability were below 10%.

6. Real-time quantitative PCR

Cells were harvested with RNAiso Plus (Takara Bio Inc., Shiga, Japan). RNA was quantified using NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After reverse transcription with oligo-dT primers using a

PrimeScriptTM 1st strand cDNA synthesis Kit (Takara Bio Inc.), Real-time quantitative RT-PCR was performed using IQ SYBR (Bio-Rad Laboratories). 2 μl of cDNA in triplicate with β-actin as internal control. Before PCR amplification, the primers were denatured at 95 °C for 3 min. Amplification was made up of 44 cycles at 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. PCR was performed by CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories). cDNA was probed by the following primer: CYP11A1 forward (5'- GAG ATG GCA CGC AAC CTG AAG -3'); CYP11A1 reverse (5'- CTT AGT GTC TCC TTG ATG CTG GC -3'); CYP17A1 forward (5'- GGC ACC AAG ACT ACA GTG ATT G -3'); CYP17A1 reverse (5'- AGA GTC AGC GAA GGC GAT AC -3'); CYP19 forward (5'- AGG TGC TAT TGG TCA TCT GCT C -3'); CYP19 reverse (5'- TGG TGG AAT CGG GTC TTT ATG G -3'); 3β-HSD2 forward (5'-TGC CAG TCT TCA TCT ACA CCA G -3'); 3β-HSD2 reverse (5'- TTC CAG AGG CTC TTC TTC

GTG -3'); 17β-HSD forward (5- TTC ATG GAG AAG GTG TTG G -3'); 17-HSD reverse (5'-

AAG ACT TGC TTG CTG TGG -3'); β-actin forward (5'- TCC TCA CCC TGA AGT ACC

CCA T -3'); β-actin reverse (5'- AGC CAC ACG CAG CTC ATT GTA -3')

7. Western blotting

After removing media, cells were lysed with lysis buffer containing 10 Mm Tris (Ph 7.5), 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 1 % Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 % glycerol and protease inhibitor cocktail tablet. The protein concentration was measured using a protein assay reagent kits as described by the manufacturer. 60 μg of protein lysates were separated electrophoretically using a 10 % SDS-polyacrylamide gel and transferred onto an Immobilon P membrane (MERK Millipore). The membrane was

blocked in 5 % fat-free milk for 1 h, and then incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualized using a chemiluminescence detection kit (GE healthcare, London, UK) after hybridization with the HRP-conjugated secondary antibody (Life technologies, Waltham, MA).

8. Cell viability assay

H295R, MCF-7, and OVCAR-8 cells were cultured in 96 well plates at a density of 4.0×10^4 cells/well. H295R cells were incubated in DMEM/F-12 supplemented with 2.5% Nu-Serum, 1% insulin/transferrin/selenium premix and 0.1% Penicillin-Streptomycin. MCF-7 and OVCAR-8 cells were incubated in DMEM supplemented with 10% FBS and 0.1% Penicillin-Streptomycin. After sample treatment, the cells were incubated for 48 h. After addition of 20 μL PMS/MTS solution per 100 μL

medium, cells were incubated for 1~4 h. The absorbance at 490 nm was then measured using a microplate reader.

9. 3T3-L1 and MCF-7 coculture

For differentiation, 3T3-L1 preadipocytes were seeded at 3.0×10^4 cells/well into 12 well plates. 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (ATCC). 3T3-L1 preadipocytes were maintained in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% Bovine Calf Serum (Gibco, Grand Island, NY, USA), under an atmosphere of 5 % CO_2 at 37 ℃ until 100% confluence. After post-confluence (day 0), the cells were differentiated for 6 days in the presence or absence of the test sample. The cells were incubated in DMEM supplemented with 10 % Fetal Bovine Serum (VWR, PA, USA) and an adipogenic

cocktail (MDI) which was a mixture of 0.5 mM IBMX (Sigma, Missouri, USA), 1 μM dexamethasone (DEX; Sigma, Missouri, USA), and 5 μg/mL^{-1} insulin for 2 days in order to induce differentiation. After 2 days, the media was changed to DMEM containing 10 % FBS and 5 μg/mL^{-1} insulin. Two days later, the media was switched to DMEM containing 10 % FBS which was replaced every two days until the preadipocytes were fully differentiated. After 4 days, MCF-7 cells were seeded at 1.5×10^4 cells/well into the upper insert of Corning Transwell system (CLS3460). Three days later, cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, USA)

10. Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics ver. 23.0 (IBM, Armonk, NY, USA). The data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD and expressed as mean ± standard error of the mean (SEM). Differences between control and sample treated group in mono-cultured adipocytes were assessed with unpaired Student's *t*-test.

III. RESULTS

1. Yak-Kong seed coat extract has the most estradiol-stimulating effect in H295R cells among soybean extracts

To identify the estradiol-stimulating effect of soybean extracts, H295R cells were treated with 0.1 % DMSO as vehicle control, 40 μg/ml of Yak-Kong seed coat extract, Yellow soybean seed coat extract, Yak-Kong embryo extract, and Yellow soybean embryo extract. Yak-Kong seed coat extract promoted production of 17β-estradiol in H295R cells compared to vehicle control and other samples. (Fig. 1A) Cell viability data had no significant differences and showed that the concentration in which samples were treated was not cytotoxic. (Fig. 1B) Considering the estradiol-simulating effect, this research was carried out further with Yak-Kong seed coat extract.

Figure 1

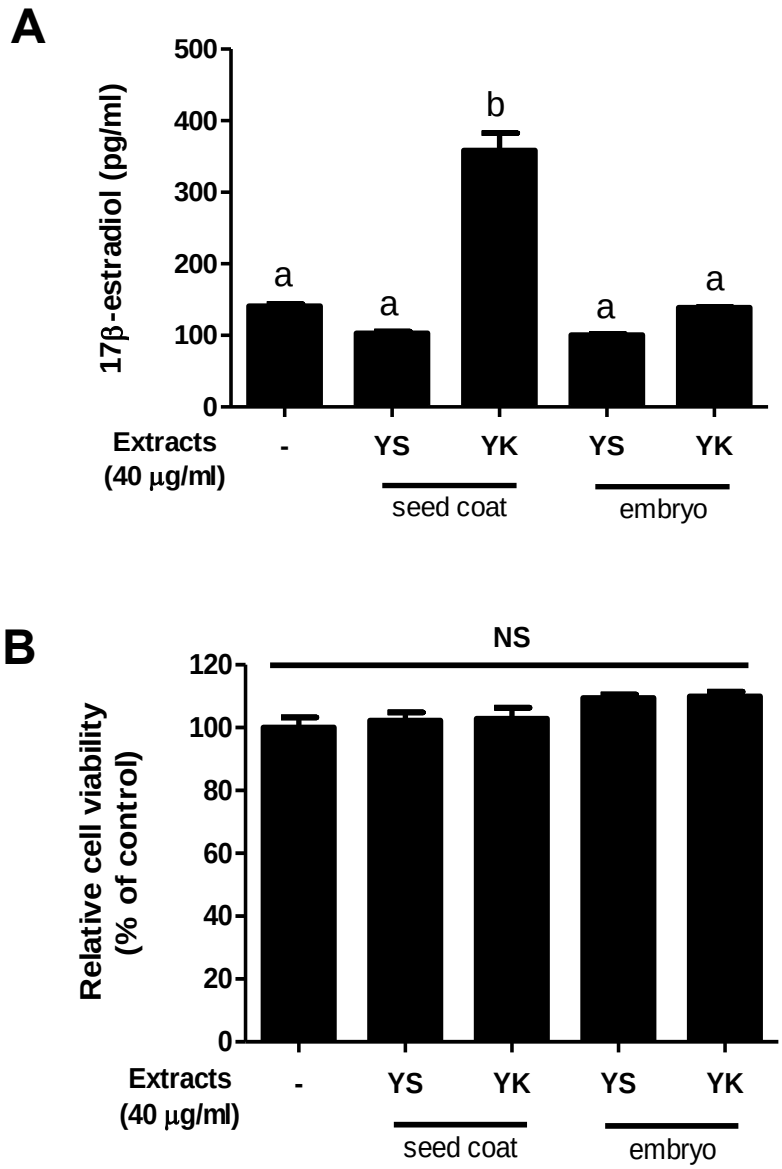

A
17β-estradiol (pg/ml)
500
400
300
200
100
0
a
a
b
a
a
Extracts
(40 μg/ml)
-
YS
YK
YS
YK
seed coat
embryo
B
NS
Relative cell viability
(% of control)
120
100
80
60
40
20
0
Extracts
(40 μg/ml)
-
YS
YK
YS
YK
seed coat
embryo

Figure 1. Effects of four soybean extracts on 17β-estradiol production in H295R cells

All soybean extracts were used at 40 μg/ml. After H295R cells were treated with four soybean extracts for 48 h, cell culture media was collected and measured 17β-estradiol by enzyme-linked immunosorbent assays. **A.** 17β-estradiol concentration of cell culture medium. Data was shown as means ± SEM with n=3. Mean values with different letters (a-b) are significantly different from each other at $p<0.05$. **B.** Cell viability evaluated by MTS assay. Data was shown as means ± SEM with n=4. Mean values were found to be non-significant ($p>0.05$). YS, yellow soybean; YK, Yak-Kong; NS, no significance.

2. Yak-Kong seed coat extract promotes production of 17β-estradiol in a dose-dependent manner in H295R cells and mouse antral follicles

To observe the estradiol-stimulating effect of Yak-Kong seed coat extract in a dose-dependent manner, H295R cells were used to measure 17β-estradiol production. The extract promoted 17β-estradiol production in a dose-dependent manner in H295R cells. Data showed significant difference at 10 and 20 μg/ml of Yak-Kong seed coat extract treatment (Fig. 2A). Yak-Kong seed coat extract was not cytotoxic up to 20 μg/ml. Cell viability data was not supplemented, but it can be deduced from Fig. 1B.

To determine the estrogen-stimulating effect of Yak-Kong seed coat extract not only in extra-gonadal site but also in gonadal site, mouse antral follicles were used to measure 17β-estradiol production. Yak-Kong seed coat extract promoted 17β-estradiol

production in mouse antral follicles. Data showed significant difference at 10 and 20 μg/ml of Yak-Kong seed coat extract treatment (Fig. 2B).

Figure 2

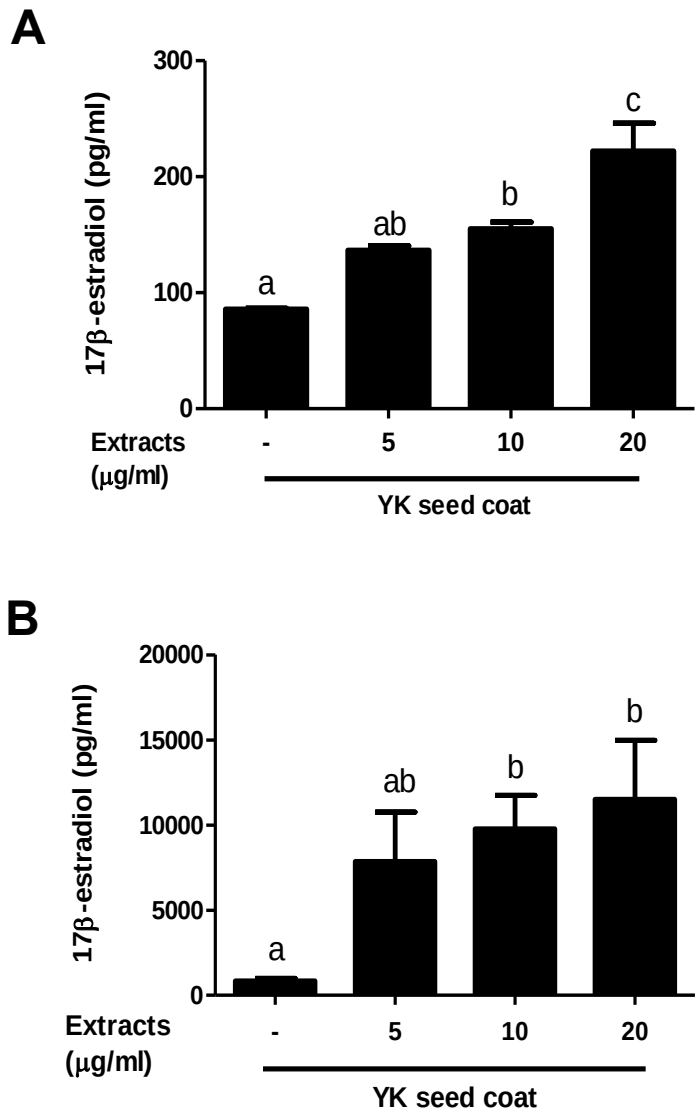

A
300
200
100
0
17β-estradiol (pg/ml)
a
ab
b
c
Extracts
(μg/ml)
-
5
10
20
YK seed coat
B
20000
15000
10000
5000
0
17β-estradiol (pg/ml)
a
ab
b
b
Extracts
(μg/ml)
-
5
10
20
YK seed coat

Figure 2. Effects of Yak-Kong seed coat extract on production of 17 β-estradiol in H295R cells and mouse antral follicles.

5, 10 and 20 μg/ml of Yak-Kong seed coat extract were treated. After H295R cells were treated for 48 h and mouse antral follicles were treated for 96 h, cell culture media was collected and measured 17β-estradiol by enzyme-linked immunosorbent assays. **A.** 17β-estradiol concentration of H295R cell culture medium. **B.** 17β-estradiol concentration of mouse antral follicle culture medium. Data was shown as means ± SEM with n=3. Mean values with different letters (a-c) are significantly different from each other at $p<0.05$. YK, Yak-Kong.

3. Yak-Kong seed coat extract increases protein and mRNA levels of CYP19A1 in H295R cells

The expression of steroidogenic enzymes was studied to determine how Yak-Kong seed coat extract increased 17β-estradiol in steroidogenesis pathway. CYP19A1 is the enzyme responsible for the conversion of androgens to estrogens. The extract increased protein levels of CYP19A1 significantly at 10 and 20 μg/ml (Fig. 3B) and mRNA levels of *CYP19A1* at 10 and 20 μg/ml (Fig. 3C).

Figure 3

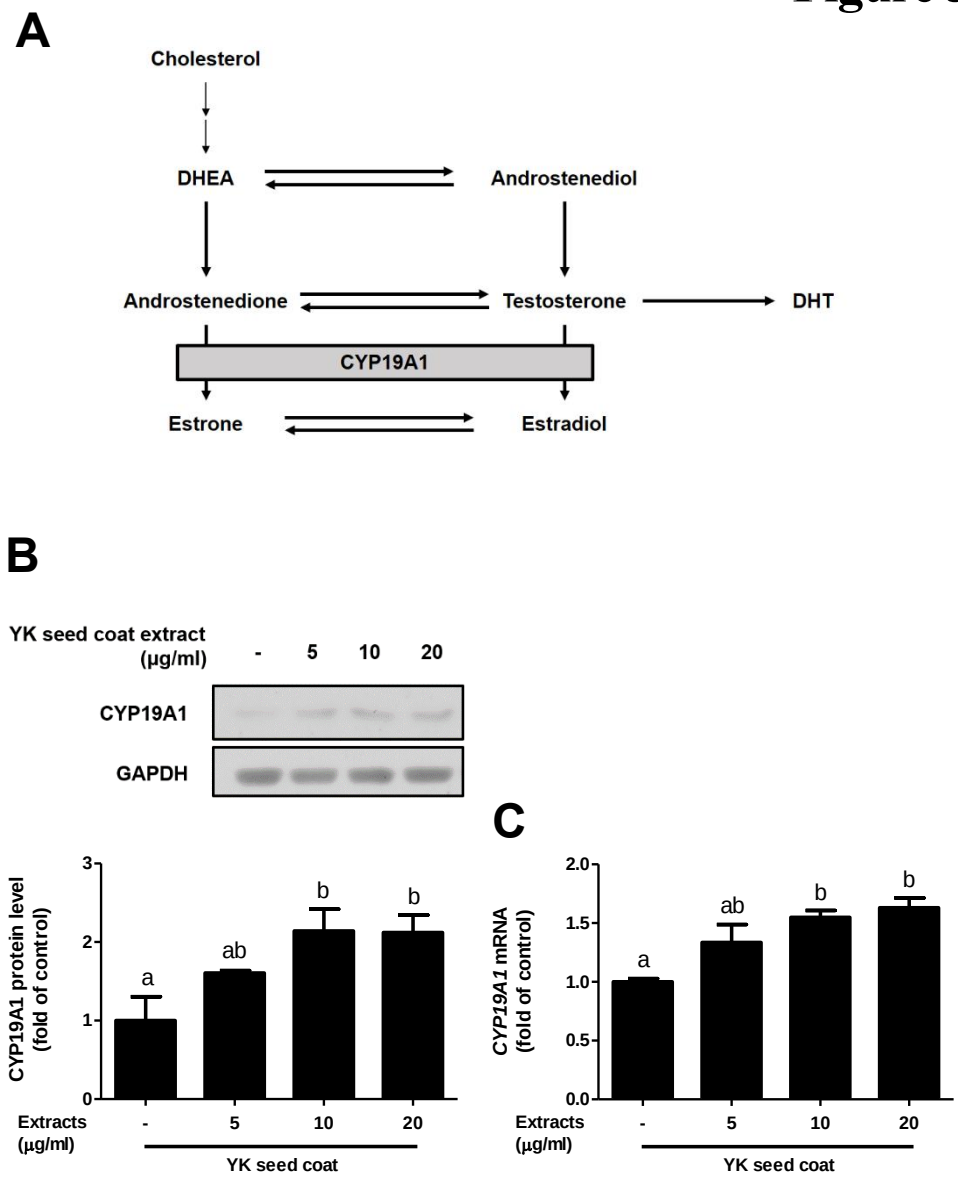

A
Cholesterol
DHEA
Androstenediol
Androstenedione
Testosterone
DHT
CYP19A1
Estrone
Estradiol
B
YK seed coat extract (μg/ml)
-
5
10
20
CYP19A1
GAPDH
C
CYP19A1 protein level (fold of control)
a
ab
b
b
Extracts (μg/ml)
YK seed coat
CYP19A1 mRNA (fold of control)

Figure 3. Effects of Yak-Kong seed coat extract on protein and mRNA levels of CYP19A1 in H295R cells

5, 10 and 20 μg/ml of Yak-Kong seed coat extract were treated. After H295R cells were treated with different concentrations for 24 h, cells were lysed and protein was extracted. The protein expression was measured by western blotting assay. **A.** CYP19A1 in steroidogenesis pathway. **B.** Protein levels of CYP19A1. The loading control is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). After H295R cells were treated with different concentrations for 12 h, RNA extraction was performed and mRNA level was measured by real-time quantitative PCR. **C.** mRNA levels of *CYP19A1*. Data was shown as means ± SEM with n=3. Mean values with different letters (a-b) are significantly different from each other at $p<0.05$. YK, Yak-Kong.

4. Yak-Kong seed coat extract increases protein and mRNA levels of 3β-HSD in H295R cells

3β-HSD is the enzyme responsible for producing androgens that are direct precursors of estrogens. Yak-Kong seed coat extract increased protein levels of 3β-HSD significantly at 20 μg/ml (Fig. 4B) and mRNA levels of *3β-HSD* at 10 and 20 μg/ml (Fig. 4C).

Figure 4

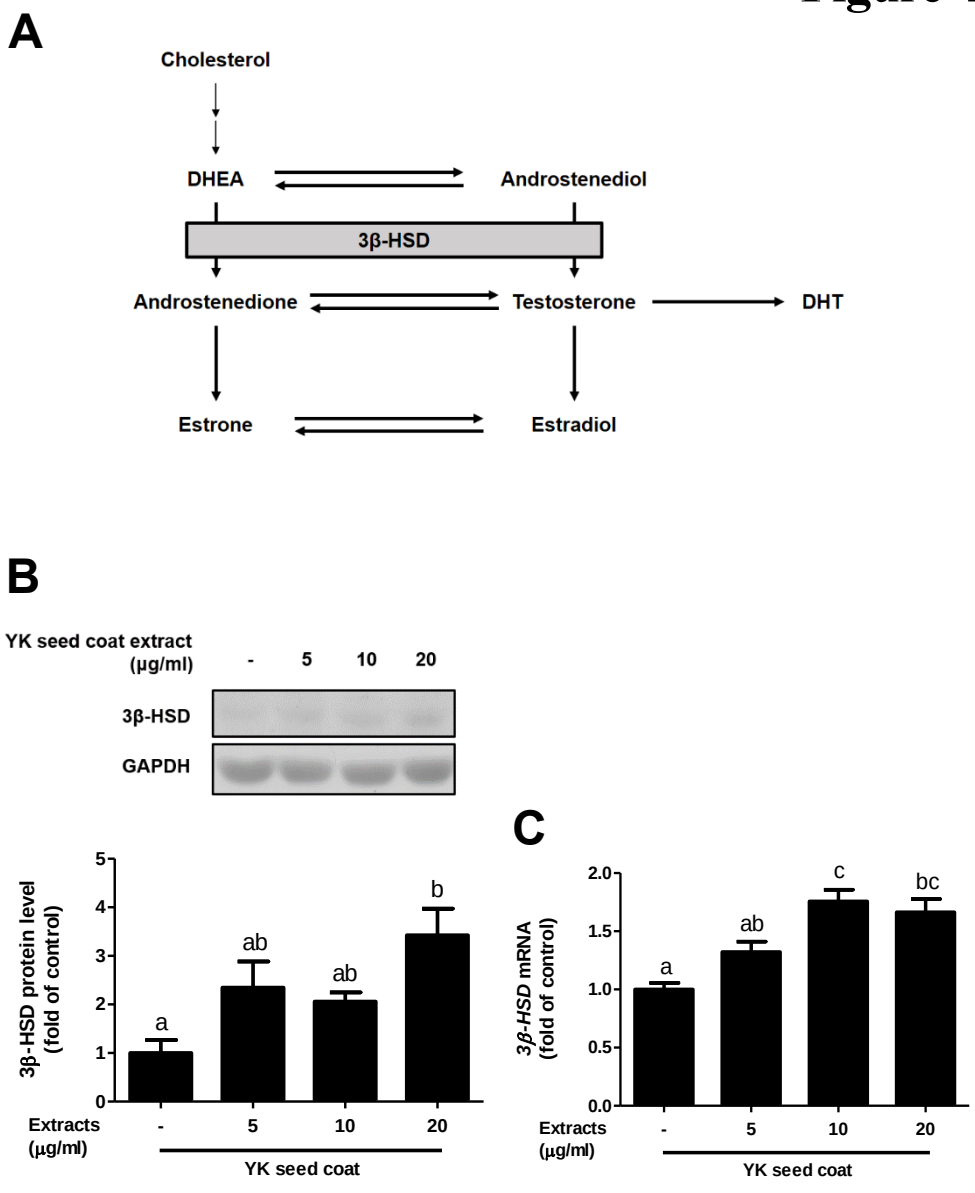

A
Cholesterol
DHEA
Androstenediol
3β-HSD
Androstenedione
Testosterone
DHT
Estrone
Estradiol
B
YK seed coat extract (μg/ml)
-
5
10
20
3β-HSD
GAPDH
3β-HSD protein level (fold of control)
a
ab
ab
b
Extracts (μg/ml)
YK seed coat
C
3β-HSD mRNA (fold of control)
a
ab
c
bc
Extracts (μg/ml)
YK seed coat

Figure 4. Effects of Yak-Kong seed coat extract on protein and mRNA levels of 3β-HSD in H295R cells

A. 3β-HSD in steroidogenesis pathway. **B.** Protein levels of 3β-HSD. The loading control is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **C.** mRNA levels of *3β-HSD*. Data was shown as means ± SEM with n=3. Mean values with different letters (a-c) are significantly different from each other at $p<0.05$. YK, Yak-Kong.

5. Yak-Kong seed coat extract does not increase protein levels of CYP11A1, CYP17A1, or 17β-HSD in H295R cells

To determine which steroidogenic enzymes were stimulated to synthesize 17β-estradiol in steroidogenesis pathway, an effect of Yak-Kong seed coat extract on the expression of other steroidogenic enzymes except CYP19A1 and 3β-HSD was studied. Yak-Kong seed coat extract did not increase protein levels of CYP11A1, CYP17A1 or 17β-HSD in steroidogenesis pathway.

Figure 5

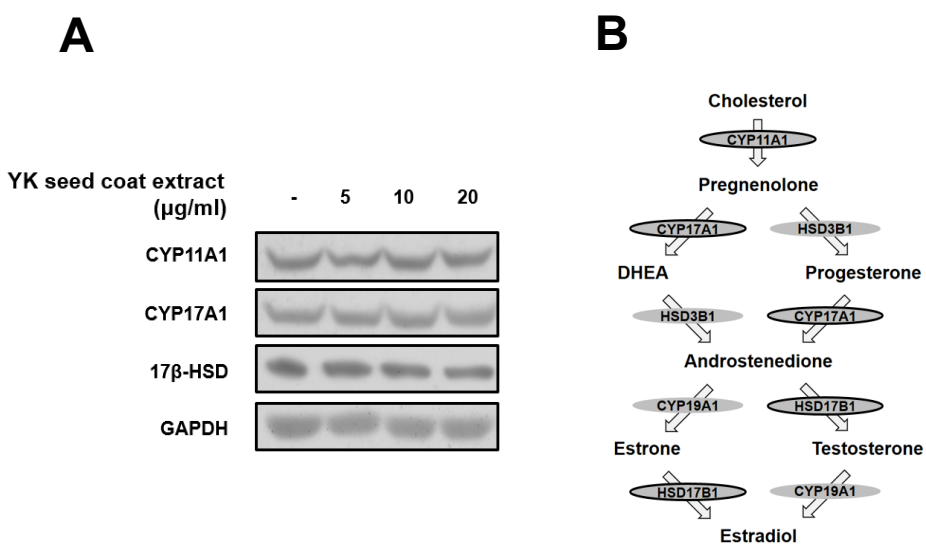


Figure 5. Effects of Yak-Kong seed coat extract on protein levels of CYP11A1, CYP17A1, or 17β-HSD in H295R cells

A. Protein levels of CYP11A1, CYP17A1, or 17β-HSD. The loading control is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **B.** CYP11A1, CYP17A1, and 17β-HSD in steroidogenesis pathway. YK, Yak-Kong.

6. Yak-Kong seed coat extract decreases viability of human breast and ovarian cancer cells

To identify the anti-estrogen dependent cancer effect of Yak-Kong seed coat extract, MCF-7 and OVCAR-8 cells were used to assess cell viability. Yak-Kong seed coat extract decreased viability of human breast cancer cells significantly at 40 μg/ml (Fig. 6A) and human ovarian cancer cells significantly at 5, 10, 20 and 40 μg/ml (Fig. 6B).

Figure 6

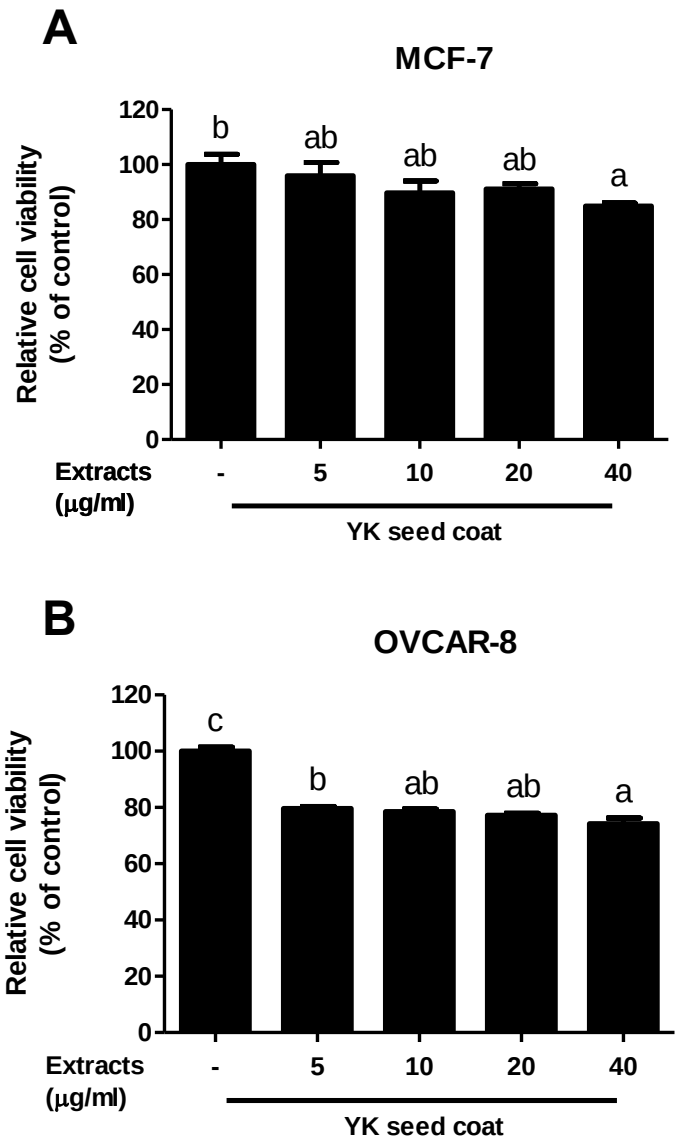

A
MCF-7
Relative cell viability (% of control)
120
100
80
60
40
20
0
b
ab
ab
ab
a
Extracts (μg/ml)
-
5
10
20
40
YK seed coat
B
OVCAR-8
Relative cell viability (% of control)
120
100
80
60
40
20
0
c
b
ab
ab
a
Extracts (μg/ml)
-
5
10
20
40
YK seed coat

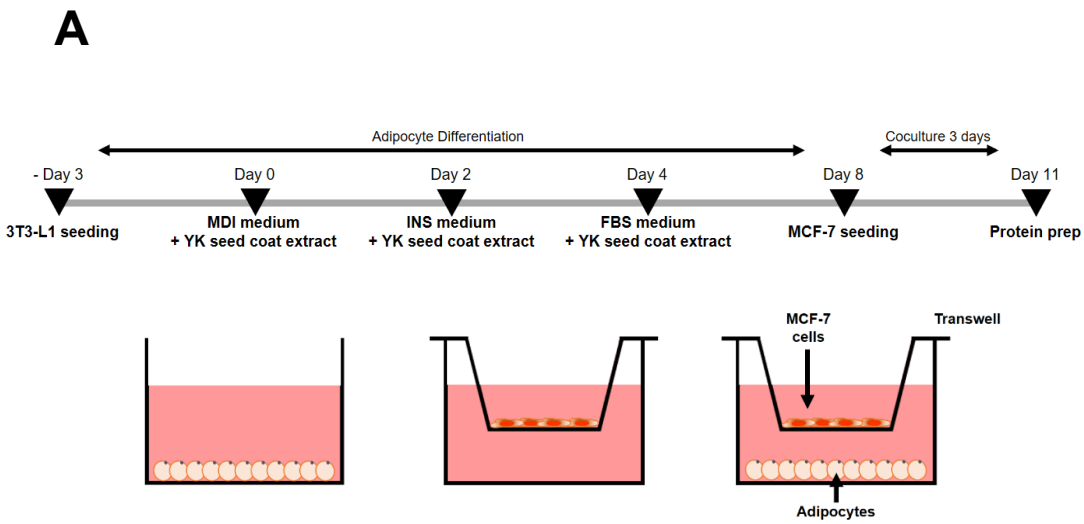
Figure 6. Effects of Yak-Kong seed coat extract on viability of MCF-7 and OVCAR-8 cells

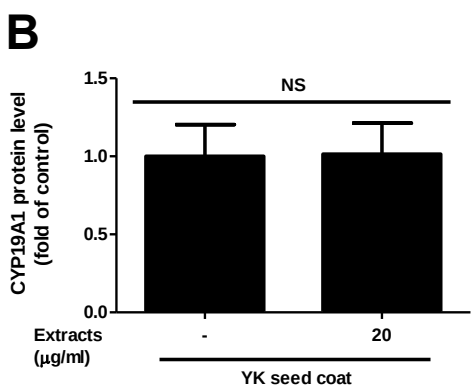
5-40 μg/ml of Yak-Kong seed coat extract were treated. After MCF-7 and OVCAR-8 cells were treated with different concentrations for 48 h, cell viability was evaluated by MTS assays. **A.** Cell viability of MCF-7 cells. **B.** Cell viability of OVCAR-8 cells. Data was shown as means ± SEM with n=5. Mean values with different letters (a-c) are significantly different from each other at $p<0.05$. YK, Yak-Kong.

7. Yak-Kong seed coat extract does not stimulate CYP19A1 expression in 3T3-L1 adipocytes, both mono- and co-cultured with MCF-7 cells

To observe the tissue-specific aromatase expression of Yak-Kong seed coat extract, 3T3-L1 cells were used in the presence or absence of breast cancer cells. Mono-cultured 3T3-L1 cells were treated with 20 μg/ml of Yak-Kong seed coat extract. 3T3-L1 cells co-cultured with MCF-7 cells were treated with different concentrations of Yak-Kong seed coat extract; 5, 10 and 20 μg/ml. Yak-Kong seed coat extract did not have any effect on protein levels of CYP19A1 both in mono-cultured adipocytes (Fig. 7B) and in adipocytes co-cultured with breast cancer cells (Fig. 7C).

Figure 7


A
Adipocyte Differentiation
Coculture 3 days
- Day 3
Day 0
Day 2
Day 4
Day 8
Day 11
3T3-L1 seeding
MDI medium
+ YK seed coat extract
INS medium
+ YK seed coat extract
FBS medium
+ YK seed coat extract
MCF-7 seeding
Protein prep
MCF-7
cells
Transwell
Adipocytes


B
CYP19A1 protein level
(fold of control)
NS
1.5
1.0
0.5
0.0
Extracts
(μg/ml)
-
20
YK seed coat

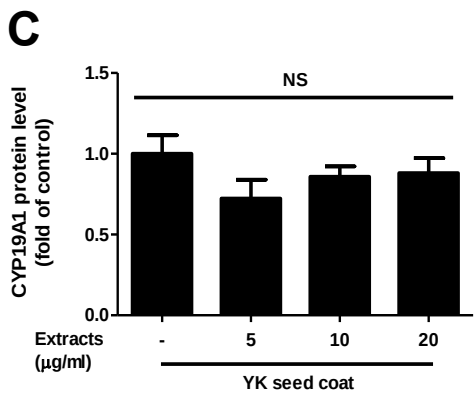

C
CYP19A1 protein level
(fold of control)
NS
1.5
1.0
0.5
0.0
Extracts
(μg/ml)
-
5
10
20
YK seed coat

Figure 7. Effects of Yak-Kong seed coat extract on protein levels of CYP19A1 in 3T3-L1 adipocytes in the presence or absence of MCF-7 cells

5-20 μg/ml of Yak-Kong seed coat extract were treated. After differentiation, 3T3-L1 adipocytes were co-cultured with MCF-7 cells for 3 days. Adipocytes were lysed and protein was extracted. The protein expression was measured by western blotting assay. **A.** Schematic illustration of adipocyte differentiation and co-culture experiment. **B.** Protein levels of CYP19A1 in mono-cultured adipocytes. **C.** Protein levels of CYP19A1 in adipocytes co-cultured with breast cancer cells. The loading control is β-actin. Data was shown as means ± SEM with n=3. Mean values were found to be non-significant ($P>0.05$) YK, Yak-Kong; NS, no significance.

IV. DISCUSSION

Soybeans are well-known as foods containing phytoestrogens [33], but the effect on estrogen biosynthesis has not been discovered. To identify estrogen stimulating effect of soybean extracts, H295R cells were cultured with four indicator extracts of soybean; yellow soybean seed coat extract, Yak-Kong seed coat extract, yellow soybean embryo extract, and Yak-Kong embryo extract. Yak-Kong seed coat extract had the most estradiol-stimulating effect in H295R cells among soybean extracts (Fig. 1). Thus, Yak-Kong seed coat extract was chosen as the study material for this project. The extract promoted production of 17β-estradiol in a dose dependent manner in H295R cells and mouse antral follicles (Fig. 2). It not only showed estradiol-stimulating effect of Yak-

Kong seed coat extract in a concentration dependent manner, but also suggested that the extract increases 17β-estradiol in ovary, the primary site of estrogen synthesis.

In steroidogenesis pathway, many steroidogenic enzymes are involved in estrogen production [23]. To determine which steroidogenic enzymes are induced by Yak-Kong seed coat extract, the protein and gene expression levels of steroidogenic enzymes were measured. Yak-Kong seed coat extract increased the expression of CYP19A1 (Fig. 3) and 3β-HSD (Fig. 4) in H295R cells. It means the extract promotes estrogen production by increasing the expression of CYP19A1, which converts more androgens into estrogens, and the expression of 3β-HSD, which increases the production of androgens as direct precursors of estrogens; androstenedione and testosterone. Yak-Kong seed coat extract did not have any effect on the rest of enzymes in steroidogenesis

pathway. (Fig. 5) It means the extract increases only the expression of CYP19A1 and 3β-HSD.

The stimulating effects of aromatase expression and estrogen production of Yak-Kong seed coat extract had to be further studied in aspect of estrogen-dependent cancer. In menopausal women, aromatase expression and estrogen production should be induced in sites with the symptoms of estrogen deprivation and inhibited in sites with risk of developing estrogen-dependent cancer. Although the stimulating effect of Yak-Kong seed coat extract to estrogen biosynthesis was demonstrated, it had to be confirmed that the extract did not develop estrogen-dependent cancer and stimulate aromatase expression at the local sites associated with estrogen-dependent cancer. To identify the anti-estrogen dependent cancer effect of Yak-Kong seed coat extract, viability of MCF-7 and OVCAR-8 cells treated the extract was evaluated. The local

estrogen stimulation promotes cell proliferation in breast epithelial cancer cell and ovarian epithelial cancer cell [20, 34]. However, even though Yak-Kong seed coat extract promoted estrogen biosynthesis and did not affect cell viability in other cells (Fig. 1 and 2), viability of estrogen-dependent cancer cells with the extract was decreased (Fig. 6). It suggests that bioactivity of Yak-Kong seed coat extract are tissue-specific [35].

To observe the tissue-specific aromatase stimulation of Yak-Kong seed coat extract, 3T3-L1 adipocytes were used in the presence or absence of breast cancer cells. In mono-cultured 3T3-L1 cells, Yak-Kong seed coat extract did not have any effect on aromatase expression (Fig. 7B). Adipose tissue adjacent to malignant breast epithelial cells accounts for most of the aromatase expression of breast tumors. Thus, it means Yak-Kong seed coat extract does not stimulate aromatase expression of adipose tissue

proximal to malignant cells. In 3T3-L1 cells co-cultured with MCF-7 cells, Yak-Kong seed coat extract also did not have any effect on aromatase expression (Fig. 7C). It suggests that the extract does not stimulate aromatase overexpression of adipose tissue in breast cancer microenvironment [36]. As a result, the effect of aromatase expression of Yak-Kong seed coat extract on 3T3-L1 cells was different from that of H295R cells.

In summary, Yak-Kong seed coat extract was discovered that can stimulate estrogen biosynthesis, suppress proliferation of estrogen-dependent cancer cells, and regulate aromatase expression tissue-specifically. These observations suggest that Yak-Kong seed coat extract could be used as an estradiol stimulating natural medicine candidate via tissue-specific regulation of aromatase expression in menopausal women.

For further study, Yak-Kong seed coat extract should be observed the stimulation of tissue-specific aromatase expression in aspect of distinct promoter regulation and tissue-specific estrogen biosynthesis.

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국문초록

에스트로겐은 여성의 생식과 성적 발달에 중요한 역할을 하는 스테로이드 호르몬이다. 여성은 노화가 진행됨에 따라 난소의 기능이 저하되고 에스트로겐의 분비가 감소하므로, 정상적인 에스트로겐 수준을 유지하는 것이 여성 건강에 중요하다. 아로마타제는 에스트로겐 합성에 필수적 효소이며, 조직 특이적으로 발현한다. 선택적 아로마타제 조절제 (selective aromatase modulator; SAM)는 에스트로겐 합성을 필요로 하는 부위에서 아로마타제를 발현할 수 있고, 에스트로겐 의존성 암 조직에서 아로마타제 과발현을 억제할 수 있다.

따라서 본 연구의 목적은 갱년기 및 폐경기 여성의 에스트로겐 저하 증상을 개선하기 위해 체내에서 에스트로겐을 근본적으로 생성하고,

에스트로겐 생성이 필수적인 조직에서 아로마타제의 발현을 증가시키는 천연물을 발견하는 것이다. 더불어 아로마타제 발현을 조직 특이적으로 조절하는 선택적 아로마타제 조절제로서의 작용을 확인하고자 한다.

본 연구를 통해, 약콩껍질추출물이 사람의 부신 피질 암세포주인 NCI-H295R 세포와 쥐의 난소에서 유래한 난포에서 17 베타-에스트라디올 생합성을 증가시키는 것을 관찰하였다. 약콩껍질추출물은 스테로이드 호르몬 생합성 경로에서 스테로이드 호르몬 생성 효소인 3β-HSD 와 CYP19A1 의 발현을 증가시킴으로써 17 베타-에스트라디올 생합성을 촉진하였다. 약콩껍질추출물은 부신 및 난소에서는 에스트로겐을 생합성 하였지만, 에스트로겐 의존성 암세포로 알려진 유방암 및 난소암세포에서는 세포 증식을 억제하였다. 더불어 H295R 세포에서 CYP19A1 의 발현을 증가시켰지만 단일 배양된 지방세포 및

유방암세포와 공동 배양된 지방세포에서 CYP19A1 의 발현에 영향을 주지 않았다.

결론적으로, 본 연구는 약콩껍질추출물이 조직 특이적으로 에스트로겐 생합성 및 아로마타제 발현을 촉진하는 에스트로겐 저하 증상 개선용 천연물 의약품 기능성 소재로 사용될 수 있음을 시사한다.