

RESEARCH ARTICLE

Inhibitory Aromatase Effects of Flavonoids from Ginkgo Biloba Extracts on Estrogen Biosynthesis

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Abstract

Ginkgo biloba extract (GBE) is a popular phytoedicine and has been used for disorders of the central nervous system, cardiovascular, renal, respiratory, and circulatory diseases. Although GBE is a complex mixture of over 300 compounds, its major components are 24% flavonoids and 6% terpene lactones. In this study, we tested the inhibitory effects of the three major flavonoids (kaempferol, quercetin, and isorhamnetin) from GBE, independently and as mixtures, on aromatase activity using JEG-3 cells (human placental cells) and recombinant proteins (human placental microsome). In both systems, kaempferol showed the strongest inhibitory effects among the three flavonoids; the flavanoid mixtures exerted increased inhibitory effects. The results of exon I.1-driven luciferase reporter gene assays supported the increased inhibitory effects of flavonoid mixtures, accompanied by suppression of estrogen biosynthesis. In the RT-PCR analysis, decreased patterns of aromatase promoter I.1 mRNA expressions were observed, which were similar to the aromatase inhibition patterns of flavonoids and their mixtures. The present study demonstrated that three flavonoids synergistically inhibit estrogen biosynthesis through aromatase inhibition, decrease CYP19 mRNA, and induce transcriptional suppression. Our results support the usefulness of flavonoids in adjuvant therapy for breast cancer by reducing estrogen levels with reduced adverse effects due to estrogen depletion.

Keywords: Aromatase - estrogen biosynthesis - breast cancer - flavonoids - ginkgo biloba extracts

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Introduction

Since estrogen is a key hormone that stimulates cancer growth during the initiation and promotion stages, regulating estrogen is important in the treatment of hormone-dependent breast cancer (Yager and Davidson, 2006). Locally produced estrogen plays a particularly important role in breast cancer promotion; therefore, lowering excessive estrogen is an important strategy for breast cancer treatment (Bernstein and Ross, 1993).

Cytochrome P450 aromatase (CYP19) is the enzyme responsible for the key step in the biosynthesis of estrogen. It catalyzes the last step of estrogen biosynthesis, the aromatization of androgens to estrogen (Chumsri et al., 2011). It has been found in various extragonadal tissues such as the brain, liver, skin fibroblasts, and adipose tissues, as well as in the ovaries and testis, which are the gonadal tissues. Higher levels of the aromatase gene and estrogen hormone were found in breast cancer tissues compared to normal breast tissues; therefore, the inhibition of aromatase is considered an effective targeted therapy for breast cancer, especially for postmenopausal breast cancer patients (Cazzaniga and Bonanni, 2012; Renoir et al., 2012). Endocrine therapy using aromatase inhibitor

can reduce the breast cancer risk, especially metastatic cancer (Behan et al., 2015; Iwase and Yamamoto, 2015; Mukhopadhyay et al., 2015).

Several studies have suggested that foods such as soy beans, whole grain cereals, red wine, citrus, and grapes are associated with lowering the risk of breast cancer when included in the diet (Adlercreutz, 1995; Eng et al., 2003; Kijima et al., 2006; Meiyanto et al., 2012; Bahadoran et al., 2013). These are plant-derived phytoestrogens that have estrogen-like biological activities that may have dual effects, acting as both antiestrogens and estrogens by competing with estrogen for binding to the estrogen receptor (Suganya et al., 2014). The aromatase inhibitory effects of phytoestrogens are emerging for the prevention or treatment of breast cancer (Hong and Chen, 2006). Food or traditional medicine-derived AIs are preferred for targeting specific tissues and are considered less toxic than other AIs currently in use (Balunas and Kinghorn, 2010).

Ginkgo biloba extract (GBE) is the most popular herbal extract. The role of GBE as an estrogen modulator in breast cancer therapy was investigated; GBE was a part of the Selective Estrogen Receptor Modulator (SERM) (Oh and Chung, 2004; Oh and Chung, 2006) and a Selective Estrogen Enzyme Modulator (SEEM) (Kim et al., 2013).

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The major components of standard GBE (EGB 761) are 24% flavonoids and 6% terpene lactones. Aromatase inhibitory effects of flavonoids from GBE (kaempferol, quercetin, and isorhamnetin) were previously reported (Khan et al., 2011; Lu et al., 2012). However, considering that flavonoids are present in mixed form, as in GBE, it is important to analyze the mixture effects of flavonoids on aromatase enzymes and estrogen biosynthesis. The aim of the present study was to elucidate the inhibitory effects of flavonoids from GBE and its mixtures, on aromatase activity and estrogen biosynthesis. The mechanism of aromatase inhibition was analyzed by measuring CYP19 mRNA expression and transcriptional activation.

Materials and Methods

Chemicals

The standard extracts of *G. biloba* (EGB 761) were provided and standardized by Yuyu Pharma, Inc. (Seoul, Korea); they were approved by the Korea Food & Drug Administration (KFDA). Kaempferol, quercetin, and isorhamnetin were obtained from Sigma-Aldrich Corp. (St Louis, Mo, USA) and were prepared in dimethyl sulfoxide (DMSO) at 0.1 M. [1β - ^3H] Androst-4-ene-3,17-dione (sp. Act.: 20.7Ci/mmol) was obtained from PerkinElmer Corp. (Waltham, MA, USA). All chemical solutions were stored at -20°C .

Cell Culture

The human placental choriocarcinoma cell line, JEG-3, was obtained from the American Tissue Culture Collection (Rockville, MD, USA). The JEG-3 cells were maintained in Dulbecco's modified Eagle's media (DMEM, GIBCO BRL, Invitrogen, Grand Island, NY, USA) supplemented with 5% Fetal Bovine Serum (FBS, Thermo Scientific Hyclone, Logan, UT, USA). Cells were incubated at 37°C in 5% $\text{CO}_2/95\%$ at 100% humidity. They were routinely sub-cultured after reaching a density of 80%.

Cell Viability Test

The cell viability assay, using JEG-3 cells, was performed according to the method presented by Perez et al. (1998). Briefly, the JEG-3 cells were harvested with 0.05% trypsin 0.53 mM EDTA \cdot 4Na (GIBCO BRL) and resuspended in 5% FBS in DMEM. The cells were seeded in 48-well plates at an initial concentration of 5,000 cells/well and allowed to attach for 48 h. To deplete the steroids from the experimental media, the seeding media (5% FBS in DMEM) was replaced with experimental media, 10% Charcoal dextran-treated FBS (CDFBS) supplemented with phenol red-free DMEM. Cell viability was determined by treating with flavonoids (10-500 μM) for 18 h. After 18 h of flavonoid treatment, the media was removed, and cells were fixed and stained with sulforhodamine-B (SRB). Cell viability was measured with an ELISA reader (Versamax, Molecular Devices, CA, USA) at 490 nm.

Aromatase enzyme activity

In vitro enzyme activity assays were performed, including a cellular aromatase assay and a recombinant

protein assay. Cellular aromatase assays were performed with JEG-3 cells using both a direct aromatase assay and an indirect aromatase assay. In the direct aromatase assay, the flavonoids were mixed with 54 nM [1β - ^3H] androstenedione; the cells were treated with this mixture for 2 h. In the indirect aromatase assay, cells were pretreated with the flavonoids for 18 h, and then 54 nM [1β - ^3H] androstenedione was added for an additional 1 h. After incubation at 37°C with 5% CO_2 , the supernatant was collected and extracted with chloroform. The aqueous supernatant was mixed with 5% charcoal/0.5% dextran and incubated for 15 min. The mixture was centrifuged at 14,000 g for 5 min, and the aromatase activity of the supernatant was measured using a liquid scintillation counter (LS-6500, Beckman counter, CA, USA) with a liquid scintillation cocktail (PerkinElmer[®], MA, USA). The level of radioactivity in [^3H] H_2O was normalized to the protein concentration.

Recombinant human CYP19 Supersomes[®] (BD Biosciences, NJ, USA) were used in NADPH solution A and NADPH solution B (BD Biosciences, NJ, USA). Solution A contained 31 mM NADP⁺, 66 mM Glucose-6-phosphate, and 66 mM MgCl_2 in H_2O ; Solution B contained 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate. Flavonoids were treated with Supersomes[®], the reaction was started by adding NADPH solutions A and B, and incubated at 37°C for 1 h according to manufacturer's protocol. The supernatant was collected, and aromatase activity was measured using a liquid scintillation counter.

RT-PCR analysis

JEG-3 cells were seeded in 6-well plates and GBE (500 $\mu\text{g}/\text{ml}$) and single flavonoids (K: 40.32 $\mu\text{g}/\text{ml}$, Q: 80.64 $\mu\text{g}/\text{ml}$, I: 5.04 $\mu\text{g}/\text{ml}$) or mixtures (M, 126 $\mu\text{g}/\text{ml}$) of the three flavonoids were added to the cells for 18 h with phenol red-free media containing 5% CDFBS-DMEM. After 18 h of incubation, the total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Three micrograms of cDNA were synthesized using Avian Myeloblastosis Virus (AMV) Reverse transcriptase (Promega, CA, USA) in a final volume of 30 μl . A polymerase chain reaction (PCR) was performed using tissue-specific primers and the mRNA expression levels of the coding domain sequence (CDS); aromatase exon I.1, I.3, and I.4 were analyzed (Mu et al., 2000). The PCR conditions were 94°C for 2 min, followed by 27 cycles of β -actin cDNA, and 35 cycles for aromatase p450arom cDNA at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 10 min. The sequences of the sense and antisense primers for the amplification of β -actin cDNA were 5'-TGG ACT TCG AGC AAG AGA TGG' and 5'-ATC TCC TTC TGC ATC CTG TCG-3'. The sequences of the sense/antisense primers for the amplification of the aromatase gene were 5'-CGG CCT TGT TCG TAT GGT CA-3' (CDS sense), 5'-GTC TCA TCT GGG TGC AAG GA-3' (CDS anti-sense), 5'-CTG GAG GGC TGA ACA CGT GG-3' (Exon I.1 sense), 5'-GCT TTC AAT TGG GAA TGC ACG-3' (Exon I.3 sense), 5'-GCA CCC TCT GAA GCA ACA GGA-3' (Exon I.4 sense), and 5'-GTG

TTC CTT GAC CTC AGA GG-3' (Exon common anti-sense). A PCR was performed using a MJ Research PCR Thermocycler (GMI Inc., MJ, USA). The primers were synthesized by Bioneer (Daejeon, South Korea). The size of the detected PCR products for CDS, exon I.1, I.3, and β -actin were 987, 113, 339, and 287 bp, respectively. The PCR-amplified products were resolved in a 2% agarose gel in 1× Tris-acetate EDTA buffer containing 5% ethidium bromide. The PCR products were identified using the Gel Documentation & Analysis System (UVP Inc., CA, USA) and quantified by Image J v1.43 software (National Institute of Health., MD, USA).

Reporter gene assay

The cytochrome P450arom promoter I.1 fragment was generated, and sequence information was obtained from the National Center for Biotechnology Information (NCBI) GenBank (NM_000103.3). A 710 bp fragment of promoter I.1 (Exon 1a; -700/+10) was prepared using the gene synthesis service from Integrated DNA Technologies, Inc. (IDT; Coralville, IA, USA). This fragment was inserted into the Kpn I and Hind III sites of the pGL4-basic vector (Promega, WI, USA). Ligation was performed using T4 ligase reagents (Promega) according to the manufacturer's instructions. JEG-3 cells were seeded in a 48-well plate at a density of 8×10^4 /well. After 18 h of incubation, the cells were washed twice with FBS-free OPTI-MEM I and 100 μ l FBS-free OPTI-MEM I media was added to each well. After mixing 120 ng/well of DNA and 4 μ l/well PLUS reagent in 100 μ l FBS-free OPTI-MEM I media, it was incubated at room temperature for 15 min. After mixing 2 μ l/well Lipofectamine and the DNA/PLUS mixture, it was incubated at room temperature for 15 min before being added to the JEG-3 cells. The resulting plasmid/Lipofectamine solution (200 μ l) was added to each well and incubated in a 5% CO₂ incubator at 37°C for 3 h. After 3 h, the cells were washed with serum-free DMEM media twice, and then treated with the flavonoids (10-100 μ M) for 18 h. The final concentration of the vehicle (DMSO) in the experimental media was adjusted to 0.1%. After 18 h of incubation, the cells were lysed with 100 μ l passive lysis buffer in a dual luciferase kit (Promega) according to the manufacturer's protocol. The luciferase activity was normalized with pRL-Tk control vector expression.

Determination of estradiol biosynthesis

JEG-3 cells were seeded in a 24-well plate and cultured overnight. The next day, GBE (500 μ g/ml), single flavonoids (K: 40.32 μ g/ml, Q: 80.64 μ g/ml, I: 5.04 μ g/ml), or mixed samples (M, 126 μ g/ml) of the three flavonoids were added to the cells with phenol red free 5% CDFBS-DMEM for 12 h. Concentrations of each flavonoid were determined by considering the amount of flavonoids in 500 μ g/ml GBE. After incubation, testosterone (10 nM) was added to each well and the cells were incubated for 12 h. At the end of this incubation, the culture supernatants were collected and stored at -20°C. Levels of 17 β -estradiol in the supernatants were quantified using a competitive enzyme-immunoassay (EIA), according to the manufacturer's instructions (Cayman chemical, Ann Arbor, MI, USA). Duplicated

samples were analyzed and the product of this enzymatic reaction was measured with an ELISA reader at 412 nm.

Data analysis

Sigma Plot 10.0 (Jandel Science Software, San Rafael, CA, USA) and Excel 2007 (Microsoft, Redmond, WA, USA) were used to analyze the data. Each *in vitro* assay was performed in triplicate. The data from each assay are expressed as mean \pm standard deviations (SD). Statistical analysis was performed using the SPSS (version 18.0 SPSS Inc., Chicago, IL, USA) program. Differences between groups were assessed by Duncan's post hoc test following one-way analysis of variance (ANOVA). Statistical significance was accepted at $p < 0.05$.

Results

Effect on viability of JEG-3 cells

Cell viability was measured to determine the concentration of flavonoids causing cytotoxicity in JEG-3 cells. Cells were exposed to the flavonoids (10-100 μ M) for 18 h and stained with the SRB method (Figure 1). Tests confirmed the acceptable treatment concentrations of the flavonoids in JEG-3 cells. The exposure concentrations (10-100 μ M) of each flavonoid were chosen, considering cytotoxicity was not observed at a rate of more than 30% higher than the cells treated with the vehicle control (DMSO).

Direct inhibitory effects on aromatase activity using recombinant protein assay and cellular aromatase assay

To evaluate the direct inhibitory effects of the three flavonoids on aromatase activity, the recombinant protein assay and cellular aromatase assay methods were employed, which have been widely used for screening aromatase activity. The aromatase assay using the recombinant protein was performed using recombinant human CYP19 Supersomes® (BD Biosciences, NJ, USA). To find out the major contributor to the inhibitory effects of GBE on aromatase activity, three major flavonoids (kaempferol, quercetin, isorhamnetin) were tested. As shown in Figure 2A, kaempferol significantly inhibited the aromatase activity at 50 μ M (** $p < 0.01$), while quercetin (** $p < 0.01$) and isorhamnetin (* $p < 0.05$) significantly inhibited activity at 100 μ M. IC₅₀ was calculated from the results and is shown in Table 1. In the recombinant protein assay, kaempferol showed the lowest IC₅₀ (87.7 μ M) compared to quercetin (141.3 μ M) and isorhamnetin (N.C).

The cellular aromatase assay was performed using JEG-3 cells, which endogenously express a large amount of the aromatase gene. To find out the direct effects of the flavonoids, tritiated androstenedione [1β -³H] was mixed with GBE (500 μ g/ml) and added to a mixture of the three flavonoids (M, 126 μ g/ml) or to single flavonoids (kaempferol: 40.32 μ g/ml, quercetin: 80.64 μ g/ml, isorhamnetin: 5.04 μ g/ml) individually. This mixture was incubated with the JEG-3 cells for 2 h. Concentrations of each flavonoid were decided by considering the amount of flavonoids present in 500 μ g/ml GBE. GBE directly inhibited the aromatase activity by approximately 59.9%

compared to the vehicle-treated group; however, mixtures of the three flavonoids showed even more potent aromatase inhibitory effects (89.9% compared to the vehicle control group). Aromatase activity was significantly inhibited by 80.64 $\mu\text{g/ml}$ of quercetin and 40.32 $\mu\text{g/ml}$ of kaempferol by approximately 66.9% and 73.8%, respectively (Figure 2B) ($p < 0.01$).

Indirect inhibitory effects on aromatase activity using cellular aromatase assay

Indirect inhibitory effects of the flavonoids were tested in JEG-3 cells. To determine the indirect effects of the flavonoids on aromatase activity, cells were treated with various concentrations (10, 50, and 100 μM) of the three flavonoids, GBE (500 $\mu\text{g/ml}$), and single flavonoids at concentrations similar to those present in GBE (kaempferol: 40.32 $\mu\text{g/ml}$, quercetin: 80.64 $\mu\text{g/ml}$, isorhamnetin: 5.04 $\mu\text{g/ml}$) or mixtures of the three flavonoids (M, 126 $\mu\text{g/ml}$) for 18 h at 37°C.

The three flavonoids caused a significant, dose-dependent decrease in aromatase activities in the JEG-3 cells (Figure 3A). Kaempferol (50 μM) decreased aromatase activity by about 94.2% compared to the

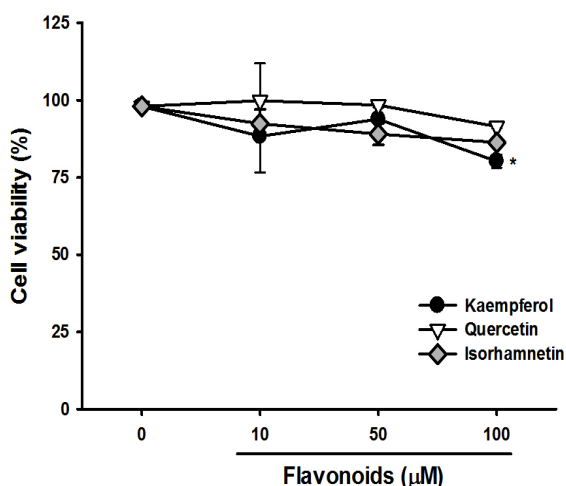


Figure 1. Effects of Flavonoids on the Viability of JEG-3 Cells. JEG-3 cells were seeded in 48-well plates, maintained in 5% charcoal-dextran supplemented with phenol red-free Dulbecco's modified Eagle's medium (DMEM), and treated with various concentrations (10, 50, and 100 μM) of the three flavonoids for 18 h. The sulforhodamine-B (SRB) assay was conducted to measure cell viability. The results are expressed as mean \pm SD of three separate experiments for each group. * Represents the statistical difference from the vehicle control [Con, 0.1 % dimethyl sulfoxide (DMSO)]: * $p < 0.05$

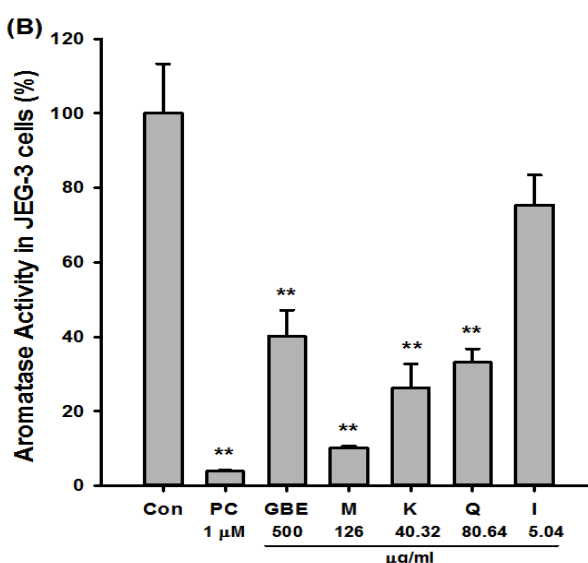
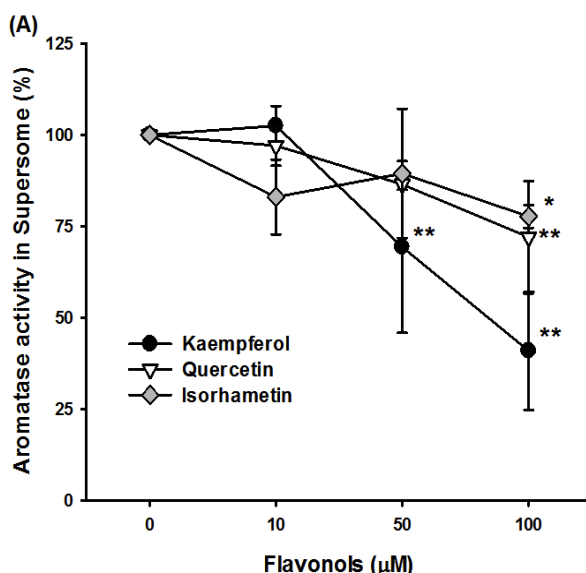


Figure 2. Direct Inhibitory Effects of Flavonoids on Aromatase Activity was Analyzed in Supersome™ (A) and JEG-3 (B) Cells. Tritiated androstenedione [1β - ^3H] was mixed with various concentrations (10-100 μM) of three flavonoids (Q: quercetin; K: kaempferol; I: isorhamnetin) and treated with recombinant CYP19 (CYP19-Supersome™) for 1 h (A). Tritiated androstenedione [1β - ^3H], Ginkgo biloba extract (GBE, 500 $\mu\text{g/ml}$), and mixed samples (M, 126 $\mu\text{g/ml}$) of the three flavonoids (K: 40.32 $\mu\text{g/ml}$; Q: 80.64 $\mu\text{g/ml}$; I: 5.04 $\mu\text{g/ml}$) were incubated in JEG-3 cells for 2 h (B). 10^{-6}M HA (4-androsten-4-ol-3, 17-dione) was used as a positive control (PC). The results are expressed as mean \pm SD of three separate experiments for each group. * Represents the statistical difference from the vehicle control [Con, 0.2% Dimethyl sulfoxide (DMSO)]: * $p < 0.05$, ** $p < 0.01$

Table 1. Aromatase Inhibitory Activity of the Flavonoids from in Ginkgo Biloba Extracts ($\text{IC}_{50} = \mu\text{M}$)

	Human microsome assay		In-cell assay		
	Human placenta microsome ^a	Present study (Recombinant protein)	JAR cells ^b	JEG-3 cells ^c	Present study (JEG-3 cells)
Kaempferol	- ^d	87.7	161.6	11.0	31.5
Quercetin	12.0	141.3	86.4	>100.0	53.3
Isorhamnetin	- ^d	N.C. ^e	- ^d	N.C. ^e	99.7

^a Kellis and Vickery, 1984; ^b Monteiro et al., 2006; ^c Saارين et al., 2001; ^d Result is not available; ^e N.C.: Not calculated

vehicle-treated group. Aromatase activity decreased with 100 μM of isorhamnetin and quercetin by approximately 49.0% and 85.4%, respectively, compared to the vehicle control group.

GBE indirectly inhibited the aromatase activity by about 38.4% compared to the vehicle-treated group in JEG-3 cells, which was less than the activity of the mixture of three flavonoids (92.3%). Aromatase activity was dramatically inhibited following 80.64 $\mu\text{g/ml}$ quercetin and 40.32 $\mu\text{g/ml}$ kaempferol by approximately 73.1% and 86.4%, respectively, compared to the vehicle control group (Figure 3B) ($p < 0.01$). However, isorhamnetin slightly increased aromatase expression, compared to the vehicle-treated group (111.8%).

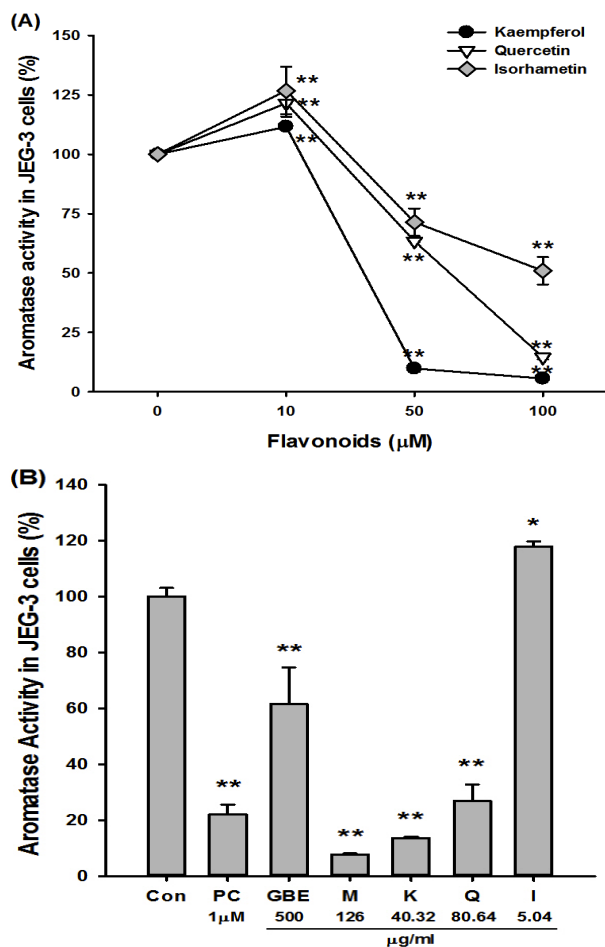


Figure 3. Indirect Inhibitory Effects of the Flavonoids on the Aromatase Activity were Analyzed in JEG-3 Cells. Tests were designed to elucidate individual effects (A) and mixed effects (B). The cells were seeded in 12-well plates, maintained in 5% charcoal-dextran supplemented with phenol red-free Dulbecco's modified Eagle's medium (DMEM) and treated with various concentrations (10, 50, and 100 μM) of the three flavonoids (Q: quercetin; K: kaempferol; I: isorhamnetin), Ginkgo biloba extract (GBE, 500 $\mu\text{g/ml}$), and single flavonoids (K: 40.32 $\mu\text{g/ml}$; Q: 80.64 $\mu\text{g/ml}$; I: 5.04 $\mu\text{g/ml}$) or mixed samples (M, 126 $\mu\text{g/ml}$) of the three flavonoids for 18 h at 37 $^{\circ}\text{C}$. 10^{-6}M HA (4-androsten-4-ol-3, 17-dione) was used as a positive control (PC). The aromatase activity was determined using the tritiated water-release assay after treating the cells with 54 nM [^3H] andro-4-ene-3, 17-dione for 1 h. The results are expressed as mean \pm SD of three separate experiments for each group. * Represents the statistical difference from the vehicle control [Con, 0.1% dimethyl sulfoxide (DMSO)]; ** $p < 0.01$

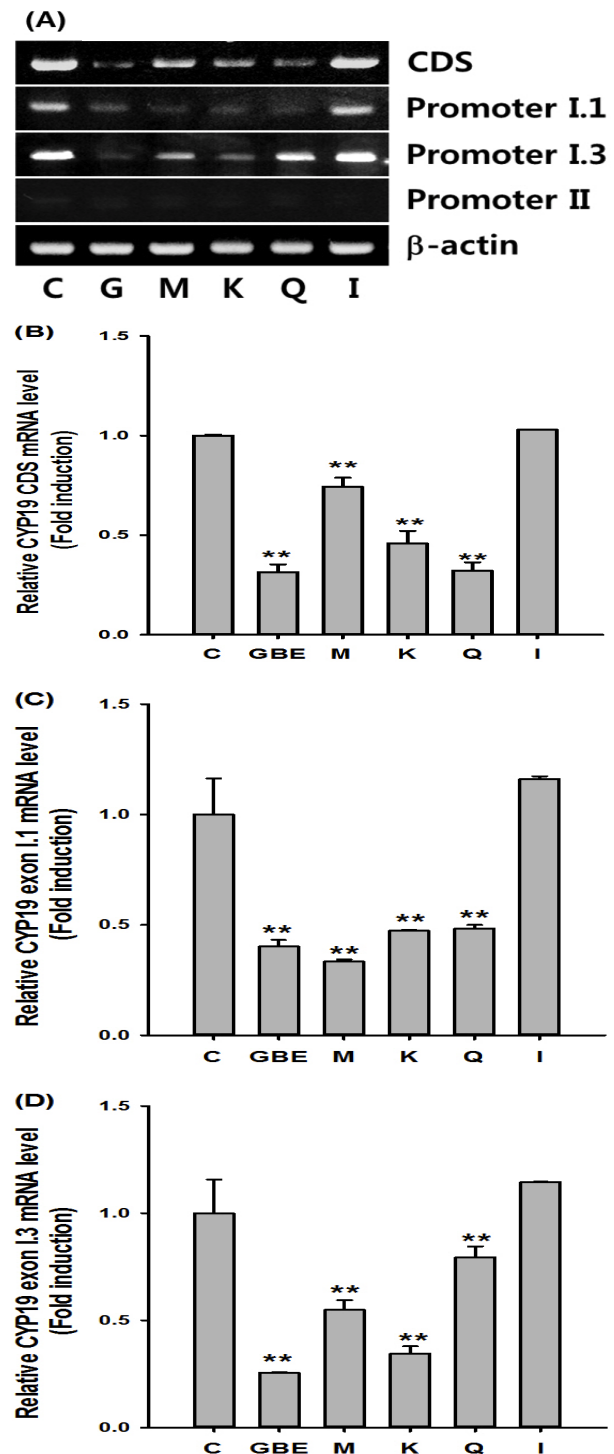


Figure 4. Inhibitory Effects of the Flavonoids on CYP19 mRNA Expression in JEG-3 Cells. The cells were treated with individual flavonoids or their mixtures (K: 40.32 $\mu\text{g/ml}$; Q: 80.64 $\mu\text{g/ml}$; I: 5.04 $\mu\text{g/ml}$) and Ginkgo biloba extract (GBE, 500 $\mu\text{g/ml}$), (C: control; G: GBE; M: mixture sample; K: kaempferol; Q: quercetin; I: isorhamnetin). After incubation for 18 h at 37 $^{\circ}\text{C}$, total RNA was extracted with a Trizol reagent. CYP19 mRNA expression was measured using reverse transcription-polymerase chain reaction (RT-PCR) analysis method (A). The mRNA expression was measured by each expression of coding domain sequence (CDS) region (B), exon I.1 (C), and exon I.3 (D). They were quantified by Image J and normalized to β -actin mRNA levels. The results are expressed as mean \pm SD of three separate experiments for each group. * Represents the statistical difference from the vehicle control [Con, 0.1% dimethyl sulfoxide (DMSO)]; * $p < 0.05$, ** $p < 0.01$

IC₅₀ was calculated from the results and shown in Table 1. IC₅₀ was lower in the JEG-3 cells than in the human microsomes, and kaempferol showed the lowest IC₅₀ value (kaempferol: 31.5, quercetin: 53.3, isorhamnetin: 99.7 μg/ml) in the indirect aromatase inhibition system.

Inhibitory effects on CYP19 mRNA expression

In RT-PCR analysis, to determine the mechanism of action of the aromatase inhibitory effects, primers were designed for the aromatase CDS and promoter I.1, I.3, and II; however, the PCR product of promoter II could not be sufficiently measured (Figure 4A). As shown in Figure 4A, the aromatase promoter I.1, I.3 mRNA expression, and coding sequence decreased following treatment with the flavonoids compared to the vehicle

control groups. Expression of mRNA was quantified by Image J and normalized to the β-actin mRNA levels. In this study, GBE, mixtures of the three flavonoids, and each individual flavonoid significantly decreased the CYP19 mRNA expression, except for isorhamnetin (Figure 4B, 4C, 4D). These results showed that the inhibitory effects on aromatase activity were accompanied by mRNA changes and mainly controlled by promoter I.1 and I.3 in JEG-3 cells.

Inhibitory effects on transcriptional activity of CYP19 promoter I.1

To figure out the mechanism of action of the aromatase inhibitory effects, CYP19 promoter regulation was measured using a luciferase reporter gene assay. Cells were treated with various concentrations (10, 50, and 100 μM) of the three flavonoids, GBE (500 μg/ml), and individual flavonoids at concentrations similar to that in 500 μg/ml GBE (K: 40.32 μg/ml, Q: 80.64 μg/ml, I: 5.04 μg/ml) or mixtures of the flavonoids (M, 126 μg/ml). Results indicated that the three flavonoids significantly suppressed activation of the CYP19 promoter I.1. Among the three flavonoids, kaempferol showed the most potent suppression effect; kaempferol significantly suppressed CYP19 promoter I.1 starting with concentrations of 10 μM, while quercetin and isorhamnetin also significantly inhibited CYP19 promoter I.1 from 40 μM (Figure 5A).

GBE suppressed activation of CYP19 promoter I.1 by about 90.1% compared to the vehicle-treated group in JEG-3 cells, while mixtures of the three flavonoids showed even more potent inhibitory effects than GBE

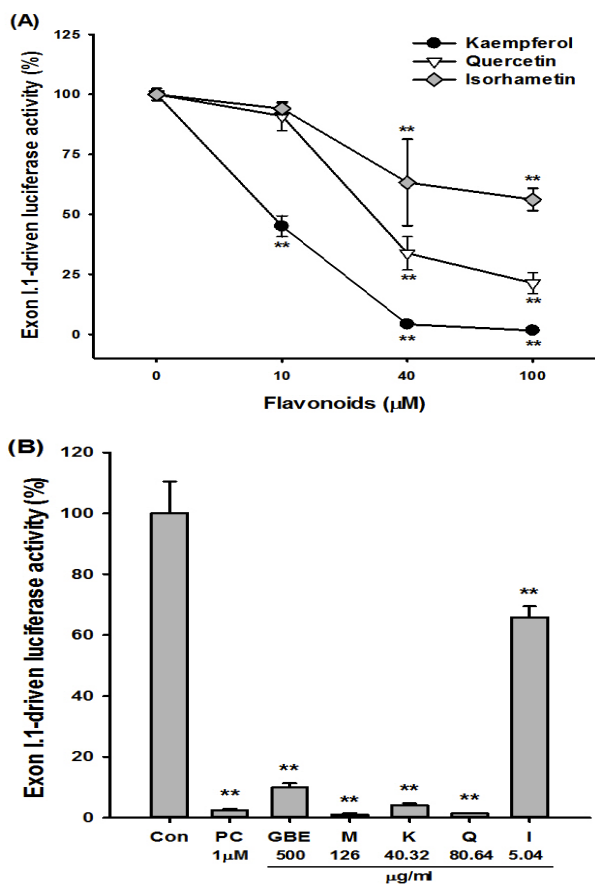


Figure 5. Inhibitory Effects of Flavonoids on Aromatase Transcriptional Activity in JEG-3 Cells. A reporter plasmid, constructed from a fragment of human CYP19 promoter I.1 from human genomic DNA, was digested and subcloned into firefly luciferase pGL3 basic reporter vector. The JEG-3 cells were transfected with the CYP19 I.1 promoter construct and pRL-TK using Lipofectamine reagent. Tests were designed to elucidate single effects (A) and mixed effects (B). The cells were treated with various concentrations (10, 40, and 100 μM) of three flavonoids (Q: quercetin; K: kaempferol; I: isorhamnetin) and Ginkgo biloba extract (GBE, 500 μg/ml), alone (K: 40.32 μg/ml; Q: 80.64 μg/ml; I: 5.04 μg/ml) or in mixed samples (M, 126 μg/ml) of three flavonoids for 18 h at 37 °C. The activities of the CYP19 promoter represented by firefly luciferase light units were then normalized to that of renilla luciferase. The results are expressed as mean ± SD of three separate experiments for each group. * Represents the statistical difference from the vehicle control [Con, 0.1% dimethyl sulfoxide (DMSO)]; * p<0.05, ** p<0.01

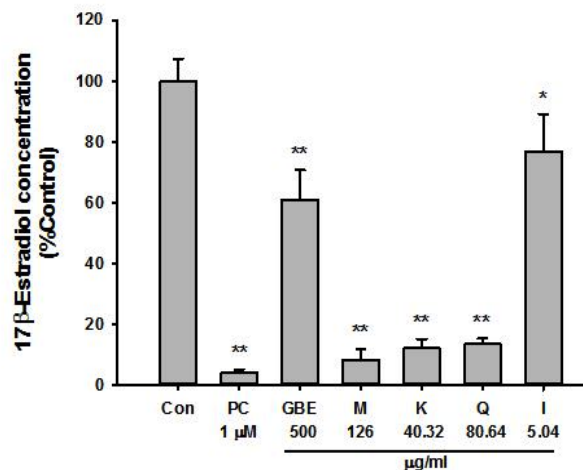


Figure 6. Inhibitory Effects of Flavonoids on 17β-estradiol (E2) Biosynthesis in JEG-3 cells. The cells were seeded in 24-well plates, maintained in 5% charcoal-dextran supplemented with phenol red-free Dulbecco's modified Eagle's medium (DMEM), and treated with Ginkgo biloba extract (GBE, 500 μg/ml) and flavonoids alone (K: 40.32 μg/ml; Q: 80.64 μg/ml; I: 5.04 μg/ml) or mixed samples (M, 126 μg/ml) of three flavonoids for 12 h at 37 °C. 10⁻⁶M HA (4-androsten-4-ol-3, 17-dione) was used as a positive control (PC). Testosterone was added for 12 h further, E2 concentrations in the culture supernatants were quantified by a E2 enzyme-immunoassay (EIA) kit. The results are expressed as mean ± SD of three separate experiments for each group. * Represents the statistical difference from the vehicle control [Con, 0.4 % dimethyl sulfoxide (DMSO)]; * p<0.05, ** p<0.01

(98.9%). CYP19 promoter I.1 activation was also dramatically inhibited with 80.64 $\mu\text{g/ml}$ quercetin and 40.32 $\mu\text{g/ml}$ kaempferol by approximately 98.5% and 95.9%, respectively, compared to the vehicle control group (Figure 5B). Isorhamnetin also showed inhibitory effects compared to the vehicle control group (34.2%) ($p < 0.01$).

Inhibitory effects on estrogen biosynthesis

To determine the effects of the flavonoids on 17β -estradiol biosynthesis, JEG-3 cells were incubated for 12 h with various concentrations of the test compounds, followed by 12 h incubation with 10 nM testosterone. Inhibitory effects on estrogen biosynthesis by the mixtures of the three flavonoids and each individual flavonoid were measured. As shown in Figure 6, GBE (500 $\mu\text{g/ml}$), kaempferol (40.32 $\mu\text{g/ml}$), and the mixtures of flavonoids (126 $\mu\text{g/ml}$) strongly inhibited estrogen biosynthesis, by about 38.9%, 87.9%, and 91.8%, respectively, compared to the control. This result indicated that GBE significantly inhibited estrogen biosynthesis by inhibiting aromatase expression; kaempferol is a major component of the aromatase inhibitory effect.

Discussion

GBE is one of the most widely used natural products for treating bronchial asthma (Mahmoud et al., 2000), memory loss (Rigney et al., 1999; Cieza et al., 2003), diabetes, and circulatory diseases (Kudolo, 2001; Kudolo and Delaney, 2001). GBE showed anticancer effects related to antioxidant, antiangiogenic, and DNA damage repair activities; however, its anticancer effects as an estrogen modulator were not well identified. Standard GBE (EGb 761) is a complex extract composed of over 300 components and contains 22% to 27% flavonoid glycosides (kaempferol, quercetin, and isorhamnetin) and 6% terpene lactones (ginkgolides, bilobalide) (Kleijnen and Knipschild, 1992; Dubber and Kanfer, 2004). Although aromatase inhibitory effects of flavonoids (kaempferol, quercetin, and isorhamnetin) were reported, all experimental studies have focused on screening aromatase inhibitors using only single phytochemicals; mixture effects have not been investigated (Khan et al., 2011; Lu et al., 2012). However, phytochemicals exist as complex mixtures in nature, and different combinations of flavonoids may alter the actions on aromatase through different mechanisms, such as different solubility or accessibility (Lu et al., 2012). In the present study, we elucidated the inhibitory effects of flavonoids from GBE, and its mixture effects were evaluated using recombinant protein (human placental microsomes) and JEG-3 cells (human placental cells). Aromatase inhibition was evaluated by treating with chemicals directly and indirectly. To determine the mechanism of aromatase inhibition, promoter-specific regulation of aromatase was assessed by measuring CYP19 mRNA expression and transcriptional activation.

Placental microsomes and human choriocarcinoma cell line, JEG-3, are widely used to screen the aromatase inhibitory effects (Kellis and Vickery, 1984; Yue and Brodie, 1997). They are rapid and inexpensive methods for

measuring the effects of chemicals on aromatase activity, which can be determined by measuring the amount of $^3\text{H}_2\text{O}$ released from [1β - ^3H]-androstenedione substrate. In this study, although the inhibitory effects on JEG-3 cells are more sensitive than that in Supersome[®], their tendency was similar; kaempferol showed the strongest effects, while isorhamnetin showed the weakest effects. From the analysis of the aromatase inhibitory effects of the three flavonoids, IC_{50} was calculated and compared with values obtained in other studies (Table 1) (Kellis and Vickery, 1984; Saarinen et al., 2001; Monteiro et al., 2006). The sensitivity of each test method was different; however, these studies indicated that kaempferol and quercetin both have strong aromatase inhibitory effects among the flavonoids.

Direct aromatase inhibition testing can evaluate the chemical's effects on the enzyme itself (Plant, 2004). To evaluate the direct inhibitory effects of test compounds on aromatase enzyme, the microsomal aromatase assay was performed using recombinant human CYP19 Supersomes[®]. This assay is a well-established source of cDNA-expressed enzymes for the study of metabolism and contains NADPH and cytochrome P450. Its advantage is that it does not depend on cytotoxic effects of chemicals (Huang et al., 2007). In microsomal aromatase assay, kaempferol and quercetin, but not isorhamnetin, significantly inhibited aromatase activities at each concentration (Figure 2A). They inhibit aromatase enzyme without contribution of other enzymes to the metabolism of compounds (Plant, 2004). This result also showed that mixture effects on direct aromatase inhibition were higher than each flavonoid (Figure 2B).

When we evaluate the inhibitory effects, both direct and indirect aromatase inhibition are important, because chemicals can act through feedback mechanisms that can result in up or downregulation of estrogen biosynthesis. To assess the indirect inhibitory effects on the aromatase enzyme, we used the human JEG-3 choriocarcinoma cell line, which has been widely used as an *in vitro* system for measuring the aromatase inhibitory activity for academic and industrial purposes (Kohler and Bridson, 1971). Kaempferol showed the strongest aromatase inhibitory effects among the three flavonoids (Figure 3A). All flavonoids increased aromatase activity slightly when added at low concentrations (10 μM); however, they significantly inhibited aromatase activity in a dose-dependent manner ($p < 0.01$). Our results indicated that inhibitory effects increased when flavonoids were mixed, and increased aromatase activity by isorhamnetin did not affect the mixture's inhibitory effects (Figure 3 B).

Aromatase has been found in various tissues and its transcription is regulated by alternative splicing using a tissue-specific exon I; tissue-specific aromatase promoters determine the aromatase activity and estrogen biosynthesis (To et al., 2015). Estrogen is involved in various physiological functions through endocrine, autocrine, and paracrine actions; therefore, tissue-specific promoter regulation is important. The aromatase gene spans a stretch of DNA of over 123 kb (Bulun et al., 2003). The placenta-specific promoter I.1 and fetal liver/skin fibroblast-specific promoter I.4 were located more than

93 and 73 kb, respectively, upstream of placental exon II, whereas the ovary-specific promoter I.3 and promoter II were located within the 1 kb region upstream of the ATG translation start site in exon II (Harada et al., 1993; Bulun et al., 2003). Based on this principle, promoter-specific aromatase regulation was measured using the RT-PCR method and analyzed using Image J (Figure 4). Our results showed that flavonoids and their mixtures decreased CYP19 CDS and promoter I.3, especially promoter I.1. Promoter I.1 is known for regulating aromatase expression in placental tissues and is the most distally located promoter (93 kb) from the translation start site in exon II (Kamat et al., 1998).

A reporter gene assay was performed using CYP19 promoter I.1 to elucidate the effects of the flavonoids on transcriptional activation. CYP19 promoter I.1 luciferase vector was designed, composed of a 5'-deleted aromatase promoter (-700/+10) fused up-stream of the luciferase reporter gene in the pGL4-basic vector. The reporter gene assay results indicated that the transcriptional activation of CYP19 promoter I.1 was significantly suppressed by the three flavonoids, even at 40 μ M (Figure 5A). Inhibitory effects of the flavonoid mixtures on aromatase transcription were higher than each individual flavonoid component (Figure 5B). Since CYP19 promoter I.1 is mainly expressed in placental cells, this result demonstrated that flavonoids could downregulate the transcriptional activity of the aromatase enzyme. These mRNA expression and reporter gene assay results indicated that flavonoids and their mixtures significantly inhibit the expression of aromatase, by inhibiting transcriptional activation through CYP19 mRNA expression. It suggests their role in reducing the estrogen levels in JEG-3 cells.

Regulation of estrogen levels is important to prevent or treat breast cancer. There is clear evidence indicating that high circulating levels of endogenous estrogens can increase breast cancer risk (Bernstein and Ross, 1993). In postmenopausal women, ovaries are no longer a major source of estrogen; rather, peripheral tissues are the major producers. AIs are known to work at the peripheral tissues and they decrease the local estrogen levels by blocking the conversion of androgen into estrogens, thereby becoming the first line of therapy for Estrogen receptor (ER)-positive postmenopausal women (Brueggemeier et al., 2005).

Although many vegetables and fruits can inhibit aromatase enzyme, it is not clear that they inhibit estrogen biosynthesis (Chen et al., 2006; Kijima et al., 2006; Wang et al., 2006). To elucidate the relationship between aromatase inhibition and estrogen biosynthesis by flavonoid treatment, the estrogen level was measured after flavonoid treatment (Figure 6). Decreased estrogen biosynthesis patterns indicate that flavonoids and their mixtures inhibited estrogen biosynthesis through aromatase enzyme inhibition, and their mixtures increased the inhibitory effects synergistically.

The aromatase enzyme has a very specific function in steroid biosynthesis and its inhibition is a targeted approach for breast cancer treatment with minimal side effects, since it would not interfere with the biosynthesis of other steroids. In the present study, we evaluated aromatase inhibitory effects of three

flavonoids and their mixture effects from GBE. GBE has preventive activity on osteoporosis, which is the major adverse effect of aromatase inhibitor (Oh et al., 2008). Our results suggest that three flavonoids from GBE synergistically inhibit estrogen biosynthesis through aromatase inhibition, decreasing CYP19 mRNA, and transcriptional suppression. Use of flavonoids to reduce breast cancer risk is still controversial. Our results support the concept that flavonoid administration may be useful for adjuvant therapy for breast cancer by reducing estrogen level with reduced risk of undesirable adverse effects due to estrogen depletion.

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