



• Original Article

Ginkgo biloba extract (EGb761) did not express estrogenic activity in an immature rat uterotrophic assay

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Ginkgo biloba is a dioecious tree that has been used in traditional Chinese medicine for about 5,000 years. In previous studies on *Ginkgo biloba* extract (EGb761) using *in vitro* systems, we confirmed that EGb761 has biphasic effects on estrogenicity. In this study, we evaluated the agonistic and antagonistic activities of EGb761 using a uterotrophic assay in immature female rats. To evaluate agonistic and antagonistic effects of EGb761 on uterus, 21-day-old immature Sprague-Dawley (SD) female rats were treated with EGb761 (100, 200, or 400 mg/kg) by oral gavage, 10 µg/kg of estradiol (E2) or 1 mg/kg tamoxifen (TM) by subcutaneous injection, or with EGb761 plus E2 or TM for 3 consecutive days. At the end of the treatment period, animals were sacrificed and their body weights and organ weights (liver, lung, spleen and kidney) were measured. In addition, estrogen-related gene expressions (IGFBP-1 in liver and CaBP-9 in uterus) were determined. During the experiment, no animal showed clinical signs, a change in body weight or died. EGb761 treatment alone had no effect on absolute/relative uterine weight, luminal epithelial cell height (LECH, µm), or luminal circumference (LC, µm). In addition, uterine weights, LECHs, and LC induced by E2 or TM were not significantly changed by EGb761 at any dose. These results collectively suggested EGb761 has no agonistic/antagonistic effects in utero.

Keywords: *Ginkgo biloba* extract, EGb761, uterus, uterotrophic assay

INTRODUCTION

Ginkgo biloba is a dioecious tree that has been used in traditional Chinese medicine for about 5,000 years and is now also commercially cultivated in France and the USA [1]. Clinically, extracts of *Ginkgo biloba* extract (GBE) of leaves have been used to treat cerebrovascular and peripheral vascular insufficiency [2] and CNS disorders such as Alzheimer's disease and cognitive deficits [3]. The standardized extracts of *Ginkgo biloba* leaves, EGb761 (trade names *Tebonin*[®], *Tanakan*[®], *Rökan*[®]), were introduced by the Dr. Willmar Schwabe group (Karlsruhe, Germany) and was developed in Europe [4]. It contains a high concentration of phytoestrogens including; flavonoid glycosides (Ginkgo-flavone glycosides, 24%) and terpenoid lactones (ginkgolides and bilobalides, 6%) [5,6].

Phytoestrogens are near ideal selective estrogen receptor modulators (SERMs) and are considered powerful herbal

remedies [7]. SERMs have organ-specific estrogenic activity and exhibit antagonistic activity in uterus and breast, and agonistic activity in bone, the cardiovascular system, and brain [8]. Furthermore, plant-derived products containing these compounds might have potential use for hormone replacement therapy (HRT) due to their ER-mediated molecular mechanisms [9]. GBE has been similarly reported to have estrogenic and antiestrogenic activities as determined by *in vitro* assay [10,11]. It has anticancer properties related to its antiangiogenic, antioxidant effects [12,13] and selective estrogen enzyme modulatory effects [14,15]. Due to these characteristics, GBE has been considered an HRT for post-menopausal women and breast cancer patients. However, several studies have reported recently HRT might have serious side effects, including endometrial cancer [16]. Therefore, to confirm the side effects on uterus of EGb761, we evaluated its agonistic and antagonistic potentials on uterus using a uterotrophic assay. Uterotrophic assays provide an internationally recognized means of determining the estrogenic and antiestrogenic potential of chemicals [17,18,19] and are based on the development of uterine tissue in the presence of estrogen. In this study, 21-day-old immature female rats were exposed to EGb761 alone or EGb761 plus 17β-estradiol (E2) or tamoxifen

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(TM). We documented changes in their uterus weights, histologically determined luminal epithelial cell heights (LECH, μm) and luminal circumferences (LC, μm), and in estrogen-related gene expressions in uterus and liver tissues.

METHODS

Chemicals

E2 and TM were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 100% ethanol to produce 0.1 and 10 mg/mL stock solutions, respectively, which were stored at -20°C prior to experiments. The standardized *Ginkgo biloba* extract (EGb761) was provided by Yuyu pharma Inc. (Seoul, Korea); it contains flavonoids (25.2%) and terpene lactones (5.8%). Prior to treatment, EGb761 diluted with water to concentrations of 10, 20, and 40 mg/mL.

Animals

Female Crj: CD rats (14 days old) were obtained from Dae-Han Bio Link Co., Ltd (Chungbuk, Korea), acclimated for 7 days and provided tap water and a commercial pellet diet (DHL, Co., Ltd, Korea) *ad libitum*. Animals were maintained in an environmentally controlled room (room temperature, $21\pm 2^{\circ}\text{C}$; room humidity, $50\pm 20\%$) under a 12-h light/dark cycle. During the acclimation and 3 days experiment, general health was monitored twice daily. Animal husbandry was complied with Korean regulations on animal welfare and all procedures were approved beforehand by Review Board in the AB solution Inc. (Suwon, Korea). Every effort was made to minimize animal suffering.

Uterotrophic assay

Uterotrophic assays were performed according to OECD Test Guideline No. 440 [19]. Briefly, to evaluate the agonist and antagonistic effects of EGb761 on uterus, the rats allocated to 15 groups of six rats (Table 1) by stratified random sampling based on body weight. E2 and TM were dissolved in corn oil at 10 $\mu\text{g}/4\text{ mL}$ and 1 mg/10 mL, respectively, and administered to the rats by subcutaneous (SC) injection (E2: 10 $\mu\text{g}/4\text{ mL}/\text{kg}$ -body weight, TM: 1 mg/4 mL/kg-bw). EGb761 (100, 200 or 400

mg/10 mL/kg) was suspended in water administered by oral gavage. Vehicle controls were administered water (oral gavage, 10 mL/kg) and corn oil (SC injection, 4 mL/kg). All administrations were made once daily for 3 consecutive days. Animals were sacrificed on experimental day 4 by exsanguination from the abdominal aorta. Uteri, including uterine horn, body and cervix, liver, lungs, spleen, and kidneys of the rats were carefully excised and weighed after removing fat and mesentery.

Clinical signs and mortality

All animals were observed at least twice daily for any clinical signs of toxicity, that is, for behavioral changes (e.g., salivation, fur loss, lethargy, and sleep patterns), for changes in physical appearance (injury, pain, and signs of illness) and for death.

Uterine histology

Excised uteruses were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. Epithelial lining cell height and luminal width were measured by using an optical system attached to a light microscope (Olympus cx31, Japan) equipped with a digital CCD camera (IMTscan cooled model, Germany).

Table 1. Experimental design 1 to evaluate estrogenic and antiestrogenic effect of *Ginkgo Biloba* extract (EGb761) in immature female rat model.

Groups	Chemicals and Dose	The number of female rats
G1	Control (corn oil/water treatment)	6
G2	17 β -Estradiol (E2, 10 $\mu\text{g}/\text{kg}$)	6
G3	EGb1 (EGb761 100 mg/kg)	6
G4	EGb2 (EGb761 200 mg/kg)	6
G5	EGb4 (EGb761 400 mg/kg)	6
G6	Control (corn oil/water treatment)	6
G7	E2 (10 $\mu\text{g}/\text{kg}$)	6
G8	E2 (10 $\mu\text{g}/\text{kg}$) + EGb1 (EGb761 100 mg/kg)	6
G9	E2 (10 $\mu\text{g}/\text{kg}$) + EGb2 (EGb761 200 mg/kg)	6
G10	E2 (10 $\mu\text{g}/\text{kg}$) + EGb4 (EGb761 400 mg/kg)	6
G11	Control (corn oil/water treatment)	6
G12	Tamoxifen (TM, 1 mg/kg)	6
G13	TM (1 mg/kg) + EGb1 (EGb761 100 mg/kg)	6
G14	TM (1 mg/kg) + EGb2 (EGb761 200 mg/kg)	6
G15	TM (1 mg/kg) + EGb4 (EGb761 400 mg/kg)	6

Table 2. Primers used for polymerase chain reaction (PCR) analysis

Genes		Primer (5'-3')	Annealing Temp. ($^{\circ}\text{C}$)	PCR Cycle	Amplicon(bp)
Insulin-like growth factor binding protein (IGFBP-1)	F	CAACAGAAAGCAGGAGATGAGA	63	27	234
	R	GAAGAAGGAGGGAGGAAACAAC			
Calcium binding protein (CaBP9)	F	TGTCTGACTCTGGCAGCACTCACTG	63	30	181
	R	CCTTCAGGAGGCTGGGAACTCTG			
Cytochrome c Oxidase I (CO1)	F	TGAGCAGGAATAGTAGGGACAGC	50	28	261
	R	GAGTAGAAATGATGGAGGAAG			

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from uteruses and livers using Trizol Reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer’s protocol and stored at -80°C until needed. First-strand complementary DNA (cDNA) was generated by reverse transcribing 1 µg of total RNA in a final volume of 20 µL containing 4 µL of 5× RT buffer, 9 U of avian myeloblastosis virus (AMV) reverse transcriptase, 20 U of the RNase inhibitor, 1 µL of 10 mM dNTPs, and 0.5 µg/mL of random hexamer. The following conditions were used for RT: room temperature for 10 min, 42 °C for 50 min and 94 °C for 5 min.

The primers used for PCR were synthesized by Bioneer (Daejeon, Korea), and are listed in Table 2. PCR was conducted with 2 µL of first-strand cDNA in a final volume of 20 µL, which contained 1 µL up- and 1 µL down-specific primer and PCR premix (Bioneer, Daejeon, Korea) dissolved in 16 µL 0.1% diethyl pyrocarbonate (DEPC) distilled water. The following PCR conditions were used: 94 °C for 2 min followed by 27-30 cycles of 94 °C for 30 s, 50 °C (Cytochrome c Oxidase I; CO1), 63 °C (Calcium Binding Protein; CaBP-9), or 53 °C (Insulin like growth factor binding protein 1; IGFBP-1) for 30 s, and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. PCR-amplified products were resolved in 1.5% agarose gels in 1 × Tris-acetate EDTA (TAE) buffer containing 5% ethidium bromide (EtBr). PCR product sizes are provided in Table 2, and products were identified using gel documentation and quantified using ImageJ 1.43u software (NIH, USA). mRNA amounts were normalized with CO1.

Table 3. Body weight, mortality, and clinical signs in immature female rats exposed to *Ginkgo Biloba* extract (EGb761) or combination of EGb761 and estradiol (E2) or tamoxifen (TM).

Groups		Initial Body weight (g)	Last Body weight (g)	Mortality	Clinical signs ^a
G1	Control	40.74 ± 3.26	44.86 ± 2.98	0 / 6	0 / 6
G2	E2	41.87 ± 4.76	44.99 ± 4.63	0 / 6	0 / 6
G3	EGb1	41.82 ± 3.02	45.89 ± 3.02	0 / 6	0 / 6
G4	EGb2	43.34 ± 3.92	44.35 ± 6.00	0 / 6	0 / 6
G5	EGb4	41.67 ± 2.33	45.10 ± 2.59	0 / 6	0 / 6
G6	Control	41.46 ± 3.25	45.61 ± 3.44	0 / 6	0 / 6
G7	E2	41.07 ± 3.37	44.57 ± 2.78	0 / 6	0 / 6
G8	E2+EGb1	41.60 ± 3.43	45.41 ± 4.80	0 / 6	0 / 6
G9	E2+EGb2	39.70 ± 3.72	43.63 ± 2.97	0 / 6	0 / 6
G10	E2+EGb4	41.59 ± 3.89	45.79 ± 4.62	0 / 6	0 / 6
G11	Control	34.92 ± 3.80	39.91 ± 3.50	0 / 6	0 / 6
G12	TM	34.96 ± 3.63	39.06 ± 3.38	0 / 6	0 / 6
G13	TM+EGb1	33.33 ± 1.98	37.60 ± 1.91	0 / 6	0 / 6
G14	TM+EGb2	34.16 ± 3.46	38.48 ± 3.58	0 / 6	0 / 6
G15	TM+EGb4	34.90 ± 2.80	39.16 ± 3.13	0 / 6	0 / 6

^abehavioral pattern (salivation, fur loss, lethargy, and sleep patterns), changes in physical appearance, injury, pain, and signs of illness; Control (corn oil/water treatment); E2: 10 µg/kg; EGb1: 100 mg/kg of EGb761; EGb2: 200 mg/kg of EGb761; EGb4: 400 mg/kg of EGb761; TM: 1 mg/kg.

Statistical analysis

Data were analyzed using Sigma Plot software (Jandel Science Software, San Rafael, CA, USA) and Excel (Microsoft, Redmond, WA, USA). Results are presented as means±standard deviation (SD). Statistical analysis was performed using SPSS version 18.0 (SPSS, Chicago, IL, USA). Levene’s test was used for confirming homogeneity of variances. Tukey’s HSD test followed by one-way analysis of variance (ANOVA) was used to analyze parametric data, and the Mann Whitney test followed by the Kruskal-Wallis test was used to analyze nonparametric data. Statistical significance was accepted for p values<0.05.

RESULTS

Body weight, clinical signs, and mortality

As shown in Table 3, there were no significant changes in initial and last body weight in any treated group as compared

Table 4. Absolute/relative organ weight in immature female rats exposed to Ginkgo Biloba extract (EGb761)

Groups		Liver	Lung	Spleen	Kidney
G1	CON	1.39 ± 0.12 (3.10 ± 0.17)	0.39 ± 0.03 (0.88 ± 0.03)	0.16 ± 0.01 (0.36 ± 0.02)	0.44 ± 0.01 (0.99 ± 0.07)
G2	E2	1.48 ± 0.18 (3.28 ± 0.10)	0.41 ± 0.04 (0.89 ± 0.06)	0.18 ± 0.02 (0.39 ± 0.03)	0.45 ± 0.05 (0.99 ± 0.05)
G3	EGb1	1.61 ± 0.21 (3.49 ± 0.24)	0.40 ± 0.04 (0.87 ± 0.03)	0.20 ± 0.04 (0.44 ± 0.06)	0.48 ± 0.07 (1.04 ± 0.11)
G4	EGb2	1.62 ± 0.13 (3.69 ± 0.48*)	0.42 ± 0.05 (0.95 ± 0.14)	0.18 ± 0.03 (0.42 ± 0.12)	0.46 ± 0.04 (1.05 ± 0.13)
G5	EGb4	1.58 ± 0.13 (3.50 ± 0.10)	0.40 ± 0.03 (0.87 ± 0.04)	0.16 ± 0.02 (0.36 ± 0.03)	0.43 ± 0.03 (0.96 ± 0.06)
G6	CON	1.61 ± 0.18 (3.53 ± 0.15)	0.39 ± 0.03 (0.87 ± 0.04)	0.18 ± 0.03 (0.39 ± 0.07)	0.47 ± 0.04 (1.03 ± 0.08)
G7	E2	1.49 ± 0.09 (3.34 ± 0.14)	0.37 ± 0.03 (0.84 ± 0.05)	0.16 ± 0.01 (0.36 ± 0.03)	0.44 ± 0.05 (0.98 ± 0.06)
G8	E2+EGb1	1.64 ± 0.22 (3.59 ± 0.16)	0.42 ± 0.03 (0.94 ± 0.15)	0.17 ± 0.02 (0.37 ± 0.02)	0.44 ± 0.05 (0.97 ± 0.07)
G9	E2+EGb2	1.48 ± 0.14 (3.40 ± 0.17)	0.38 ± 0.04 (0.87 ± 0.06)	0.15 ± 0.03 (0.35 ± 0.05)	0.41 ± 0.03 (0.95 ± 0.08)
G10	E2+EGb4	1.70 ± 0.23 (3.69 ± 0.19)	0.42 ± 0.05 (0.92 ± 0.03)	0.16 ± 0.03 (0.34 ± 0.05)	0.44 ± 0.05 (0.97 ± 0.04)
G11	CON	1.30 ± 0.13 (3.29 ± 0.54)	0.38 ± 0.05 (0.97 ± 0.19)	0.18 ± 0.04 (0.46 ± 0.12)	0.40 ± 0.03 (1.02 ± 0.14)
G12	TM	1.20 ± 0.13 (3.06 ± 0.11)	0.35 ± 0.02 (0.90 ± 0.05)	0.17 ± 0.01 (0.43 ± 0.04)	0.40 ± 0.05 (1.03 ± 0.06)
G13	TM+EGb1	1.22 ± 0.09 (3.24 ± 0.18)	0.36 ± 0.03 (0.96 ± 0.07)	0.14 ± 0.02 (0.38 ± 0.05)	0.36 ± 0.01 (0.95 ± 0.03)
G14	TM+EGb2	1.31 ± 0.20 (3.39 ± 0.23)	0.36 ± 0.02 (0.95 ± 0.07)	0.15 ± 0.03 (0.39 ± 0.07)	0.35 ± 0.06 (0.93 ± 0.20)
G15	TM+EGb4	1.39 ± 0.16 (3.55 ± 0.33)	0.35 ± 0.03 (0.92 ± 0.07)	0.16 ± 0.05 (0.40 ± 0.09)	0.40 ± 0.03 (1.02 ± 0.03)

21-day-old female Crj:CD (SD) rats were treated with EGb761 once daily for 3 days. Each group consisted of 6 animals. The animals were sacrificed at the age of 24 days. Values in parentheses are relative organ weights (organ weight per body weight, %). The results are expressed as the mean ± SD. Values significantly different from the control are indicated by an asterisk (*p < 0.05). Control (corn oil/water treatment); 17β-estradiol (E2): 10 µg/kg; EGb1: 100 mg/kg of EGb761; EGb2: 200 mg/kg of EGb761; EGb4: 400 mg/kg of EGb761; Tamoxifen (TM): 1 mg/kg. Organ weight of kidney is value for sum of right and left organ.

with vehicle controls. During the 4-day experimental period, no clinical sign or mortality was observed in any group of EGb761 exposure (Table 3).

Organ weights

Livers, lungs, spleens, and kidneys were excised on experimental day 4, trimmed free of fat, and weighed. Weights of lungs, spleens, and kidney were not changed by EGb761 exposure, except for a significant increase in relative liver weight in the 200 mg/kg EGb761 group (G4) (Table 4).

Uterine weights and histology

Uteri were pierced, blotted to remove excess fluid, and weighed. As shown in Figure 1, the positive control, E2 (10 µg/kg) showed a significant increase in absolute and relative uterine weights (**p < 0.01), whereas EGb761 did not show a significant increase in uterine weight at any concentration (100,

200 or 400 mg/kg) (Figures 1A and 1B). Absolute and relative weights of uteri increased by E2 (10 µg/kg) or TM (10 mg/kg) were not significantly decreased by EGb761 at any concentration (Figures 1C, 1D, 1E, and 1F).

For morphometric analysis of cross sections of uterus mid-horns, LECH and LC were measured at a magnification of × 400 (Figure 2). Increases in LECH and LC of uterus are histological hallmarks of uterotrophy, which can be induced by estrogen [20]. As shown in Figure 2, both E2 and TM significantly increased (**p < 0.01) LECH and LC normalized with vehicle controls, whereas EGb761 did not even at its highest concentration (400 mg/kg). In addition, EGb761 (400 mg/kg) did not cause a significant reduction in E2- or TM-induced increases in LECH or LC, which indicated EGb761 had no estrogenic and antiestrogenic activity.

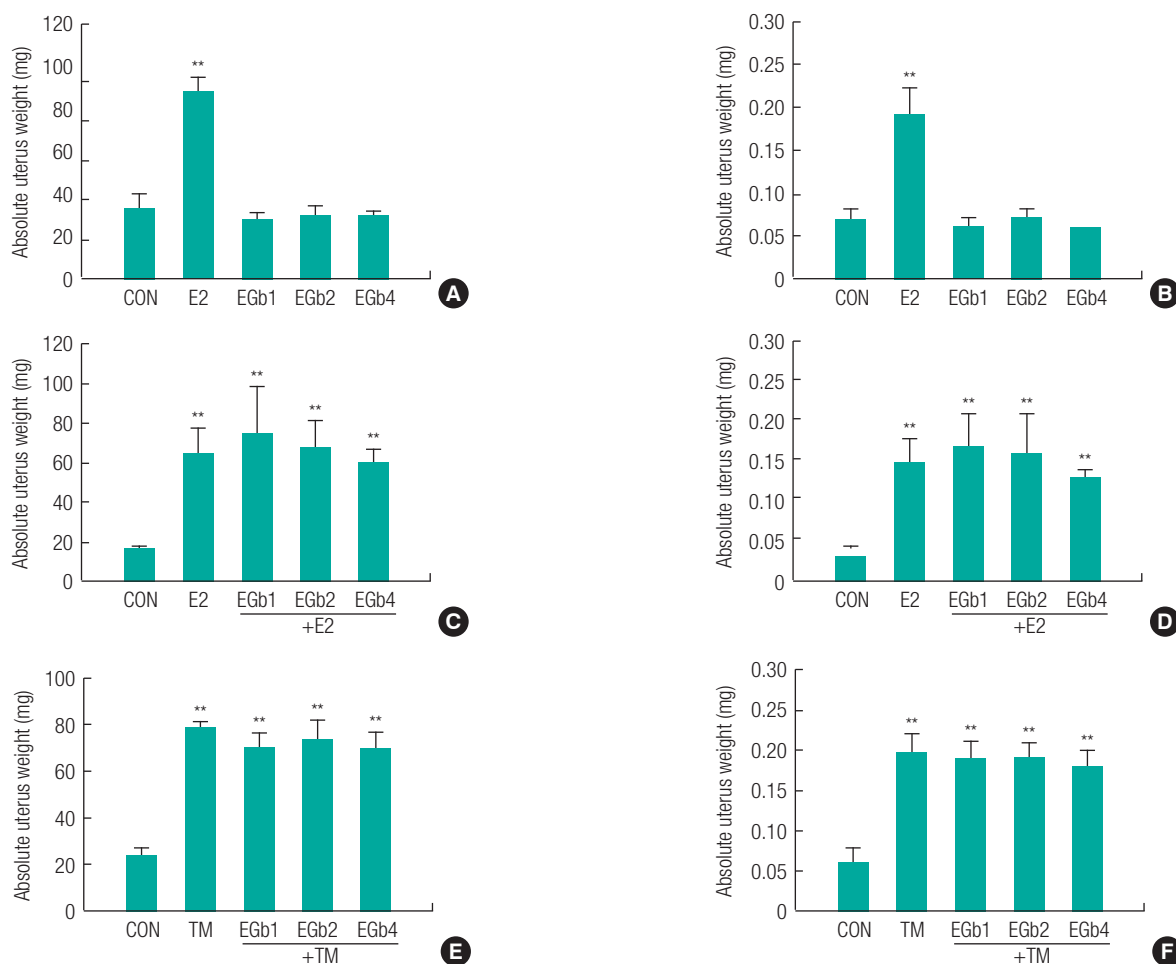


Figure 1. Estrogenic and antiestrogenic effect on uterine weight in immature female rats exposed to Ginkgo biloba extract (EGb761) or combination of EGb761 and 17β-estradiol (E2) or tamoxifen (TM). 21-day-old female Crj:CD (SD) rats (n = 6) were treated with E2 (10 mg/kg) or TM (1 mg/kg) as a positive control, EGb761 (EGb1 = 100 mg/kg, EGb2 = 200 mg/kg, EGb4 = 400 mg/kg) (A, B), combination of EGb761 (EGb1, EGb2, EGb4) and E2 (C, D) or TM (E, F) once daily for 3 days. The animals were sacrificed at the age of 24 days. The results are expressed as the mean ± SD. Values are significantly different from the control (**p < 0.01).

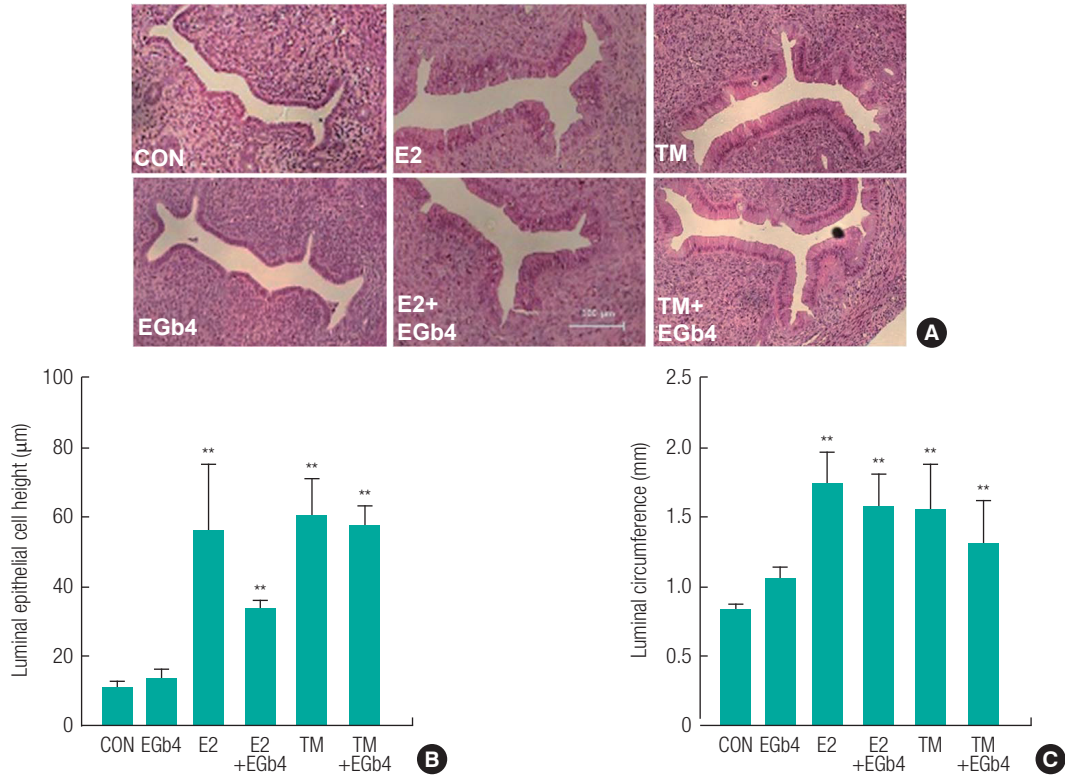


Figure 2. Estrogenic and antiestrogenic activity on uterine in immature female rats exposed to Ginkgo biloba extract (EGb761). 21-day-old female Crj: CD (SD) rats (n = 6) were treated with 17β-estradiol (E2, 10 mg/kg) or tamoxifen (TM, 10 mg/kg) as a positive control and EGb761 (400 mg/kg) once daily for 3 days. Excised uteri from the rats were fixed in 10% buffered formalin, processed to slide for morphology, and stained with hematoxylin-eosin (A). Luminal epithelium is indicated by arrows (Magnification, ×400). Each group consisted of 3 animals. Luminal epithelial lining cell height (LECH) (B) and luminal circumference(LC) (C) is expressed as a relative measurement based on calibrations in an ocular micrometer on the microscope (Magnification, ×400). The results are expressed as the mean ± SD of three separate experiments for each group. Values are significantly different from the CON (control group) (**p < 0.01).

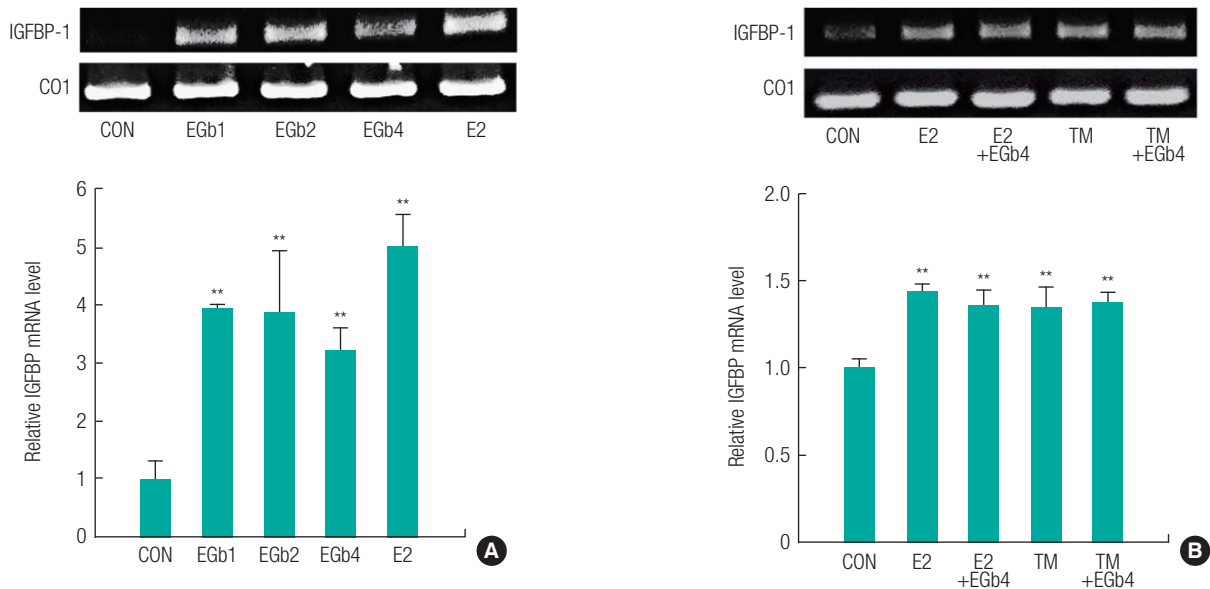


Figure 3. Relative mRNA expression of estrogen-related genes in liver from immature female rats exposed to Ginkgo biloba extract (EGb761) alone (A) or the combination of EGb761 and 17β-estradiol (E2) or tamoxifen (TM) (B). The total RNA was extracted using TRIzol in liver obtained from the immature female rats exposed to respective compounds. The mRNA levels (IGFBP) were measured using RT-PCR along with cytochrome c Oxidase I (CO1) mRNA as the internal standard. The PCR product was identified using a gel documentation and quantified by ImageJ 1.43u. The results are expressed as mean ± SD of three separate experiments for each group. Values significantly different from the control are indicated by an asterisk (**p < 0.01).

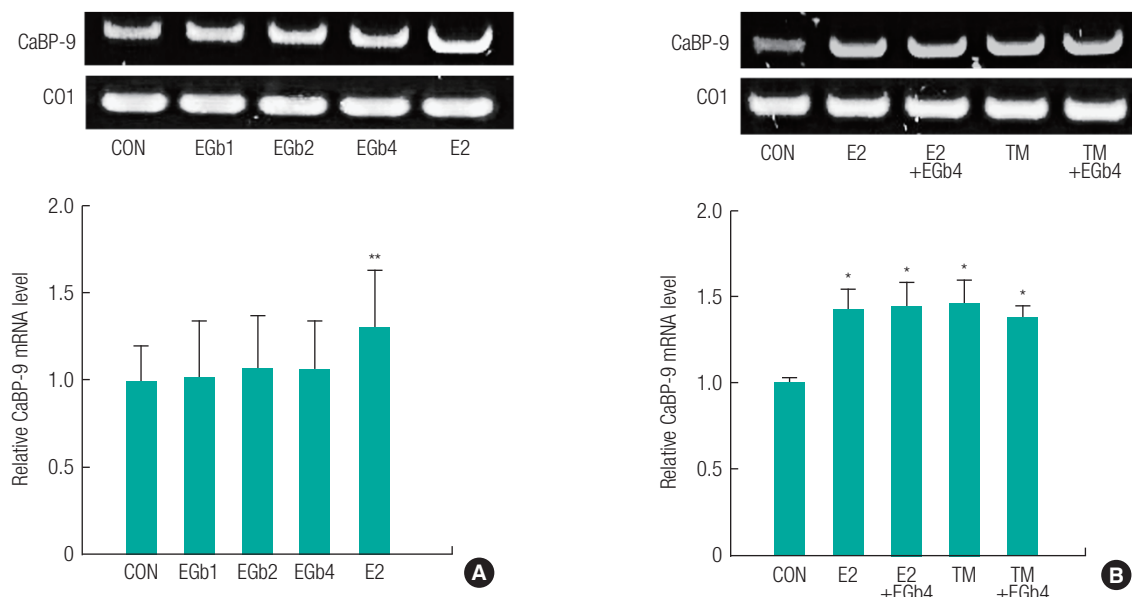


Figure 4. Relative mRNA expression of estrogen-related genes in uterus from immature female rats exposed to Ginkgo biloba extract (EGb761) alone (A) or the combination of EGb761 and 17β-estradiol (E2) or tamoxifen (TM) (B). The total RNA was extracted using TRIzol in liver obtained from the immature female rats exposed to respective compounds. The mRNA levels of CaBP-9 were measured using RT-PCR along with cytochrome c Oxidase I (CO1) mRNA as the internal standard. The PCR product was identified using a gel documentation and quantified by ImageJ 1.43u. The results are expressed as mean ± SD of three separate experiments for each group. Values significantly different from the control are indicated by an asterisk (*p < 0.05, **p < 0.01).

Estrogen relative gene expressions in liver and uterus

To further examine the estrogenic and antiestrogenic activities of EGb761, we evaluated the gene expression levels of IGFBP-1 on liver (Figure 3) and CaBP-9 in uterus (Figure 4). In liver tissues, E2 (the positive control) significantly induced a 3.99-fold increase in the expression of IGFBP-1 (estrogen responsive gene). EGb761 significantly increased IGFBP mRNA levels by 3 to 4-fold all concentrations normalized with vehicle controls. However, IGFBP mRNA upregulations by E2 or TM were not diminished by EGb761 (Figure 3B). In uterine tissues, E2 significantly increased CaBP-9 mRNA levels by 1.31-fold normalized with vehicle controls, whereas EGb761 did not at any concentration (Figure 4A). In addition, E2 or TM-induced CaBP-9 mRNA level increases were unaffected by EGb761 (Figure 4B). These results indicate EGb761 had an agonistic effect in liver, but not in uteri, and no antagonistic effect on either tissue type.

DISCUSSION

The uterotrophic assay using immature female SD rats recommended by the Endocrine disruptors screening and testing advisor committee (EDSTAC) has been used as *in vivo* short-term screening assay to evaluate estrogenic and antiestrogenic activities [18,19,21,22]. The endpoint of the uterotrophic assay is an

increase in uterine weight caused by ER-mediated water imbibition and cellular proliferation in uterine tissues [23]. In this study, E2 (10 µg/kg, i.p. injection, positive control) significantly increased absolute and relative uterus weights, whereas EGb761 (100, 200, or 400 mg/kg) did not do so as compared with vehicle controls (Figure 1). LECH and LC are hallmarks of the estrogen activity in rodents and are correlated with increases in uterine wet weights [20,24]. However, in the present study, uterine LECHs, LC and uterine weights were not significantly increased by EGb761 in the immature female rats (Figure 2). These results indicate that EGb761 had no agonistic effect on uterus.

TM is a SERM and has been used to treat ER-positive breast cancer. Although TM has an antagonistic effect on breast cancer cells, it acts as a full agonist in utero and increase the risk of endometrial cancer development [25]. Therefore, we evaluated effects of EGb761 on the agonistic effects of TM and of E2. When rats were exposed to EGb761 plus E2 or TM, EGb761 had no observed antagonistic effect on absolute or relative uterus weight, LECH, or LC increases by E2 or TM. These results indicate that EGb761 does not act as an agonist or antagonist in utero.

Several plant extracts known to contain flavonoids have been studied with respect to uterotrophic response. Neither onion extract, garlic extract [26], *Epimedium koreanum* extract [27,28], nor *Tropaeolum majus* L. extract (3-300 mg/kg) [29] elicited any agonistic or antagonistic effect on uterus as deter-

mined by uterotrophic assay. Although icariin (10 or 20 mg/kg) and icaritin (10 or 20 mg/kg), which are major components of *E. koreanum* extract, have agonistic and antagonistic effects on uterus, *E. koreanum* extract (100 mg/kg) did not exert any agonistic or antagonistic effect [28]. Quercetin and kaempferol, which are major components of EGb761, have been reported to have agonistic and antagonistic effects on estrogenicity (determined using an *in vitro* assay) [10,11]. In an uterotrophic assay using ovariectomized (OVX) rats, 3.55 or 18.42 mg/kg quercetin did not induce any estrogenic effect on uteri [30], whereas 100 mg/kg/day quercetin exerted estrogenic effects [31]. In addition, 10 mg/kg/day quercetin exhibited antiestrogenic effects. These reports indicate quercetin could exert estrogenic or antiestrogenic effects on uterus depending on the exposure dose. The second major flavonoid in EGb761, kaempferol (1,000 mg/kg) was found to have no effects on agonistic and antagonistic responses in utero in OVX mice [23,32]. Stroheker et al. [33] reported that kaempferol elicits no agonistic (25-200 mg/kg) or antagonistic (100 mg/kg) response in immature female rats or in OVX rat models. In the present study, EGb761 was found to contain 25.2% by weight of flavonoids, which included 64% quercetin and 32% kaempferol, that is, 100-400 mg EGb761 contained 16-64 mg and 8-32 mg of quercetin and kaempferol, respectively. However, EGb761 did not exert either of these effects. Oh et al. [34] reported that GBE (50-200 mg/kg) administered to OVX rats for 6 weeks did not show agonistic effects on uterus. In addition, *Opuntia ficus-indica* (L.) Mill (250 and 500 mg/kg) extract, which contained quercetin, kaempferol, and isorhamnetin, had no agonistic effects on uterus in OVX rat models [9]. On the other hand, onion extract (30 mg/kg), which contained the same flavonoids as EGb761 (quercetin, kaempferol, and isorhamnetin), was found to have agonistic effects on the uteri of female Wistar immature mice [26]. These reports suggest that complex plant extracts may affect the uterus in ways quite different from single major components.

To evaluate the estrogenic and antiestrogenic potentials of EGb761 in an immature uterotrophic model further, we examined the gene expression of CaBP-9 in uterine tissues using real-time PCR. The estrogen-dependent response of CaBP-9 in rat uterus is regulated by imperfect palindromic estrogen response elements (ERE) [35,36] and CaBP-9 is a known valuable biomarker of potential estrogenic activity [37]. In the present study, EGb761 treatment did not significantly change CaBP-9 mRNA levels in uterine tissues, which also suggests EGb761 had no estrogenic or antiestrogenic potential in utero.

However, unlike that observed in uterine tissues, EGb761 induced IGFBP-1 (a hepatic estrogenic marker) in liver. The up-

regulation of IGFBP-1 mRNA in liver is a clear marker of estrogenic regulation *in vivo* and *in vitro* [38,39,40]. IGFBP-1 has been reported to play a central role as a hepatic survival factor for liver injury of carbon tetrachloride [41] and as an early detection of alcohol-induced liver damage [42]. Tada et al. [43] reported that long-term feeding of GBE in aged, spontaneously hypertensive rats significantly increased liver weights and serum alanine aminotransferase levels. Hepatoblastomas and non-neoplastic lesions were observed in the livers of rats exposed to GBE for 2 years [44]. However, several other studies have reported GBE has hepatic protective effects for obstructive jaundice [45], hepatocarcinogenesis [46], and liver fibrosis [47]. In addition, Lieberman et al. [48] reported that ginkgo intake (EGb761) as typically consumed by American adults showed no significant adverse effects on liver function. Based on the controversial results of the GBE, further studies were needed to clarify the effects of GBE on liver function.

CONCLUSION

Ginkgo biloba has been used as a traditional Chinese medicine for treating cerebrovascular and peripheral vascular insufficiencies. GBE (a SERM) has been studied in the contexts of HRT and its anticarcinogenic effects in breast tissues. However, HRT might have serious side effects, including endometrial cancer. In a uterotrophic assay, EGb761 (100, 200, or 400 mg/kg) did not show any change of uterine weight, estrogen-related gene expression (CaBP-9), and histopathological effects (LECH and LC) on the uterus. These results suggested that EGb761 has no agonistic and antagonistic effects on uterus.

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CONFLICT OF INTEREST

The authors have no conflict of interest associated with the material presented in this paper.

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