In-vitro Estrogenic Effect of the bis(4-hydroxybenzyl)ether Extracted from *Gastrodia elata* Blume

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Abstract A decline in the function of the ovaries and decrease in the production of sex hormones with aging cause hormonal deficiencies, leading to female menopause. Natural products have been investigated to develop estrogen-like substances to improve menopausal conditions in women. Using MCF-7 cells, a female breast cancer cell line, we investigated whether *Gastrodia elata* Blume (GEB) extract had an effect similar to that of estrogen (E2). TFF-1 and PR were upregulated in the GEB extract-treated cells similar to those under E2 treatment. Moreover, the levels of phosphorylated MAP kinase kinases 1/2, extracellular signal-regulated kinase 1/2, estrogen receptor (ER) alpha, and ER beta were upregulated. Using an ER antagonist, ICI 182.780, we found that GEB extract induces a downstream response via the ER pathway. Additionally, GEB extract were fractionated according to solvent polarity. Estrogen-like effects were found in the dichloromethane fraction and bis(4-hydroxybenzyl)ether was identified as an effective compound by qTOF analysis and standard chemical compound. In this study, we report that GEB extract showed an estrogen-like effect in vitro and bis(4-hydroxybenzyl)ether was identified as an effective compound.

Keywords: estrogenic effect, *Gastrodia elata* Blume extract, bis(4-hydroxy benzyl)ether

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1. Introduction

Menopausal diseases are common in most women aged 50 to 60 years. Aging gradually deteriorates the functioning of the uterus and ovaries leading to menopause. The representative symptoms include hot flashes, decreased libido, depression, and osteoporosis [1,2]. The leading cause of these menopausal disorders is the decreased secretion of sex hormones owing to reduced ovarian function [3]. Estrogen and progesterone are typically produced in the ovaries of women, and estrogen plays an important role in metabolism [4]. Cells in the body have estrogen receptors (ERs), and estrogen synthesized in the body binds to these ERs. This mechanism helps maintain a normal life and regulates metabolism by transmitting a number of vital signals [5].

Estrogen functions specifically in several tissues. For example, in breast cancer, estrogen plays an important role. FoxA1 has been shown to be very important for breast cancer cells to respond to estrogen. Estrogen induces osteoclast apoptosis through upregulation of FasL.

Although the exact mechanism has not been elucidated, the absence of estrogen in the brain's hema indicates depression and anxiety. The results suggest that upregulation of estrogen is effective in treating menopausal symptoms. [6,7,8,9]

However, owing to the loss of function of the ovaries during menopause, estrogen production decreases and the body cannot supply the required amount, resulting in hormonal control disorders [10]. Estrogens, which are important for menopausal women, are largely divided into three types: estradiol, estrone, and estriol.

Menopausal diseases are experienced by almost all middle-aged women, whether healthy or not. Therefore, their treatment or prevention methods have been extensively studied. One of the common treatments is to take estrogen-like compounds present in natural products that can replace the effects of estrogen [11]. A representative example is legume extract (soy beans, lentils, mung beans extract etc.), containing phytoestrogens, which has an estrogen-like effect. Despite the body’s estrogen deficiency, these phytoestrogens are capable of binding to ERs and transmit biological signals. Phytoestrogens have the potential to treat and prevent menopausal diseases [12]. Such phytoestrogens have fewer adverse effects than synthetic
2. Materials and Methods

2.1. Extraction of GEB

A 10-fold volume of 30% ethanol solution (Daehan Ethanol Life, Hwaseong, Korea) was added to GEB and extracted at 85–90°C for 3 h. This process was repeated twice. The extract was filtered through a 1-µm filter and evaporated at 60–70°C and spray-dried with 30% maltodextrin.

2.2. Cell Culture

MCF-7 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). MCF-7 cells were incubated at 37°C under 5% CO₂ for 6 days; the medium was changed every 3 days. The medium used was RPMI 1640 (Welgene, Gyeongsan-si, Korea) containing 25 mM HEPES, L-glutamine, 1% penicillin-streptomycin (Hyclone, MA, USA), and 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) treated with charcoal dextran (Sigma-Aldrich) and ER-related genes. The total RNA (500 ng) was reverse transcribed into complementary DNA using the ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). Relative gene expression levels were measured using a Step-One Plus™ Real-Time PCR system (Applied Biosystems, CA, USA). The housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used for normalization as a reference gene. TFF-1, PR, and HPRT primers, purchased from Cosmo Genetech (Seoul, Korea), are shown in Table 1. qRT-PCR was performed using the THUNDERBIRD™ SYBR® qPCR Mix (Toyobo). The housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used for normalization as a reference gene. TFF-1, PR, and HPRT primers, purchased from Cosmo Genetech (Seoul, Korea), are shown in Table 1. qRT-PCR was performed using a Step-One Plus™ Real-Time PCR system (Applied Biosystems, CA, USA) using the following conditions: holding stage: 1 min at 95°C; cycling stage: 15 s at 95°C to 30 s at 58°C, total 40 cycles; melt curve stage: 15 s at 95°C and 30 s at 60°C, measured every 0.5°C.
from Santa Cruz Biotechnology. Peroxidase-linked secondary antibodies were purchased from Abcam (Cambridge, UK). Horseradish peroxidase-linked antibodies were purchased from Santa Cruz Biotechnology (Bevery, MA, USA). ER α and ER β (ERK1/2) were purchased from Cell Signaling Technology (Dallas, TX, USA). MEK1/2 and extracellular signal-regulated kinase 1/2 (ERK1/2) were purchased from Cell Signaling Technology (Beverly, MA, USA). β-Actin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.6. Immunoblotting

Cell pellets were lysed with radioimmunoprecipitation buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). To quantify the total protein in the cell pellet, a bicinchoninic acid assay was performed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the proteins by size. The amount of protein in each sample was set to 20 μg. The sample was loaded onto a 10% acrylamide gel and subjected to electrophoresis. The proteins in the gel were transferred on to a polyvinylidene difluoride membrane (Amersham Hybond-P; GE Healthcare, Buckinghamshire, UK) using a semi-dry transfer method, according to the manufacturer’s instructions. After the transfer, the membrane was immersed in 1% skim milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 1 h at 20–30°C for blocking. The membrane was incubated with primary antibodies for 16 h at 4 °C, washed three times with TBS-T, and incubated with horseradish peroxidase-linked secondary antibodies for 1 h at 20–30°C. The membrane was then washed three times with TBS-T and the protein bands were visualized using the WesternBright Peroxide chemiluminescent detection reagent (Advansta, Menlo Park, CA, USA). The membrane was then stripped and incubated with horseradish peroxidase-linked secondary antibodies for 1 h at 20–30°C. The membrane was then washed three times with TBS-T and the protein bands were visualized using the WesternBright Peroxide chemiluminescent detection reagent (Advansta, Menlo Park, CA, USA). The membrane was then stripped with a stripping buffer (10% SDS, 0.5 M Tris-Cl, and 0.8% β-mercaptoethanol) for 10 min at 60°C and reacted with other primary antibodies. Band intensity was quantified using Imagej 1.53e Java 1.8.0_172 (NIH software, Bethesda, MD, USA) and expressed as a ratio to β-actin band intensity. Specific primary antibodies for total and phosphorylated MAP kinase kinases 1/2 (MEK1/2) and extracellular signal-regulated kinase 1/2 (ERK1/2) were purchased from Cell Signaling Technology (Beverly, MA, USA). ER α and ER β antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). β-Actin antibody was purchased from Abcam (Cambridge, UK). Horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology.

2.7. Influence of ICI 182.780 on Cell Proliferation

ICI 182.780 (Sigma-Aldrich), an ER antagonist, was used to confirm whether any effect of GEB extract on cell proliferation was mediated by the ER. After 21 h of cell seeding, the cells (2 × 10^5 cells/mL) were treated with 1 μM ICI 182.780 and then incubated for 3 h. After the cell treatment, cell proliferation, RNA isolation, qRT-PCR, and immunoblotting were performed as described above.

2.8. qTOF for Identification of Effective Substance

Distilled water was added to GEB extract in a 10-fold volume for fraction. The solvents such as hexane, dichloromethane, ethyl acetate and n-butanol were added to the re-dissolved GEB extract and concentrated separately. The dichloromethane GEB fraction was re-fractrationed by open column chromatography with a mixed solvent of chloroform and acetone. Fraction with estrogen-like effect was concentrated on qTOF analysis. qTOF analysis was performed in Korea Basic Science Institute Chuncheon Center (KBSI, https://www.kbsi.re.kr/). Chemical composition in GEB fraction was determined by QTOF-MS/MS analysis using Waters Acquity UPLC I-Class system (Water Corp., Milford, MA, USA) coupled Waters Xevo G2 QTOF MS (Waters MS Technologies, Manchester, UK) with ESI (electrospray ionization) interface. In brief, the GEB fraction was dissolved in 70% methanol (final concentration was 200 ppm) and filtered through a PTFE syringe filter (0.2 μm). The 2 μL of GEB fraction were injected (10°C) with the flow rate of 300 µL/min and column oven temperature was 40°C. The analysis was carried out by Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm) (Waters Co., USA). The mobile phase was used as 0.1% formic acid in H2O and 0.1 % in acetonitrile. Compound identifications were achieved by UNIFI 1.8 (Waters, Milford, MA, USA). In-house library in UNIFI consisted of more than 2400 natural products compounds. The library was enriched with compounds that were either isolated or analyzed from the plant by searching databases such as Dictionary of Natural product (CRC, 2009), SciFinder Scholar of the American Chemical Society, and Natural Product Activity & Species Source Database. The Natural Products Application Solution with UNIFI along with in-house library were verified the compounds MS/MS fragmentations with MassFragment. MassFragment is an in silico fragmentation tool that uses a systematic bond disconnection approach to identify possible structures from the parent structure. It removes the possible false positives and allows higher confidence for identifying chemical components in complex mixtures such as natural products. We tentatively identified compounds and later confirmed bis(4-hydroxybenzyl)ether with purchased standard (Simson Pharma Limited, Mumbai, India).

2.9. Statistical Analysis

The results of each experiment are expressed as mean ± standard deviation (SD). The one-way ANOVA (Dunnett’s test, SPSS, v.23, for Windows) was used to compare the group means. Values were considered significant when P < 0.05.

Table 1. Primers used in the study

| Gene   | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|--------|--------------------------|--------------------------|
| HPRT   | CCT GGC GTC GTG ATT AGTG | TGA GGA ATA AAC ACC CTT TCC A |
| TFF-1  | GAC AGA GAG GTG TAC AGT GG | GGA TAG AAG CAC CAG GGG AC |
| PR     | AGT TGT GAG AGC ACT GGA TGC | GAT CTG CCA CAT GGT AAGGC |
3. Results

3.1. Effect of GEB Extract on Cell Proliferation

To confirm the effect of GEB extract on MCF-7 cell proliferation, MCF-7 cells were treated with GEB extract in a concentration-dependent manner (50, 100, 200, and 400 μg/mL). At 100 μg/mL GEB extract, a cell proliferation effect was observed (viability = 192%); the proliferation rate increased in a concentration-dependent manner. The cell proliferation effect of 400 μg/mL GEB extract (viability = 373%) was similar to that of 17β estradiol (E2, 10^{-10} M, viability = 385%), which was used as a positive control. To confirm whether GEB extract exerts an estrogenic effect in MCF-7 cells via the ER pathway, the ER antagonist ICI 182.780 (1 μM) was first used to inhibit the ER, and then all experiments were repeated. When the cells were first treated with ICI 182.780 and then with E2 or GEB extract, there was no cell proliferation effect (E2 = 96%, GEB extract = 98%) (Figure 1).

![Figure 1](image1.png)

**Figure 1.** Effect of GEB extract on cell proliferation (The cell proliferation rates are expressed as percentages. A sample treated with 0.3% EtOH was used as a control and the result was normalized to 100%. Cell proliferation rates were analyzed by treating MCF-7 cells with GEB extract in a concentration-dependent manner after 144hr. Values are mean ± SD of three experiments. ** means significantly different from the control (p < 0.05).)

![Figure 2](image2.png)

**Figure 2.** Effect of GEB extract on estrogen receptor-related gene expression (Analysis of mRNA expression in MCF-7 cells. Cells were treated with GEB extract (200 μg/mL) for 48, 96, 144 h. The relative gene expression level is expressed as fold change. A vehicle sample treated with 0.3% EtOH was used as the control and the result was normalized to 1.0. The expression levels of TFF-1 and PR were analyzed and showed as fold change (48, 96, and 144 h). Values are mean ± SD of three experiments. ** means significantly different from the control (p < 0.05).)
3.2. Effect of GEB Extract on Estrogen Receptor-related Gene Expression

To confirm the expression of genes regulated by GEB extract, the expression levels of related genes TFF-1 and PR were measured by qRT-PCR. After 48 h (two-fold = 2.9), 96 h (two-fold = 6.7), and 144 h (two-fold = 9.1) of treatment with GEB extract (200 μg/mL), the TFF-1 level increased in the same manner as that after treatment with E2 (10⁻¹⁰ M, two-fold = 5.2, 23.6, and 34.5, respectively) with increased treatment time. The expression of another related gene PR also increased (two-fold = 4.6, 5.4, and 7.7, respectively). When the cells were treated with GEB extract for 144 h, the highest gene expression (TFF-1 gene two-fold = 9.1, PR gene two-fold = 7.7) was observed (Figure 2).
Figure 3. ER pathway up-regulated by GEB extract. (A) The protein expression levels of the total and phosphorylated forms of MEK1/2, ERK1/2, estrogen receptor alpha and beta, and beta-actin were visualized by immunoblotting. MCF-7 Cells were treated with GEB extract (200 μg/mL) for 48, 96, and 144 h. (B) The P-ERK band intensity was quantified using imageJ software and expressed as a ratio to the β-actin band intensity. (C) The P-MEK band intensity was quantified using imageJ software and expressed as a ratio to the β-actin band intensity. (D) Estrogen receptor alpha and beta band intensity was quantified using imageJ software and expressed as a ratio to the β-actin band intensity. Values are mean ± SD of three experiments. ** means significantly different from the control (p < 0.05).
We confirmed the ER pathway with protein level in MCF-7 cells treated with GEB extract (200 μg/mL) was separated by SDS-PAGE, and the related proteins were identified by immunoblotting. After 48, 96, and 144 h treatment, the proteins of downstream ER pathway were investigated for total and phosphorylated forms of MEK1/2 and ERK1/2. The phosphorylated form of MEK1/2 (P-MEK1/2) proteins was hardly expressed in the control (vehicle, 0.3% EtOH) but conspicuously expressed with E2 (10^{-10} M) (144 h P-MEK/β-actin = 5.3, MEK/β-actin = 1.0) and GEB extract (144 h P-MEK/β-actin = 2.4, MEK/β-actin = 1.0) treatment. The expression of P-ERK1/2 also increased after treatment with E2 (144 h P-ERK/β-actin = 1.6, MEK/β-actin = 1.0) and GEB extract (144 h P-ERK/β-actin = 1.3, MEK/β-actin = 1.0) compared with that in the control. The expression of ER α and ER β increased after treatment with E2 (144 h ER α/β-actin = 3.4, ER β/β-actin = 11.2) and GEB extract (144 h ER α/β-actin = 1.7, ER β/β-actin = 5.7) compared with that in the control (Figure 3).

3.3. Effect of GEB Extract in the Presence of Estrogen Receptor Antagonists, ICI182.780

To confirm whether GEB extract has an estrogenic effect in MCF-7 cells via the ER pathway, the antagonist ICI 182.780 (1 μM) was used to inhibit the ER. The ER pathway-related gene expression levels were measured by qRT-PCR after treatment with the ER antagonist ICI 182.780. The expression of ER pathway-related genes, TFF-I and PR, increased after E2 (10^{-10} M) (TFF-I gene two-fold = 34.5, PR gene two-fold = 16.0) or GEB extract (200 μg/mL) (TFF-I gene two-fold = 9.1, PR gene two-fold = 7.7) treatment. When the cells were treated with ICI 182.780 followed by E2 or GEB extract, there was no related gene expression (E2 TFF-I gene two-fold = 1.1, PR gene two-fold = 1.2) (GEB extract TFF-I gene two-fold = 1.2, PR gene two-fold = 1.2) (Figure 4A). The proteins downstream of the ER pathway were identified after treatment with the ER antagonist ICI 182.780 to confirm the effect of GEB extract on MCF-7 cell proliferation. Immunoblotting confirmed the phosphorylation of MEK1/2, ERK1/2, and ER α and β proteins. The expression of the downstream proteins of the ER pathway increased after E2 (144 h P-MEK/β-actin = 2.3, MEK/β-actin = 1.0, P-ERK/β-actin = 1.2, ERK/β-actin = 9.6, ER β/β-actin = 22.0) or GEB extract (144 h P-MEK/β-actin = 1.9, MEK/β-actin = 1.0, P-ERK/β-actin = 1.1, ERK/β-actin = 1.0, ER α/β-actin = 7.5, ER β/β-actin = 13.5) treatments. However, there was no significant difference in levels of phosphorylated proteins when cells were treated with ICI 182.780 (144 h P-MEK/β-actin = 1.1, MEK/β-actin = 1.0, P-ERK/β-actin = 0.9, ERK/β-actin = 1.0, ER α/β-actin = 1.1, ER β/β-actin = 1.0) or GEB extract (144 h P-MEK/β-actin = 1.0, MEK/β-actin = 1.0, P-ERK/β-actin = 1.0, ERK/β-actin = 0.9, ER α/β-actin = 1.0, ER β/β-actin = 1.0) (Figure 4B).
### B

| ICI 182.780 (1 μM) treatment | Control | E2 | GEB | Control | E2 | GEB |
|-----------------------------|---------|----|-----|---------|----|-----|
| Sample                      |         |    |     |         |    |     |
| P-MEK1/2 (45 kDa)           |         |    |     |         |    |     |
| MEK1/2 (45 kDa)             |         |    |     |         |    |     |
| P-ERK (44, 42 kDa)          |         |    |     |         |    |     |
| ERK (44, 42 kDa)            |         |    |     |         |    |     |
| ER α (66 kDa)               |         |    |     |         |    |     |
| ER β (56 kDa)               |         |    |     |         |    |     |
| β-actin (43 kDa)            |         |    |     |         |    |     |

### C

![Graph showing comparison between Control, E2, and GEB conditions.](image)

| Condition | Control | E2 | GEB |
|-----------|---------|----|-----|
| ICI 182.780 | -       | -  | -   | 1 μM |
| 144 h     |         |    |     |      |
Figure 4. Inhibitory effect of ER antagonist ICI 182.780 on GEB extract. ((A) The relative gene expression level is expressed as fold change. A sample treated with 0.1% dimethyl sulfoxide (vehicle) was used as the control and the result was normalized to 1.0. After treatment with GEB extract at the same time (144 h) in MCF-7 cells, the expression levels of TFF-1 and PR were analyzed. Values are mean ± SD of three experiments. ** means significantly different from control (p < 0.05). (B) The total and phosphorylated protein expression levels of MEK1/2, ERK1/2, and estrogen receptor alpha and beta proteins were visualized by immunoblotting. (C) The P-ERK band intensity was quantified using imageJ software and expressed as a ratio to the β-actin band intensity. (D) The P-MEK band intensity was quantified using imageJ software and expressed as a ratio to the β-actin band intensity. (E) Estrogen receptor alpha and beta band intensity was quantified using imageJ software and expressed as a ratio to the β-actin band intensity. Values are mean ± SD of three experiments. ** means significantly different from control (p < 0.05).)

3.4. Identification of Effective Compound in GEB Extract

Polar solvent fractionation and mass analysis were performed to identify the effective compound in GEB extract. Polar solvent fractions obtained (hexane, dichloromethane, ethyl acetate and n-butanol) were tested for efficacy on MCF-7 cells and dichloromethane fraction showed the highest effect (viability = 416.6%) (Figure 5A). For identification of effective compound in the dichloromethane fraction, it was fractionated using a silica gel open column. Collected fractions were tested for cell proliferation (data not shown) and the fraction having the efficacy was concentrated with
The concentrated material was analyzed against chemical library-based qTOF and several compounds were identified in this fraction (Figure 5B, Table 2).
**Figure 5.** Identification of effective compound in GEB extract by qTOF mass. (A) Polar solvent fraction was tested in cell proliferation assay with MCF-7 cells. A sample treated with 0.1% dimethyl sulfoxide (vehicle) was used as the control and the result was normalized to 1.0. After treatment with fractionated materials at the same time (144 h) in MCF-7 cells, the cell proliferation levels were analyzed. Values are mean ± SD of three experiments. ** means significantly different from control (p < 0.05). (B) qTOF mass chromatogram showed analyzed candidate effective substances. (C) Mass of bis(4-hydroxybenzyl)ether standard product showed exactly same with qTOF chromatogram of effective fraction’s one. (D) Bis(4-hydroxybenzyl)ether standard product showed increased cell proliferation effect in MCF-7 cell.

Table 2. Result from Chemical library-based qTOF analysis

| RT (min) | Tentative identification | Formula | m/z [M-H]- | Mass error (ppm) | Response | Fragmentation (m/z) | Reference |
|---------|--------------------------|---------|------------|-----------------|----------|---------------------|-----------|
| 2.02    | 4-Hydroxybenzaldehyde    | C7H6O2  | 121.0292   | -2.4            | 474457   | 92.0275             | [22-25]   |
| 2.33    | Vanillin                 | C8H8O3  | 151.0402   | 1.0             | 2343     | 136.0168            | [22]      |
| 2.82    | trans-p-coumaric acid    | C9H8O3  | 163.0398   | -1.4            | 4454     | 119.0503            | [22]      |
| 3.95    | Unknown                  | C15H14O3| 241.0871   | 0.5             | 276053   | -                   |           |
| 4.10    | 4-Hydroxybenzaldehyde    | C7H6O2  | 121.0292   | -2.4            | 43357    | 92.0268, 121.0294   | [22-24]   |
| 4.24    | Resveratrol              | C14H12O3| 227.0715   | 0.5             | 835554   | 143.0499, 185.0595  | [26]      |
| 4.37    | Bis-(4-hydroxybenzyl)ether | C15H16O5| 275.0932   | 2.5             | 547395   | -                   |           |
| 4.37    | Bis-(4-hydroxybenzyl)ether | C14H14O3| 229.0876   | 2.3             | 2514205  | 93.0342, 107.0501, 121.0294 | [24-25] |
| 4.54    | Unknown                  | C15H14O3| 241.0874   | 1.7             | 99164    | -                   |           |
| 5.12    | 5-[4'-4'-4'-hydroxybenzyl]-3'-hydroxybenzoylmethyl-furan-2-carbaldehyde| C20H18O5| 337.1087 | 1.5 | 140252 | - | [27] |
| 6.53    | Unknown                  | C13H22O2| 209.1546   | -0.4            | 132819   | -                   |           |
| 7.02    | Bis-(4-hydroxybenzyl)ether | C14H14O3| 229.0869   | -0.3            | 131412   | 93.0340, 107.0500, 121.0291 | [24-25] |
| 7.23    | Unknown                  | C17H26O4| 293.1758   | 0.0             | 305133   | -                   |           |
| 11.16   | Unknown                  | C15H22O3| 249.1500   | 1.5             | 140869   | -                   |           |

The bis(4-hydroxybenzyl)ether was identified with standard compound purchased from Simson Pharma Limited company by comparing its retention time and mass spectrum (Figure 5C). The bis(4-hydroxybenzyl)ether in retention time of 4.35 was shown [M-H]- ion at m/z 229.0861 including fragmentation at m/z 93.0342, 107.0501 and 121.0294 [23,24]. Detected substances were tested cell proliferation assay and showed 4-hydroxybenzyl aldehyde has no effect and bis(4-hydroxybenzyl)ether has strong effect with 2μM concentration (Figure 5D).

### 4. Discussion

Various effects are known for Gastrodia elata Blume. GEB extract inhibits angiogenesis and reduces the production of nitric oxide through inhibition of iNOS and COX-2 expression. [21] Compounds contained in GEB have been found to reduce levels of ROS and MDA, and to regulate genes related to antioxidants. [22] Gastrodin contained in GEB is known as a major physiologically active ingredient. Gastrodin has antioxidant and anti-inflammatory properties.
It also modulates neurotransmitters, and it has been shown that Gantrodin may be effective against diseases of the general central nervous system, including epilepsy, Alzheimer's disease and Parkinson's disease. [23]

We studied the estrogen-like effects of GEB extract. In addition, active substances contained in the GEB extract were identified. To confirm the effect of GEB extract on MCF-7 cell proliferation, MCF-7 cells were treated with GEB extract in a concentration-dependent manner. From 100 μg/mL GEB extract, the proliferation rate increased in a concentration-dependent manner. The cell proliferation effect of 400 μg/mL GEB extract was similar to that of 17β estradiol. And we show that GEB extract shows its effect on cell proliferation through the ER metabolic pathway, the expression of related genes and related proteins was confirmed through this study. Expression of TFF-1 and PR in the ER metabolic pathway was shown to have increased significantly. In addition, the phosphorylation of MEK1/2, ERK1/2, and ER α and β proteins in the ER metabolic pathway increased, resulting in cell proliferation. To demonstrate that GEB extract effects through the ER metabolic pathway, the cells were treated with the ER antagonist ICI 182.780, followed by cell proliferation and gene and protein expression studies. When not treated with an antagonist, the cell proliferation rate increased. There was a corresponding increase in the levels of related genes and protein. However, when the cells were pre-treated with ICI 182.780, there were no changes compared with those in the control. It shows that GEB extract has estrogenic effect through the ER pathway.

Additionally, to identify substances with estrogen-like effects, GEB extract was fractionated into polar solvents. The dichloromethane fraction had the highest estrogen-like effect. Furthermore, the dichloromethane fraction was fractionated using silica open column chromatography and the fraction with efficacy was confirmed. QTof mass was performed to identify the effective compound from this fraction. Substances such as 4-hydroxybenzaldehyde, resveratrol, bis(4-hydroxybenzyl)ether were identified as effective candidates. Bis(4-hydroxybenzyl)ether has shown cell proliferation effect in MCF-7 cell. In addition, it was confirmed that the analyzed candidate substance was a bis(4-hydroxybenzyl)ether based on the standard test.

In conclusion, we verified the estrogen-like activity of GEB extract using the in-vitro system, and this efficacy confirmed the molecular mechanism that appears through the ER pathway. In addition, various components contained in the GEB extract were analyzed using QTof mass. The standard chemical were purchased and efficacy evaluation was conducted, and bis(4-hydroxybenzyl)ether proved to have estrogen-like activity.

### Declarations of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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