韓藥作誌(Korean J. Medicinal Crop Sci.) 30(4) : 233 – 246 (2022)
ISSN(Print) 1225-9306
ISSN(Online) 2288-0186
http://www.medicinalcrop.org
http://dx.doi.org/10.7783/KJMCS.2022.30.4.233

천마 추출물의 에스트로겐 유사효과

임해령[1,2] · 이재훈[3] · 박선주[4] · 지은수[5] · 이득찬[6†]

Estrogenic Effect of *Gastrodia elata* Blume Extract

Hae Ryeong Lim[1,2], Jae Hoon Lee[3], Seon Ju Park[4], Eun Su Ji[5] and Deug Chan Lee[6†]

Received: 2022 July 4
1st Revised: 2022 August 11
2nd Revised: 2022 August 22
3rd Revised: 2022 August 22
Accepted: 2022 August 22

ABSTRACT

Background: A decline in the function of the ovaries and a decrease in the production of sex hormones with aging causes hormonal deficiencies, leading to female menopause. Natural products may be developed into estrogen-like substances to improve menopausal conditions in women.
Methods and Results: We investigated whether *Gastrodia elata* Blume (GEB) extract could produce a similar effect to estrogen (E2) on MCF-7 cells, from a female breast cancer cell line. Trefoil factor 1 (*TFF-1*) and progesterone receptor (*PR*) were upregulated in the GEB extract-treated cells, like the E2 treatment. Moreover, the levels were upregulated in the phosphorylated mitogen-activated protein (MAP) kinase kinases 1/2, extracellular signal-regulated kinase 1/2, estrogen receptor (ER) alpha, and ER beta. An ER antagonist (ICI 182.780) with the GEB extract induceds a downstream response via the ER pathway. A qTOF analysis using a standard chemical compound revealed bis(4-hydroxybenzyl)ether was an effective compound.
Conclusions: GEB extract had an estrogen-like effects *in vitro* and bis(4-hydroxybenzyl)ether was an effective compound.

***Key Words*:** *Gastrodia elata* Blume, Bis(4-hydroxy benzyl)ether, Estrogenic Effect

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 (Gold *et al*., 2001; Dratva *et al*., 2009). The leading cause of these menopausal disorders is the decreased secretion of sex hormones owing to reduced ovarian function (Sørensen *et al*., 2001).

Estrogen and progesterone are typically produced in the ovaries of women, and estrogen plays an important role in metabolism (Lee *et al*., 1998). 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 (Moy and Goss, 2006).

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

†Corresponding author: (Phone) +82-33-250-6488 (E-mail) dclee@kangwon.ac.kr
[1]강원대학교 의생명과학대학 의생명융합학부 석사 / Master's degree, Department of Biomedical Technology, Kangwon National University, Chuncheon 24341, Korea.
[2]더밸류바이오텍 연구원 / Researcher, Thevaluebiotech, Chuncheon 24232, Korea.
[3]강원대학교 의생명과학대학 의생명융합학부 석사과정생 / Master's degree student, Department of Biomedical Technology, Kangwon National University, Chuncheon 24341, Korea.
[4]한국기초과학지원연구원 춘천센터 센터장 / Head of center, Korea Basic Science Institute (KBSI), Chuncheon 24341, Korea.
[5]더밸류바이오텍 연구소장 / Head of research institute, Thevaluebiotech, Chuncheon 24232, Korea.
[6]강원대학교 의생명과학대학 의생명융합학부 교수 / Professor, Department of Biomedical Technology, Kangwon National University, Chuncheon 24341, Korea.

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 (Carroll *et al*., 2005; Morrison *et al*., 2006; Krum *et al*., 2008; Wend *et al*., 2012).

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 (Chakravarti *et al*., 1976). 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 (Zava *et al*., 1997). 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 (Boué *et al*., 2003). Such phytoestrogens have fewer adverse effects than synthetic estrogens; thus providing consumers with reliable alternatives (Tham *et al*., 1998).

MCF-7 cells are a human female breast cancer cell line. In particular, they contain many ERs and are highly sensitive to estrogen. When 17-β estradiol (E2) bind to its receptors, the rate of cell proliferation increases via the transmission of signals through the ER metabolic pathway. MCF-7 cells have favorable conditions for screening natural products containing estrogen-like substances; when estrogens, such as estradiol and similar estrogen substances, are present, cell proliferation increases (Villalobos *et al*., 1995).

Gastrodia elata Blume (GEB) is a perennial, parasitic plant belonging to the Orchidaceae family that grows and reproduces by receiving nutrition from plants of the genus *Morus*. GEB has several beneficial properties, including antioxidant (Han *et al*., 2014), anti-cardio-cerebrovascular disease (Zeng *et al*., 2006), anti-inflammatory and analgesic (Ahn *et al*., 2007), and memory-improving and anti-aging activities (Chen *et al*., 2011; Farooq *et al*., 2019).

In this study, the changes in the MCF-7 cell proliferation rate and expression levels of ER pathway-related genes and proteins were measured after GEB extract application. Furthermore, we identified the substance in GEB extract that exhibits estrogen-like effect.

MATERIALS AND METHODS

1. Extraction of GEB

A product grown in Gimcheon, Korea, and dried by slicing *Gastrodia elata* Blume harvested in autumn was purchased (Samdobong Cheonma-Farm, Gimcheon, Korea) and it was extracted by a 3 h sedimentation method using 30% ethanol.

Gastrodin and gastrodigenin were set and analyzed as raw material quality management indicators. The extract was concentrated using batch concentrator at 60 - 70℃ (Chuncheon Bioindustry Foundation, Chuncheon, Korea) and dried by spray dryer (Ein system, Seoul, Korea).

GEB extract was used sample for cell proliferation assay and qTOF analysis.

2. Cell culture

MCF-7 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). MCF-7 cells were incubated at 37℃ under 5% CO_2 for 6 days; the medium was changed every 3 days.

The medium used was RPMI 1640 (Welgene, Gyeongsan, Korea) containing 25 mM HEPES, L-glutamine, 1% penicillin-streptomycin (Hyclone, Marlborough, MA, USA), and 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Marlborough, MA, USA) treated with charcoal dextran (Sigma-Aldrich Co., St. Louis, MO, USA). The cells were subcultured to 8×10^5 cells/10㎖ in 100 ㎜ culture dishes.

3. Cell proliferation assay

The sulforhodamine B (Sigma-Aldrich, Saint Louis, MO, USA) assay was performed to measure the cell proliferation effect. The MCF-7 cells were seeded at a density of 1×10^4 cells/㎖ in 24-well culture plates and incubated at 37℃ under 5% CO_2 for 24 h.

After removing the medium, the cells were washed twice with phosphate-buffered saline (PBS). The samples were added to phenol red-free Dulbecco's modified Eagle medium (DMEM; Welgene, Gyeongsan, Korea) containing 25 mM HEPES, L-glutamine, 1% penicillin–streptomycin, and 10% charcoal dextran (Sigma-Aldrich Co., St. Louis, MO, USA)-

treated heat-inactivated FBS.

The sample is GEB extract and the maximum concentration is 400 ㎍/㎖. After incubation for 144 h, the medium was removed and the cells were washed with PBS, loaded with 400 ㎕ of 10% trichloroacetic acid (Sigma-Aldrich, Saint Louis, MO, USA), and fixed at 4℃ for 30 min. The solution was removed and the cells were washed twice with PBS. All wells containing the cells were dried at 20℃–30℃. The wells were then loaded with 400 ㎕ of 4% sulforhodamine B (in 1% acetic acid) and allowed to stand for 15 min to stain the cells. The mixture was washed three times with 1% acetic acid solution and dried at 20–30℃.

Finally, the cells were mixed with 200 ㎕ of 10 mM Tris (pH 9.5) (Sigma-Aldrich Co., St. Louis, MO, USA) and supernatant were transferred to a 96-well plate. The absorbance of the samples was measured at 490 ㎚ using an Epoch microplate spectrophotometer (Biotek, Santa Barbara, CA, USA) to confirm the cell proliferation rate. The control group for statistical inference was used based on cells that did not treat E2 or GEB extract.

Cell viability (%) : A_{sample} / $A_{control}$ × 100

4. RNA isolation

The total RNA was isolated from cells to confirm the differences in the expression levels of genes related to the ER. MCF-7 cells were seeded at a density of 2 × 10^5 cells/㎖ in 60-mm culture dishes and incubated for 24 h.

After removing the medium, the cells were washed twice with PBS. The samples were treated in each dish with 200 ㎍/㎖ of test samples and incubated for 144 h. After removing the medium, the cells were washed with 4℃ PBS, detached the cells with a cell scraper, and harvested into an Eppendorf tube.

The total cellular RNA was isolated from MCF-7 cells using AccuZol total RNA extraction solution (Bioneer, Daejeon, Korea). RNA concentration was measured using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA). Briefly, 2 ㎕ of RNA samples were loaded onto the Take3 Micro-Volume Plate. The wavelengths of 230, 260, and 280 ㎚ were read and the concentration of RNA was calculated.

5. Quantitative real time-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed to evaluate the expression of trefoil factor 1 (*TFF-1*), progesterone receptor (*PR*), and ER-related genes.

The total RNA (500 ng) was reverse transcribed into complementary DNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan). Relative gene expression levels were measured using the THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Osaka, Japan). 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) under the following conditions: holding stage: 1 min at 95℃; cycling stage: 15 s at 95℃ to 30 s at 58℃, total 40 cycles; melt curve stage: 15 s at 95℃ and 30 s at 60℃, measured every 0.5℃

6. Immunoblotting

Cell pellets were lysed with radioimmunoprecipitation buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific Inc., Waltham, MA, USA).

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 ㎍. 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, England) 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

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 GAC 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

(TBS-T) for 1 h at 20 – 30℃ for blocking. The membrane was incubated with primary antibodies for 16 h at 4℃, washed three times with TBS-T, and incubated with horseradish peroxidase-linked secondary antibodies for 1 h at 20 – 30℃. 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 using a stripping buffer (10% SDS, 0.5 M Tris-HCl, and 0.8% β-mercaptoethanol) for 10 min at 60℃ 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 mitogen-activated protein (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, England). Horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

7. Influence of ICI 182.780 on cell proliferation

ICI 182.780 (Sigma-Aldrich Co., St. Louis, MO, USA), 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/㎖) 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.

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-fractionated 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 Co., Milford, MA, USA) coupled Waters Xevo G2 QTOF MS (Waters MS Technologies, Manchester, Engalnd) 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 ㎛). The 2 ㎕ of GEB fraction were injected (10℃) with the flow rate of 300 ㎕/min and column oven temperature was 40℃. The analysis was carried out by Acquity UPLC BEH C18 column (50 ㎜ × 2.1 ㎜, 1.7 ㎛) (Waters Co., Milford, MA, USA). The mobile phase was used as 0.1% formic acid in H_2O 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 and 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).

9. Statistical analysis

The results of each experiment are expressed as means ± standard deviation (SD). The One-way ANOVA (Dunnett's test, Statistical package for social sciences, version 23.0, SPSS Inc., Chicago, IL, USA) was used to compare the group means. Values were considered significant when $p < 0.05$.

RESULTS

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

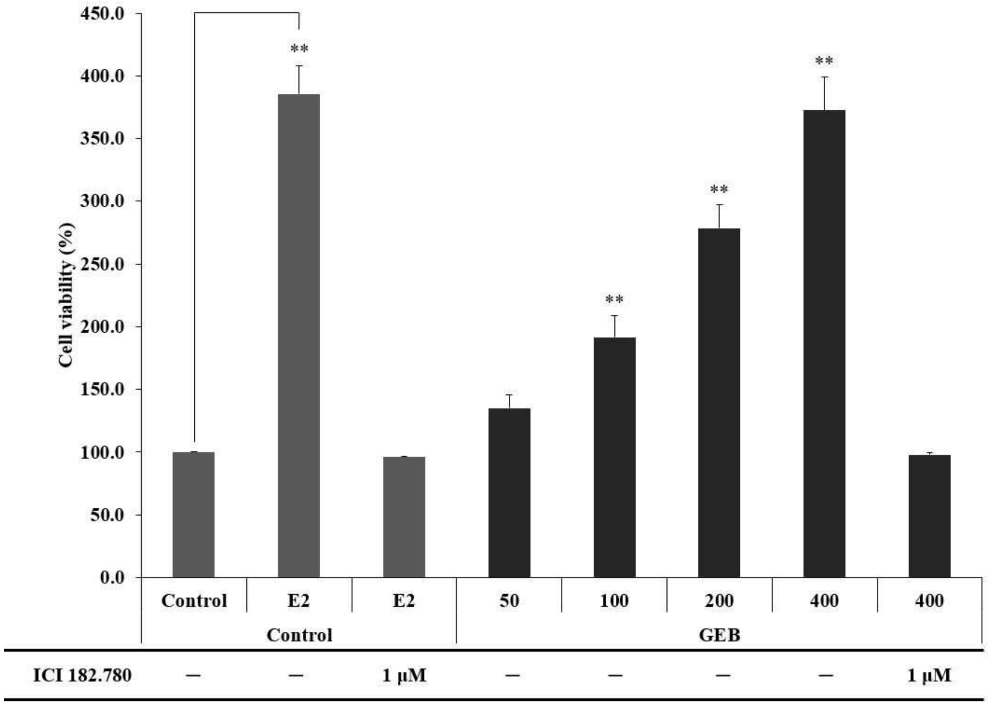

Fig. 1. Effect of GEB extract on cell proliferation. The cell proliferation rates are expressed as percentages. A sample treated with 0.3% EtOH was used as a control and the result was normalized to 100%. Cell proliferation rates were analyzed by treating MCF-7 cells with GEB extract in a concentration-dependent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

concentration-dependent manner (50, 100, 200, and 400 ㎍/㎖).

At 100 ㎍/㎖ 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 ㎍/㎖ GEB extract (viability = 373%) was similar to that of 17-β 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%) (Fig. 1).

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 ㎍/㎖), 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 (Fig. 2).

We confirmed the ER pathway with protein level in MCF-7 cells treated with GEB extract (200 ㎍/㎖) 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

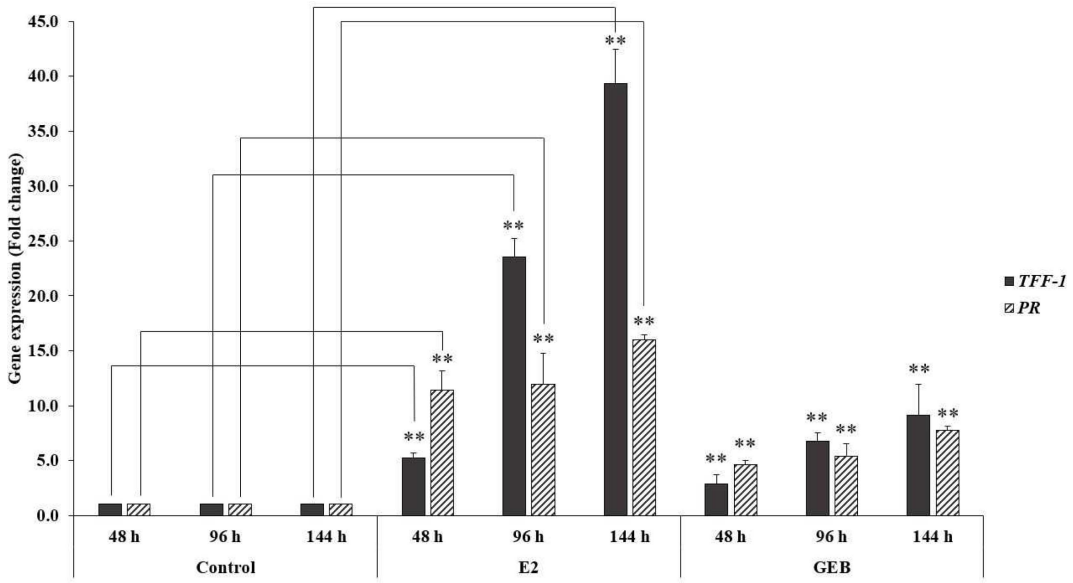

Fig. 2. Effect of GEB extract on estrogen receptor-related gene expression. Analysis of mRNA expression in MCF-7 cells. Cells were treated with GEB extract (200 ㎍/㎖) for 48, 96, 144 h. The relative gene expression level is expressed as fold change. A vehicle sample treated with 0.3% EtOH was used as 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 means ± SD of three experiments. **Means significantly different from the control ($p < 0.05$).

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 (Fig. 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

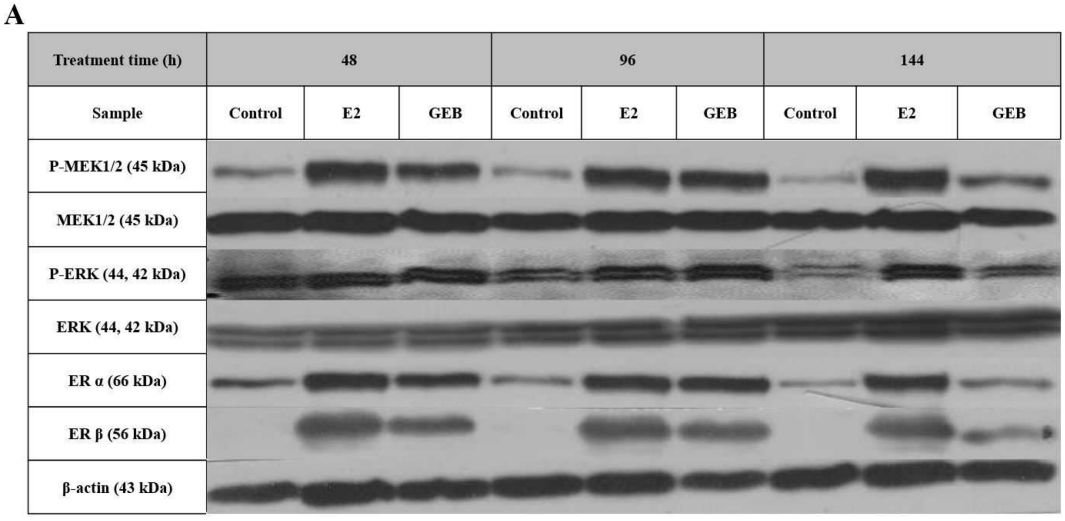

Fig. 3. ER pathway up-regulated by GEB extract. (A) The protein expression levels of the total and phosphorylated forms of MEK1/2, ERK1/2, estrogen receptor alpha and beta, and beta-actin were visualized by immunoblotting. MCF-7 Cells were treated with GEB extract (200 ㎍/㎖) for 48, 96, and 144 h (B) 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 means ± SD of three experiments. **Means significantly different from the control ($p < 0.05$).

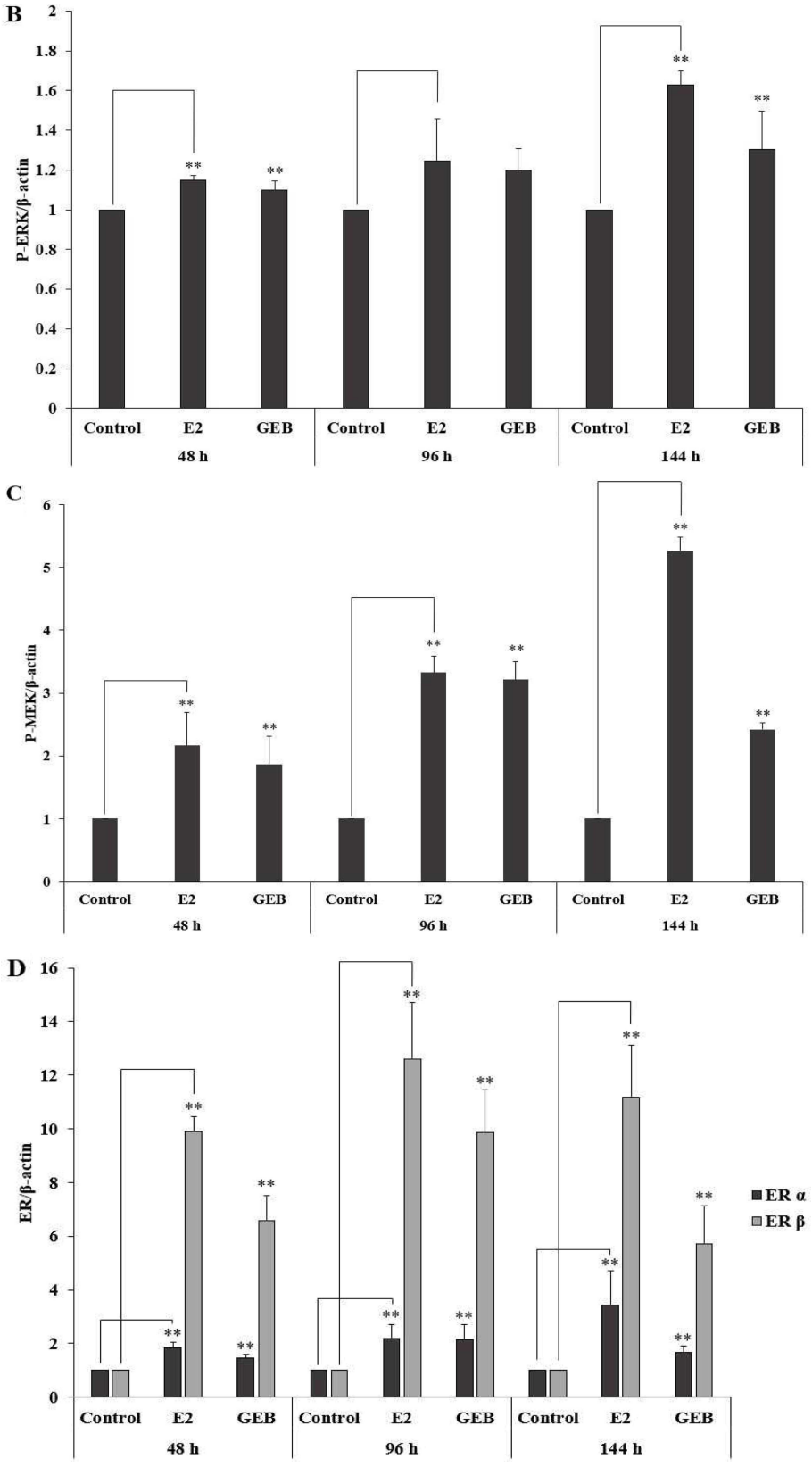

Fig. 3. Continued.

(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-1* and *PR*, increased after E2 (10^{-10} M) (*TFF-1* gene two-fold = 34.5, *PR* gene two-fold = 16.0) or GEB extract (200 ㎍/㎖) (*TFF-1* gene two-fold = 9.1, *PR* gene two-fold = 7.7) treatment. When the cells were treated with ICI 182.780

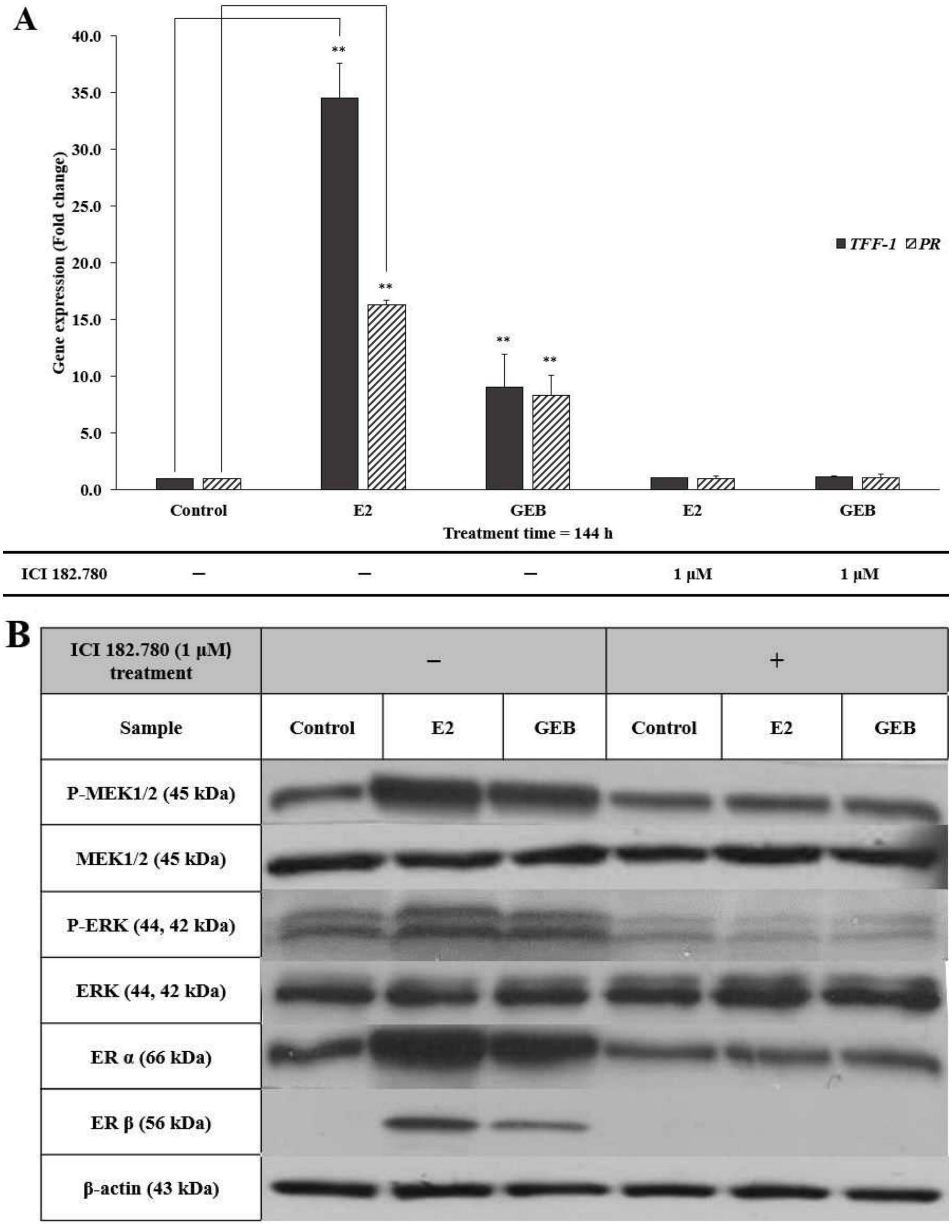

Fig. 4. Inhibitory effect of ER antagonist ICI 182.780 on GEB extract. (A) The relative gene expression level is expressed as fold change. A sample treated with 0.1% dimethyl sulfoxide (vehicle) was used as the control and the result was normalized to 1.0. After treatment with GEB extract at the same time (144 h) in MCF-7 cells, the expression levels of *TFF-1* and *PR* were analyzed. Values are means ± SD of three experiments. **Means significantly different from the 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 means ± SD of three experiments. **Means significantly different from the control ($p < 0.05$).

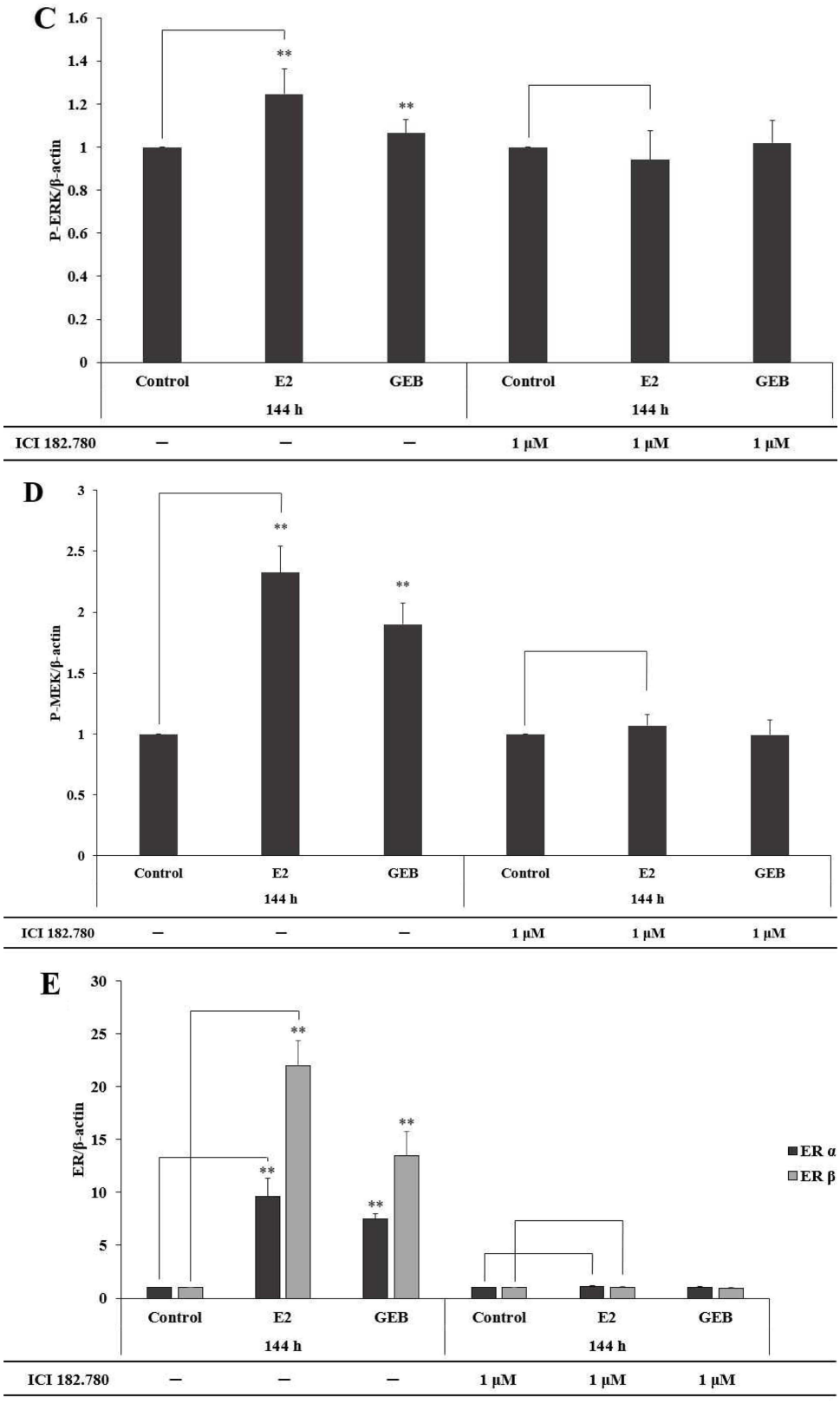

Fig. 4. Continued.

followed by E2 or GEB extract, there was no related gene expression (E2 *TFF-1* gene two-fold = 1.1, *PR* gene two-fold = 1.2) (GEB extract *TFF-1* gene two-fold = 1.2, *PR* gene two-fold = 1.2) (Fig. 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 = 0.9, ER α/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) (Fig. 4).

4. Identification of effective compound from 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%) (Fig. 5A). The yield of fractions using the properties according to the polarity of the material was confirmed to be 9.75% of hexane, 7.98% of dichloromethane, 5.32% of ethylacetate, 19.5% of butanol, respectively.

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 nitrogen gas. The concentrated material was

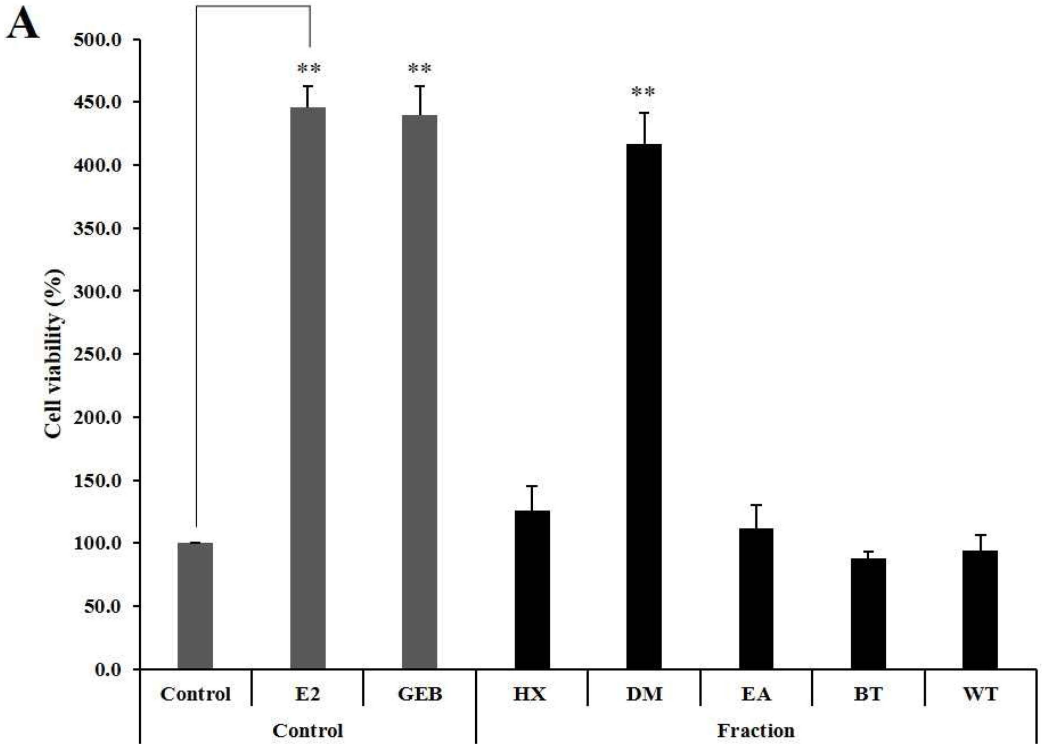

Fig. 5. Identification of effective compound in GEB extract by qTOF mass. (A) Polar solvent fraction was tested cell proliferation assay with MCF-7 cells. A sample treated with 0.1% dimethyl sulfoxide (vehicle) was used as the control and the result was normalized to 1.0. After treatment with fractionated materials at the same time (144 h) in MCF-7 cells, the cell proliferation levels were analyzed. Values are means ± SD of three experiments. **Means significantly different from the 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.

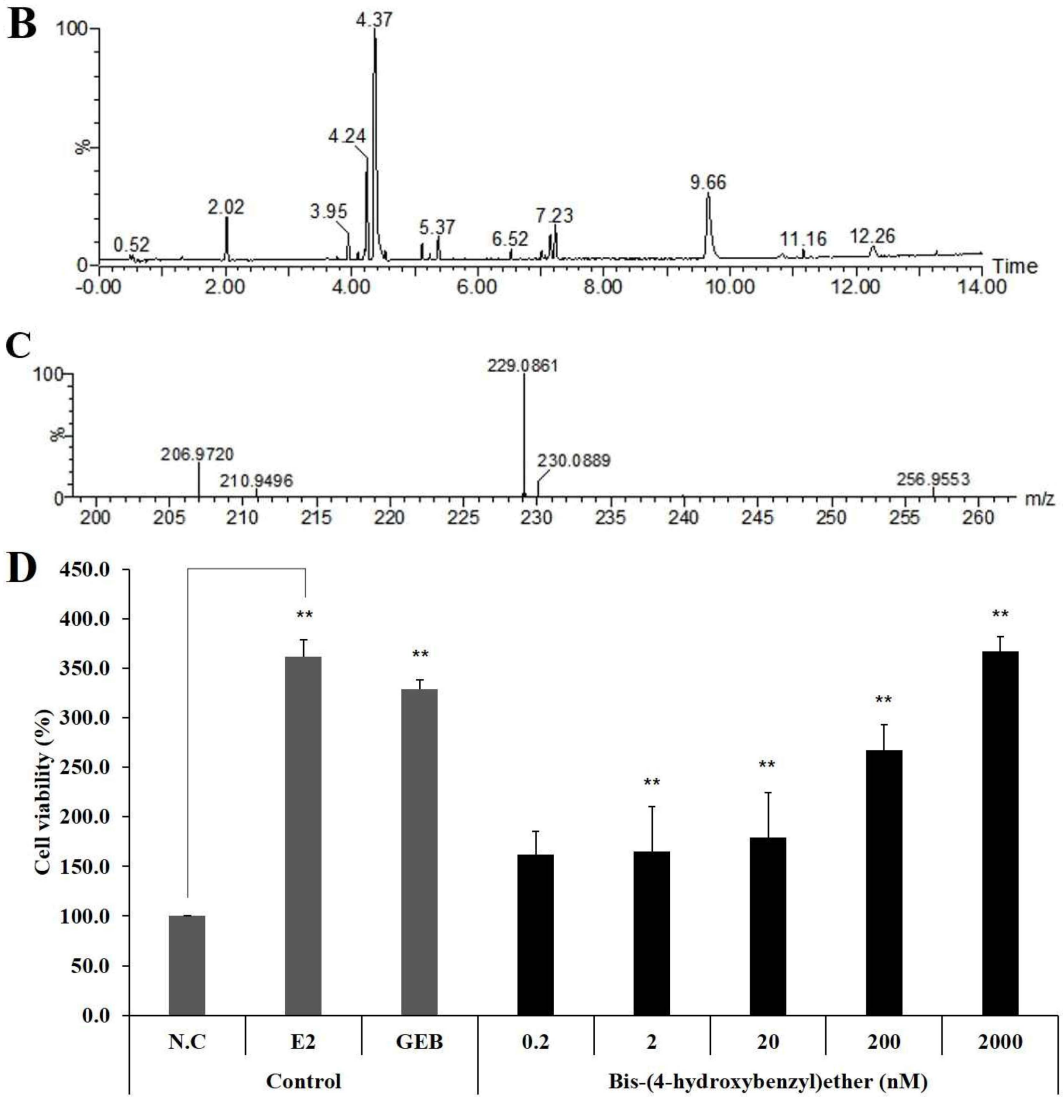

Fig. 5. Continued.

analyzed against for chemical library-based qTOF and several compounds were identified in this fraction (Fig. 5B and Table 2).

The bis(4-hydroxybenzyl)ether was identified with standard compound purchased from Simson Pharma Limited company by comparing its retention time and mass spectrum (Fig. 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 (Ha *et al*., 2000, Tang *et al*., 2015). In cell proliferation assay, bis(4-hydroxybenzyl)ether showed a strong effect equivalent to E2, a control reagent, at a concentration of 2 μM (Fig. 5D). The quantitative content of bis(4-hydroxybenzyl)ether in GEB extract was analyzed to be 19.861 ㎍/㎖.

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 (Ahn *et al*., 2007). Compounds contained in GEB have been found to reduce levels of ROS and MDA, and to regulate genes related to antioxidants (Kim *et al*., 2014).

Gastrodin contained in GEB is known as a major physiologically active ingredient. Gastrodin has antioxidant and anti-inflammatory properties. It also modulates neurotransmitters,

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	Fu *et al*., 2019; Ha *et al*., 2000; Tang *et al*., 2016; Kim *et al*., 2020
2.33	Vanillin	C8H8O3	151.0402	1.0	2343	136.0168	Fu *et al*., 2019
2.82	trans-p-coumaric acid	C9H8O3	163.0398	-1.4	4454	119.0503	Fu *et al*., 2019
3.95	Unknown	C15H14O3	241.0871	0.5	276053	–	–
4.10	4-Hydroxybenzaldehyde	C7H6O2	121.0292	-2.4	43357	92.0268, 121.0294	Fu *et al*., 2019; Ha *et al*., 2000; Tang *et al*., 2016
4.24	Resveratrol	C14H12O3	227.0715	0.5	835554	143.0499, 185.0595	Qiao *et al*., 2020
4.37	Unknown	C15H16O5	275.0932	2.5	547395	–	–
4.37	Bis-(4-hydroxybenzyl)ether	C14H14O3	229.0876	2.3	2514205	93.0342, 107.0501, 121.0294	Tang *et al*., 2016; Kim *et al*., 2020
4.54	Unknown	C15H14O3	241.0874	1.7	99164	–	–
5.12	5-[4'-(4"-hydroxybenzyl)-3'-hydroxybenzyloxymethyl]-furan-2-carbaldehyde	C20H18O5	337.1087	1.5	140252	–	Huang *et al*., 2015
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	Tang *et al*., 2016; Kim *et al*., 2020
7.23	Unknown	C17H26O4	293.1758	0.0	305133	–	–
11.16	Unknown	C15H22O3	249.1500	1.5	140869	–	–

and it has been shown that Gastrodin may be effective against diseases of the general central nervous system, including epilepsy, Alzheimer's disease and Parkinson's disease (Liu *et al*., 2018).

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 ㎍/㎖ GEB extract, the proliferation rate increased in a concentration-dependent manner. The cell proliferation effect of 400 ㎍/㎖ GEB extract was similar to that of 17-β estradiol. It was confirmed that the estrogen-like effect of the *Gastrodia elata* Blume extract showed similar activity to that of the red bean and black bean extracts (Data not shown). 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.

Among these candidate materials, bis(4-hydroxybenzyl)ether has shown cell proliferation effect in MCF-7 cell. Bis(4-

hydroxybenzyl)ether in the *Gastrodia elata* Blume was first reported in 1981 (Takuchi *et al.*, 1980). Since then, there have been reports of bis(4-hydroxybenzyl)ether substances containing in the *Gastrodia elata* Blume, but no estrogen-like effects have been reported.

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. And 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.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Small and Medium-sized Enterprises (SMEs) and Startups (MSS), Korea, under the "Regional Specialized Industry Development Plus Program (R&D, S2910423)" supervised by the Korea Institute for Advancement of Technology (KIAT) and the Research Grant from Kangwon National University (grant number 520170412).

REFERENCES

Ahn EK, Jeon HJ, Lim EJ, Jung HJ and Park EH. (2007). Anti-inflammatory and anti-angiogenic activities of *Gastrodia elata Blume*. Journal of Ethnopharmacology. 110:476-482.

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

Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA and Brown M. (2005). Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell. 122:33-43.

Chakravarti S, Collins WP, Forecast JD, Newton JR, Oram DH and Studd JW. (1976). Hormonal profiles after the menopause. British Medical Journal. 2:784-787.

Chen PJ, Liang KC, Lin HC, Hsieh CL, Su KP, Hung MC and Sheen LY. (2011). Gastrodia elata Bl. Attenuated learning deficits induced by forced-swimming stress in the inhibitory avoidance task and Morris water maze. Journal of Medicinal Food. 14:610-617.

Dratva J, Gómez Real FG, Schindler C, Ackermann-Liebrich, U, Gerbase MW, Probst-Hensch NM, Svanes C, Omenaas ER, Neukirch F, Wjst M, Morabia A, Jarvis D, Leynaert B and Zemp E. (2009). Is age at menopause increasing across Europe? Results on age at menopause and determinants from two population-based studies. The Journal of The North American Menopause Society. 16:385-394.

Farooq U, Pan Y, Lin Y, Wang Y, Osada H, Xiang L and Qi J. (2019). Structure characterization and action mechanism of an antiaging new compound from *Gastrodia elata Blume*. Oxidative Medicine and Cellular Longevity. 2019:5459862. https://www.hindawi.com/journals/omcl/2019/5459862/ (cited by 2022 April 1).

Fu Y, Shan M, Hu M, Jiang Y, Chen P, Chi Y, Yu S, Zhang L, Wu Q, Zhang F and Mao Z. (2019). Chemical profiling of Banxia-Baizhu-Tianma decoction by ultra-fast liquid chromatography with tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis. 174:595-607.

Gold EB, Bromberger J, Crawford S, Samuels S, Greendale GA, Harlow SD and Skurnick J. (2001). Factors associated with age at natural menopause in a multiethnic sample of midlife women. American Journal of Epidemiology. 153:865-874.

Ha JH, Lee DU, Lee JT, Kim JS, Yong CS, Kim JA, Ha JS and Huh K. (2000). 4-Hydroxybenzaldehyde from *Gastrodia elata* B1. is active in the antioxidation and GABAergic neuromodulation of the rat brain. Journal of Ethnopharmacology. 73:329-333.

Han YJ, Je JH, Kim SH, Ahn SM, Kim HN, Kim YR, Choi YW, Shin HK and Choi BT. (2014). *Gastrodia elata* shows neuroprotective effects via activation of PI3K signaling against oxidative glutamate toxicity in HT22 cells. The American Journal of Chinese Medicine. 42:1007-1019.

Huang LQ, Li ZF, Wang Q, Cui YR, Ou YH, Xiong SS, Huang WP, Feng YL and Yang SL. (2015). Two new furaldehyde compounds from the rhizomes of *Gastrodia elata*. Journal of Asian Natural Products Research. 17:352-356.

Kim HM, Kwon JY, Lee KR, Lee JW, Jang DS and Kwon HC. (2020). Constituents of *Gastrodia elata* and their neuroprotective effects in HT22 hippocampal neuronal, R28 retinal cells, and BV2 microglial cells. Plants. 9:1051. https://www.mdpi.com/2223-7747/9/8/1051 (cited by 2022 April 1).

Kim MH, Kim JG and Choi JH. (2014). Antioxidant activity and changes in major functional components of fermented *Gastrodia elata Blume*. The Korean Journal of Food and Nutrition. 27:684-691.

Krum SA, Miranda-Carboni GA, Hauschka PV, Carroll JS, Lane TF, Freedman LP and Brown M. (2008). Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. EMBO Journal. 27:535-545.

Lee SJ, Lenton EA, Sexton L, and Cooke ID. (1988). The effect of age on the cyclical patterns of plasma LH, FSH, oestradiol and progesterone in women with regular menstrual cycles. Human Reproduction. 3:851-855.

Liu Y, Gao J, Peng M, Meng H, Ma H, Cai P, Xu Y, Zhao Q and Si G. (2018). A review on central nervous system effects of gastrodin. Frontiers in Pharmacology. 9:24. https://www.frontiersin.org/articles/10.3389/fphar.2018.00024/full (cited by 2022 April 1).

Morrison JH, Brinton RD, Schmidt PJ and Gore AC. (2006).

Estrogen, menopause, and the aging brain: How basic neuroscience can inform hormone therapy in women. Journal of Neuroscience. 26:10332-10348.

Moy B and Goss PE. (2006). Estrogen receptor pathway: Resistance to endocrine therapy and new therapeutic approaches. Clinical Cancer Research. 12:4790-4793.

Qiao M, Guang C, Ding L, Shi X, Chai L and Feng X. (2020). Metabolic study of resveratrol in rat biosamples by UPLC-Q-TOF/MS. Cement and Concrete Research. 2:96-109.

Sørensen MB, Rosenfalck AM, Højgaard L and Ottesen B. (2001). Obesity and sarcopenia after menopause are reversed by sex hormone replacement therapy. Obesity Research and Clinical Practice. 9:622-626.

Taguchi H, Yosioka I, Yamazaki K, and Kim IH. (1981). Studies on the constituents of *Gastrodia elata* Blume. Chemical and Phamaceutical Bulletin. 29:55-62.

Tang C, Wang L, Liu X, Cheng M and Xiao H. (2016). Chemical fingerprint and metabolic profile analysis of ethyl acetate fraction of *Gastrodia elata* by ultra performance liquid chromatography/quadrupole-time of flight mass spectrometry. Journal of Chromatography B. 1011:233-239.

Tham DM, Gardner CD and Haskell WL. (1998). Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence. The Journal of Clinical Endocrinology Metabolism. 83:2223–2235.

Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM and Pedraza V. (1995). The E-screen assay: A comparison of different MCF7 cell stocks. Environmental Health Perspectives. 103:844-850.

Wend K, Wend P and Krum SA. (2012). Tissue-specific effects of loss of estrogen during menopause and aging. Frontiers in Endocrinology. 3:19. https://www.frontiersin.org/articles/10.3389/fendo.2012.00019/full (cited by 2022 April 1).

Zava DT, Blen M and Duwe G. (1997). Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture. Environmental Health Perspectives. 105:637-645.

Zeng X, Zhang S, Zhang L, Zhang K and Zheng X. (2006). A study of the neuroprotective effect of the phenolic glucoside gastrodin during cerebral ischemia *in vivo* and *in vitro*. Planta Medica. 72:1359-1365.