ABSTRACT: Based on a previous study, glabridin displayed a dose-dependent increase in estrogenic activity and cell proliferative activity in Ishikawa cells. However, when treated in combination with 17β-E2, synergistic estrogenic effect was observed but without the same synergistic increase in cell proliferative effect. This study aimed to identify the estrogen and nonestrogen-regulated activities induced by glabridin and in combination with 17β-E2 in comparison with 17β-E2. The results showed that 10µM glabridin and the combination treatment of 100nM glabridin with 1nM 17β-E2 regulated both the genomic and nongenomic estrogen pathways to possibly provide benefits of estrogens in cardiovascular, circulatory, and vasculature systems. Meanwhile, the combination of 100nM glabridin with 1nM 17β-E2 seems to be more suitable to be used as an estrogen replacement. Finally, the results of this study have added on to the present knowledge of glabridin’s function as a phytoestrogen and suggested new ideas for the usage of glabridin.

KEYWORDS: Glabridin, microarray, Ishikawa cells

Background
Licorice root of the Glycyrrhiza plant has been used for medicinal purposes for more than 4000 years.1 The plant is widely used in Asia as herbal medicine, flavoring agent, and sweetener.2 As a medicinal plant, licorice root has been used for menopausal symptoms in women.3,4 The estrogenicity of licorice has been attributed to the presence of phytoestrogens such as glabridin, a prenylated isoflavonoid.5,6

According to Botta et al,7 prenylation of an isoflavonoid modulates its response toward estrogen receptors (ER). In 2000, Tamir et al showed that glabridin expressed ER-dependent growth-promoting effect at 10 to 100nM and ER binding activity in T47D human breast ductal carcinoma cells with an IC50 of 5nM. The ER binding activity of glabridin was found to be similar to values of other known phytoestrogens, such as genistein, which is 104 lower than that of 17β-E2. At the same time, glabridin also had ER-independent antiproliferative activity at concentrations of >15mM. Tamir et al8 also showed that glabridin activated creatine kinase (CK) activity in skeletal and cardiovascular tissues while increasing uterine weight of rats displaying the potential of glabridin to bring out the positive effect of 17β-E2 in the bone, cardiovascular tissues, and the uterus. This selective ER modulator activity of glabridin showed that it acts as a phytoestrogen and suggested that glabridin may serve as a natural estrogen agonist in preventing the symptoms and diseases associated with estrogen deficiency.

Later studies have shown more similarities between glabridin and 17β-E2. In 2004, Somjen et al discovered that both glabridin and 17β-E2 stimulated DNA synthesis in human endothelial cells and exhibited biphasic effect on proliferation of human vascular smooth muscle cells. Meanwhile, in animal studies, both glabridin and 17β-E2 also stimulated the activity of CK in the aorta and left ventricle of the heart of both intact and ovariectomized female rats.8 Later on, Choi9 discovered that glabridin (1–10µM) significantly increased alkaline phosphatase (ALP) activity, cell proliferation, collagen content, and osteocalcin secretion in MC3T3-E1 osteoblast cells, supporting its use for the prevention of osteoporosis and inflammation in the bone. The increase in ALP activity and collagen was thought to be related to estrogenic effects as they were inhibited by 1µM tamoxifen.9

In the previous study,10 it was found that glabridin displayed a dose-dependent increase in ALP activity from 1pM to a maximum induction at 10µM. At 10µM, glabridin induced cell proliferation of 25.04±3.33% increase from the untreated control. When treated in combination with 17β-E2, synergistic effect was observed and the greatest synergy occurred at the combination treatment of 100nM glabridin with 1nM 17β-E2 (synergy quotient [SQ] of 1.217). At this combination treatment, there was a slight decrease in cell proliferation (16.98±3.76% increase from control) compared with the combination treatment of 100nM glabridin with 1nM 17β-E2 (synergy quotient [SQ] of 1.217). At this combination treatment, there was a slight decrease in cell proliferation (16.98±3.76% increase from control) compared with the combination treatment of 100nM glabridin with 1nM 17β-E2 (synergy quotient [SQ] of 1.217). At this combination treatment, there was a slight decrease in cell proliferation (16.98±3.76% increase from control) compared with the combination treatment of 100nM glabridin with 1nM 17β-E2 (synergy quotient [SQ] of 1.217). At this combination treatment, there was a slight decrease in cell proliferation (16.98±3.76% increase from control) compared with the combination treatment of 100nM glabridin with 1nM 17β-E2 (synergy quotient [SQ] of 1.217).

The results showed that 10µM glabridin and the combination treatment of 100nM glabridin with 1nM 17β-E2 seems to be more suitable to be used as an estrogen replacement. Finally, the results of this study have added on to the present knowledge of glabridin’s function as a phytoestrogen and suggested new ideas for the usage of glabridin.
downstream study. The treatments selected were the untreated control, 10 μM glabridin, 100 nM glabridin, 1 nM 17β-E2, and the combination treatment of 100 nM glabridin with 1 nM 17β-E2. The cells were treated for 72 hours and the RNA was extracted for microarray and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis to evaluate on the estrogen responsive genes and possible activities.

**Materials and Methods**

**Chemicals**

Minimum Essential Medium (MEM), 17β-E2, and dextran-coated charcoal (DCC) were purchased from Sigma, USA. Fetal bovine serum (FBS), fetal calf serum (FCS), antibiotic-antimycotic, and l-glutamine were obtained from Gibco, USA. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) was sourced from Nacalai Tesque, Japan. Glabridin was purchased from Wako, Japan. Phosphate-buffered saline was from OXOID, Lenexa, KS, USA. Thiazolyl blue tetrazolium bromide (MTT) was obtained from Amresco, USA. Premarin was obtained from Wyeth Pharmaceuticals, USA. Receptor cofactor assay was purchased from Cosmo Bio Co., Ltd, Japan. All other chemicals were purchased from Merck, Germany.

**Cell culture and maintenance of Ishikawa cells**

Ishikawa cells (99040201; European Collection of Authenticated Cell Cultures [ECACC]), which are human endometrial adenocarcinoma cells, were maintained in MEM supplemented with 10% FBS, 1% antibiotic-antimycotic, and 1% l-glutamine. Cells were passaged twice weekly. Before 2 days of the start of the experiment, near-confluent cells were changed to an estrogen-free basal medium (EFBM). The EFBM consists of DMEM/F-12 media supplemented with 5% DCC-stripped FCS, 1% antibiotic-antimycotic, and 1% l-glutamine. After 24 hours, the cells were harvested with 0.25% EDTA-trypsin and seeded in 96-well flat-bottomed microtiter plates, in 100 μL of EFBM per well.

**Preparation of test compounds**

All the test compounds were dissolved in ethanol (EtOH) and aliquoted accordingly. Prior to use, the test compounds were diluted appropriately with EFBM until the final concentration of EtOH was at 0.1% (v/v). All test compounds were stored at −20°C.

**Treatment**

Approximately 1.0 × 10^6 cells was seeded into T-75 flasks in 10 mL of EFBM. After 24 hours, when the cells had attached, the EFBM was replaced with 10 mL of EFBM containing test compounds at the appropriate concentrations. After addition of test compounds, the cells were incubated at 37°C in a 5% CO2 incubator for 72 hours. All experimental conditions were assayed in triplicate. The test compounds treated to the cells were as follows: untreated control (MEM with 0.1% EtOH), 10 μM glabridin, 100 nM glabridin, 1 nM 17β-E2, and 100 nM glabridin and 1 nM 17β-E2. All treatments were performed in triplicate.

**RNA extraction**

RNA extraction was performed using the QIAshredder spin column (Qiagen, USA) and RNeasy Mini Kit (Qiagen, USA) as per manufacturer’s protocol. The eluted RNA was quantified using DU 730 Life Science UV/Vis Spectrophotometer (Beckman Coulter, USA). Only RNA with an A_{260}/A_{280} ratio of 1.9 to 2.1 and A_{260}/A_{230} ratio of 1.7 to 2.1 was used for subsequent experiments.11

**Deoxyribonuclease treatment**

The extracted RNA was subjected to deoxyribonuclease (DNase) treatment using the RapidOut DNA Removal Kit (Thermo Scientific, USA) as per manufacturer’s protocol. Briefly, 10 μg of RNA in nuclease-free water was added to 5 μL of 10× DNase buffer with MgCl₂, 2.5 μL of RNase-free DNase I and adjusted with nuclease-free water up to 50 μL.

**Microarray analysis**

The extracted messenger RNA (mRNA) (250 ng) was converted to complementary DNA (cDNA) using GeneChip 3′ IVT Express Kit (Affymetrix, USA) as per manufacturer’s protocol (Affymetrix, USA). Next, antisense RNA (aRNA) amplification was performed using the GeneChip 3′ IVT Express Kit (Affymetrix, USA). After aRNA synthesis, the aRNA was placed on ice and quantified immediately for fragmentation. Hybridization was performed using the GeneChip PrimeView array (Affymetrix, USA) in the Affymetrix GeneChip Hybridization Oven 645 (Affymetrix, USA). After washing and staining, the probe arrays were scanned using the Affymetrix GeneChip Scanner 3000. A total of 3 biologically independent assays were performed.12

**Microarray data analysis**

For the analysis, each probe set was analyzed as an individual entity, based on its Affymetrix ID number (Affy ID), regardless of the multiplicity of probe sets representing any given gene product and were considered as representing an individual gene until the completion of the analysis.12 For analysis of gene expression, GeneSpring GX 12.6.1 Software (Agilent, USA) was used. The microarrays were analyzed via robust
evaluate the stability of the candidate reference genes. The Cq values collected across all samples were used to

Selection of a suitable reference gene

The Cq values collected across all samples were used to evaluate the stability of the candidate reference genes.

Quantitative reverse transcription polymerase chain reaction

The mRNA extracted was converted to cDNA using Omniscript Reverse Transcripase (Qiagen, USA), according to manufacturer’s protocol. The RT-qPCR was performed using the qPCRBIOSY Green Mix Lo-ROX 2X master mix (PCR Biosystems, UK) following manufacturer’s protocol and conditions, as shown in Table S3. All experiments were performed using a Mastercycler RealPlex (Eppendorf, USA). No RT and no DNA template control were included to ensure that the samples were not contaminated with either genomic DNA or primer dimers. All RT-qPCRs were run in triplicate.

RT-qPCR data analysis

Quantification cycle (Cq) values for all the genes from all the samples were compiled and imported to qbasePLUS data analysis software (Biogazelle, Belgium). Each gene was adjusted for amplification efficiency according to their own standard curve and was scaled to the untreated control. The lower and upper boundaries of Cq range for positive controls were 5 and 35, respectively. The acceptable replicate variability (difference in Cq) was <0.5. For normalization, the genes of interest were normalized with the 2 reference genes that were chosen from 8 different housekeeping genes. For statistical analysis, unpaired t test was performed with multiple correction testing on the RT-qPCR data after normalization with the reference genes.

Primer design

Primers specific for the gene of interest was designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The details of primers designed are listed in Table S1 for the genes of interest and Table S2 for the candidate reference genes.

Several programs were used for the identification of suitable reference genes used in this study: (1) BestKeeper (http://www.gene-quantification.com/bestkeeper.html), (2) NormFinder (http://moma.dk/normfinder-software), and (3) geNorm in qbasePLUS (Biogazelle, Belgium). The suitable reference genes were selected based on the results generated from all 3 programs.

Results and Discussions

Microarray analysis showed that the treatment of 10 μM glabridin was relatively similar to 1 nM 17β-E2

Based on the results from ALP and MTT assay from a previous study, glabridin exhibited weak estrogenic activity by increasing ALP activity and it also increased cell proliferation. However, in the presence of 17β-E2, the activity of glabridin differed from other isoflavones in that it decreased cell proliferation while increasing ALP activity. The presence of synergism suggests that mechanisms of action of glabridin and 17β-E2 are likely to occur at different sites and/or through different pathways. As this is the first study to investigate the use of glabridin and 17β-E2 as an estrogen replacement therapy (ERT) agent, additional work is required to determine the activities of each of the drugs alone before activities of their synergistic effects can be determined. Hence, microarray was used to study the mechanisms and therapeutic effects exhibited by glabridin and the combination treatment in comparison with 17β-E2. Five different treatments were chosen for further downstream study via microarray. The treatments selected were the untreated control, concentration of glabridin which induced the highest estrogenic activity (10 μM glabridin), and the combination treatment of 100 nM glabridin with 1 nM 17β-E2 which induces a synergistic increase in estrogenic activity (SQ = 1.217) but with a lower induction of cell proliferation as compared with 17β-E2 alone, 100 nM glabridin, and 1 nM 17β-E2. The cells were treated for 72 hours and the RNAs were extracted for microarray and RT-qPCR analyses. All RNA samples had an RNA integrity number greater than 9.4 (Figure S1).

The principal component analysis plot (Figure 1) shows that most of the replicates were strongly correlated with each other. Replicates in each of the treatment groups clustered together and away from other treatments, except for 100 nM glabridin which seemed closely correlated with the control. The heatmap (Figure 2) suggests that the overall gene expression profile of 10 μM glabridin was similar to 1 nM 17β-E2, whereas 100 nM glabridin was similar to the control. Displaying similar activities as 17β-E2 suggests that glabridin could exert similar activities as 17β-E2. This supports many of the past studies which have suggested that glabridin displayed similar effects to 17β-E2. Volcano plot was used to identify the set of genes that were differently regulated from the control cells with the
inclusion criteria set as ≥1.5 fold change with a P value of ≤.05. The differentially regulated probe sets were deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE68333 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68333). The volcano plot showed that there was no significant difference in any probe sets between 100 nM glabridin and the control. Among the 49,395 probe sets on the GeneChip PrimeView array, a total of 8,204 probe sets were regulated by treatment of glabridin (10 μM and 100 nM), 1 nM 17β-E2, and the combination treatment by 100 nM glabridin with 1 nM 17β-E2.

**HPRT1 and PPIA represent suitable reference gene**

For the determination of the most suitable reference genes, geNorm (based in qbasePLUS), NormFinder, and BestKeeper were used. Based on geNorm, all the candidate reference genes were stably expressed (average geNorm M ≤0.2) (Figure S2). The most stable reference gene was PPIA, followed by B2M, GAPDH, and RPL13A. Meanwhile, HSP90AB1, ACTB, and TBP represented the 3 least stable reference genes. In general, geNorm V should be <0.15. In this study, geNorm V was <0.15 in general, but when comparing a normalization factor based on the 2 or 3 most stable targets with that of 3 or 4 targets, there was not much added value of including an additional reference gene. Therefore, the use of only 2 reference genes was sufficient.

Based on BestKeeper, PPIA (R = 0.859, P = .001) and HPRT1 (R = 0.743, P = .002) represent the top 2 most stably expressed reference genes based on the coefficient of correlation (R), as shown in Tables S1 and S2. The most stable reference genes were chosen based on the following 3 criteria: (1) high R value, (2) power (x-fold) of lesser than 2, and (3) standard deviation (±Cq) of lesser than 1. According to NormFinder, PPIA represents the most stable gene (M = 0.003), followed by HPRT1 (M = 0.005), as shown in Table S3. Also, the best combination of 2 genes which had the most stable value was PPIA and HPRT1, with a stability value of 0.003. Based on the results from geNorm (qbasePLUS), NormFinder, and BestKeeper, it was concluded that the 2 most suitable reference targets to be used for this study were HPRT1 and PPIA.

**Validation of microarray by RT-qPCR**

To validate the results obtained from the microarray, 12 genes—9 upregulated genes and 3 downregulated genes—were selected to be tested by RT-qPCR. The comparison of the relative expressions of the target genes from microarray and RT-qPCR is tabulated in Table 1. Consistent with the result of the microarray, PGR represented the most highly expressed gene and it was expressed more in 1 nM 17β-E2 than the combination treatment of 100 nM glabridin with 1 nM 17β-E2. As observed in Table 1, the trend of the genes of interest correlated with the microarray data. Hence, it was concluded that the microarray result shown in the study was suitably validated by RT-qPCR.
Upregulation of PGR confirms activation of classical estrogen signaling of glabridin

To further classify the effects of the different treatments that were not regulated by 17\(\beta\)-E\(_2\), the probe sets were compared via a Venn diagram. The Venn diagram in Figure 3 shows the probe sets that are overlapping between the different treatments on Ishikawa cells. Among the 38 commonly regulated probe sets (Figure 3), PGR represents the most upregulated gene. They were significantly involved in gene ontologies such as blood vessel development, multicellular organismal development, multicellular organismal process, developmental process, single-multicellular organism process, positive regulation of cell cycle, and regulation of epithelial cell proliferation. These probe sets were also involved in the pathways of the nuclear receptors and metabolism of steroid hormone pathways. Among the pathways that were regulated, ovarian infertility genes’ pathway was the most significantly regulated.

PGR which codes for progesterone receptor was the most upregulated gene in all the different treatment groups (Table 2). The increase in PGR expression by 17\(\beta\)-E\(_2\) had been well established in many steroid-sensitive tissues. In Ishikawa cells, the upregulation of PGR indicates estrogenic response as past literature showed that 17\(\beta\)-E\(_2\) upregulates PGR, whereas progesterone downregulates it.\(^{20,21}\) The upregulation of PGR also represents a marker for estrogen action that can occur via the classical estrogen signaling through ER-\(\alpha\), ER-\(\beta\) or as a result of mechanical stimulation by progesterone.\(^{22,23}\) Similarly, in this study, PGR was upregulated a lot higher compared with ESR1. The upregulation of PGR by the different treatments confirmed that they displayed estrogenic effects in Ishikawa cells via the classical estrogen pathway. This also showed that PGR is more suitable as a gene marker for detecting estrogenic activity in Ishikawa cells, compared with ESR1.

For global functional classifications, functional annotations derived from GeneSpring’s Gene Ontology Analysis

### Table 1. Comparison of the relative expressions of the target genes from microarray and quantitative reverse transcription polymerase chain reaction.

| GENES | MICROARRAY | COMBINATION OF 1 NM 17\(\beta\)-E\(_2\) AND 100 NM GLABRIDIN | QBASEPLUS | COMBINATION OF 1 NM 17\(\beta\)-E\(_2\) AND 100 NM GLABRIDIN |
|-------|------------|-------------------------------------------------|------------|-------------------------------------------------|
| ALDH3A1 | — | 4.08 | — | <1.19> | 3.33 | <1.34> |
| ALPL2 | 6.79 | — | 4.88 | 6.39 | <1.17> | <4.87> |
| ANPEP | 12.05 | 1.61 | 11.2 | 16.15 | <0.98> | 11.22 |
| C-FOS | — | −1.58 | −2.06 | <1.18> | <1.24> | 0.48 |
| C-JUN | — | — | −2.17 | <0.88> | <0.89> | 0.58 |
| COL1A2 | 5.26 | 1.94 | 5.59 | 2.58 | <2.06> | <1.94> |
| DHRS3 | 7.83 | 1.88 | 6.16 | 6.92 | 2.06 | 8.9 |
| ESR1 | 1.59 | — | 1.56 | 2.05 | <0.893> | <1.81> |
| HES2 | 6.66 | 1.53 | 6.85 | <1.89> | <0.51> | 6.6 |
| NRIP1 | 2.96 | — | 3.21 | 3.22 | 2.23 | <2.48> |
| PGR | 120.41 | 8.16 | 117.95 | 47.3 | 5.37 | 44.86 |
| PPARGC1A | −1.78 | −1.61 | −1.94 | 0.47 | 0.66 | 0.38 |

— represents values that were not available as they either had fold change <1.5 or the P value was >.05, whereas values in < > represent values that were not statistically significant at P<.05.
database were used. Figure 4 shows the effect of the distribution of the different treatments on biological processes, cellular components, and molecular functions in Ishikawa cells. Focusing on the probe sets regulated, Tables 2 and 4 presented the top 10 most highly upregulated and downregulated probe sets, respectively, by the treatment. PGR represents the most highly regulated gene in all treatments. Most of the genes, such as PGR, ANPEP, OLFM1, HES2, and DHRS3, were similarly regulated in both the combination treatment and 1 nM 17β-E2. This could be because their effect was highly influenced by the presence of 1 nM 17β-E2. Except for PGR, there were no other similarities between the top 10 highly upregulated genes between the 3 different treatments. Meanwhile, most of the most highly downregulated genes were by 10 μM glabridin. PPARGC1A was the only most highly downregulated gene that was common to all treatments.

Table 2. A list of the top 10 highly upregulated and downregulated probe sets in Ishikawa cells based on microarray from the different treatments that were significant compared with the control.

| PROBE SET ID | ENTREZ GENE | GENE SYMBOL | FOLD CHANGE |
|-------------|-------------|-------------|-------------|
|             |             |             | 100NM GLABRIDIN WITH 1 NM 17β-E2 | 1 NM 17β-E2 | 10 NM GLABRIDIN |
| Top 10 upregulated probe sets |
| 11726168_at | 5241 | **PGR** | 117.95 | 120.41 | 8.16 |
| 11755866_a_at | 10439 | **OLFM1** | 20.31 | 20.12 | — |
| 11717127_a_at | 290 | **ANPEP** | 11.20 | 12.05 | — |
| 11728894_at | 54626 | **HES2** | 6.85 | 6.66 | — |
| 11724762_a_at | 64798 | **DEPTOR** | 6.47 | — | — |
| 11716835_a_at | 9249 | **DHRS3** | 6.16 | 7.83 | — |
| 11721092_a_at | 7057 | **THBS1** | — | 7.53 | — |
| 11723334_at | 251 | **ALPPL2** | — | 6.79 | — |
| 11726360_a_at | 218 | **ALDH3A1** | — | — | 4.08 |
| 11754344_s_at | 81848 | **SPRY4** | — | — | 3.56 |
| Top 10 downregulated probe sets |
| 11746122_s_at | 9877 | **ZC3H11A** | — | — | 3.82 |
| 11716915_a_at | 57509 | **MTUS1** | — | — | 3.34 |
| 11755635_s_at | 728642///984 | **CDK11A///CDK11B** | — | — | 3.14 |
| 11729051_a_at | 8496 | **PPFIBP1** | — | — | 3.01 |
| 11731416_at | 4719 | **NDUFS1** | — | — | 2.76 |
| 11723403_at | 10891 | **PPARGC1A** | 1.94 | 2.97 | 2.69 |
| 11718954_at | 57532 | **NUFIP2** | — | — | 2.34 |
| 11718505_a_at | 222166 | **C7orf41** | — | — | 2.29 |
| 11764058_at | 5621 | **PRNP** | — | 2.88 | — |
| 11744091_s_at | 552889 | **ATXN7L3B** | — | — | 2.28 |

— represents values that were not available as they either had fold change <1.5 or the P value was >.05.

Table 3 tabulates the effect of the different treatments on gene ontologies related to activities in the circulatory, cardiovascular, and vascular systems. These activities had been, in the past, associated with its estrogenic action. As shown in Table 1, the combination treatment significantly regulated the most number of gene ontologies compared with 10μM glabridin and 1nM 17β-E2. Meanwhile, Table 4 shows the regulation of NRIP1 by the combination treatment and 1 nM 17β-E2 alone. This gene was not regulated by 10μM glabridin. This gene is involved in the estrogen pathway and was one of the genes chosen for validation of microarray.
Glabridin at 10 µM displayed similar effect on the estrogen pathway as 1 nM 17β-E2.

For the curation of biological pathways of the differentially regulated probe sets, WikiPathways (www.wikipathways.org) was used. Similar gene expression profiles between 10 µM glabridin and 1 nM 17β-E2 as compared with the control suggested that their activities might be analogous. Estrogen pathway is one of the commonly regulated pathways by 10 µM glabridin and 1 nM 17β-E2. From Figure 5, it could be seen that 10 µM glabridin and 1 nM 17β-E2 were clustered close together in comparison with the control, whereas the combination treatment of 100 nM glabridin with 1 nM 17β-E2 regulated the genes in a different manner.

Many of the genes are regulated in a similar manner between 10 µM glabridin and 1 nM 17β-E2. Based on the gene list—GSE68333, the effect of glabridin and the combination treatment on the estrogen pathway which could be classical, estrogen response element (ERE)–independent genomic, or nongenomic were drawn out in the form of pathways with the regulation of the different genes involved marked accordingly (Figure 6A and B). Although 10 µM glabridin did not upregulate ESR1, in a previous study, we found that glabridin induced a dose-dependent increase in ER-α–SRC-1 and coactivator complex activity.10 Hence, the proposed pathway for estrogenic action of glabridin in Ishikawa cells is shown in Figure 6A. Moreover, 10 µM glabridin regulated several downstream effectors that are involved in the classical and ERE-independent pathways,25 which were also regulated by 1 nM 17β-E2, such as SP-1 of the classical pathway and PGR which is involved with the ER pathway in general.

C-FOS, C-JUN, and NRIP1 might be involved in the decrease in cell proliferation in the combination treatment of glabridin with 17β-E2.

Based on a past study,10 the synergistic effect on ALP induction could have occurred via the ER-α–SRC-1 and coactivator. Meanwhile, glabridin and 17β-E2 induced dose-dependent

Table 3. Gene ontologies (GO) of 10 µM glabridin, 1 nM 17β-E2, and the combination treatment of 100 nM glabridin with 1 nM 17β-E2 that have been associated with its estrogenic action.

| GO ID | GO ACCESSION | GO TERM | 10 µM GLABRIDIN | COMBINATION OF 1 NM 17β-E2 AND 100 NM GLABRIDIN | 1 NM 17β-E2 |
|-------|--------------|---------|----------------|-----------------------------------------------|-------------|
| 957   | GO:0001568   | blood vessel development | 1.40E–02 | 1.14E–06 | 2.97E–03 |
| 1269  | GO:0001944   | vasculature development | 2.35E–02 | 1.95E–06 | — |
| 24971 | GO:0048514   | blood vessel morphogenesis | — | 4.22E–05 | — |
| 32123 | GO:0072358   | cardiovascular system development | 3.35E–04 | 1.96E–05 | 1.25E–03 |
| 32124 | GO:0072359   | circulatory system development | 3.35E–04 | 1.96E–05 | 1.25E–03 |

Table 4. Regulation of NRIP1 gene by the combination of 100 nM glabridin with 1 nM 17β-E2 and 1 nM 17β-E2.

| PROBE SET ID | COMBINATION OF 100 NM GLABRIDIN WITH 1 NM 17β-E2 | 1 NM 17β-E2 | GENE SYMBOL | ENTREZ GENE |
|--------------|--------------------------------------------------|--------------|-------------|-------------|
| 11720153_s_at | .01 | 3.212 | Up | .01 | 2.960 | Up | NRIP1 | 8204 |
increase in both ALP activity and cell proliferation in Ishikawa cells, and the combination study results showed an increase in ALP activity but with a lower induction of cell proliferation. This suggested that glabridin could act in a different manner from 17β-E2. Therefore, it was postulated that this difference could be via ER-α–independent mechanisms in Ishikawa cells.

Compared with the difference in gene expressions, the combination treatment of 100 nM glabridin with 1 nM 17β-E2 downregulated genes, such as C-FOS and C-JUN, which are transcription factors that heterodimerize to form a part of the nuclear activator protein 1 (AP-1) complex that regulates the expression of genes involved in many rapid cellular events such as proliferation, differentiation, and apoptosis apoptosis.26 Although 10 μM glabridin also decreased C-FOS expression, the induction of cell proliferation was comparable with that of 17β-E2 treatment. This suggests both that C-FOS and C-JUN have to decrease in expression to reduce the induction of cell proliferation. Table S4 shows that C-JUN represented the most highly regulated probe set among the probe sets that were differentially regulated by the combination of 1 nM 17β-E2 and 100 nM glabridin alone that was not regulated by others. The probe sets that were only regulated by the combination treatment were observed to regulate gene ontologies such as the regulation of cell death and the regulation of programmed cell death (Table S5). Similarly, Table S6 also shows that pathways such as regulation of cell death and regulation of programmed cell death were involved.

The microarray result also showed that the combination upregulated NRIP1 (Table 4), which is a ligand-dependent corepressor for most of the nuclear receptors. NRIP1 had also been shown to act as a corepressor that specifically antagonizes ER transcription via interaction with AP-1 protein in vivo.27,28 Previously, it had been reported that retinoic acid could inhibit cell proliferation in Ishikawa cells and NRIP1 represents 1 of the 4 most highly regulated genes that was upregulated.29 Similarly, in a separate study, upregulation of NRIP1 was found to inhibit the proliferation of microglioma cells.30 Therefore, the downregulation of C-JUN and upregulation of NRIP1 by the combination of 100 nM glabridin with 1 nM 17β-E2 might be the cause of the slight decrease in induction of cell proliferation as compared with 1 nM 17β-E2. Combining with the previous postulation, it was suggested that the downregulation of C-FOS, C-JUN and upregulation of NRIP1 could have occurred via ER-α–independent mechanisms.

Possible effects of the combination treatment on ovulation

Table 4 shows that similar to 1 nM 17β-E2, the combination treatment of 100 nM glabridin with 1 nM 17β-E2 upregulated NRIP1. NRIP1 plays an important role in the ovary. Expression of NRIP1 in the ovary is essential for oocyte release from mature follicles during ovulation, but not for luteinization of mature ovarian follicles.31,32 This could highlight the importance of NRIP1 in female fertility. NRIP1 was also found to have a role in the maintenance of pregnancy.33 Another gene which was also involved in ovulation from the ovarian follicle is PGR.23

The gene ontology analysis also showed that the combination treatment displayed effects on ovulation. Among the different treatments, only the combination treatment had an effect on reproduction and ovulation. Meanwhile, 10 μM glabridin had no effect on both reproduction and ovulation, and 1 nM 17β-E2 only regulated ovulation. The gene ontologies related to steroid and developmental activity based on probe sets that were differently regulated in the combination of 100 nM glabridin with 1 nM 17β-E2 are shown in Tables 5 and 6, respectively. Together with the gene analysis, this suggested that there is a possibility of the combination treatment to be explored for using in aiding ovulation.

Possible regulation of glabridin and the combination treatment on cardiovascular, circulatory, and vasculatory development

Past studies had shown that premenopausal women had lower chances of cardiovascular diseases as compared with menopausal women, and this effect could be reversed with the intake of ERT.34,35 This is as estrogen has a significant impact on heart and vasculature.14 Gene ontology analysis in this study showed

![Figure 5. Hierarchical clustering of the estrogen pathway of the different treatments (untreated control, 1 nM 17β-E2, 10 μM glabridin, and the combination treatment of 100 nM glabridin with 1 nM 17β-E2).](image-url)
that similar to $17\beta$-E$_2$, both 10$\mu$M glabridin and the combination treatment regulated genes involved in the vasculature development, cardiovascular, and circulatory systems that had been associated with estrogenic action (Table 3). Meanwhile, the combination treatment of 100 nM glabridin with 1 nM $17\beta$-E$_2$ could regulate blood vessel morphogenesis, vasculature development, cardiovascular, and circulatory systems.

This is consistent with a past study by Tamir et al. which showed that glabridin activated CK activity in skeletal and cardiovascular tissues. Even in animal studies, glabridin had been shown to stimulate the activity of CK in the aorta and left ventricle of the heart of both intact and ovariectomized female rats. Somjen et al. (2004) also showed that glabridin stimulated DNA synthesis in human endothelial cells and displayed biphasic effect on proliferation of human vascular smooth muscle cells similar to that of $17\beta$-E$_2$. The result of this study together with past studies provides evidence that 10$\mu$M glabridin and the combination treatment could bring

Figure 6. (A) Possible effects of 10$\mu$M glabridin and (B) 100 nM glabridin with 1 nM $17\beta$-E$_2$ on the estrogen pathway after 72 hours treatment on Ishikawa cells. The pathways drawn were adapted from ER signaling pathways from WikiPathways and Ingenuity Target Explorer.

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about the positive effects of 17β-E2 in the cardiovascular, circulatory, and vasculature systems.

**Possible regulation of glabridin and the combination treatment in improving polycystic ovary syndrome conditions**

Polycystic ovary syndrome (PCOS) is a condition correlated with molecular defects of insulin signaling pathway at the postreceptor level in tissues such as adipocytes, muscles, and ovary. In this study, both 10 μM glabridin and the combination treatment were reported to play a role in regulating the insulin signaling pathway, MAPK signaling pathways, and adipogenesis (Table 7), which are all related to metabolic syndromes and PCOS.37

Besides, this study found that 10 μM glabridin increased PPARG expression by 1.562-fold \((P = .01)\), thus suggesting that it could help with metabolic disorders by improving insulin

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**Table 5.** Gene ontologies (GO) related to steroid activity based on probe sets that were differently regulated in the combination of 100 nM glabridin with 1 nM 17β-E2.

| GO ID | GO ACCESSION | GO TERM                                                                 | P VALUE  |
|-------|--------------|-------------------------------------------------------------------------|----------|
| 3     | GO:0000003   | reproduction                                                             | .03      |
| 933   | GO:0001542   | ovulation from ovarian follicle                                          | .01      |
| 1221  | GO:0001890   | placenta development                                                    | .05      |
| 2266  | GO:0003006   | developmental process involved in reproduction                          | .02      |
| 2704  | GO:0003707   | steroid hormone receptor activity                                        | .03      |
| 5328  | GO:0007292   | female gamete generation                                                | .02      |
| 5548  | GO:0007548   | sex differentiation                                                      | .02      |
| 6054  | GO:0008585   | female gonad development                                                | .03      |
| 7002  | GO:0009725   | response to hormone stimulus                                            | 1.44E−04 |
| 12453 | GO:0022414   | reproductive process                                                    | .02      |
| 12458 | GO:0022602   | ovulation cycle process                                                 | .03      |
| 13158 | GO:0030728   | ovulation                                                               | 1.44E−04 |
| 17513 | GO:0035239   | tube morphogenesis                                                       | .02      |
| 17568 | GO:0035295   | tube development                                                         | .01      |
| 19740 | GO:0042698   | ovulation cycle                                                          | .04      |
| 20380 | GO:0043401   | steroid hormone mediated signaling pathway                               | .04      |
| 20596 | GO:0043627   | response to estrogen stimulus                                            | 2.13E−04 |
| 21871 | GO:0045137   | development of primary sexual characteristics                           | 7.61E−03 |
| 23151 | GO:0046545   | development of primary female sexual characteristics                     | .04      |
| 23255 | GO:0046660   | female sex differentiation                                               | .04      |
| 25000 | GO:0048545   | response to steroid hormone stimulus                                     | 1.95E−06 |
| 25047 | GO:0048598   | embryonic morphogenesis                                                  | .03      |
| 25051 | GO:0048608   | reproductive structure development                                       | 8.39E−04 |
| 25172 | GO:0048732   | gland development                                                        | .02      |
| 28359 | GO:0060135   | maternal process involved in female pregnancy                           | .01      |
| 28784 | GO:0060562   | epithelial tube morphogenesis                                            | .02      |
| 28966 | GO:0060745   | mammary gland branching involved in pregnancy                           | .05      |
| 29392 | GO:0061180   | mammary gland epithelium development                                     | .04      |
| 29669 | GO:0061458   | reproductive system development                                          | 8.39E−04 |
| 37089 | GO:2000241   | regulation of reproductive process                                       | .03      |
Table 6. Gene ontologies (GO) related to developmental activity based on probe sets that were differently regulated the combination of 100 nM glabridin with 1 nM 17β-E2.

| GO ID | GO ACCESSION | GO TERM                                           | P VALUE  |
|-------|--------------|---------------------------------------------------|----------|
| 957   | GO:0001568   | blood vessel development                           | 1.14E−06 |
| 1009  | GO:0001655   | urogenital system development                      | 1.05E−03 |
| 1011  | GO:0001657   | ureteric bud development                           | .03      |
| 1153  | GO:0001822   | kidney development                                 | .05      |
| 1220  | GO:0001889   | liver development                                  | .03      |
| 1221  | GO:0001890   | placenta development                               | .05      |
| 1269  | GO:0001944   | vasculature development                            | 1.95E−06 |
| 2266  | GO:0003006   | developmental process involved in reproduction     | .02      |
| 2454  | GO:0003205   | cardiac chamber development                         | .03      |
| 2480  | GO:0003231   | cardiac ventricle development                      | .01      |
| 2528  | GO:0003279   | cardiac septum development                         | 1.54E−03 |
| 2529  | GO:0003281   | ventricular septum development                     | 5.38E−04 |
| 5311  | GO:0007275   | multicellular organismal development                | 3.63E−09 |
| 5416  | GO:0007399   | nervous system development                         | .02      |
| 5927  | GO:0008406   | gonad development                                  | 4.03E−03 |
| 6054  | GO:0008585   | female gonad development                            | .03      |
| 7151  | GO:0009888   | tissue development                                  | 1.05E−03 |
| 14851 | GO:0032502   | developmental process                               | 4.77E−11 |
| 17568 | GO:0035295   | tube development                                    | .01      |
| 21705 | GO:0044767   | single-organism developmental process               | 2.17E−06 |
| 21871 | GO:0045137   | development of primary sexual characteristics       | 7.61E−03 |
| 23151 | GO:0046545   | development of primary female sexual characteristics| .04      |
| 24927 | GO:0048468   | cell development                                    | 4.35E−03 |
| 28651 | GO:0060429   | epithelium development                              | 1.49E−03 |
| 29228 | GO:0061008   | hepaticobiliary system development                 | .03      |
| 29392 | GO:0061180   | mammary gland epithelium development                | .04      |
| 29519 | GO:0061307   | cardiac neural crest cell differentiation involved in heart development | .05 |
| 29520 | GO:0061308   | cardiac neural crest cell development involved in heart development | .04 |
| 29669 | GO:0061458   | reproductive system development                    | 8.39E−04 |
| 31770 | GO:0072001   | renal system development                            | .01      |
| 31775 | GO:0072006   | nephron development                                 | .01      |
| 31778 | GO:0072009   | nephron epithelium development                     | .01      |
| 31840 | GO:0072073   | kidney epithelium development                       | .04      |
| 31907 | GO:0072141   | renal interstitial cell development                 | .04      |
| 32123 | GO:0072358   | cardiovascular system development                  | 1.96E−05 |
| 32124 | GO:0072359   | circulatory system development                     | 1.96E−05 |
| 36879 | GO:2000026   | regulation of multicellular organismal development | 1.99E−04 |
sensitivity and increase uptake of glucose by fat cells via the PPARG pathway. This was contradictory to a past study where Lee et al found that glabridin did not affect the expression of PPARG or adipogenesis in adipose tissue of glabridin-treated obese mice. However, in a later study, Ahn et al showed that glabridin and glabridin-rich licorice extract effectively inhibited adipogenesis in 3T3-L1 cells and hence were effective anti-obesity agents.

Another past study had suggested that the upregulation of PPARG was indicative of its effect in maintaining glucose homeostasis and improving insulin sensitivity. In addition, Nelson-Degrave et al found that the inactivation of MAPK pathway was implicated in the pathogenesis of excessive ovarian androgen production in PCOS women. Overall, the upregulation of PPARG by glabridin together with the regulation of pathways associated with adipogenesis, insulin resistance, and gluconeogenesis, which are metabolic disorders related to PCOS, suggests that further studies on 10μM glabridin and the combination in these areas could be explored for the improvement of the PCOS-related metabolic syndromes. This is the first study to show that glabridin could regulate pathways related to PCOS and hence suggests the use of glabridin for further study as ERT for patients with PCOS. From this study, it could be observed that although glabridin exhibited a wide array of activities, they seem to be interconnected and results mostly from its ability to act on ER.

### Table 7. Various pathways related to the metabolic syndrome and MAPK pathways that were regulated by the different treatments.

| PATHWAYS | 10μM GLABRIDIN | COMBINATION OF 1NM 17β-E2 AND 100NM GLABRIDIN | 1NM 17β-E2 |
|----------|----------------|----------------------------------------------|------------|
| P VALUE  | P VALUE        | P VALUE                                     |            |
| Metabolic syndrome | Insulin signaling | 3.85E−10 | .01 | 1.05E−06 |
|          | Adipogenesis     | 4.09E−05 | 7.24E−03 | .03 |
| MAPK pathways | MAPK cascade    | 9.19E−05 | — | 9.76E−06 |
|          | MAPK targets—nuclear events mediated by MAPKs | 1.74E−02 | 2.49E−04 | — |
|          | MAPK signaling pathway | 5.53E−06 | 1.31E−04 | 5.72E−04 |
|          | p38 MAPK signaling pathway | 1.17E−02 | — | — |

### Conclusions

The microarray results showed that 10μM glabridin and the combination treatment of 100nM glabridin with 1nM 17β-E2 regulated both the genomic and nongenomic estrogen pathways in Ishikawa cells. Based on the results, both 10μM glabridin and the combination could possibly provide benefits of estrogens in ERT. The combination might be more suitable as an ERT, whereas glabridin could be explored for the improvement of PCOS-related metabolic syndromes.

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Author Contributions
PSWM, CYY, and VN conceived and designed the experiments. PSWM analyzed the data and wrote the first draft of the manuscript. PSWM, CYY, and YVCP contributed to the writing of the manuscript. PSWM, CYY, YVCP, and VN agree with manuscript results and conclusions. PSWM, CYY, YVCP, and VN jointly developed the structure and arguments for the paper. PSWM and CYY made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics
As a requirement of publication, author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in a blind manner. The external blind peer reviewers report no conflicts of interest.

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