Introduction

Estrogens play important roles in reproductive physiology and multiple diseases, including breast and endometrial cancers, cardiovascular disease, osteoporosis, and Alzheimer’s disease [1, 2]. The most active form of endogenous estrogen, 17β-E2, is mostly synthesized in the ovaries of premenopausal women. To exert its effect, 17β-E2 binds to specific ESR1 and ESR2, after which the activated estrogen-ESR complex is transported to the nucleus, where it binds to the ERE in the promoter regions of estrogen-dependent genes, thereby altering gene expression [3]. The maintenance of appropriate levels of estrogen is important for reducing post-menopausal symptoms; however, the hypoestrogenic state is associated with an increased risk of cardiovascular disease, osteoporosis, and complications in preg-
nancy and lactation, while the hyperestrogenic state promotes cancer cell proliferation [4,5].

Estrogen replacement therapy (ERT) appears to reduce the risk of osteoporosis, colorectal cancer, and the severity of postmenopausal symptoms [6–9]. Nevertheless, the safety of ERT is a major concern due to the higher incidence of breast cancer, cardiovascular disease, and stroke among postmenopausal women undergoing therapy [8–10]. In this respect, phytoestrogens, a diverse group of plant-derived nonsteroidal compounds that mimic 17β-E2 because of their structural similarity to mammalian estrogens, could be alternatives to conventional ERT [11]. Phytoestrogens, including glycinol in soybean and resveratrol in grapes, are reported to exert estrogenic effects [12,13]. Lignans are thought to exert protective effects against cancer; however, the anticancer effects of glabridin, genistein, quercetin, and resveratrol are inconsistent [14–16]. Furthermore, phytoestrogens act as both estrogen agonists and antagonists, and differ in their levels of estrogenic activity [17,18]. In addition, herbal plants, such as kudzu root, chasteberry, red clover, hops, and agrimony, show estrogenic activities in the uterus and in breast cancer cell lines [19–21]. Among those, hops and red clover contain 8-prenylnaringenin and genistein, respectively, as the major estrogenic compounds [21,22]. Ginseng, which contains ginsenosides as active compounds, prevents postmenopausal osteoporosis and cancer cell proliferation by modulating ESR1 activity [23,24]. SJW (Hypericum perforatum L., Clusiaceae) contains several bioactive compounds, the main ones being hyperforin and hypericin. SJW extract has been reported to have a therapeutic effect on mild-to-moderate depression, relieve the psychological and psychosomatic symptoms of menopause, and possess antimicrobial, anti-inflammatory, antioxidant, and free radical scavenging properties [25–28]. Hypericin has been found to show antiretroviral activity, while hyperforin has antidepressant and anti-inflammatory activities [29–31]. Moreover, several studies evaluated the role and mechanism of SJW for the treatment of estrogen-mediated menopausal symptoms. A previous study reported that SJW regulates the genes related to antidepressant activity [32]. Liu et al. presented the possible mechanisms of SJW extract for the reduction of menopausal symptoms, including depression and hot flashes [33]. Although many studies have dealt with the estrogen-related activities of SJW and its constituents, the mechanisms of the activities of hyperforin have not yet been fully characterized.

In the present study, we assessed the estrogenic activities of hyperforin by measuring evolved ERE-luciferase activity and cell proliferation in MCF-7 cells. In addition, we used proteomic approaches to examine the modulation of proteomic profiles and their post-translational modifications in cells treated with hyperforin and 17β-E2 to identify the underlying mechanism.

Results and Discussion

The components of SJW extract were separated as a total ion chromatogram using UFLC-MS (Fig. 1A, Supporting Information). To confirm the identification, the electrospay ionization (ESI) mass spectra of the compound was obtained from the sample under similar operating conditions, and was found to be comparable to the spectra of the authentic reference sample. Among the different peaks, the largest peak area was identified as hyperforin (denoted by the green chromatogram) and had a retention time of 9.0. The mass spectra for hyperforin showed ionic species at m/z 535.4 (Fig. 1A, B, Supporting Information), which confirms its presence in SJW. The amount of hyperforin and hypericin in the extract was 3.5 mg/g and 2.6 mg/g, respectively. The estrogenic activities of SJW extract and hyperforin in MCF-7 cells transfected with ESR1 or ESR2 were tested using ERE-luciferase activity assays. The results are expressed as percentages of the induced luciferase activity (RLA) compared to the untreated control, and for comparison, a positive control was also prepared by treating the cells with 10−2 M 17β-E2. Relative luciferase activities were calculated as percentages of the induced luciferase relative to control after normalization to the Renilla luciferase activity. Bars represent the averages of triplicate determinations. Asterisks indicate significant differences from the control (Ctrl) determined using Dunnett’s multiple comparison t-test (*p < 0.05 and ***p < 0.001).

Fig. 1 Effects of St John’s wort (SJW) extract, hyperforin (HF), and 17β-estradiol (17β-E2) on ERE-luciferase activity in MCF-7 cells. The luciferase activities in MCF-7 cells transfected with ERO (A, C) or ERO (B, D) and an ERE reporter plasmid were measured after treatment with 0.2 to 20 µg/mL SJW extract (A, B) or 10−5 to 10 µM HF (C, D). A positive control was prepared by treating the cells with 10−2 M 17β-E2. Relative luciferase activities were calculated as percentages of the induced luciferase relative to control after normalization to the Renilla luciferase activity. Bars represent the averages of triplicate determinations. Asterisks indicate significant differences from the control (Ctrl) determined using Dunnett’s multiple comparison t-test (*p < 0.05 and ***p < 0.001).
ing that the EC50 of hyperforin was lower than that of 17β-E2. Similarly, the Emax of hyperforin was lower than that of 17β-E2 (Emax: hyperforin, 112.7% vs. 17β-E2, 128.5%). These results indicated that hyperforin possessed lower potency (EC50 value) and efficacy (Emax value) to that of 17β-E2 for inducing cell proliferation (Fig. 2B).

To determine whether the cell proliferation induced by SJW extract and hyperforin reflected their estrogenic activities, we next examined the effects of the ER antagonist ICI 182,780 on the response. The proliferation of cells treated with SJW extract (20 µg/ml) and hyperforin (10 µM) increased significantly when compared to the control cells in the absence of ICI 182,780 (p = 0.000 and p = 0.001, respectively). However, we found that the ER antagonist ICI 182,780 significantly inhibited cell proliferation induced by the same concentration of SJW extract and hyperforin by 16.9% (p = 0.000) and 6.6% (p = 0.007), respectively, when compared to the corresponding sample without ICI 182,780 (Fig. 3), indicating that the induced cell proliferation was related to their estrogenic activities. Notably, ICI 182,780 had a 1.2-fold and 3.1-fold stronger effect on 17β-E2–induced proliferation than it did on SJW-induced and hyperforin-induced proliferation, respectively. The inhibitory effect of ICI 182,780 on 17β-E2–induced cell proliferation has also been reported in the rat uterus, which could be due to multiple steps, including binding to the ESR by ICI 182,780 followed by disrupting ESR nuclear localization, and reducing binding of ESR to ERE [35, 36].

Proteomic profiles of three samples, including control, 17β-E2–treated, and hyperforin–treated cells, were generated by nano-UPLC-MS analysis. A total of 453 proteins were identified in the three samples, and among them, 282 proteins were differentially modulated in the hyperforin–treated cells compared to the 17β-E2–treated cells (Table 1S, Supporting Information). The proteomic data were further integrated into a knowledge database supported by Ingenuity pathway analysis (IPA) to visualize the interaction network composed of identified and predicted proteins. (Fig. 4) shows the networks of proteins that were differentially expressed in response to hyperforin treatment relative to 17β-E2 treatment in MCF-7 cells. Mainly, hyperforin was predicted to be more inhibitive of ESR1 activity than 17β-E2, leading to the downregulation of cell proliferation. However, hyperforin was predicted to activate ESR2 activity, leading to the reduced production of reactive oxygen species (ROS).

In our study, cell proliferation–related proteins, mainly CCND1, ERK, IL6ST, and AKR1C3, were downregulated by hyperforin treatment compared to 17β-E2 treatment, indicating that hyperforin less likely leads to cancer progression. A previous study reported that activation of ESR1 by 17β-E2 treatment subsequently triggered ERK signaling, which supported the upregulation of the expression of CCND1 and further induced cell proliferation [37, 38]. Similar to CCND1, CTSD, a prognostic biomarker of cancer cell proliferation, was downregulated by hyperforin compared to 17β-E2 [39]. Downregulation of IL6ST by hyperforin suggests that hyperforin abrogates cancer cell proliferation by impairing CCND1 and ERK expression. Furthermore, 17β-E2–stimulated AKR1C3 also was downregulated by hyperforin, which suggests that cell proliferation is inhibited by controlling the activities of estrogen and progesterone [40]. AKR1C3 induces cell proliferation by catalyzing the reaction either to reduce estrone to the more potent 17β-E2 or to reduce progesterone to the less potent pregnanediol [41]. AKR1C3 controls concentrations of estrogens and progesterone, which further regulates the activities of ESR1 and ESR2.

To protect against the harmful consequences of oxidative stress, IPA predicted that a number of prooxidant and antioxidant enzymes, including CAT, NOX, PRDX1, and PRDX6 were modulated by hyperforin and 17β-E2 through the activation of ESR2. Hyperforin and 17β-E2 affect oxidative stress by increasing CAT activity, which is dependent on the ratio of ESR1 and ESR2 in the cancer cell. Moreover, hyperforin, similar to other phytoestrogens, acts as an inhibitor of NOX, resulting in the decreased production of ROS via ESR2 [42]. IPA also showed that the activity of NOX is dependent on PRDX6 [43]. Hyperforin suppressed PRDX6 expression, which subsequently decreased NOX activity. Additionally, IPA indicated that the hyperforin–induced activation of ESR2 reduced oxidative stress relative to 17β-E2. A previous study also reported that a low ESR1/ESR2 ratio is a hallmark for the protection of cancer cells against oxidative stress [44].

Along with protein expression, ER function is also regulated by PTMs of the proteins. In this study, proteomic analysis identified 59 post-translationally modified proteins. Among the PTMs, phosphorylation, acetylation, and oxidation were observed in 47, 15, and 3 proteins, respectively. Proteins that were phosphorylated or acetylated included heat shock protein 90B (HSP90B), PRDX2, and isoform 2 of the NUMA, which are all involved in cell proliferation and ROS production (Fig. 4S and Table 2S, Supporting Information). However, notably, phosphorylation of NUMA was significantly inhibited by hyperforin treatment when compared to 17β-E2 treatment (hyperforin/17β-E2 ratio, 0.867). NUMA is crucial for mitotic spindle formation during progression of the cell cycle. Localization of NUMA to the mitotic spindle is regulated by its phosphorylation, which transforms NUMA into a soluble component of the mitotic pole at the onset of mitosis [45]. Our PTM analysis showed lower levels of phosphorylated NUMA in hyperforin–treated cells than in 17β-E2–treated cells, indicating that cancer cell proliferation was inhibited by blocking mitosis.

A limitation of this study is that our in vitro findings represent a portion of the animal’s metabolic system, and does not address the bioavailability of the compounds. The effects of a compound in an in vitro study may not be directly replicated in an animal model. Therefore, these compounds that showed estrogenic potential in vitro need to be tested in vivo for their efficacy.

In conclusion, hyperforin can mimic the estrogenic activity of 17β-E2 by showing comparable effects on ERE-luciferase activity in vitro, in MCF-7 cells. In this study, for the first time, we attempted to identify comparative protein expression in hyperforin and 17β-E2 treatment. The expression of proteins related to cell proliferation and ROS production was differentially regulated. Hyperforin treatment induced less cancer cell proliferation than 17β-E2 treatment, possibly by downregulating CCND1 and ERK expression through ESR1. At the same time, hyperforin-treated cells showed lower rates of ROS production than 17β-E2-treated cells, possibly by the downregulation of NOX through ESR2. Hence, this study will aid in the identification and development of a safe alternative method for estrogenic regulation for the purpose of reducing postmenopausal symptoms in women.

Materials and Methods

Sample preparation
The leaves and flowers of SJW obtained from a local producer (Cooperative of Daeho-dong, Naju, Korea) were used for this study because the compounds, including hyperforin, hypericin, chlorogenic acid, hyperoside, and quercetin, are mainly present in those parts of the plant [46]. A voucher specimen (identification number: JBF-FRL-B-2012-0001) of the plant used in our experiment was deposited at the Jeonnam Biofood Technology Center, Korea. First, 500 g of dried leaves and flowers of SJW were extracted in 5 L of 75% ethanol for 8 h and extracted again in 2.5 L 75% ethanol for 5 h, with stirring in the dark. The resultant mixture was filtered through a bar filter (1 µm), after which the solvent was evaporated at 42°C using a rotary vacuum evaporator (Daesin Machine Industry). The remaining extract was then freeze-dried, packed into screw-capped vials under nitrogen, and stored at -20°C for later use. Stock solutions of SJW extract and 17β-E2 (Sigma Aldrich, purity ≥ 98%) were prepared in DMSO (purity ≥ 99.7%, Sigma-Aldrich) at concentrations of 200 µg/mL and 10 mM, respectively, Stock solutions of hyperforin (Cayman Chemical Co., purity ≥ 90%) were prepared in methanol and stored at -20°C.

Transient transfection and luciferase activity assay
For transient transfection, MCF-7 cells (Korean Cell Line Bank) were cultured in DMEM/Nutrient Mixture F-12 medium (DMEM/F-12, Gibco Life Technologies) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, and 1% bovine insulin as previously described [20]. The cells were plated in 96-well plates at a density of 5 × 10^4 cells/well. After 24 h, the cells was transfected using FuGENEHD transfection reagent (Promega) according to the manufacturer’s protocol. Briefly, Opti-MEM was used to dilute ERα (pEGFP-C1-ERα, Addgene), ERβ (pCDNA Flag ERβ, Addgene), ERE (3× ERE TATA luc, Addgene), and Renilla luciferase (pRL-SV40, Promega) prior to the addition of the transfection reagent. The cells were incubated for 24 h at 37°C under a humidified 5% CO2 atmosphere [47,48] in phenol red-free DMEM/F-12 containing 5% charcoal-dextran-stripped fetal bovine serum with 0.2, 2.0, or 20 µg/mL SJW extract or 10⁻³, 10⁻², or 10⁻¹ M hyperforin. Following the incubation, the cells were washed twice with PBS and lysed in 20 µL of passive lysis buffer (Promega). Luciferase activity was then measured using Luciferase Assay Reagent (Promega) in a GloMax Multi Microplate Luminometer (Promega) according to the manufacturer’s protocol. The RLA was calculated as previously described [49]. The positive control was prepared by treating the cells with 10⁻² M 17β-E2, while the negative control was prepared by using the vehicle solvent only.

Cell proliferation assay
MCF-7 cells were seeded into 96-well microplates at a density of 5 × 10^4 cells/well in culture medium. After 24 h, the medium was replaced with estrogen-free, phenol red-free DMEM/F-12 containing 5% charcoal-dextran-stripped fetal bovine serum. Different concentrations of SJW extract or hyperforin were added to the medium as described in the luciferase activity assay, and the cells were cultured for 96 h. In addition, to investigate the effect of an estrogen antagonist, duplicate test samples were prepared using 20 µg/mL SJW extract, 10 µM hyperforin, and 10 µM 17β-E2, with or without the ER-antagonist ICI 182,780 (10⁻² M). The positive control was prepared by treating the cells with 10⁻³, 10⁻², and 10⁻¹ M 17β-E2, while the negative control was prepared by using the vehicle solvent only [20]. Cell proliferation was assessed using a Cell Counting Kit-8 (Dojindo) as described by Nakagawa et al. [50]. The proliferation was expressed as a percentage compared to the negative control. Concentration-response curves were plotted and the EC₅₀ and E₅₀ were estimated using GraphPad Prism version 5.01 (GraphPad Software, Inc.).

Protein extraction and tube-gel protein digestion
Proteins were extracted as previously described [51]. The protein was quantified using a Detergent Compatible Protein Assay Kit (Bio-Rad). A tube-gel digestion protocol was adopted as described previously [51].
Nano-UPLC-HDMSE
Tryptic peptide mixtures were separated using nano-ACQUITY ultra-performance liquid chromatograph (UPLC) equipped with a Synapt G2-Si HDMS System (Waters Corp.), a previously described method with optimization of the mobile phase system [51]. A gradient elution program was conducted for chromatographic separation with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) as follows: 97% mobile phase A initially, 90% mobile phase A for 3 min, 65% mobile phase A for 150 min, gradual decrease to 20% mobile phase A over 160 min, and a sharp increase to 97% mobile phase A for the last 10 min.

Identification and relative quantification of protein
For identification of proteins, MS spectra of peptides were aligned using Progenesis QI for Proteomics (QIP) version 2.0 (Nonlinear Dynamics), a previously adopted method with modifications in criteria for protein identification [51], and the spectra were matched to human proteins using the International Protein Index (IPI) human database (v.3.87). The criteria for protein identification were set as follows: ≥3 fragment per peptide, ≥7 fragments per protein, and ≥2 peptides per protein. Carbamidomethylation of cysteine was set as fixed, and oxidation of methionine and phosphorylation of serine/threonine/tyrosine were set as variable modifications.

Bioinformatics analysis
Ingenuity pathway analysis (IPA version 9.0; Ingenuity Systems, Inc.) was used to perform knowledge-based network analysis of proteomics data.

Data analysis
Data are shown as the mean ± SD. All experiments were performed in triplicate. Statistical significance among multiple treatment groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Student’s t-test was performed for comparison between two treatment groups. The analysis was performed using SPSS Version 21 (IBM). A difference was considered statistically significant at p < 0.05.

Supporting information
A list of proteins and mean relative ratios, a list of PTM, target peptide sequence of modified proteins, and mean relative ratios, ion chromatogram and mass spectra of compounds present in St. John’s wort extract, networks derived comparing proteins expression, and three-dimensional spectra are available as Supporting Information.

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Conflict of Interest
The authors declare no conflict of interest.

Affiliations
1 Korea Basic Science Institute, Daejeon, Republic of Korea
2 Department of Food Science and Technology and BK21 Plus Program, Chonnam National University, Gwangju, Republic of Korea
3 Jeonnam Biofood Technology Center, Naju, Republic of Korea
4 Bioenergy Research Center, Chonnam National University, Gwangju, Republic of Korea
5 Foodborne Virus Research Center, Chonnam National University, Gwangju, Republic of Korea
6 Jeonnam Biofood Technology Center, Naju, Republic of Korea

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