

# Estrogenic Activity of Hyperforin in MCF-7 Human Breast Cancer Cells Transfected with Estrogen Receptor

## Authors

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- estrogenic activity
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- Proteomics
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## Abstract

Hyperforin, a major active compound of St. John's wort extract, affects estrogenic activity. In this study, the compound evoked estrogen response element-dependent luciferase activity and cell proliferation in MCF-7 cells. Hyperforin-induced cell proliferation was significantly inhibited by the estrogen receptor antagonist ICI 182,780. These results suggested that hyperforin had estrogenic and cell proliferation activities, which were stimulated via the estrogen receptor. Compared to 17 $\beta$ -estradiol, hyperforin showed significantly lower estrogenic activity and cell proliferation. The mechanism underlying the estrogenic activity of hyperforin was unknown, therefore, in this study, for the first time, the expression and post-translational modification of proteins were determined and compared among control, 17 $\beta$ -estradiol-treated, and hyperforin-treated cells using proteomic techniques. A total of 453 proteins were identified, of which 282 proteins were significantly modulated in hyperforin-treated cells compared to 17 $\beta$ -estradiol-treated cells. Ingenuity pathway analysis also demonstrated that hyperforin treatment induced less cell proliferation than 17 $\beta$ -estradiol by downregulating estrogen receptor 1. Protein network analysis showed that cell proliferation was regulated mainly by cyclin

D1 and extracellular signal-regulated kinases. In conclusion, although, hyperforin exhibited lower estrogenic activity than 17 $\beta$ -estradiol, the compound induced lower levels of cancer cell proliferation *in vitro*.

## Abbreviations

17 $\beta$ -E2:	17 $\beta$ -estradiol
AKR1C3:	aldo-keto reductase family 1 member C3
CAT:	catalase
CCND1:	cyclin D1
CTSD:	cathepsin D
ERE:	estrogen response element
ERK:	extracellular signal-regulated kinases
ESR:	estrogen receptor
IL6ST:	interleukin-6 signal transducer
NOX:	NADPH oxidase
NUMA:	nuclear mitotic apparatus protein 1
PRDX1:	peroxiredoxin 1
PRDX2:	peroxiredoxin 2
PRDX6:	peroxiredoxin 6
PTM:	post-translational modification
SJW:	St. John's wort

Supporting information available online at <http://www.thieme-connect.de/products>

## 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, 17 $\beta$ -E2, is mostly synthesized in the ovaries of

premenopausal women. To exert its effect, 17 $\beta$ -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-

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nancy and lactation, while the hyperestrogenic state promotes cancer cell proliferation [4,5].

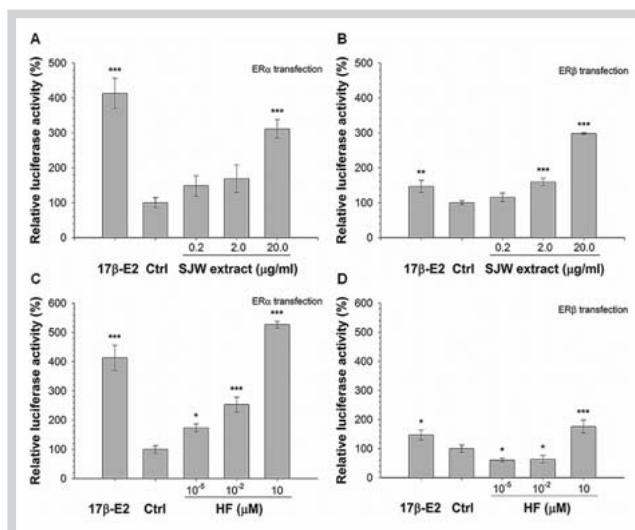
Estrogen replacement therapy (ERT) appears to reduce the risk of osteoporosis, colorectal cancer, and the severity of postmenopausal symptoms [6–9]. Nevertheless, the safety of ERT is a major concern due to the higher incidence of breast cancer, cardiovascular disease, and stroke among postmenopausal women undergoing therapy [8–10]. In this respect, phytoestrogens, a diverse group of plant-derived nonsteroidal compounds that mimic  $17\beta$ -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 evoked 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  $17\beta$ -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. 1S A, Supporting Information). To confirm the identification, the electrospray 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



**Fig. 1** Effects of St John's wort (SJW) extract, hyperforin (HF), and  $17\beta$ -estradiol ( $17\beta$ -E2) on ERE-luciferase activity in MCF-7 cells. The luciferase activities in MCF-7 cells transfected with ER $\alpha$  (A, C) or ER $\beta$  (B, D) and an ERE reporter plasmid were measured after treatment with 0.2 to 20  $\mu$ g/mL SJW extract (A, B) or  $10^{-5}$  to 10  $\mu$ M HF (C, D). A positive control was prepared by treating the cells with  $10^{-2}$   $\mu$ M  $17\beta$ -E2. Relative luciferase activities were calculated as percentages of the induced luminance 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).

at  $m/z$  535.4 (Fig. 1S A, 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 relative luciferase activity (RLA) compared to the untreated control, and for comparison, a positive control was also prepared by treating the transfectants with  $10^{-2}$   $\mu$ M  $17\beta$ -E2. The RLA of the transfected cells after treatment with SJW extract (0.2 to 20  $\mu$ g/mL) and hyperforin ( $10^{-5}$  to 10  $\mu$ M) is shown in Fig. 1. Both samples significantly increased the RLA in cells transfected with ESR1 or ESR2 in a concentration-dependent manner. At a concentration of 20  $\mu$ g/mL, SJW extract elicited responses that were 1.3-fold smaller (RLA: 311.2% vs. 412.9%) or 2.0-fold larger (297.8% vs. 146.7%) than those elicited by  $10^{-2}$   $\mu$ M  $17\beta$ -E2 in ESR1 or ESR2 transfectants, respectively (Fig. 1A, B). In contrast, irrespective of whether the cells were transfected with ESR1 or ESR2, RLA levels elicited by  $10^{-2}$   $\mu$ M hyperforin (RLA: ESR1, 252.8% and ESR2, 63.4%) were lower than those elicited by the same concentration of  $17\beta$ -E2 (RLA: ESR1, 412.9% and ESR2, 146.7%) (Fig. 1C, D). This finding is consistent with that of Harris et al. who observed a difference between RLAs elicited by  $17\beta$ -E2 and phytoestrogen in breast cancer cells transfected with either ESR1 or ESR2 [34].

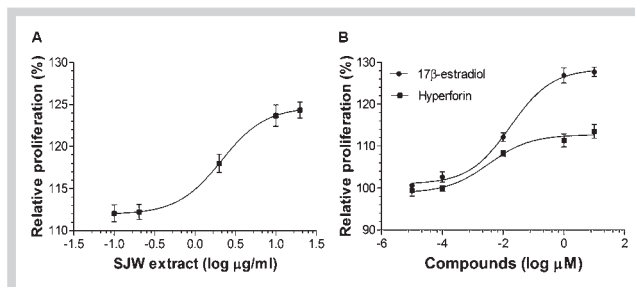
Fig. 2 shows the concentration-dependent effects of the SJW extract, hyperforin, and  $17\beta$ -E2 on the proliferation of MCF-7 cells. SJW extract induced cell proliferation with a 50% effective concentration ( $EC_{50}$ ) of 2.1  $\mu$ g/mL and maximum response ( $E_{max}$ ) of 124.7% of the control (Fig. 2A). The  $EC_{50}$  of hyperforin and  $17\beta$ -E2 was  $3.3 \times 10^{-3}$   $\mu$ M and  $1.8 \times 10^{-2}$   $\mu$ M, respectively, indicat-

ing that the  $EC_{50}$  of hyperforin was lower than that of  $17\beta$ -E2. Similarly, the  $E_{max}$  of hyperforin was lower than that of  $17\beta$ -E2 ( $E_{max}$ : hyperforin, 112.7% vs.  $17\beta$ -E2, 128.5%). These results indicated that hyperforin possessed lower potency ( $EC_{50}$  value) and efficacy ( $E_{max}$  value) to that of  $17\beta$ -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  $\mu$ g/mL) and hyperforin (10  $\mu$ M) 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  $17\beta$ -E2-induced proliferation than it did on SJW-induced and hyperforin-induced proliferation, respectively. The inhibitory effect of ICI 182,780 on  $17\beta$ -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,  $17\beta$ -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  $17\beta$ -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  $17\beta$ -E2 treatment in MCF-7 cells. Mainly, hyperforin was predicted to be more inhibitive of ESR1 activity than  $17\beta$ -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  $17\beta$ -E2 treatment, indicating that hyperforin less likely leads to cancer progression. A previous study reported that activation of ESR1 by  $17\beta$ -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  $17\beta$ -E2 [39]. Downregulation of IL6ST by hyperforin suggests that hyperforin abrogates cancer cell proliferation by impairing CCND1 and ERK expression. Furthermore,  $17\beta$ -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  $17\beta$ -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.



**Fig. 2** Concentration-response curves of St. John's wort (SJW) extract, hyperforin, and  $17\beta$ -estradiol for MCF-7 cell proliferation. MCF-7 cells were cultured in estrogen-free medium, treated with 0.2–20  $\mu$ g/ml SJW extract or with  $10^{-5}$ –10  $\mu$ M of hyperforin or  $17\beta$ -estradiol. Cell proliferation was determined using the Cell Counting Kit-8, and calculated as percentage of cell proliferation relative to untreated control. The curves of cell proliferation versus logarithm of concentration of SJW extract (A), hyperforin, or  $17\beta$ -estradiol (B) were plotted. The curves represent the averages of five determinations expressed as percentages.

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  $17\beta$ -E2 through the activation of ESR2. Hyperforin and  $17\beta$ -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  $17\beta$ -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  $17\beta$ -E2 treatment (hyperforin/ $17\beta$ -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  $17\beta$ -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  $17\beta$ -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  $17\beta$ -E2 treatment. The expression of proteins related to cell proliferation and ROS production was differentially regulated. Hyperforin treatment induced less cancer cell proliferation than  $17\beta$ -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  $17\beta$ -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.

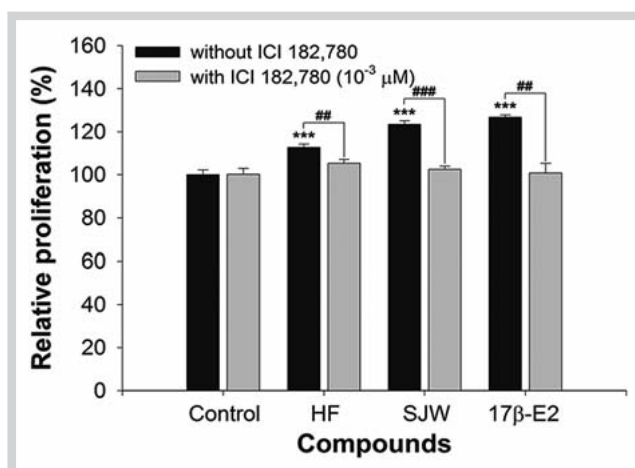
## Materials and Methods

### 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-FRI-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 bag filter (1  $\mu$ m), 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  $17\beta$ -E2 (Sigma Aldrich, purity  $\geq$  98%) were prepared in DMSO (purity  $\geq$  99.7%, Sigma-Aldrich) at concentrations of 200  $\mu$ g/mL and 10 mM, respectively. Stock solutions of hyperforin (Cayman Chemical Co., purity  $\geq$  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  $2.0 \times 10^4$  cells/well. After 24 h, the cells were transfected using FuGENEHD transfection reagent (Promega) according to the manufacturer's protocol. Briefly, Opti-MEM was used to dilute ER $\alpha$  (pEGFP-C1-ER $\alpha$ , Addgene), ER $\beta$  (pcDNA Flag ER $\beta$ , Addgene), ERE (3 $\times$  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% CO<sub>2</sub> 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  $\mu$ g/mL SJW extract or  $10^{-5}$ ,  $10^{-2}$ , or 10  $\mu$ M hyperforin. Following the incubation, the cells were washed twice with PBS and lysed in 20  $\mu$ L 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 de-



**Fig. 3** Inhibitory effect of ICI 182,780 on MCF-7 cell proliferation induced by St. John's wort (SJW) extract, hyperforin (HF), or  $17\beta$ -estradiol ( $17\beta$ -E2). MCF-7 cells were cultured for 96 h in estrogen-free medium and treated with 20  $\mu$ g/mL of SJW extract, 10  $\mu$ M HF, or 10  $\mu$ M  $17\beta$ -E2 in the presence and absence of ICI 182,780. Cell counts were normalized to an untreated control sample. Bars represent the averages of triplicate determinations expressed as percentages. Asterisk (\*) indicates significant differences when compared to the untreated control (without ICI 182,780) determined using Dunnett's multiple comparison-test (\*\*\*)  $p < 0.001$ , and hash (#) indicates significant difference when compared to the corresponding sample without ICI 182,780 (##  $p < 0.01$  and ###  $p < 0.001$ ) determined using Student's t-test.

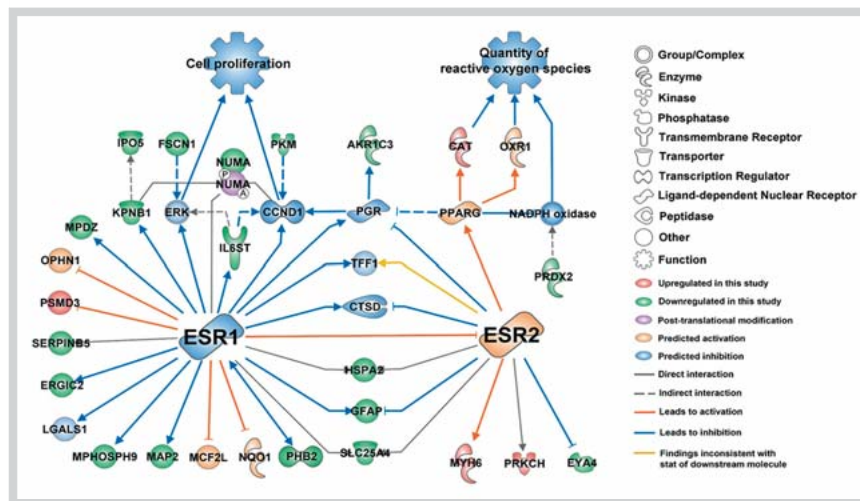
scribed [49]. The positive control was prepared by treating the cells with  $10^{-2}$   $\mu$ M  $17\beta$ -E2, while the negative control was prepared by using the vehicle solvent only.

### Cell proliferation assay

MCF-7 cells were seeded into 96-well microplates at a density of  $5 \times 10^3$  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  $\mu$ g SJW extract, 10  $\mu$ M hyperforin, and 10  $\mu$ M  $17\beta$ -E2, with or without the ER-antagonist ICI 182,780 ( $10^{-3}$   $\mu$ M, Santa Cruz Biotechnology, purity  $\geq$  98%). The positive control was prepared by treating the cells with  $10^{-5}$ ,  $10^{-2}$ , and 10  $\mu$ M  $17\beta$ -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 EC<sub>50</sub> and E<sub>max</sub> 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].



**Fig. 4** Network derived with comparing proteins expressed in hyperforin-treated and 17 $\beta$ -estradiol (17 $\beta$ -E2)-treated MCF-7 cells. The proteins shown in **Tables 1S** and **2S**, Supporting Information, were imported into IPA, and specific proteins related to cell proliferation and ROS generation were selected for the network using the Ingenuity knowledge database. Significantly upregulated and downregulated ( $p$  value  $< 0.05$ ) proteins in hyperforin-treated cells compared to 17 $\beta$ -E2-treated cells are shown in red and green, respectively. The proteins, which were predicted to be activated and inhibited, are shown in orange and blue, respectively. Post-translational modification, phosphorylation (P), and acetylation (A) of proteins are indicated by enclosed text. (Color figure available online only.)

### Nano-UPLC-HDMS<sup>E</sup>

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:  $\geq 3$  fragment per peptide,  $\geq 7$  fragments per protein, and  $\geq 2$  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  $\pm$  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.

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### References

- <sup>1</sup> Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Ström A, Treuter E, Warner M, Gustafsson JA. Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* 2007; 87: 905–931
- <sup>2</sup> Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. *Physiol Rev* 2001; 81: 1535–1565
- <sup>3</sup> Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. *Curr Genomics* 2006; 7: 497–508
- <sup>4</sup> Dalal PK, Agarwal M. Postmenopausal syndrome. *Indian J Psychiatry* 2015; 57: S222–S232

- 5 Zanetta GM, Webb MJ, Li H, Keeney GL. Hyperestrogenism: a relevant risk factor for the development of cancer from endometriosis. *Gynecol Oncol* 2000; 79: 18–22
- 6 Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med* 2000; 133: 933–941
- 7 Moreira AC, Silva AM, Santos MS, Sardão VA. Phytoestrogens as alternative hormone replacement therapy in menopause: What is real, what is unknown. *J Steroid Biochem Mol Biol* 2014; 143: 61–71
- 8 Warren MP. A comparative review of the risks and benefits of hormone replacement therapy regimens. *Am J Obstet Gynecol* 2004; 190: 1141–1167
- 9 Davis SR, Lambrinoudaki I, Lumsden M, Mishra GD, Pal L, Rees M, Santoro N, Simoncini T. Menopause. *Nat Rev Dis Primers* 2015; 1: 15004
- 10 Chen CL, Weiss NS, Newcomb P, Barlow W, White E. Hormone replacement therapy in relation to breast cancer. *JAMA* 2002; 287: 734–741
- 11 Borrelli F, Ernst E. Alternative and complementary therapies for the menopause. *Maturitas* 2010; 66: 333–343
- 12 Boué SM, Tilghman SL, Elliott S, Zimmerman MC, Williams KY, Payton-Stewart F, Miraflores AP, Howell MH, Shih BY, Carter-Wientjes CH, Segar C, Beckman BS, Wiese TE, Cleveland TE, McLachlan JA, Burow ME. Identification of the potent phytoestrogen glycinol in elicited soybean (*Glycine max*). *Endocrinology* 2009; 150: 2446–2453
- 13 Henry LA, Witt DM. Resveratrol: phytoestrogen effects on reproductive physiology and behavior in female rats. *Horm Behav* 2002; 41: 220–228
- 14 Adlercreutz H. Phytoestrogens: epidemiology and a possible role in cancer protection. *Environ Health Perspect* 1995; 103: 103–112
- 15 Mense SM, Hei TK, Ganju RK, Bhat HK. Phytoestrogens and breast cancer prevention: possible mechanisms of action. *Environ Health Perspect* 2008; 116: 426–433
- 16 Tamir S, Eizenberg M, Somjen D, Izrael S, Vaya J. Estrogen-like activity of glabrene and other constituents isolated from licorice root. *J Steroid Biochem Mol Biol* 2001; 78: 291–298
- 17 Kostelac D, Rechkemmer G, Briviba K. Phytoestrogens modulate binding response of estrogen receptors  $\alpha$  and  $\beta$  to the estrogen response element. *J Agric Food Chem* 2003; 51: 7632–7635
- 18 Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor  $\beta$ . *Endocrinology* 1998; 139: 4252–4263
- 19 Boué SM, Wiese TE, Nehls S, Burow ME, Elliott S, Carter-Wientjes CH, Shih BY, McLachlan JA, Cleveland TE. Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. *J Agric Food Chem* 2003; 51: 2193–2199
- 20 Lee YM, Kim JB, Bae JH, Lee JS, Kim PS, Jang HH, Kim HR. Estrogen-like activity of aqueous extract from *Agrimonia pilosa* Ledeb. in MCF-7 cells. *BMC Complement Altern Med* 2012; 12: 260
- 21 Liu J, Burdette JE, Xu H, Gu C, van Breemen RB, Bhat KPL, Booth N, Constantinou AI, Pezzuto JM, Fong HHS, Farnsworth NR, Bolton JL. Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J Agric Food Chem* 2001; 49: 2472–2479
- 22 Overk CR, Yao P, Chadwick LR, Nikolic D, Sun Y, Cuendet MA, Deng Y, Hedayat A, Pauli GF, Farnsworth NR. Comparison of the *in vitro* estrogenic activities of compounds from hops (*Humulus lupulus*) and red clover (*Trifolium pratense*). *J Agric Food Chem* 2005; 53: 6246–6253
- 23 Lee HY, Park SH, Chae SW, Soung NK, Oh MJ, Kim JS, Kim YO, Chae HJ. Aqueous ginseng extract has a preventive role in RANKL-induced osteoclast differentiation and estrogen deficiency-induced osteoporosis. *J Funct Foods* 2015; 13: 192–203
- 24 Zhu Y, Xu J, Li Z, Xie S, Zhou J, Guo X, Zhou X, Li G, Zhong R, Ma A. Ginsenoside Rh2 suppresses growth of uterine leiomyoma *in vitro* and *in vivo* and may regulate ER $\alpha$ /c-Src/p38 MAPK activity. *J Funct Foods* 2015; 18 (Part A): 73–82
- 25 Grube B, Walper A, Wheatley D. St. John's wort extract: efficacy for menopausal symptoms of psychological origin. *Adv Ther* 1999; 16: 177–186
- 26 Hunt E, Lester C, Lester E, Tackett R. Effect of St. John's wort on free radical production. *Life Sci* 2001; 69: 181–190
- 27 Tripathi YB, Pandey E. Role of alcoholic extract of shoot of *Hypericum perforatum* Linn on lipid peroxidation and various species of free radicals in rats. *Indian J Exp Biol* 1999; 37: 567–571
- 28 Barnes J, Anderson LA, Phillipson JD. St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties. *J Pharm Pharmacol* 2001; 53: 583–600
- 29 Chatterjee SS, Bhattacharya SK, Wonnemann M, Singer A, Müller WE. Hyperforin as a possible antidepressant component of hypericum extracts. *Life Sci* 1998; 63: 499–510
- 30 Koeberle A, Rossi A, Bauer J, Dehm F, Verotta L, Northoff H, Sautebin L, Werz O. Hyperforin, an anti-inflammatory constituent from St. John's wort, inhibits microsomal prostaglandin E2 synthase-1 and suppresses prostaglandin E2 formation *in vivo*. *Front Pharmacol* 2011; 2: 7
- 31 Meruelo D, Lavie G, Lavie D. Therapeutic agents with dramatic anti-retroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. *Proc Natl Acad Sci U S A* 1988; 85: 5230–5234
- 32 Butterweck V. Mechanism of action of St John's wort in depression. *CNS Drugs* 2003; 17: 539–562
- 33 Liu YR, Xiao BK, Yang JY, Guo CH, Shen SJ, Tang ZS, Dong JX, Huang RQ. <sup>1</sup>H-NMR and HPLC-MS/MS-based global/targeted metabolomic evaluation of *Hypericum perforatum* L. intervention for menopause. *J Funct Foods* 2015; 17: 722–741
- 34 Harris D, Besselink E, Henning S, Go V, Heber D. Phytoestrogens induce differential estrogen receptor  $\alpha$ - or  $\beta$ -mediated responses in transfected breast cancer cells. *Exp Biol Med (Maywood)* 2005; 230: 558–568
- 35 Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. *Cancer Res* 1991; 51: 3867–3873
- 36 Howell A, Osborne CK, Morris C, Wakeling AE. ICI 162,760 (Faslodex™). *Cancer* 2000; 89: 817–825
- 37 Lucas TFG, Royer C, Siu ER, Lazari MFM, Porto CS. Expression and signaling of G protein-coupled estrogen receptor 1 (GPER) in rat Sertoli cells. *Biol Reprod* 2010; 83: 307–317
- 38 Royer C, Lucas TFG, Lazari MFM, Porto CS. 17 $\beta$ -estradiol signaling and regulation of proliferation and apoptosis of rat Sertoli cells. *Biol Reprod* 2012; 86: 108
- 39 Rochefort H, Garcia M, Glondu M, Laurent V, Liaudet E, Rey JM, Roger P. Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 overview. *Clin Chim Acta* 2000; 291: 157–170
- 40 Fung K, Samara E, Wong C, Metwalli A, Krlin R, Bane B, Liu C, Yang J, Pitha J, Cullin D. Increased expression of type 2 3 $\alpha$ -hydroxysteroid dehydrogenase/type 5 17 $\beta$ -hydroxysteroid dehydrogenase (AKR1C3) and its relationship with androgen receptor in prostate carcinoma. *Endocr Relat Cancer* 2006; 13: 169–180
- 41 Rižner TL, Penning TM. Role of aldo-keto reductase family 1 (AKR1) enzymes in human steroid metabolism. *Steroids* 2014; 79: 1–37
- 42 McCarty MF, Barroso-Aranda J, Contreras F. Genistein and phycocyanobilin may prevent hepatic fibrosis by suppressing proliferation and activation of hepatic stellate cells. *Med Hypotheses* 2009; 72: 330–332
- 43 Ambruso DR, Ellison MA, Thurman GW, Leto TL. Peroxiredoxin 6 translocates to the plasma membrane during neutrophil activation and is required for optimal NADPH oxidase activity. *Biochim Biophys Acta* 2012; 1823: 306–315
- 44 Nadal-Serrano M, Sastre-Serra J, Pons DG, Miro AM, Oliver J, Roca P. The ER- $\alpha$ /ER $\beta$  ratio determines oxidative stress in breast cancer cell lines in response to 17 $\beta$ -estradiol. *J Cell Biochem* 2012; 113: 3178–3185
- 45 Compton DA, Luo C. Mutation of the predicted p34cdc2 phosphorylation sites in NuMA impair the assembly of the mitotic spindle and block mitosis. *J Cell Sci* 1995; 108: 621–633
- 46 Cirak C, Radusiene J, Stanius Z, Camas N, Caliskan O, Odabas MS. Secondary metabolites of *Hypericum orientale* L. growing in Turkey: variation among populations and plant parts. *Acta Physiol Plant* 2012; 34: 1313–1320
- 47 Felekis KN, Narsimhan RP, Near R, Castro AF, Zheng Y, Quilliam LA, Lerner A. AND-34 activates phosphatidylinositol 3-kinase and induces anti-estrogen resistance in a SH2 and GDP exchange factor-like domain-dependent manner. *Mol Cancer Res* 2005; 3: 32–41
- 48 Mirebeau-Prunier D, Le Pennec S, Jacques C, Gueguen N, Poirier J, Malthiery Y, Savagner F. Estrogen-related receptor  $\alpha$  and PGC-1-related coactivator constitute a novel complex mediating the biogenesis of functional mitochondria. *FEBS J* 2010; 277: 713–725
- 49 Chou J, Lin JH, Brenot A, Kim JW, Provot S, Werb Z. GATA3 suppresses metastasis and modulates the tumour microenvironment by regulating microRNA-29b expression. *Nat Cell Biol* 2013; 15: 201–213
- 50 Nakagawa Y, Suzuki T, Tayama S. Metabolism and toxicity of benzophenone in isolated rat hepatocytes and estrogenic activity of its metabolites in MCF-7 cells. *Toxicology* 2000; 156: 27–36
- 51 Jeong EH, Vaidya B, Cho SY, Park MA, Kaewintajuk K, Kim SR, Oh MJ, Choi JS, Kwon J, Kim D. Identification of regulators of the early stage of viral hemorrhagic septicemia virus infection during curcumin treatment. *Fish Shellfish Immunol* 2015; 45: 184–193