

The Estrogenic Effect of *Lysiphyllum strychnifolium* (Craib) A. Schmitz Leaf Water Extract in MCF-7 Cells

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Abstract

Lysiphyllum strychnifolium (Craib) A. Schmitz (LS) has been widely used as traditional medicine in the northeast region of Thailand to stimulate the production of breast milk. There are no known studies on the estrogenic action of LS on the stimulation of breast milk production. Hence, the present study aimed to investigate the estrogenic effect of LS leaf water extract compared with quercetin, one of the compounds in LS, in human breast cancer cells (MCF-7). The effect of LS leaf water extract and quercetin on cell viability of MCF-7 cells was studied by MTT assay at a concentration range of 0 to 500 µg/mL. The expression of estrogen-dependent genes, the *pS2*, *ERα*, and *ERβ* was also examined by real-time RT-PCR, and the expression of *ERα* protein was detected by Western blotting. The quercetin content in the LS water extract was 285.67 ± 0.11 µg/g of dry extract. MCF-7 cells treated with LS leaf water extract (20 µg/mL) showed an upregulation of *pS2* gene expression similar to that of the treatment with E2 (10^{-9} M) compared with that of the untreated control. The *ERα* gene expression was found to be upregulated by quercetin (0.16 µg/mL) and E2 (10^{-5} M) compared with the untreated control. In addition, quercetin (0.16 µg/mL) and LS extract (0.8, 4, and 20 µg/mL) decreased the phosphorylation of *ERα* at Ser167. LS extract (20 µg/mL) decreased *ERα*, but there was no significant effect on the *ERα* at Ser118 protein expression. This study provided scientific evidence for the potential estrogenic activities of LS leaf water extract. This indicates that ER-dependent pathways in MCF-7 cells served as mediators, facilitating the estrogenic properties of LS and its compounds to function effectively. Since LS induced *pS2* gene transcription, it was confirmed that this could affect the transcription of estrogen-responsive genes, causing estrogenic effects.

Keywords: *Lysiphyllum strychnifolium*, Ya Nang Daeng, Estrogenic activity, 17β-estradiol (E2)

Introduction

Lysiphyllum strychnifolium (Craib) A. Schmitz (LS) (synonym *Bauhinia strychnifolia*), which belongs to the Fabaceae family, is a woody climbing plant known as Ya Nang Daeng or Khayan in Thai. LS grows in forests full of tropical trees in northern Thailand [1]. Local people have consumed LS as a traditional medicinal plant to relieve alcohol impacts, treat poisoning, eliminate pesticides, relieve the effects of poisonous mushrooms, stimulate breast milk production in women after delivery, promote health, nourish, and reduce fatigue [2]. The therapeutic properties of a decoction of LS leaf include antioxidant [3] and anti-hyperuricemic activity [4]. Therefore, the decoction or herbal tea drink of its roots, leaves, and vines is used in traditional medicine to treat fever, diarrhea, toxic substances, and food poisoning in Kutchum District, Yasothon Province, Northeast Thailand [5]. Thailand is renowned for traditional Thai medicines, including herbal drugs used as galactagogues. There are a wide variety of local herbal drugs in Thailand but scientific evidence to verify and validate their uses and therapeutic properties is still lacking. Current literature has reported that LS, one of the Thai herbal drugs with bioactive compounds, has been consumed as a galactagogue to boost lactation among breastfeeding mothers in the northeastern region of Thailand [2]. The ethanol extract from LS vines, leaves, and stems was composed of quercetin, 3,5,7,3',5'-pentahydroxy-flavanonol-3-O-α-L-rhamnopyranoside, 3,5,7-trihydroxy-chromone-3-O-α-L-rhamnopyranoside, β-sitosterol, and stigmasterol [6, 7]. Quercetin was also found in water extracts of LS

leaves [8]. Interestingly, quercetin is a phytoestrogen, a chemically diverse group of flavonoid compounds that work as plant metabolites that possess estrogenic effects. A previous study reported that phytoestrogenic activity induced by herbal galactagogue effects seemed to be correlative to an endogenous estrogen known as 17 β -estradiol (E2), which enhances the mammary epithelial cells [9].

The phytoestrogen molecules with their activities similar to E2 may trigger the prolactin (PRL) receptor to express itself. E2 works through the intracellular receptor E2 (E2R), which boosts PRL levels and promotes breast milk secretion. This route caused by α isoform of the membrane-associated estrogen receptor (mE2R) facilitates such effects [10]. Estrogen affects different tissues in many physiological processes and establishes and regulates the reproductive organs in both genders, namely in the gonads or the mammary glands [11]. Estrogen and phytoestrogen share structural and/or chemical similarities. Thus, phytoestrogen, which can bind itself to α and β estrogen receptors (ER), may possess estrogenic properties [12]. Estrogen can also indirectly induce gene expression by ER binding to other transcription factors such as activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B), and stimulating protein-1 (SP-1). Estrogen action is mediated through the intracellular estrogen receptor (ER), on which estrogen binds DNA directly in gene promoter regions known as the estrogen response elements (EREs) and subsequent gene transcription modulation [10,13]. In general, phytoestrogens bind better to ER α compared to ER β . Divergent phytoestrogens determine how transactivation of ER α induced transcription differs from ER β induced transcription [14]. Estrogen receptor beta (ER β) acts as a dominant negative regulator of E2 signaling and in many instances, ER β opposes the actions of ER α [15]. An estrogen-responsive gene known as the *pS2* gene can be found in breast cancer cells, but it does not exist in normal mammary cells. A transcription of the single-copy *pS2* gene is activated by MCF-7 breast cancer cells exposed to E2, leading to higher levels of *pS2* mRNA. The findings of transfection assays revealed that any defective ERE can cause estrogen responsiveness to this gene. Therefore, general mechanisms for the regulation of estrogen-responsive genes and how the *pS2* gene is regulated in human breast cancer cells can be studied by examining the *pS2* gene in MCF-7 cells [16]. ER α is combined with phosphoric acid in many amino acid residues. The activation function 1 (AF1) region of ER α contains Serine 118 and 167, whose phosphorylation offers a significant mechanism that synchronizes AF1 activity. Human ER α is combined with phosphoric acid on Ser118 to react to estradiol attachment. Breast cancer cell development can be affected by estrogens whose effects are expressed through binding to the ER. When the mitogen-activated protein kinase (MAPK) pathway is stimulated, phosphorylation is initiated in Ser118 and Ser167 [17, 18].

Previous studies found that quercetin specifically promotes mammary gland development and lactation yield in milk-deficient mice, probably via stimulating prolactin expression as well as inducing prolactin receptor expression in primary mammary epithelial cells [19]. Besides, folk medicine in the northeast of Thailand has used LS to stimulate breast milk production. However, the estrogenic effect of LS extract containing quercetin, one of the compounds, has not been reported. Here, we evaluate the estrogenic effect of water extract from LS leaves compared with quercetin on ER-positive MCF-7 cells. The expression of *pS2*, *ER α* , and *ER β* genes was also examined by real-time RT-PCR, and ER α protein expression was detected by Western blotting.

Materials and methods

Plant material and leaf extraction

The fresh leaves of *Lysiphyllum strychnifolium* (LS) were collected in November 2017 from Kut Chum District, Yasothon Province of Thailand. The plant was identified by a botanist from the Plant Varieties Protection Office and the voucher specimen (BK No. 069394) was deposited at the Plant Varieties Protection Office, Department of Agriculture, Bangkok, Thailand. The leaves were dried in a hot air oven at 50 °C for 5 days, ground to a coarse powder, and boiled at 100 °C for 15 min. The samples were filtered and placed in a freeze drier for 3 days at -45 °C. The samples were then ground and stored at -20 °C until further analysis.

High-performance liquid chromatography (HPLC) analysis

The crude extract was dissolved in DMSO and filtered through a 0.45 μ m nylon membrane. The HPLC (Shimadzu, Japan) instrument was equipped with a model series LC-10AT VP pump, SCL-10A VP system controller with an SPD-10A VP UV detector. The quantification was made on a Thermo Hypersil gold C18 reversed-phase column (Thermo Fisher Scientific, Waltham, MA, USA). The analysis was performed at a flow rate of 1.0 mL/min, detecting a wavelength at $\lambda = 350$ nm. The mobile phase consisted of 0.3% formic acid in water and acetonitrile. The injection volume was 10 μ L. Since it was not yet known which of the substances in the LS extract was the key ingredient, then quercetin (Sigma-Aldrich, Oakville,

ON, Canada) was used as a trace standard and as an extraction quality control. The crude extract was quantified from the linear equation prepared from the quercetin standard solutions.

Cell lines and culture conditions

The human breast cancer cell lines (MCF-7) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (PR-free DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Merck KGaA, Darmstadt, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ incubator.

Cell viability assay

Cell viability was measured using MTT assay. MCF-7 cells were seeded into a 96-well plate at a density of 7×10^3 cells/well in 100 µL culture medium. The cells were treated with various concentrations (0, 0.16, 0.8, 4, 20, 100, and 500 µg/mL) of either LS extract or quercetin (Sigma-Aldrich, Oakville, ON, Canada). After 24 h, the cells were incubated with 10 µL (5 mg/mL) of MTT for 2 h at 37 °C with 5% CO₂. The formazan crystals produced by the viable cells were dissolved in 100 µL DMSO. The absorbance was recorded at 570 nm by a microplate reader (Varioskan Flash reader, Thermo electron corporation, Finland) and the percentage of cell viability (CV) was calculated using the following formula:

$$\% CV = \frac{\text{Average absorbance of sample}}{\text{Average absorbance of control}} \times 100$$

Estrogen-responsive element (ERE) reporter assay

The activity of the estrogen receptor signaling pathway was assayed with Cignal ERE reporter kit (Qiagen, Maryland, USA). MCF-7 cells (2×10^4 cells/well) were seeded into a 96-well culture plate with 100 µL phenol red-free DMEM (Gibco BRL, Grand Island, NY) containing 5% FBS. Then cells were transfected with the ERE-responsive firefly luciferase construct and *Renilla* luciferase construct. After 24 h of transfection, LS extract (20 µg/mL), quercetin (0.16 µg/mL), or 17β-estradiol (E2) (10^{-9} M) treatment was performed for 24 h. The firefly luciferase activity, which was normalized to *Renilla* luciferase activity, was measured using the dual luciferase assay (Promega, Madison, WI, USA) by a microplate reader (Varioskan Flash reader, Thermo electron corporation, Finland).

Real-time RT-PCR

MCF-7 cells (8×10^5 cells/dish) were seeded onto 60-mm dishes in DMEM supplemented with 10% FBS for 24 h. Each of these treatments, namely quercetin (0.16 µg/mL), LS extract (0.8, 4, and 20 µg/mL), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M), were added. After 24 h, total RNA was purified from the cells using illustra™ RNA spin Mini Isolation kit (GE Healthcare, UK). For cDNA, total RNA (50 ng) was reverse transcribed using iScript™ Reverse Transcription Supermix (BIO-RAD, California, USA). Quantitative real-time RT-PCR reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc). The target genes and the internal reference gene primer are *pS2* forward 5'-TTTGGAGCAGAGAGGAGGCAATGG-3', reverse 5'-TGGTATTAGGATAGAAGCACCAGGG-3'; *ERα* forward 5'-CAGACACTTTGATCCACCTGA-3', reverse 5'-CTCCAGCAGCAGGTCATAGA-3'; *ERβ* forward 5'-AAGAATATCTGTGTCAAGGCCATG-3', reverse 5'-GGCAATCACCCAAACCAAAG-3', and *GAPDH* forward 5'-AGGTCGGAGTCAACGGATT-3', reverse 5'-TAGTTGAGGTCAATGAAGGG-3'. The PCR amplification was analyzed by CFX96 Touch™ Real-Time PCR Detection System with CFX Manager™ Software (Bio-Rad Laboratories, Inc., CA, USA). The mRNA levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct$ was the (Ct, target - Ct, *GAPDH*) treatment group - (Ct, target - Ct, *GAPDH*) control group. Each sample was assayed from 3 replicates.

Western blot analysis

MCF-7 cells were seeded onto 60-mm dishes at a density of 8×10^5 cells/dish and treated with LS extract (0.8, 4, and 20 µg/mL), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) in Phenol red-free DMEM with 3% FBS for 24 h. The whole cells were collected and lysed with RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, and 0.5 % Triton X-100) containing protease inhibitors (Roche Diagnostics GmbH, Germany). Cell lysate supernatants were collected by centrifugation at 12,000 rpm for 30 min at 4 °C. The protein concentration was determined by using Bio-Rad® protein assay (Bio-Rad Laboratories, USA). Proteins were separated on 10% acrylamide gel and transferred onto a PVDF membrane (Merck

Millipore Corporation, Merck KGaA, Darmstadt, DE). The transferred membrane was blocked with 5% skimmed milk in TBS-Tween buffer and then incubated with primary antibody (1:1,000 Cell Signaling Technology, Beverly, MA.) overnight at 4 °C. The membranes were subsequently washed in TBST and HRP-conjugated secondary antibodies (1:10,000 dilutions) (Cell Signaling Technology, Beverly, MA). The blots were detected using Immobilon™ Western Chemiluminescent HRP substrate (Merck Millipore) in a chemiluminescent imaging system (GE Healthcare UK Limited, Amersham, UK). Intensity analysis of protein bands was performed using ImageJ software (National Institutes of Health, USA). The data were normalized to the level of GAPDH and analyzed for the relative values of the treated group.

Statistical analysis

Statistical analysis was performed with the software SPSS version 21. All data were expressed as mean \pm SEM and were analyzed by ANOVA followed by LSD or Dunnett's T3 test. Significant statistical differences in all tests were considered when p -values < 0.05 .

Results and discussion

Results

Quercetin content in LS leaf water extract by high-performance liquid chromatography (HPLC)

The yield of LS leaf water extract was 7.65 %. The HPLC chromatogram of LS extract showed a prominent peak at a retention time of 28.348 ± 0.03 min, which corresponds to the quercetin reference standard. A highly significant correlation between added quercetin (x) and the chromatographic peak area (y) was represented by the equation; $y = 24,939x - 5457.1$ ($R^2 = 0.999$). The content of quercetin in LS crude extract was 285.67 ± 0.11 $\mu\text{g/g}$ of dry extract.

Effect of LS leaf water extract on cell viability of MCF-7 cells

After the treatment of MCF-7 cells with a water extract of LS leaves or quercetin at 0.16 - 500 $\mu\text{g/mL}$ for 24 h, the percentage of viable cells was calculated and compared to the number of surviving cells in the control group. In **Figure 1**, LS extract did not affect cell viability at concentrations of 0.16 to 500 $\mu\text{g/mL}$, while treatment with quercetin of 20 $\mu\text{g/mL}$ or more decreased cell viability. The concentrations of LS leaf water extract chosen for further experiments were 0.8, 4, and 20 $\mu\text{g/mL}$, and quercetin was 0.16 $\mu\text{g/mL}$.

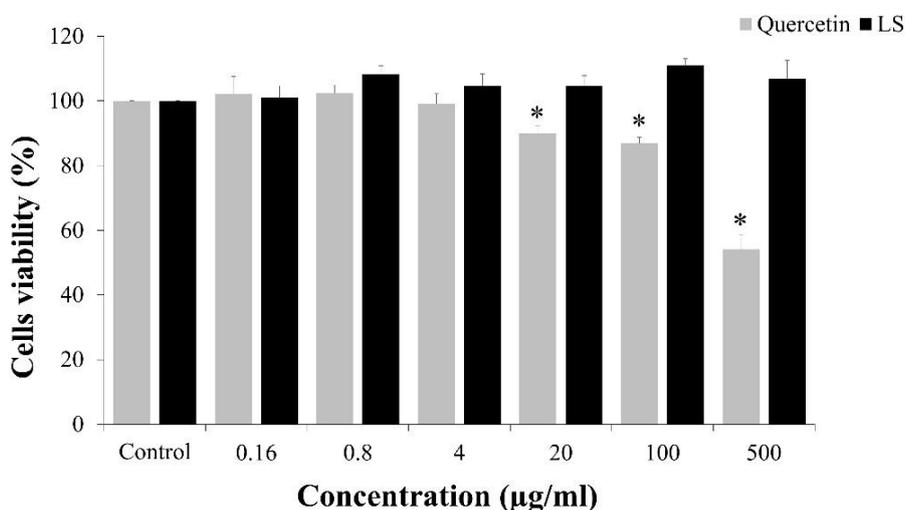


Figure 1 Effect of LS leaf extract and quercetin on cell viability of MCF-7 cells. The * mark represents $p < 0.05$ compared to the untreated control.

Effect of LS leaf water extract on the ERE-dependent transcriptional activity in MCF-7 cells

Figure 2 demonstrates the effect of control, LS extract (20 $\mu\text{g/mL}$), E2 (10^{-9} M) and quercetin (0.16 $\mu\text{g/mL}$) on the luciferase activity of MCF-7 cells transfected with EREs. E2 was used as a control for agonistic activity. There was an increase in ER α -dependent reporter activity without statistical significance when compared to untreated cells in MCF-7 cells transfected with ERE-responsive luciferase construct after 24 h of treatment. These results indicated that LS extract and quercetin tended to cause ER-mediated ERE transcriptional activity with a potent estrogenic effect on MCF-7 cells.

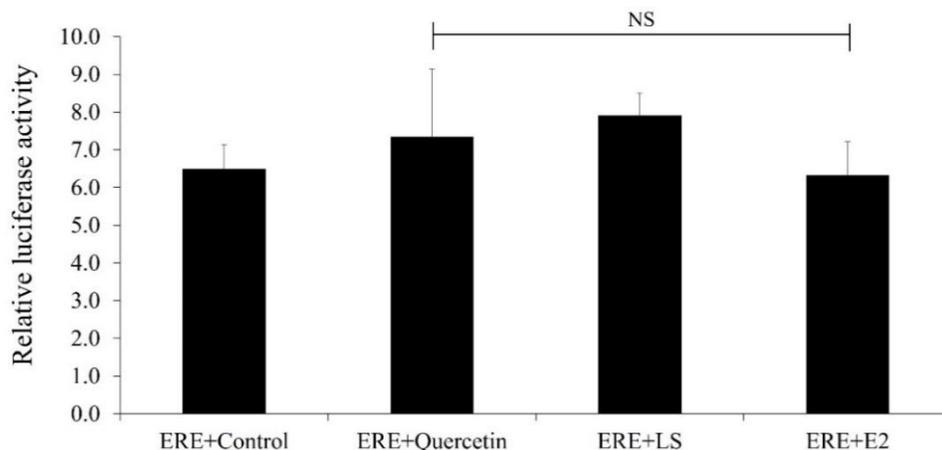


Figure 2 The effect of quercetin (0.16 $\mu\text{g/mL}$), LS (20 $\mu\text{g/mL}$), and E2 (10^{-9} M) on ERE-dependent transcriptional activity in MCF-7 cells. NS = non-significant.

Effect of LS leaf water extract on *pS2*, *ER α* , and *ER β* gene expression in MCF-7 cells

The density ratio of *pS2*, *ER α* , and *ER β* gene expression normalized to that of endogenous control *GAPDH* in comparison with the untreated control group, as shown in **Figure 3**. MCF-7 cells treated with LS (20 $\mu\text{g/mL}$) for 24 h showed an upregulation of *pS2* gene expression as compared with that of the untreated control, similar to that of the treatment with E2 (10^{-9} M) (**Figure 3(A)**). The *ER α* gene expression after treatment with quercetin (0.16 $\mu\text{g/mL}$) showed a significant increase in gene expression similar to that of the E2 (10^{-9} M) treatment (**Figure 3(B)**). The expression of the *ER β* gene after treatment with LS extract (0.8, 4, and 20 $\mu\text{g/mL}$) or quercetin (0.16 $\mu\text{g/mL}$) showed an increase in gene expression similar to that of the E2 (10^{-9} M) treatment. However, the increases were not statistically significant compared to the control (**Figure 3(C)**). The results showed that LS extract at 20 $\mu\text{g/mL}$, which induced *pS2* transcription, affected the transcription of estrogen-responsive genes, causing estrogenic effect. It was also shown that quercetin upregulates *ER α* gene expression at 24-hour treatment.

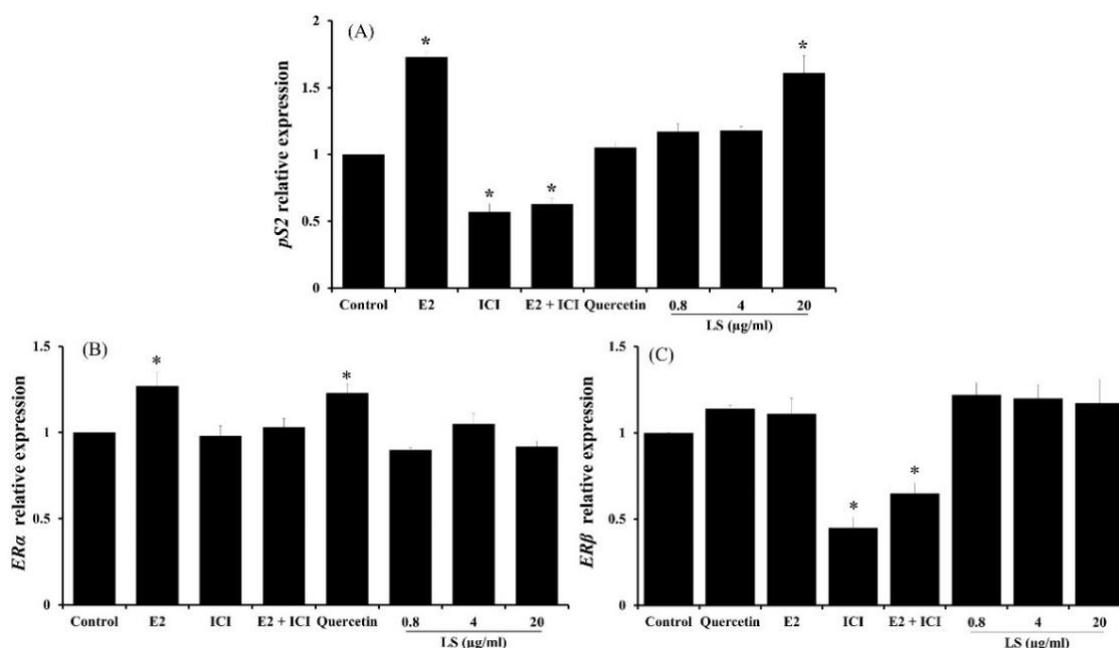


Figure 3 Effects of quercetin (0.16 $\mu\text{g/mL}$), LS leaf water extract (0.8, 4, and 20 $\mu\text{g/mL}$), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) on gene expression in MCF-7 cells. The * mark represents $p < 0.05$ compared to the untreated control.

Effect of LS leaf water extract on protein expression of ER α and phosphorylated forms in MCF-7 cells

The effect of control, quercetin (0.16 $\mu\text{g/mL}$), LS extract (0.8, 4 and 20 $\mu\text{g/mL}$), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) with or without E2 (10^{-9} M) on protein expression of total ER α and phosphorylated forms (Ser118 and Ser167) in MCF-7 cells was investigated by Western blotting. As shown in **Figures 4(A) - 4(B)**, the pER α Ser118 protein expression was found to be significantly upregulated in E2 compared with the untreated control. Additionally, treatment with quercetin or LS (0.8 and 4 $\mu\text{g/mL}$) compared with the untreated control found that the expression of pER α at Ser118 was also upregulated but was not statistically significant. In contrast, pER α Ser167 was hardly detectable in MCF-7 treated with E2. All the treatments decreased the pER α Ser167. In addition, this protein expression was found to be significantly downregulated by LS extract (20 $\mu\text{g/mL}$) compared with untreated control and was similar to that of the E2 treatment, and protein expression of cells treated with LS extract (0.8 and 4 $\mu\text{g/mL}$) was similar to that of the quercetin treatment. Moreover, the degrees of decreased phosphorylation of ER α at Ser167 protein expression seen from the LS extract (0.8, 4, and 20 $\mu\text{g/mL}$), quercetin, and E2 treatment were less than those induced by the treatment with the ICI 182 - 780 (**Figures 4(A) - 4(C)**). The expression of ER α protein treated with LS extract (20 $\mu\text{g/mL}$), E2, or ICI 182 - 780 decreased statistically significantly compared to the control. Decreased ER α protein expression was seen from the LS extract (20 $\mu\text{g/mL}$) and E2 treatment less than that caused by ICI 182,780 treatment (**Figures 4(A) - 4(D)**).

Ser167 is another important site of ER α phosphorylation that influences ER activity. These results showed that quercetin and LS extract treatment downregulated the phosphorylation of ER α at Ser167. In addition, these results indicated that the effects of LS extract (20 $\mu\text{g/mL}$) and E2 on ER α protein expression were similar since both suppressed the expression of this receptor.

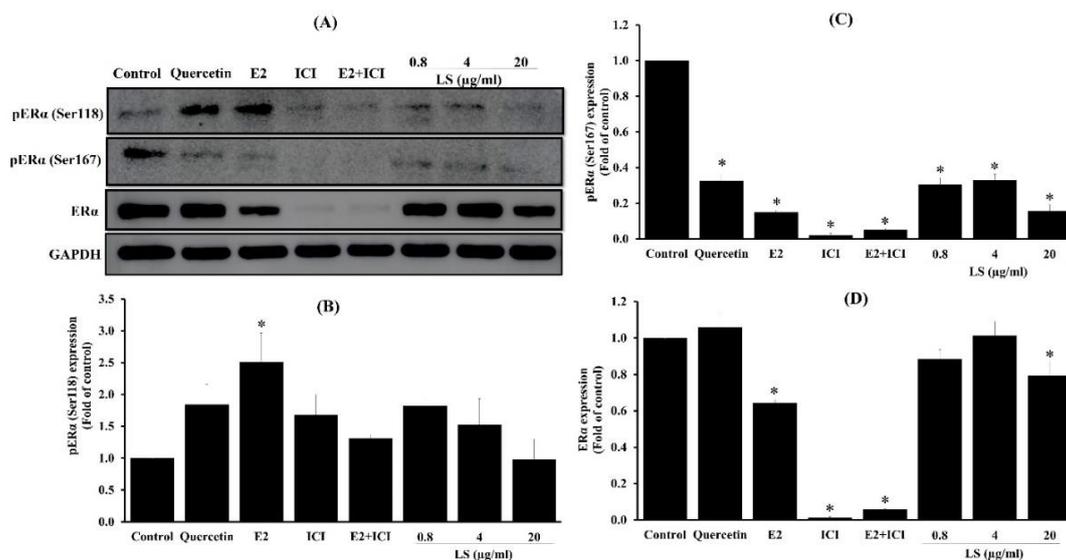


Figure 4 The effect of quercetin (0.16 $\mu\text{g/mL}$), LS leaf water extract (0.8, 4, and 20 $\mu\text{g/mL}$), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) with or without E2 (10^{-9} M) on ER α protein expression and phosphorylation (Ser118 and Ser167) in MCF-7 cells. The * mark represents $p < 0.05$ when compared to the untreated control.

Discussion

LS has been used in Northeast Thailand to promote lactation in breastfeeding women [2]. According to the literature review, LS leaf water extract contains quercetin [8], which is a phytoestrogen. Phytoestrogenic activity could trigger the herbal galactagogue effect, and certain molecules may share comparable effects resembling E2, an endogenous estrogen that promotes the proliferation of mammary epithelial cells. The phytoestrogen molecules which possess specific activities similar to E2 could cause prolactin receptors to express [20]. Estrogen stimulates the ductal epithelial cells to elongate, the primary role of which appears to be stimulating the production of prolactin [21]. Our previous clinical study found that LS tea is a promising natural galactagogue that tends to increase breast milk volume in the immediate postpartum period, and LS tea tends to stimulate breast milk to be secreted earlier [22].

After estrogen (E2) attaches to the estrogen receptor (ER), E2 switches to the nucleus, which ties to estrogen response elements (EREs) in target genes [23]. Estrogen receptor alpha (ER α) functions by triggering various estrogen response element (ERE)-reporter constructs in cell lines to express [15]. The non-cytotoxic concentrations of LS leaf water extract selected for the experiments were 0.8, 4, and 20 $\mu\text{g/mL}$, and quercetin was 0.16 $\mu\text{g/mL}$. This study found that MCF-7 cells transfected with ERE-responsive luciferase construct and treated with LS extract (20 $\mu\text{g/mL}$) and quercetin (0.16 $\mu\text{g/mL}$) tended to be upregulated because LS extract and quercetin may cause ER-mediated ERE transcriptional activity with potent estrogenic effects on MCF-7 cells.

In MCF-7 cells, the *pS2* gene, detected as an estrogen-inducible transcript, was converted into a secretory protein [13]. The *pS2* gene expression is often employed as an indicator of measuring the estrogenicity of different compounds [24]. E2 seemed to upregulate *pS2* gene expression levels while ER antagonist ICI 182,780 seemed to downregulate *pS2* gene expression levels compared with the untreated control. Similarly, LS extract (20 mg/mL) also induced an increase in the *pS2* gene. Since LS extract (20 mg/mL) upregulated *pS2* gene transcription, it was confirmed that LS possesses certain latent compounds that could cause the upregulation of ER-mediated transcription and trigger the estrogen activities in breast tissue. A previous study conducting *in vitro* assays reported that fenugreek seeds contained specific compounds similar to estrogen, which could trigger *pS2* expression in MCF-7 cells. The results found that the chloroform extracts of fenugreek seeds induced the expression of estrogen-responsive gene *pS2* in MCF-7 cells [24].

ER α and ER β converted by various genes demonstrate certain expressions in terms of tissue type or cell type. ER α is associated with certain types of estrogen-dependent breast cancer. On the other hand, ER β expression can inhibit the motility and invasion of cells in the breast [25]. ER α presumably mediates the mammary gland response to estrogens [26]. In this study, ER α gene expression treated with quercetin (0.16 $\mu\text{g/mL}$) increased significantly, and this increase was similar to that of the E2 (10^{-9} M) treatment, but the water extract of LS leaves did not, as LS extract was a crude extract that contained many compounds, and may show different effects compared to quercetin, the pure compound. ER β gene expression is found in approximately 80% of normal breast epithelial cells, and there is decreased expression of this receptor in breast cancer cells [27]. MCF-7 cells treated with ICI 182,780 showed downregulation of ER β gene expression, but ER β gene expression after treatment with LS extract or quercetin tended to increase, and this increase was similar to that of the E2 treatment.

Phosphorylation of serine residues in the activation function 1 (AF-1) domain of ER α seems to affect coactivators, leading to increased ER-mediated transcription. Phosphorylation, which develops on Ser118 and Ser167, is the process that responds to the stimulation of the mitogen-activated protein kinase (MAPK) [18]. Serine 118 and 167 can be found in the AF-1 region of ER α and their phosphorylation can be considered as a significant mechanism that controls AF-1 activity. The kinetics of E2 binding by ER α lead to the kinetics of serine 118 phosphorylation reacting to E2. In contrast, phosphorylation of ER α was not generated by the complete antagonist ICI 182,780 [28]. In this study, MCF-7 cells treated with quercetin or LS extract (0.8 and 4 $\mu\text{g/mL}$) tended to upregulate the expression of pER α at Ser118 compared with untreated control. This was interesting given that E2, quercetin, and LS extract (0.8 and 4 $\mu\text{g/mL}$) were able to activate AF-1 while ICI was not. Consequently, E2, quercetin and LS extract (0.8 and 4 $\mu\text{g/mL}$) binding seem to cause the receptor protein to express the conformational changes that facilitate transactivation by AF-1 and trigger the process of phosphorylation in Ser118. In contrast, MCF-7 cells treated with quercetin or LS extract downregulated the phosphorylation of ER α at Ser 167. Huderson *et al.* [29] reported that Ser118 is a major site of ER α phosphorylation in response to estradiol stimulation as well as a substrate for many other kinases. But pER α at Ser167 is not contained within a Ser-Pro consensus motif and phosphorylation of Ser167 by multiple kinases indicates that several signaling pathways regulate these sites.

Conclusions

This study provided scientific evidence for the potential estrogenic activities of LS leaf water extract. This indicates that ER-dependent pathways in MCF-7 cells served as mediators which facilitate the estrogenic properties of LS and its compounds to function effectively. MCF-7 cells were transfected with ERE-responsive luciferase construct and treated with LS leaf water extract and quercetin, tended to be upregulated because LS extract and quercetin may cause ER-mediated ERE transcriptional activity with potent estrogenic effects on MCF-7 cells. It was confirmed that LS-induced *pS2* gene transcription could affect the transcription of estrogen-responsive genes, causing estrogenic effects.

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