The estrogenic effect of Lysiphyllum strychnifolium (Craib) A. Schmitz water extracts in MCF-7 cells

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Abstract

Background

Lysiphyllum strychnifolium (Craib) A. Schmitz (LS) has been used to stimulate breast milk production in women after giving birth in the Northeast of Thailand. However, very little information is known about its estrogenic effect. The present study investigated the estrogenic effect of LS leaves water extracts compared with quercetin, one of the major compounds in LS, in MCF7 human breast cancer cells

Methods

The effect of LS leaves water extracts and quercetin on cell proliferation of estrogen receptor (ER)-positive breast cancer cells, MCF-7, was studied by MTT assay at a concentration range of 0 to 500 µg/ml. The expression of estrogen-dependent genes, the *pS2*, *ERα*, *ERβ* and *Bcl-xL*, were also examined by real time RT-PCR, and the expression of ERα protein was detected by Western blotting.

Results

The quercetin content in LS water extract was 285.67 ± 0.11 ug/g. The expression levels of the *pS2* and *Bcl-xL* genes in MCF-7 cells were found to be upregulated by LS leaves water extract (20 µg/ml) and 17β-estradiol (E2) (10^{-5} M) compared with 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, 20 µg/ml) decreased the phosphorylation of ERα at Ser167 (pERα (ser167)) and LS extract (20 µg/ml) decreased ERα, but there was no significant effect on the ERα at Ser118 (pERα (ser 118)) protein expression.

Conclusions

This study provided evidence for the potential estrogenic activities of LS leaves water extract. Since LS extract induced *pS2* gene transcription, it was confirmed that the extract could affect the transcription of estrogen responsive genes causing estrogenic effects. The results also showed that quercetin, one of the LS compounds, upregulated ERα gene expression at 24-hour treatment.

1. Background

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 the forests full of tropical trees in northern Thailand [1]. Local people have consumed LS as a traditional medicinal plant to relieve alcohol impacts [2], to treat poisoning, to eliminate pesticides, to relieve an

effect of poisonous mushrooms, to stimulate breast milk in women after giving birth, to promote health, to nourish and to reduce fatigue [3]. A Thai medical textbook states that LS is also used for fever relief and detoxification [4]. The therapeutic properties from a decoction of LS leaves include antioxidant [5] and anti-hyperuricemic activity [6] while those from its stems include anticancer [7], anti-HIV-1 integrase and anti-allergic activities [8]. 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 of Thailand [9]. Its leaves and vines are also used for muscle pain relief, muscle relaxation and blood circulation stimulation in Mahasarakham Province [10].

The ethanol extract from LS vines, leaves and stems were 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 [11, 12]. Quercetin was also found in water and ethanol extracts of LS stems and leaves [13–15]. Various fruits and vegetables contain quercetin, a carbohydrate-free flavonoid, and its effects include neuroprotective, antioxidant, immune system regulation, cardioprotective and anti-inflammatory [16]. In particular, quercetin is a flavonoid characterized by plant-derived compound called phytoestrogens with estrogenic effects. A previous study reported that phytoestrogenic activity induced by herbal galactagogue effects seemed to be correlative to an endogenous estrogen known as 17β-estradiol (E2) which enhances the mammary epithelial cells [17].

Estrogen is a female steroid hormone and is also found to be produced in other organs, including the adrenal glands, placenta, breast, and hippocampus. Estrogen has various functions in the reproductive system, such as the breast, uterus, and ovaries, and in other systems such as the bone, central nervous system and cardiovascular system [18]. Estrogen affecting different tissues in many physiological processes establishes and regulates the reproductive organs in both genders namely the gonads or the mammary gland [19]. Estrogen and phytoestrogen share a similar chemical structure. Thus, phytoestrogen which can bind itself to α and β estrogen receptors (ER) may possess estrogenic properties [20].

Estrogen works through estrogen receptors (ER) in the nucleus and on the cell membrane to regulate the activity of target cells. The estrogen receptors in the nuclear nucleus include the estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). The intracellular estrogen receptor (ER) contact estrogen response elements (EREs) in target genes leading to estrogenic responses to generate changes in gene transcription [21]. Estrogen can also indirectly induce gene expression by ER binding to other transcription factors such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB) and stimulating protein-1 (SP-1). If estrogen binds to the membrane ER, it leads to activation of the intracellular signaling pathway and the regulation of gene expression and intracellular protein function [22].

ERα and β mediate estrogen signaling. ER ligand and estrogen response element (ERE) sequences cause conformational changes in ERs transformed from an inactive form to a transcriptionally active state. ERα and ERβ can be attached to an ERE separately from ER ligands. The changes also induce

phosphorylation of multiple serine residues along with the protein. The activation and regulation of ERα activity require phosphorylation of serine 118 and serine 167 [22, 23].

Breast cancer cells express an estrogen-responsive gene known as the *pS2* gene. Transcription of the single-copy *pS2* gene is activated by MCF-7 breast cancer cells exposed to 17β-estradiol (E2) leading to higher levels of *pS2* mRNA and secreted protein. The findings on transfection assays revealed that any defective ERE can cause estrogen-responsiveness to this gene. Moreover, the *pS2* gene expression is applied as a reliable indicator of health issues ranging from illness severity, estrogen responses in breast cancer cells containing ER to achievement in antiestrogen therapy among patients with breast cancer. Thus, general mechanisms on the regulation of estrogen-responsive genes and how the *pS2* gene is regulated in human breast cancer cells can be studied through examining the *pS2* gene in MCF-7 cells [24].

The action of estrogen through its binding to the receptor (ER) shows both positive and negative effects. Because estrogen has an effect on cell proliferation in both normal cells and breast cancer cells [25]. Depending on the amounts of hormones received, estrogen induced Bcl-2 and Bcl-xL, anti-apoptotic members of the mammalian B-cell lymphoma-2 (Bcl-2) protein family, expression to control cell death in MCF-7 cells [26, 27].

Previous studies found that quercetin specifically promotes mammary gland development and lactation yield in milk deficient mice, probably via stimulating prolactin (PRL) expression and release from the pituitary gland, as well as inducing prolactin receptor (PRLR) expression in primary mammary epithelial cells [28]. The aim of this study is to evaluate the estrogenic effects of water extract of LS leaves and quercetin on ER positive MCF-7 cells. The expression of *pS2*, *ERα*, *ERβ* and *Bcl-xL* genes was also examined by real-time RT-PCR, and ERα protein expression was detected by Western blotting.

2. Methods

2.1 Plant and extraction

The fresh leaves of *Lysiphyllum strychnifolium* (LS) were collected from Kut Chum District, Yasothon Province of Thailand. After its specimen verification, the herbarium of the Plant Varieties Protection Office, Department of Agriculture, Bangkok, Thailand, issued the voucher specimen (BK No. 069394). 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 minutes. They were filtered and dried with a freeze dryer. Samples were placed in an aluminum foil and treated at − 20°C for 24 hours. The samples were then placed in freeze drier for 3 days at -45°C. The samples were then ground and stored in air-tight containers at -20°C until further analysis.

2.2 High-performance liquid chromatography (HPLC) analysis

The extract was dissolved in water and filtered through a 0.45 µm nylon membrane. The HPLC (Shimadzu, Japan) instrument was equipped with a model series LC-10AT VP pump, SCL-10A VP system controller with a SPD-10A VP UV detector. The quantification was made on a Thermo Hypersil gold C18 reverseed-phase column (Thermo Fisher Scientific, Waltham, MA, USA). The analysis was performed at a flow rate 1.0 ml/min, detecting wavelength at λ = 350 nm. The mobile phase consisting of 0.3% formic acid in water and acetonitrile. The injection volume was 10 µl. Since it was not yet known which of the substances in LS extract was the key ingredient, then quercetin (Sigma-Aldrich, Oakville, ON, Canada) was used as a trace standard and may be used as an extraction quality control.

2.3 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), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 incubator.

2.4 Cell viability assay

Cell viability was measured using 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (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 hours of treatment, the media was added with MTT solution (10 µl per well of 5 mg/ml MTT dissolved in PBS) and incubated for 2 hours at 37°C in a humidified atmosphere of 5% CO_2 incubator. The yellow MTT dye was reduced to purple formazan crystals by succinate dehydrogenase in the mitochondria. The viable cell number was directly proportional to the production of formazan following solubilization with DMSO. After adding the solubilizing agent, the plate was gently incubated for a few minutes and absorbance was recorded at 570 nm by microplate reader (Varioskan Flash reader, Thermo electron corporation, Finland) and the percentage of cell viability (CV) was calculated using the following formula:

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

2.5 Estrogen responsive element (ERE) reporter assay

The activity of estrogen receptor signaling pathway was assayed with Cignal ERE reporter kit (Qiagen, Maryland, USA) and seeded the MCF-7 cells in 2×10^4 cells per well in a 96-well culture plate with 100 µl Phenol red-free DMEM (Gibco BRL, Grand Island, NY) containing 5% FBS. Twenty-four hours later, the researchers transfected the cells by applying ERE-responsive firefly luciferase construct and *Renilla* luciferase construct. The researchers used the constitutively expressed GFP luciferase construct as a positive control and the non-inducible firefly luciferase construct as a negative control. After 24 hours of

transfection, LS extract (20 µg/ml), quercetin (0.16µg/ml) or 17β-estradiol (E2) (10^{-9} M) treatment was performed for 24 hours. The firefly luciferase activity that 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).

2.6 Real-time RT-PCR

MCF-7 cells (8×10^5 cells/dish) were seeded onto 60 mm dishes and used in DMEM supplemented with 10% FBS (Merck KGaA, Darmstadt, Germany) for 24 hours before treatment. After that, each of these treatments namely quercetin (0.16 µg/ml), LS extract (0.8, 4, 20 µg/ml), E2 (10^{-9} M), or ICI 182,780 (10^{-5} M) was added. After incubating for 24 hours, total RNA was purified from the cells using illustra RNA spin Mini Isolation kit (GE Healthcare, UK). Regarding reverse transcription, cDNA was synthesized from 50 ng of total RNA using iScript™ Reverse Transcription Supermix for RT-qPCR kit (BIO-RAD, California, USA). The cDNA was used in quantitative real-time PCR analyses. qPCR reactions were performed using the iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc). The target gene and the internal reference gene primer are listed in Table 1.

Table 1
The primers used in real-time RT-PCR

Genes	Primers	Annealing temp. (°C)	Product size (bp)
pS2 [29]	F- 5´TTTGGAGCAGAGAGGAGGCAATGG3´	62	240 bp
	R- 5´TGGTATTAGGATAGAAGCACCAGGG3´		
ERα [30]	F- 5´CAGACACTTTGATCCACCTGA3´	55	179 bp
	R- 5´CTCCAGCAGCAGGTCATAGA3´		
ERβ [31]	F- 5´AAGAATATCTCTGTGTCAAGGCCATG3´	65	143 bp
	R- 5´GGCAATCACCCAAACCAAAG3´		
Bcl-xL [32]	F- 5´GGAGCTGGTGGTTGACTTTCT3´	55	379 bp
	R- 5´CCGGAAGAGTTCATTCACTAC3´		
GAPDH [33]	F- 5´AGGTCGGAGTCAACGGATTT3´	55	112 bp
	R- 5´TAGTTGAGGTCAATGAAGGG3´		
F, forward primer; R, reverse primer			

The PCR amplification was analyzed by CFX96 Touch™ Real-Time PCR Detection System with CFX Manager™ Software (Bio-Rad Laboratories, Inc., CA, USA) to analyze the PCR amplification. The mRNA

levels of genes were calculated using the $2^{-\Delta\Delta C}{}_t$ method, where $\Delta\Delta C_t$ was the (C_t, target - C_t, GAPDH) treatment group – (C_t, target - C_t, GAPDH) control group. Each sample was assayed in three examples.

2.7 Western blot analysis

MCF-7 cells were seeded into 60 mm dishes at a density of 8×10^5 cells/dish and treated with LS extract (0.8, 4, 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 protein expression was determined by Western blot. 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) with protease inhibitor (Roche Diagnostics GmbH, Germany) to collect and lyse the whole cells homogenized by sonication and centrifugation at 12,000 rpm for 30 min at 4°C. The extracted protein concentration was determined by using Bio-Rad® protein assay (Bio-Rad Laboratories, USA) based on the method of Bradford's with bovine serum albumin (BSA) as a standard protein. Twenty micrograms of cell lysate protein were separated on 10% acrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore Corporation, Merck KGaA, Darmstadt, DE). The transferred membrane was blocked with 5% skimmed-milk in TBS-Tween buffer for 1 h at room temperature and then incubated with the appropriate primary antibody concentration (Cell Signaling Technology, Beverly, MA.) (1:1,000 dilution for estrogen receptor alpha (ERα), phospho-estrogen receptor α serine 118 (pERα (Ser118)), phospho-estrogen receptor α serine 167 (pERα (Ser167)) and GAPDH overnight at 4°C. The processed membranes were subsequently washed in TBS-Tween buffer and then incubated at room temperature with anti-rabbit or anti-mouse immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilutions) (Cell Signaling Technology, Beverly, MA). After the membranes were exposed to the secondary antibodies, the signals were developed using Immobilon™ Western Chemiluminescent HRP substrate (Merck Millipore) and detected using a chemiluminescent imaging system (GE Healthcare UK Limited, Amersham, UK). Intensity analysis of protein bands were performed using ImageJ software (National Institutes of Health, USA). Data were normalized to the level of GAPDH, and analyzed for the relative values of the treated group.

2.8 Statistical analysis

Statistical analysis was performed with the software SPSS version 21. All data were expressed as mean ($\bar{x}$) ± standard error of the mean (SEM) and were analyzed by ANOVA followed by LDS or Dunnett's T3 test. Significant statistical differences in all tests were considered when p-values < 0.05.

3. Results

3.1 Quercetin content in LS water extracts by high-performance liquid chromatography (HPLC)

The yield of LS water extracts was 7.65%. The HPLC chromatogram of LS extract showed a prominent peak at the retention time of 28.348 ± 0.03 min, which corresponds to quercetin reference standard. A

highly significant correlation between added quercetin (x) and chromatographic peak area (y) was represented by the equation: $y = 24939x - 5457.1$ ($R^2 = 0.999$). The content of quercetin in LS extract was 285.67 ± 0.11 ug/g.

3.2 Effect of LS leaves extract on cell viability of MCF-7 cells

After the treatment of MCF-7 cells with water extract of LS leaves or quercetin at the concentrations ranged over 0.16–500 µg/ml for 24 h, the percentage of viable cells was calculated and compared to the number of surviving cells in the control group (no treatment). In Fig. 1, LS extract did not affect the cell viability at the concentrations of 0.16 to 500 µg/ml, while the treatment with quercetin of 20 µg/ml or more decreased cell viability. Thus, the non-cytotoxic concentrations of LS leaves water extract chosen for further experiments were 0.8, 4, 20 µg/ml, and quercetin was 0.16 µg/ml.

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

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

3.4 Effect of LS leaves water extract on pS2, ERα, ERβ and Bcl-xL gene expression in MCF-7 cells

Five µg of total RNA were extracted from MCF-7 cells with untreated as control, and treated with LS extract (0.8, 4, 20 µg/ml), quercetin (0.16 µg/ml), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) with or without E2 (10^{-9} M). Then, *pS2*, *ERα*, *ERβ* and *Bcl-xL* mRNA levels were determined by real-time RT-PCR and normalized using *GAPDH* mRNA as an internal standard. Figure 3 shows the density ratio of *pS2*, *ERα*, *ERβ* and *Bcl-xL* gene expression normalized to that of endogenous control *GAPDH* in comparison with the untreated control group and it represents mean ± SEM of three replicates. MCF-7 cells treated with LS (20 µg/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). The *pS2* gene expression levels were found to be upregulated by E2 (10^{-9} M) and downregulated by ER antagonist ICI 182,780 (10^{-5} M) (Fig. 3 (A)). *Bcl-xL* gene expression levels were found to be significantly upregulated in LS (20 µg/ml), E2 (10^{-9} M), or ICI 182–780 (10^{-5} M) with or without E2 (10^{-9} M) compared with untreated control (Fig. 3 (B)). The *ERα* gene expression after treatment with quercetin (0.16 µg/ml) showed an insignificant increase in gene

expression similar to that of the E2 (10^{-9} M) treatment (Fig. 3 (C)). The expression of *ERβ* gene after treatment with LS extract (0.8, 4, 20 μg/ml) or quercetin (0.16 μg/ml) showed an insignificant 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. MCF-7 cells treated with ICI 182,780 (10^{-5} M) with or without E2 (10^{-9} M) for 24 h showed downregulation of ERβ gene expression as compared with that of the untreated control (Fig. 3 (D)). The results showed that LS extract at 20 μg/ml, which induced *pS2* transcription, affected the transcription of estrogen responsive genes causing estrogenic effects. The results also showed that quercetin, one of the LS compounds, upregulated ERα gene expression at 24-hour treatment.

3.5 Effect of LS leaves water extract on protein expression of ER_{α} and phosphorylated forms in MCF-7 cells

The effect of untreated control, quercetin (0.16 μg/ml), LS extract (0.8, 4, 20 μg/ml), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) with or without E2 (10^{-9} M) on protein expression of total ERα and phosphorylated forms (Ser118 and Ser167) in MCF-7 cell line was investigated by Western blotting. As shown in Fig. 4 (A), the phosphorylation of ERα at Ser118 protein expression was found to be significantly upregulated in E2 compared with untreated control. Additionally, treatment with quercetin and LS (0.8, 4 ug/ml) compared with untreated control found that the expression of ERα at Ser 118 was also upregulated but was not statistically significant. In contrast, phosphorylated ERα (Ser167) was hardly detectable in MCF-7 treated with E2. All the treatments decreased the phosphorylation of ERα at Ser167. In addition, this protein expression was found to be significantly downregulated by LS extract 20 μg/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, 4 μg/ml) was similar to that of the quercetin treatment. Moreover, the degrees of decreased phosphorylation of ERα at Ser167 protein expression was seen from the LS extract (0.8, 4, 20 μg/ml), quercetin and E2 treatment were less than those induced by the treatment with the ICI 182–780 with or without E2. (Fig. 4 (B)). The ERα protein expressions of the cells which were treated by LS extract (20 μg/ml), E2 or ICI 182–780 with or without E2 decreased statistically significantly compared to the control. Decreased ERα protein expression was seen from the LS extract (20 μg/ml) and E2 treatment less than that caused by ICI 182,780 treatment (Fig. 4 (C)). Ser167 is another important site of ERα phosphorylation that influences ER activity. These results showed that quercetin and LS extract treatment downregulated the phosphorylation of ERα at Ser167. In addition, these results indicated that the effects of LS extract (20 μg/ml) and E2 on ERα protein expression were similar, since both suppressed the expression of this receptor. Interestingly, this study found that quercetin could upregulate ERα expression both at the mRNA level and tended to upregulate protein level in MCF-7 cells after 24-hour treatment.

4. Discussion

LS has been used in Northeast Thailand to promote lactation in breastfeeding women [3]. According to the literature review, LS leaves water extract contains quercetin [13], which is a phytoestrogen.

Phytoestrogenic activity could trigger the herbal galactogogue effect and certain molecules may share comparable effects resembling E2 which is an endogenous estrogen enhancing reproduction of mammary epithelial cells [34]. The phytoestrogen molecules which possess specific activities similar to E2 could cause prolactin receptors to express [35, 36]. Estrogen stimulates the ductal epithelial cells to elongate, their primary role appears to be stimulating the production of prolactin [37]. The aim of this study was to investigate the potential of quercetin and LS leaves water extract on the estrogenic effect. The non-cytotoxic concentrations of LS leaves water extract selected for the experiments were 0.8, 4, 20 µg/ml, and quercetin was 0.16 µg/ml.

The pathways for estrogen receptor (ER) action through genomic DNA dependent interaction. After Estrogen (E2) attaches to the estrogen receptor (ER), E2 switch to the nucleus which ties to estrogen response elements (EREs) in target genes [38]. Estrogen receptor alpha (ERα) functions by triggering various estrogen response element (ERE)-reporter constructs in cell lines to express [39]. This study found that MCF-7 cells, which were transfected with ERE-responsive luciferase construct and treated with LS extract (20 µg/ml) and quercetin (0.16 µg/ml), tended to be upregulated since LS extract and quercetin may cause ER-mediated ERE transcriptional activity with potent estrogenic effects on MCF-7 cells.

In breast cancer cells including MCF-7 cells, the *pS2* gene detected as an estrogen inducible transcript was converted for a secretary protein from these cells [21]. The *pS2* gene expression is often employed as an indicator of measuring the esteogenicity of different compounds [29]. 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 expression compare with the untreated control. Since LS extract (20 mg/ml) upregulated *pS2* gene transcription, it was confirmed that LS possesses certain latent compounds which 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 consisted of 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 [29].

Bcl-2 is found to be abundant in 75% of breast cancer while its family members are found in mammary gland development and breast cancer [40]. Apoptosis can be regulated positively or negatively by Bcl-2 family proteins considered as structurally related molecules. Bcl-xL which belongs to the Bcl-2 family is anti-apoptotic. The Bcl-xL protein is chiefly related to invasive breast cancer and found to be abundant in 43% of invasive breast tumors [38]. In this study, the researchers found that LS extract (20 µg/ml) treatment increased the expression of Bcl-xL in MCF-7 cells significantly. These results suggested that Bcl-2 family members could play a major role in LS extract induced anti-apoptosis in breast cancer cells.

ERα and ERβ converted by various genes demonstrate certain expressions in terms of tissue type or cell type [41]. Estrogen stimulation of ERα is known to stimulate cell proliferation in breast tissue [42]. ERα is thought mediation of mammary gland response to estrogens [43]. In this study, *ERα* gene expression

treated with quercetin (0.16 μg/ml) increased significantly and this increase was similar to that of the E2 (10^{-9} M) treatment. Therefore, quercetin may stimulate cell proliferation in breast tissue. *ERβ* gene expression is found in approximately 80% of normal breast epithelial cells, and there is decreased expression of this receptor in breast cancer cells [44]. MCF-7 cells treated with ICI 182,780 showed downregulation of *ERβ* gene expression compared with that of the control, but *ERβ* gene expression after treatment with LS extract or quercetin tended to increase and this increase was similar to that of the E2 treatment compared to the control.

Phosphorylation of serine residues in the 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 responding to the stimulation of the mitogen-activated protein kinase (MAPK) [45]. Transcription activation by estrogen receptor occurs through the actions of a N-terminal transcription activation function AF-1. Serine 118 and 167 can be found in the activation function (AF) 1 region of ER-α and their phosphorylation can be considered as a significant mechanism which controls AF-1 activity. The kinetics of serine 118 phosphorylation in response to E2 follow the kinetics of E2 binding by ERα. However, the complete antagonist ICI 182,780 did not induce phosphorylation of ERα [46]. In this study, MCF-7 cells treated with quercetin or LS extract (0.8 and 4 ug/ml) tended to upregulate the expression of ERα at Ser 118 compared with untreated control. This was interesting given that E2, quercetin and LS extract (0.8, 4 μg/ml) were able to activate AF-1 while ICI was not. Consequently, E2, quercetin and LS (0.8, 4 μg/ml) binding except ICI seems to cause the receptor protein to express the conformational changes which facilitate transactivation by AF-1 and trigger the process of phosphorylation in serine 118.

Conclusions

In conclusion, this study provided evidence for the potential estrogenic activities of quercetin and LS leaves water extract. Moreover, in the model cells, there were also ERE transcriptional activity increases and cell proliferation inductions due to LS and its compounds. This indicated that estrogenic properties of LS extract and its compounds were mediated through ER-dependent pathways in MCF-7 cells. Since LS extract induced *pS2* gene transcription, it was confirmed that this could affect the transcription of estrogen responsive genes causing estrogenic effects. Interestingly, this study occasionally found that quercetin could upregulate ERα expression both at the mRNA level and protein level in MCF-7 cells after 24-hr treatment.

Abbreviations

LS: *Lysiphyllum strychnifolium* (Craib) A. Schmitz; E2:17β-estradiol; ER: estrogen receptors; ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; EREs: estrogen response elements; AP-1: activator protein-1; MAPK: mitogen-activated protein kinase.

Declarations

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Authors' contributions

AK., SM., NE. and SK. designed the study; AK., SM., NE., RM. and TN. methodology; AK., SM., NE. and RM.; analyzed the data, AK. and SM.; drafted the manuscript, AK. and SM.; writing - review and editing, AK. and SM.; revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets during and/or analyzed during the current study available form the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no conflict of interests.

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Figures

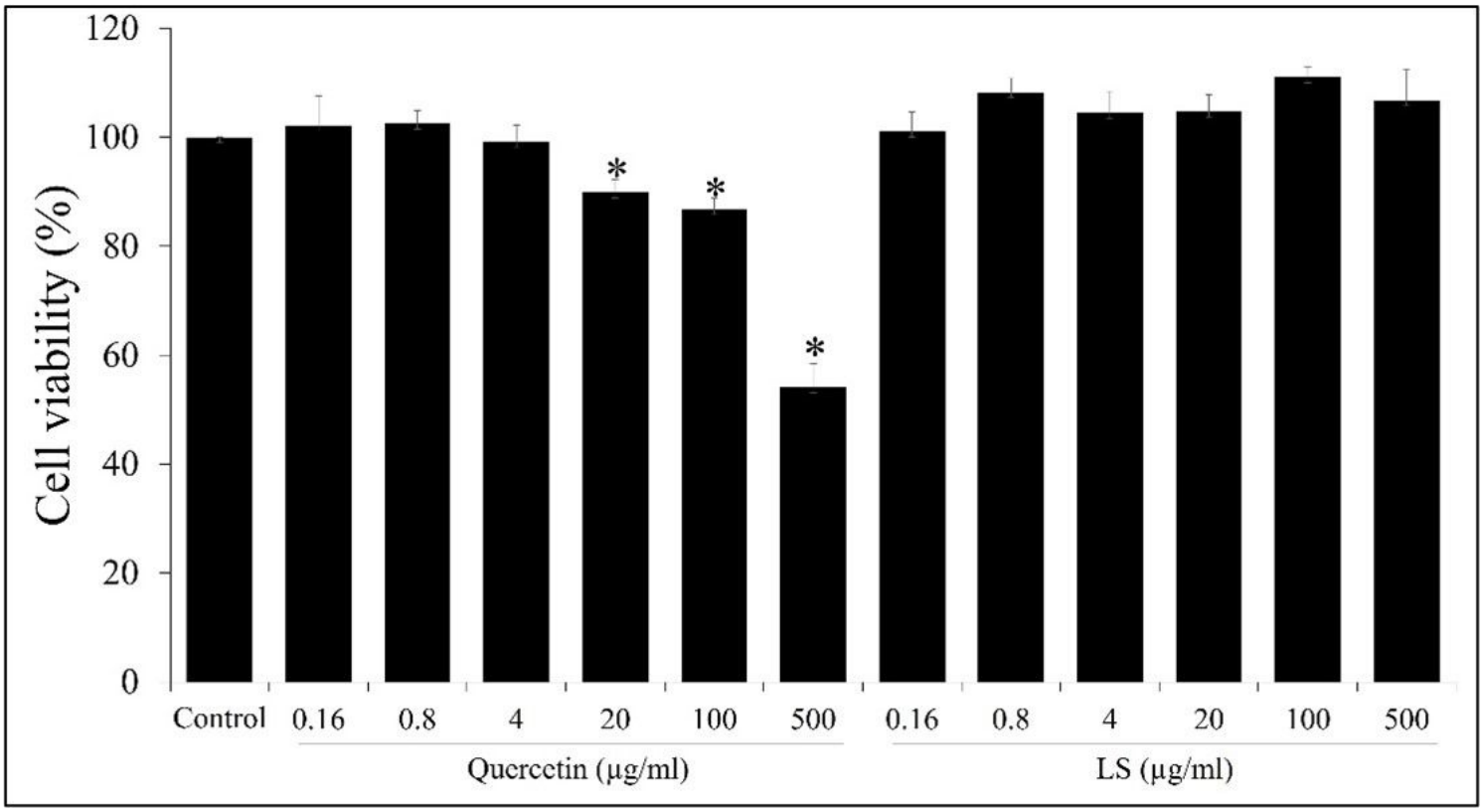

Figure 1

Effect of water extract of LS leaves or quercetin at various concentrations on the viability of MCF-7 cells. After 24 h of treatment, cell viability was quantified by MTT assay, expressed as a percentage of cell viability, and shown as the mean ± SEM of experiment (n=3). The * mark represents $p < 0.05$ compared to the untreated control.

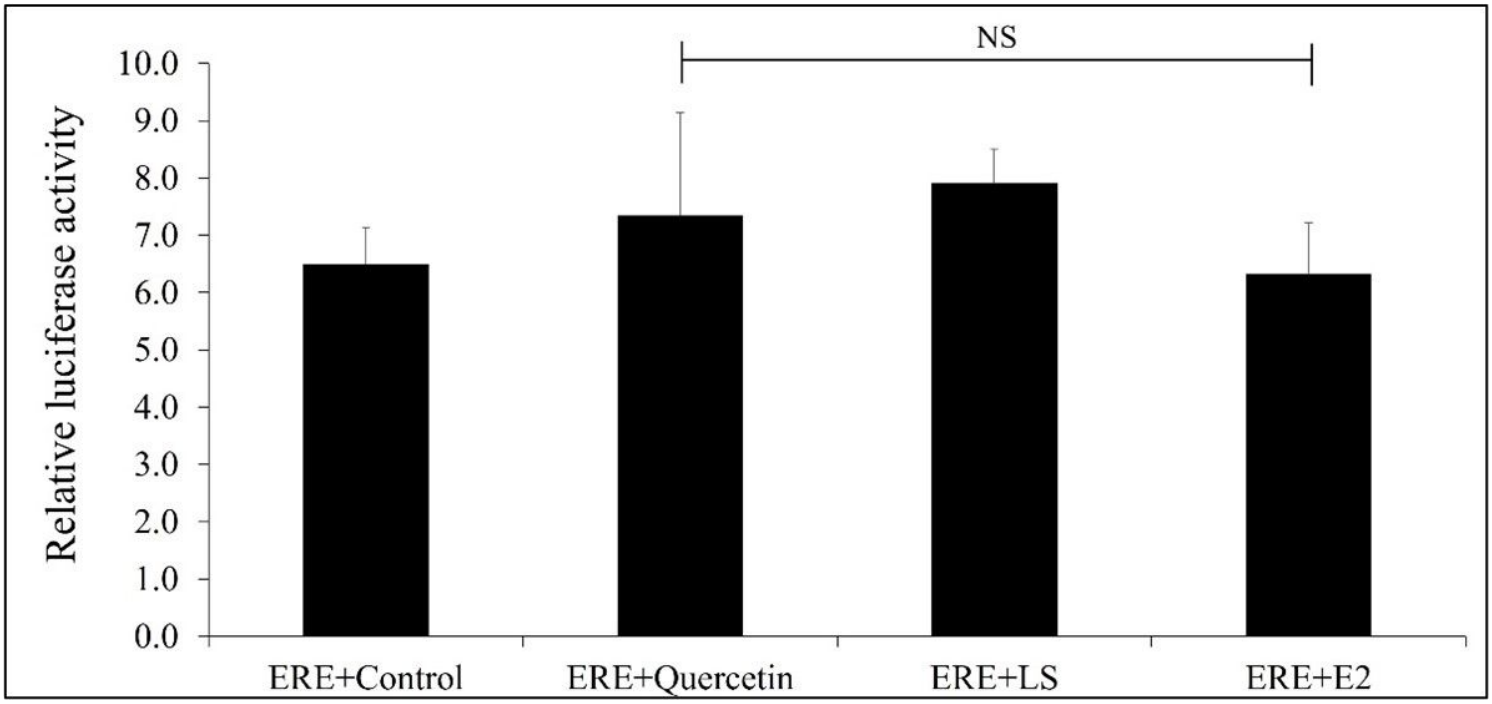

Figure 2

The effect of quercetin (0.16 μg/ml), LS (20 μg/ml) and E2 (10^{-9} M) on ERE-dependent transcriptional activity in MCF-7 cells were assayed by luciferase reporter assay. Results from three independent experiments are presented as the mean fold induction compared to control ± SEM. NC = negative control, NS = non-significant

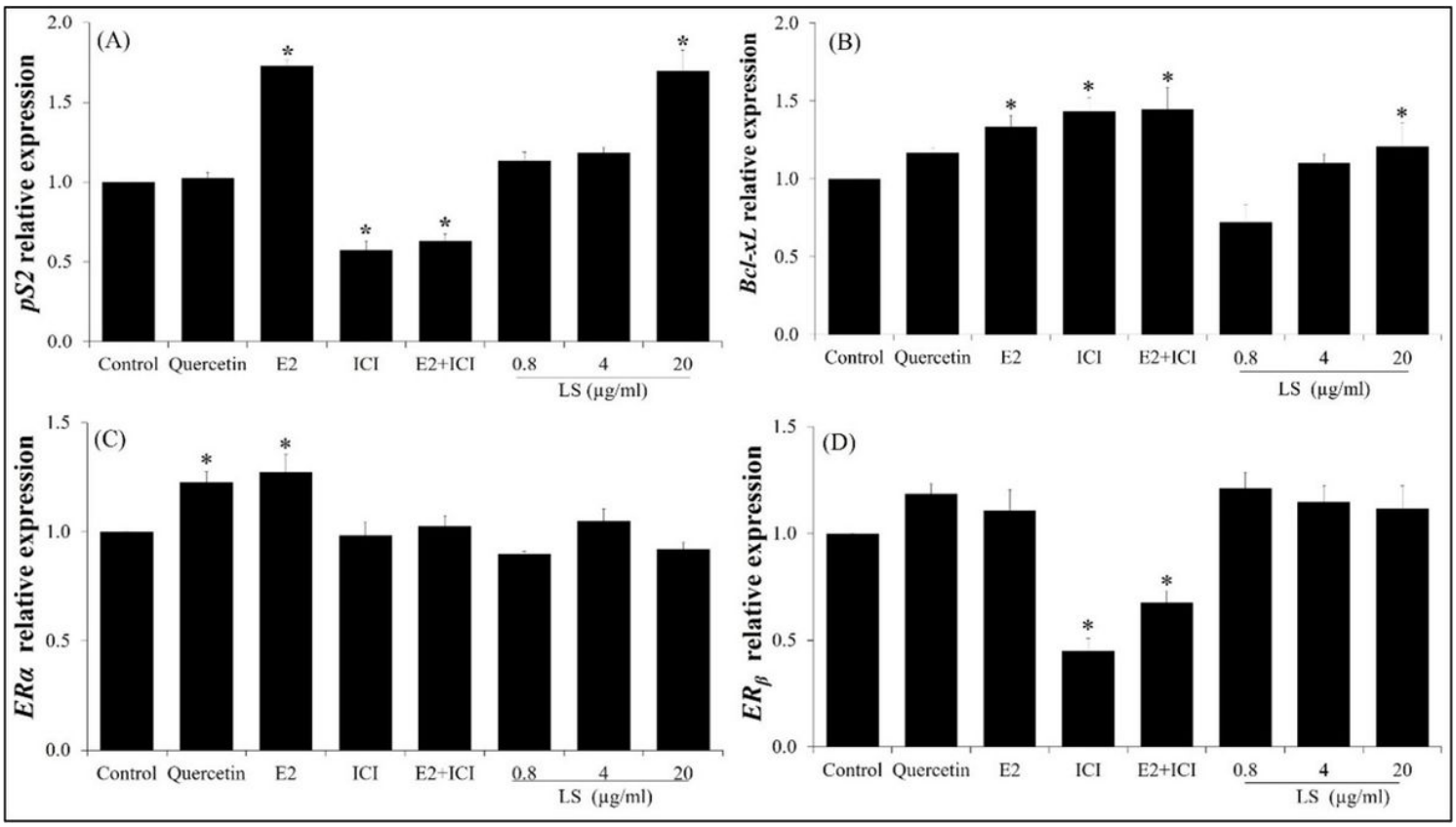

Figure 3

Effects of quercetin (0.16 μg/ml), LS leaves water extract (0.8, 4, 20 μg/ml), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) on gene expression in MCF-7 cells. After 24 h of treatment, *pS2*, *ERα*, *ERβ* and *Bcl-xL* gene expression were determined by real-time RT-PCR and normalized using GAPDH gene as an internal standard. The density ratio of **(A)** *pS2*, **(B)** *Bcl-xL*, **(C)** *ERα*, and **(D)** *ERβ* gene expression normalized to that of endogenous control *GAPDH* and represents mean ± SEM of experiment (n=3). The * mark represents $p < 0.05$ compared to the untreated control.

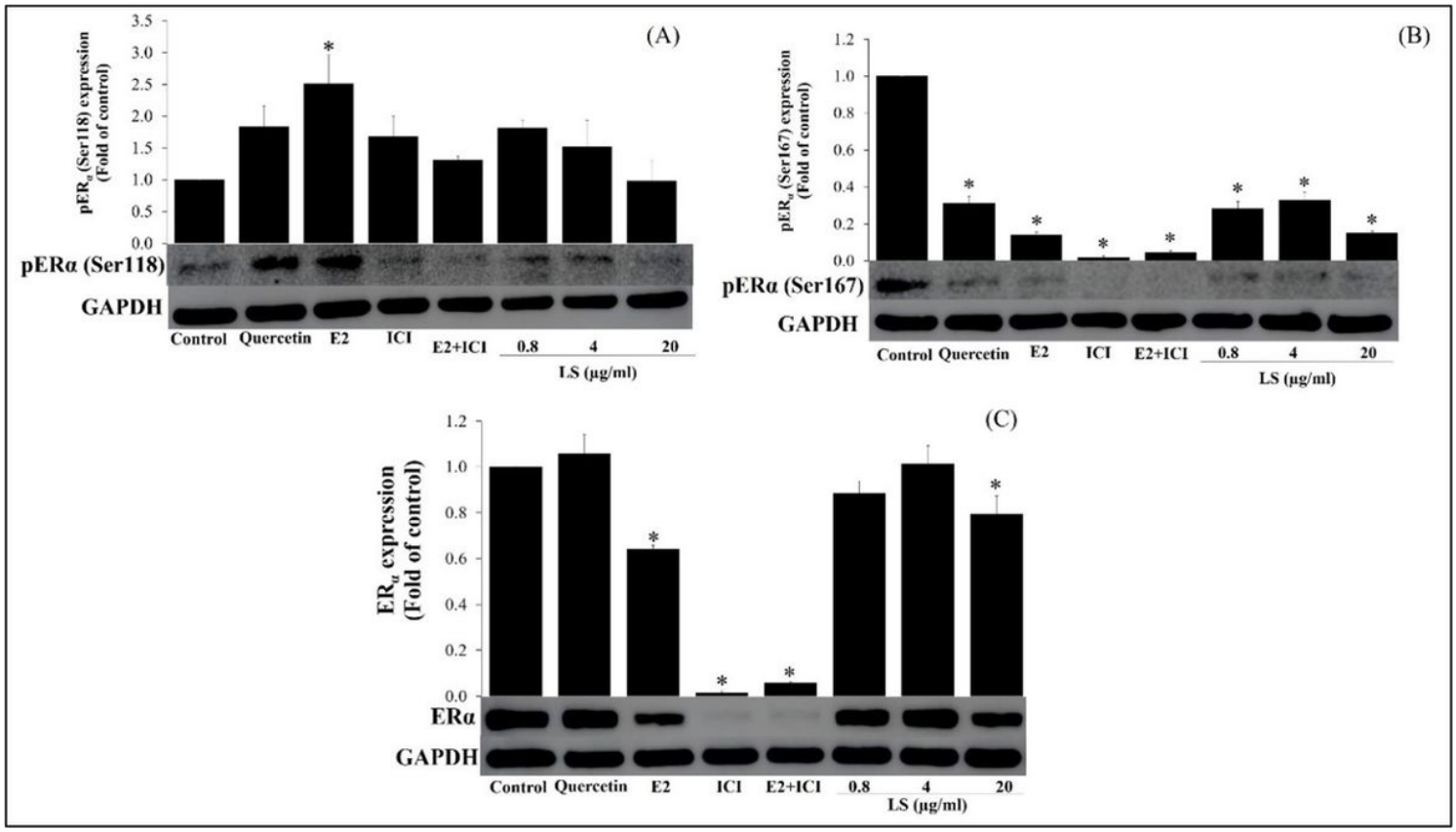

Figure 4

The effect of quercetin (0.16 μg/ml), LS (0.8, 4, 20 μg/ml), E2 (10^{-9} M) or ICI 182,780 (10^{-5} M) with or without E2 (10^{-9} M) on ERα protein expression and phosphorylation (Ser118 and Ser167) in MCF-7 cells. The protein expression of pERα (Ser118) **(A)**, pERα (Ser167) **(B)**, and total form of ERα **(C)** were analyzed by Western blotting. It represents mean ± SEM of three replicates. The * mark represents $P < 0.05$ when compared to the untreated control.