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Luteolin-7-methylether from *Leonurus japonicus* inhibits estrogen biosynthesis in human ovarian granulosa cells by suppression of aromatase (CYP19)

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ABSTRACT

Leonurus japonicus (motherwort) has been widely used to treat gynecological disorders, in which estrogen is often dysregulated, for a long time in China and other Asian countries. However, the chemical constituents and mechanisms underlying the activity of this medicinal plant are not fully understood. Seventeen of forty-six tested natural products from *L. japonicus* showed stimulatory or inhibitory effects on estrogen biosynthesis with different potency in human ovarian granulosa-like KGN cells. Luteolin-7-methylether (XLY29) potently inhibited 17β-estradiol production (IC_{50}: 5.213 μM) by decreasing the expression of aromatase, the only enzyme in vertebrates that catalyzes the biosynthesis of estrogens, but had no effect on the catalytic activity of aromatase. XLY29 decreased the expression of aromatase promoter I.3/II, and suppressed the phosphorylation of cAMP response element-binding protein. XLY29 potently inhibited phosphorylation of p38 mitogen-activated protein kinase and AKT but had no effect on phosphorylation of extracellular signal-regulated kinase and c-Jun N-terminal kinase. XLY29 also decreased the serum 17β-estradiol level and disturbed estrous cycle in mice. These results suggest that modulation of estrogen biosynthesis is a novel effect of *L. japonicus*, and XLY29 warrants further investigation as a new therapeutic means for the treatment of estrogen-related diseases.

1. Introduction

Estrogen plays an important role in cancer development (Hewitt et al., 2005). Aromatase is the rate-limiting enzyme that catalyzes the biosynthesis of estrogens (Simpson, 2000). Aromatase inhibitors (AIs), such as letrozole (Let) etc., have been developed and used as first-line treatment against hormone-dependent breast cancer with superior efficacy as compared to tamoxifen (Johnston and Dowsett, 2003). Moreover, AIs have been used in clinical settings for ovulation induction (Palomba, 2015). However, whole body estrogen deprivation increased the risk of osteoporosis and cardiovascular disease (Smith and Dowsett, 2003). Thus, new AIs are needed to offer greater clinical efficacy.

In humans, aromatase expression is regulated by tissue-specific promoters (Bulun et al., 2003). The major site of estrogen biosynthesis in premenopausal women is the ovary, where aromatase expression results from the activation of its proximal promoter (promoter I.3/II). The cAMP-dependent protein kinase A (PKA)/cAMP response element-binding protein (CREB) pathway is the primary signaling cascade through which this promoter is regulated (Hunzicker-Dunn and Maizels, 2006). CREB is the principal component in the regulation of the aromatase gene, and is stimulated by follicle-stimulating hormone

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(FSH)-activated cAMP-dependent PKA. FSH stimulation of aromatase is also mediated by the activation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and AKT/phosphoinositide 3-kinase (PI3K) signaling (Hunzicker-Dunn and Maizels, 2006; Stocco, 2008). In patients with breast cancer, the main promoter of aromatase is switched from I.4 to I.3/II in the breast and adjacent adipose tissue (Subbaramaiah et al., 2012). Therefore, inhibition of promoter II-induced aromatase expression might be an attractive strategy to inhibit estrogen production.

To date, about 300 natural compounds, mainly flavonoids, have been examined for their effects on aromatase activity in non-cellular, cellular, and *in vivo* studies (Balunas and Kinghorn, 2010), suggesting that natural products are valuable aromatase modulators candidates (Khan et al., 2011; Lu et al., 2012; Yang et al., 2013; Guo et al., 2014). Motherwort has a long history of medicinal use, which was originated in Central Europe (*Leonurus cardiaca*) and Asia (*Leonurus japonicus* and *Leonurus sibiricus*). *L. japonicus* has been used for thousands of years in China to treat various diseases in women, such as menoxenia, dysmenorrhea, amenorrhea, lochia, edema of the body, oliguresis, sores, and ulcerations (Shang et al., 2014). To date, approximately 140 chemical components have been isolated and identified from *L. japonicus* (Shang et al., 2014). Motherwort ethanol extract markedly suppressed the development of uterine adenomyosis and mammary cancers, whereas it may also promote pregnancy-dependent mammary tumors (Nagasawa et al., 1990, 1992; Tao et al., 2009). *L. japnonicus* also shows remarkable effects on women's health and diseases, indicating that it may be involved in the modulation of estrogen production; however, this is still unclear (de Boer and Cotingting, 2014). In the present study, we investigated the effects of natural products isolated from *L. japonicus* on the biosynthesis of estrogen in human ovarian granulosa cells, and further examined the mechanism whereby these potent compounds may regulate aromatase expression.

2. Materials and methods

2.1. Extraction and isolation of natural compounds

The aerial parts of *L. japonicus* were collected in Xichang County, Sichuan Province, P. R. China, in June 2012. A voucher specimen (KIB, 20120601) was identified by Prof. Xi-Wen Li, a senior botany taxonomist, and was deposited in Kunming Institute of Botany, Chinese Academy of Sciences (Li and Hedge, 1994; Liu et al., 2014; Zhang et al., 2018). A photo of this plant is shown in Fig. S1. The dried aerial parts of *L. japonicus* (15.0 kg) were extracted with 95% EtOH (40 L) three times at room temperature, and then partitioned with EtOAc or n-butyl alcohol. The EtOAc extract was chromatographed on a silica gel column eluting with $CHCl_3$–Me_2CO (1:0 → 0:1, v/v) to afford fractions A–F. The n-butyl alcohol extract was then fractionated by a silica gel column eluting with $CHCl_3$–MeOH (20:1 → 0:1, v/v) to afford fractions H–K. Fr. B was applied to RP-18 and silica gel columns to generate the following compounds: XLY5 (50.6 mg), XLY6 (10.2 mg), XLY7 (11.1 mg), XLY8 (5.0 mg), XLY9 (7.0 mg), XLY10 (15.1 mg), XLY11 (60.8 mg), XLY12 (105.0 mg), XLY13 (5.5 mg), XLY14 (30.2 mg), XLY15 (5.0 mg), XLY16 (5.1 mg), XLY17 (19.1 mg), XLY18 (8.9 mg), XLY19 (30.0 mg), XLY29 (3.1 g), XLY30 (10.0 mg), and XLY45 (6.5 mg). Fr. C was applied to RP-18 and silica gel columns to yield the following compounds: XLY21 (3.0 mg), XLY22 (3.9 mg), XLY23 (4.7 mg), XLY24 (19.9 mg), XLY31 (50.2 mg), and XLY32 (3.1 mg). Fr. D was applied to silica gel columns to purify the following compounds: XLY1 (5.7 mg), XLY2 (9.4 mg), XLY3 (3.5 mg), XLY4 (4.9 mg), XLY20 (7.1 mg), XLY25 (9.8 mg), XLY26 (60.9 mg), XLY27 (6.9 mg), XLY28 (17.9 mg), XLY33 (40.0 mg), and XLY34 (20.1 mg). Fr. E was applied to silica gel and RP-18 columns to yield the following compounds: XLY35 (9.7 mg), XLY36 (15.2 mg), and XLY37 (19.4 mg). Fr. F was further chromatographed on silica gel columns to produce the compounds XLY38 (6.1 mg), XLY39 (8.6 mg), XLY40 (2.4 mg), XLY41 (22.3 mg), XLY42 (30.7 mg), XLY43 (10.3 mg), XLY44 (8.9 mg), and XLY46 (7.7 mg). Their structures were elucidated by comparison of their nuclear magnetic resonance and mass spectrometry spectra with those reported in the literature (see Supplemental information).

2.2. Reagents

Testosterone, Letrozole, sildenafil, and forskolin (FSK) were purchased from Sigma-Aldrich (Shanghai, China). SB203580, PD98059, and LY294002 were purchased from Selleck (Shanghai, China). Aromatase (CYP19A1) antibody was purchased from Abcam (Cambridge, MA, USA). GAPDH and Myc antibodies were purchased from HuaBio (Cambridge, MA, USA). Phospho-CREB (Ser133), CREB (48H2), phospho-ERK1/2, ERK, phospho-p38, p38, phospho-JNK, JNK, phospho-AKT, AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The magnetic particle-based 17β-estradiol enzyme-linked immunosorbent assay (ELISA) kit was purchased from Bio-Ekon Biotechnology (Beijing, China). Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum were from Gibco-Invitrogen (Carlsbad, CA, USA).

2.3. Cell culture

Human ovarian granulosa-like KGN cells (kindly supplied by Prof. Yiming Mu, Chinese PLA General Hospital, Beijing, China) and the primary granulosa cells isolated from Sprague-Dawley rats (purchased from Dashuo Biological Company, Chengdu, China) were maintained in DMEM/F12 medium containing 5% (v/v) fetal bovine serum (Gibco-Invitrogen), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. Aromatase-overexpressing human embryonic kidney 293A (HEK293Aaro) cells were established and maintained as described before (Pu et al., 2016). Human Choriocarcinoma JEG3 cells and Mouse calvaria-derived MC3T3-E1 cells (purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were maintained in MEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37 °C, 5% CO_2 in incubator.

2.4. Cell-based estrogen biosynthesis assay

Estrogen biosynthesis assay was conducted as described (Lu et al., 2012). KGN cells were seeded in 24-well plates and cultured overnight. The next day, the medium was replaced with serum-free medium and the cells were pretreated for 24 h with the test chemicals. Testosterone (10 nM) was then added to each well and the cells were incubated for another 24 h. The culture supernatants were collected and stored at −20 °C. The 17β-estradiol in the supernatants was quantified by magnetic particle-based ELISA according to the manufacturer's instructions (Bio-Ekon Biotechnology, China). Optical densities were measured at 550 nm using a Varioskan Flash multimode reader (Thermo Fisher Scientific, Waltham, MA, USA). Results were normalized to the total cellular protein content and expressed as percentage of 17β-estradiol production in comparison with the control samples. The protein content was determined using a bicinchoninic acid (BCA) protein assay kit (BestBio, Shanghai, China).

2.5. Cell viability assay

KGN cells were seeded in 96-well plates at a density of 5×10^3 cells/well in 100 μl of media. Cultured cells were treated with tested compounds at 25 μM for 24 h. Then, 10 μl of Alamar Blue reagent was added to the media, and incubated for another 2–4 h until the development of a pink color. The relative fluorescence intensity was measured using a Varioskan Flash multimode reader (Thermo Fisher Scientific, USA).

2.6. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Total RNA (2 μg) was reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with oligo (dT) 18 primers. Equal amounts of cDNA were subjected to qRT-PCR with the fluorescent dye, SYBR Green I, using a Chromo4 detection system (Bio-Rad, Hercules, CA, USA). Reaction mixtures contained 12.5 μl of 2X TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China), 0.5 μl of each primer (0.2 μM), and 1 μl of template cDNA. Sterile distilled water was added to a final volume of 25 μl. The location of aromatase promoters and their primers is depicted in Fig. S2. The primer pairs were as follows: aromatase, 5′-ATCCTCAATACCAGGTCCTGGC-3' (sense) and 5′-AGAGATCCAGAC TCGCATGAATTCT-3' (antisense); GAPDH, 5′-CCACCCATGGCAAATTC CATGGCA-3' (sense) and 5′-GGTGGACCTGACCTGCCGTCTAGA-3' (antisense); aromatase promoter I.3, 5′-GTCTAAAGGAACCTGAGACTCT ACC-3' (sense) and 5′-ACGATGCTGGTGATGTTATAATGT-3' (antisense); aromatase promoter I.4, 5′-CACTGGTCAGCCCATCAA-3' (sense) and 5′-ACGATGCTGGTGATGTTATAATGT-3' (antisense); and aromatase promoter II, 5′-CCCTTTGATTTCCACAGGAC-3' (sense) and 5′-CCCATGCAGTAGCCAGGAC-3' (antisense). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 54 °C for 15 s, and 72 °C for 15 s. Standard curves were established for each primer set and both reference and target gene reactions were performed for each sample. The relative quantity (n-fold) of aromatase mRNA was calculated by the ΔΔCt method using the GAPDH amplified from the same sample as a reference.

2.7. Western blotting

Cells cultured in 6-well plates were lysed with RIPA lysis buffer (Beyotime, Haimen, China) supplemented with protease inhibitor cocktail (Sigma). Aliquots of total cell lysates (40 μg protein) were mixed in loading buffer, boiled for 5 min, and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. EasySee western markers (TransGen Biotech, Beijing, China) were included in each gel. Proteins were blotted onto nitrocellulose membranes. After blotting, the membranes were incubated overnight at 4 °C with primary antibodies. Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed using enhanced chemiluminescence detection (Amersham Bioscience, Piscataway, NJ, USA).

2.8. In vivo mice treatment

KM female mice (Kunming) purchased from Dashuo Biological Company (Chengdu, China), which were fed at 25 °C, free access to food and water. All the experiments and procedures were approved by the Institute of Laboratory Animals, Sichuan Academy of Medical Sciences (No. 2018–0219) in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No.8023, revised 1978). The mice were injected intraperitoneally (i.p.) with olive oil (control), 50 mg/kg XLY29 or 25 mg/kg Let once a day for two weeks. Estrous cycles were monitored by daily vaginal smears. After treatment, each mice weight was measured and whole blood was collected, then centrifuged at 4000 × *g* for 10 min to separate serum. Serum samples were stored at −20 °C until quantificated for 17β-estradiol concentration. Mice ovaries were collected, and fixed in fixative solution. Embedded tissues were sectioned into 5–7 μm slices by rotary microtome. For histological analysis, ovary slices were stained with hematoxylin-eosin (H&E) and examined under an inverted microscope (× 100 magnification). Immunohistochemistry (IHC) of ovary slices was performed using a primary antibody against aromatase.

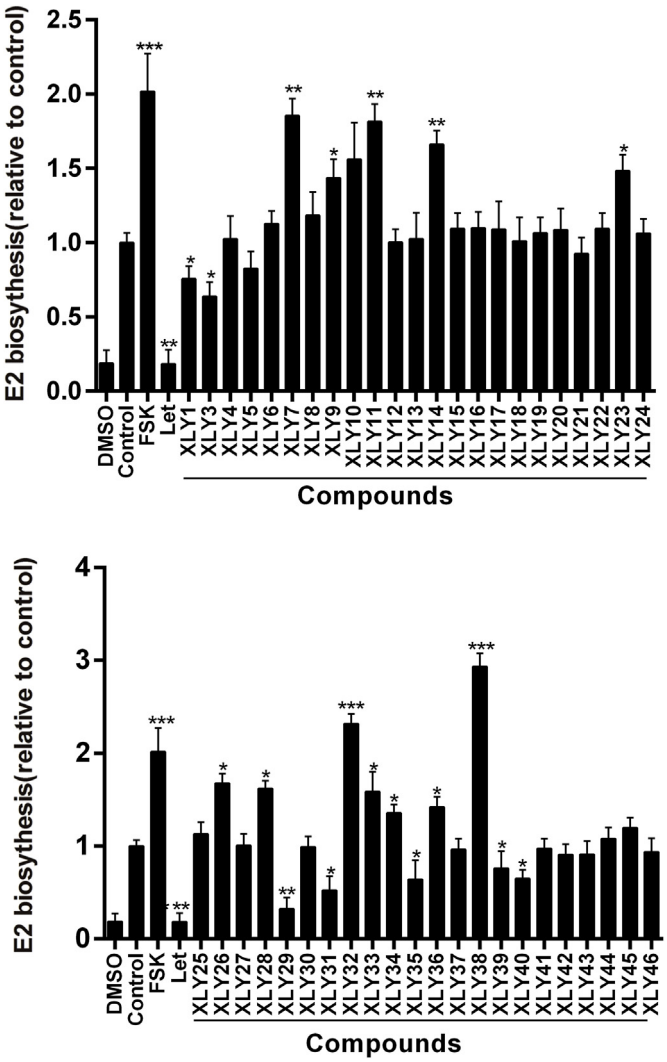

Fig. 1. Effect of natural products from *L. japonicus* on estrogen biosynthesis in KGN cells. KGN cells were seeded in 24-well plates, incubated overnight, and pretreated with the test compounds (25 μM) for 24 h. Subsequently, the cells were supplemented with 10 nM testosterone for another 24 h. The concentration of 17β-estradiol in the culture medium was quantified using a 17β-estradiol magnetic particle-based ELISA kit. FSK, 10 μM forskolin; Let, 10 nM Letrozole. (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ compared with control ($n = 3$).

2.9. Statistical analysis

Statistical analyses were performed with the GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA). The results are expressed as the mean ± standard deviation of individual values from three independent experiments. Data were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of L. japonicus extract on estrogen biosynthesis in human ovarian granulosa-like KGN cells

To examine the effects of the 46 natural products isolated from *L. japonicus* on estrogen biosynthesis, we treated human ovarian granulosa-like KGN cells separately with the natural compounds (25 μM) for

24 h. The structures of these natural products are illustrated in Fig. S1. As shown in Fig. 1, FSK, an adenylate cyclase agonist that activates the PKA/CREB pathway, significantly promoted 17β-estradiol production, whereas Let, an AI, significantly inhibited 17β-estradiol production. Among these tested compounds, 12 compounds, including leoheteronin D (XLY7), 3*α*-acetoxy-7*β*-hydroxy-9*α*,13; 15,16-diepoxy-15*β*-methoxylabdan-6-one (XLY9), (*R*)-(−)-3,7-dimethylocta-1,6-diene-3,8-diol (XLY11), tricyclohumuladiol (XLY14), 1*H*-indole-3-aldehyde (XLY23), 4-(2-formylpyrrol-1-yl)butyric acid (XLY26), methyl-5-hydroxy-2-pyridinecarboxylate (XLY28), 5, 7-dihydroxychromone (XLY32), 2-hydroxybenzoic acid (XLY33), 4-hydroxy-3-methoxycinnamic acid (XLY34), trigonelline (XLY36), and 2-furancarboxylic acid (XLY38) showed stimulatory effects on 17β-estradiol biosynthesis. In addition, 7 compounds, including (−)-zuonin-A (XLY1), scopoletin (XLY3), 7-methoxyl-luteolin (XLY29), kaempferol (XLY31), lactate (XLY35), 5-hydroxymethyl-furan-2-carboxylic acid (XLY39), and uracil (XLY40) showed inhibitory effects on 17β-estradiol production. Although phthalic acid butyl isobutyl ester (XLY2) showed complete inhibition on 17β-estradiol biosynthesis, it was found to exhibit very strong cytotoxic effect on KGN cells (data not shown).

3.2. Effect of natural compounds on KGN cell viability

To investigate the effect of the tested compounds on cell viability, we treated KGN cells with the isolated compounds at 25 μM for 24 h. As shown in Figs. 2 and 5 compounds, including (8*R*,8′*S*)-4,4′,8-trihydroxyl-3,3′-dimethoxyl-9′-lignanolide (XLY16), methyl-4-(2-formylpyrrol-1-yl)butanoate (XLY25), XLY28, XLY33, and *N*-ethylformamide (XLY45) promoted KGN cell viability, whereas 3 compounds, including 6(*R*)-hydroxy-2,6-dimethyl-2(*E*)-7-octadienoic acid (XLY12), (2S,3S)-2α-(4″-hydroxy-3″-methoxybenzyl)-3β-(40-hydroxy-3′-methoxybenzyl)-γ-butyrolactone (XLY15), and 4-methoxy-4-oxobutanoic acid (XLY44) showed inhibitory effects on KGN cell viability. Among the 7 compounds with inhibitory effects on estrogen biosynthesis, none of them decreased KGN cell viability. Among the 12 compounds with promotive effects on estrogen biosynthesis, only two compounds (XLY28 and XLY33) increased KGN cell viability. These results indicated that the effects of the 17 active compounds except XLY28 and XLY33 on 17β-estradiol production were not caused by their cytotoxicity in KGN cells.

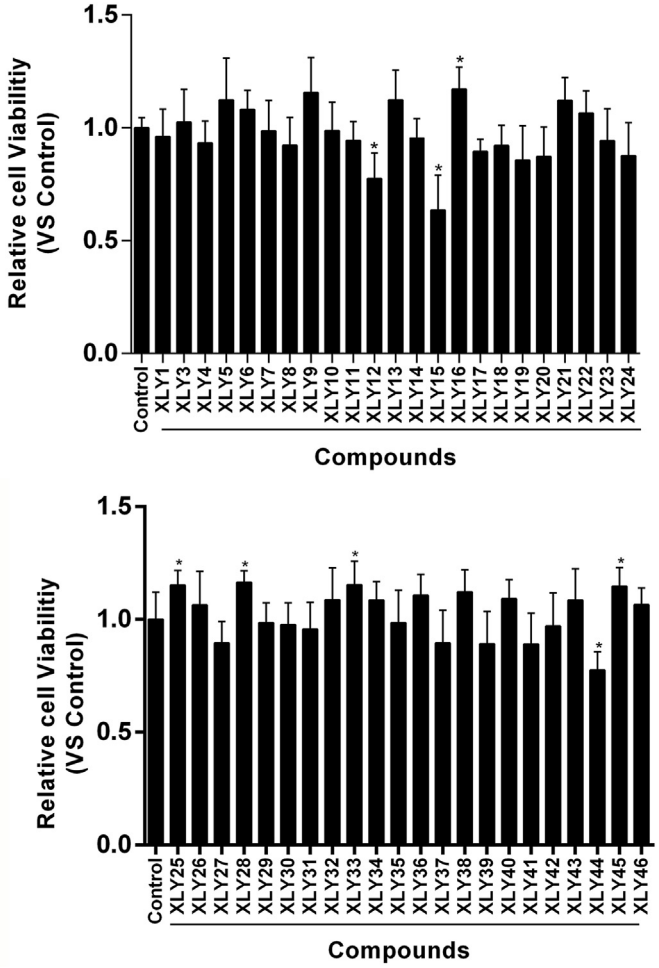

Fig. 2. Effects of the isolated natural products on KGN cell viability. KGN cells seeded in 96-well plates were pretreated with the test compounds (25 μM) for 24 h. Cells were then incubated with Alamar Blue reagent (10 μl/well) for an additional 4 h, and the fluorescence intensities were measured. (*) $P < 0.05$ compared with control ($n = 3$).

3.3. Effect of XLY29 on estrogen biosynthesis

Considering that XLY29 potently inhibited estrogen biosynthesis and was isolated in large quantities, we selected XLY29 for further mechanism studies. The chemical structure of XLY29 is illustrated in Fig. 3A. KGN cells were treated with XLY29 at various concentrations for 24 h. As shown in Fig. 3B, XLY29 at 10 μM inhibited about 70% of the 17β-estradiol production in KGN cells as compared with non-treated cells. XLY29 also significantly inhibited forskolin-stimulated 17β-estradiol production. XLY29 inhibited 17β-estradiol production in a concentration-dependent manner, with an IC_{50} value of 5.213 ± 0.257 μM (Fig. 3C). We further investigated the tissue-specificity of XLY29 on 17β-estradiol production. As shown in Fig. S3. XLY29 also inhibited significantly 17β-estradiol production in primary rat granulosa cells and human placental choriocarcinoma JEG3 cells, but had no effect on estrogen biosynthesis in mouse osteoblastic MC3T3-E1 cells. XLY29 did not show cytotoxicity in KGN cells at the concentrations examined (Fig. S4). Further studies showed that XLY29 suppressed 17β-estradiol biosynthesis in a time-dependent manner (Fig. 3D). The inhibition of 17β-estradiol production in KGN cells by XLY29 at 5 μM was first evident at 12 h and sustained up to 48 h, when 17β-estradiol production was inhibited by approximately 50% as compared with control. Furthermore, 17β-estradiol biosynthesis resumed after XLY29 was removed for 12 h, suggesting the inhibition of estrogen biosynthesis by XLY29 is reversible (Fig. S5). These results indicated that XLY29 potently inhibited the biosynthesis of estrogen in KGN cells in a concentration- and time-dependent manner.

3.4. XLY29 suppresses aromatase expression in KGN cells

KGN cells lack endogenous 17α-hydroxylase and cannot synthesize androgens or estrogens de novo (Nishi et al., 2001). Therefore, the effect of XLY29 on 17β-estradiol biosynthesis in the presence of testosterone might be caused by aromatase, the only enzyme that is able to convert testosterone to 17β-estradiol. To examine whether the inhibitory activity of XLY29 on 17β-estradiol production was caused by the regulation of aromatase expression, we investigated aromatase mRNA and protein expression in KGN cells treated with XLY29 at the indicated concentrations. As shown in Fig. 4A, FSK significantly increased aromatase mRNA expression in KGN cells, consistent with previous results (Gonzalez-Robayna et al., 1999). XLY29 remarkably decreased aromatase mRNA expression in a concentration-dependent manner. At a concentration of 10 μM, XLY29 decreased aromatase mRNA levels by 80% as compared with DMSO-treated control cells. Consistent with its inhibitory effect on aromatase transcription, XLY29 decreased aromatase protein expression in a concentration-dependent manner (Fig. 4B). XLY29 also decreased the protein expression of aromatase in a time-dependent manner, consistent with its inhibitory effect on estrogen biosynthesis (Fig. 4C). These results indicated that

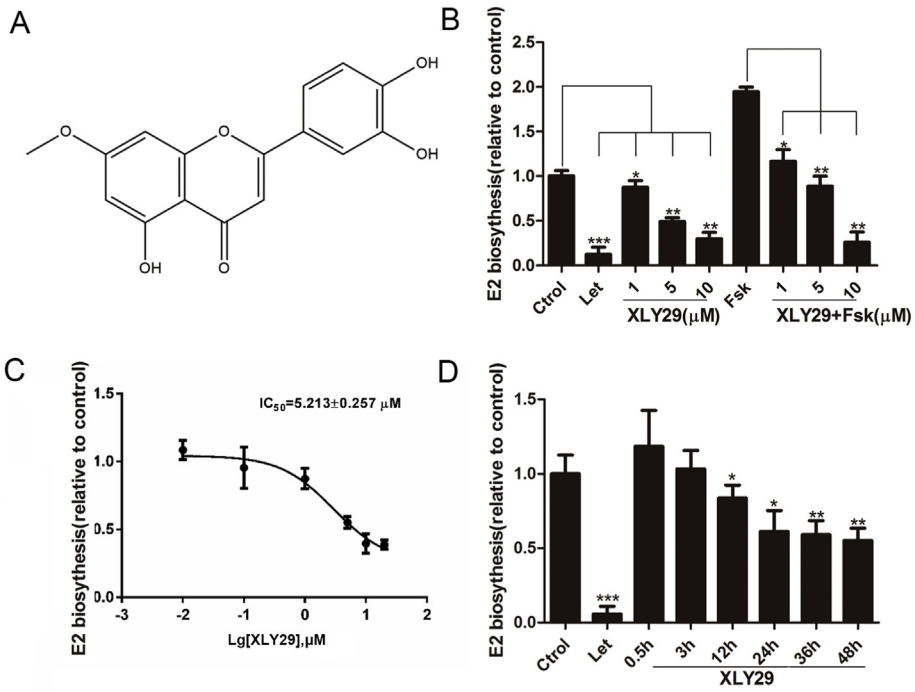

Fig. 3. Effects of XLY29 on estrogen biosynthesis. (A) The chemical structure of XLY29. (B) KGN cells seeded in 24-well plates were pretreated with XLY29 at the indicated concentrations for 24 h. Testosterone (10 nM) was added for a further 24 h of incubation. (C) Concentration-response curve of XLY29 for inhibition of estrogen biosynthesis in KGN cells. (D) The time course for the inhibition of estrogen biosynthesis by XLY29 (5 μM) in KGN cells. Ctrol, DMSO control; Let, 10 nM letrozole; FSK, 10 μM forskolin. (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ compared with control or FSK-treated cells (n = 3).

XLY29 suppresses 17β-estradiol production by inhibiting aromatase expression in KGN cells.

3.5. Effect of XLY29 on aromatase catalytic activity

To examine whether XLY29 inhibits estrogen biosynthesis through direct effect on the catalytic activity of aromatase protein, we treated aromatase-overexpressing HEK293A cells with XLY29 for 24 h. As shown in Fig. 5A, Let significantly inhibited 17β-estradiol biosynthesis, but XLY29 had no effect on 17β-estradiol production in aromatase-overexpressing HEK293A cells. Moreover, XLY29 have no obvious effect on aromatase protein expression in aromatase-overexpressing HEK293A cells (Fig. 5B). These results suggested that XLY29 does not directly inhibit the catalytic activity of aromatase protein.

3.6. XLY29 inhibits promoter I.3/II-driven aromatase transactivity

The regulation of aromatase transcript is highly tissue- and promoter-specific in humans, and the expression of aromatase transcript is mainly controlled by type I.3/II promoter in the ovary. To investigate whether XLY29 suppresses aromatase expression by inhibiting specific promoters, we treated KGN cells with different concentrations of XLY29 for 24 h, and then examined the transcription activity of different promoters. As shown in Fig. 6A, XLY29 had no effect on promoter 1.4. However, XLY29 at 10 μM decreased promoter I.3 transcript by 20% (Fig. 6B). Interestingly, XLY29 significantly suppressed promoter II transcript in a concentration-dependent manner (Fig. 6C). XLY29 at 10 μM decreased promoter II transcript by more than 50%. Thus, these results suggested that XLY29 suppresses aromatase expression by decreasing promoter I.3/II transactivity, with promoter II playing a more prominent role. CREB is the key transcription factor controlling transcription of aromatase in the ovary by binding to cAMP-response element contained in the promoter II of aromatase. To investigate whether XLY29 inhibits CREB to decrease the transcription of aromatase, KGN cells were treated with XLY29 at various concentrations for 24 h. As shown in Fig. 6D, XLY29 significantly reduced the phosphorylation of CREB in a concentration-dependent manner, but had no effect on CREB protein expression. These results suggested that XLY29 might suppress aromatase expression by inhibiting CREB activation.

3.7. Effect of XLY29 on protein kinase signaling

Since the activity of CREB is regulated by phosphorylation, we then investigated the effects of XLY29 on the activation state of the related

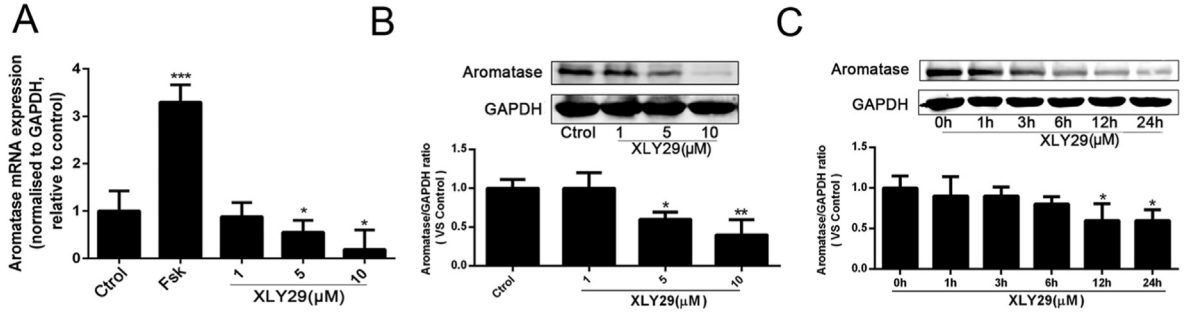

Fig. 4. XLY29 inhibits aromatase expression in KGN cells. KGN cells were treated with the indicated concentrations of the test compounds for 24 h. (A) Aromatase mRNA was measured in total cellular RNA by using qRT-PCR. The results are expressed as fold increase relative to levels in untreated cells. GAPDH was used as an internal control. (B) Cell lysates were immunoblotted with anti-aromatase or anti-GAPDH antibodies. The quantitative results are depicted. (C) The time course for the inhibition of aromatase protein expression by XLY29 (5 μM) in KGN cells. The quantitative results are depicted. Ctrol, DMSO control; FSK, 10 μM forskolin. (*) $P < 0.05$ and (***) $P < 0.001$ compared with control (n = 3).

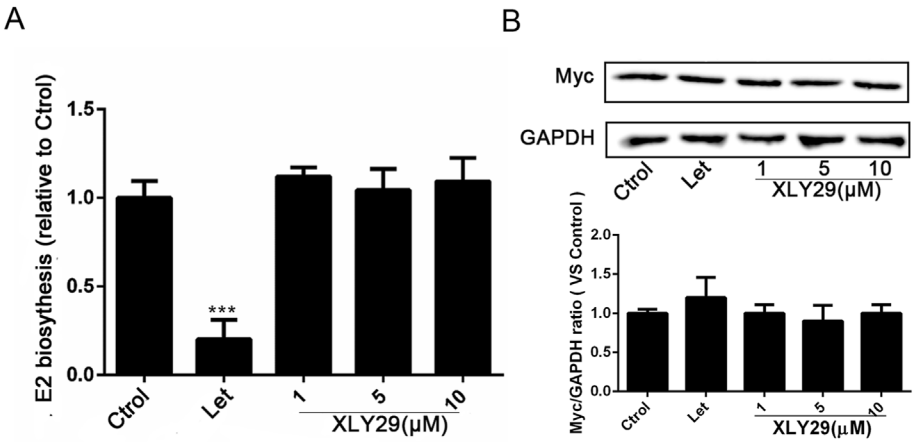

Fig. 5. Effects of XLY29 on aromatase catalytic activity. HEK293A cells overexpressing myc-aromatase were incubated with various concentrations of XLY29 for 24 h, and then treated with testosterone (10 nM) for another 24 h. The level of 17β-estradiol in the culture supernatants was quantified by ELISA (A). Cell lysates were immunoblotted with anti-Myc or anti-GAPDH antibodies. The quantitative results are depicted (B). Ctrol, DMSO control; Let, 10 nM letrozole. (***) $P < 0.001$ compared with control ($n = 3$).

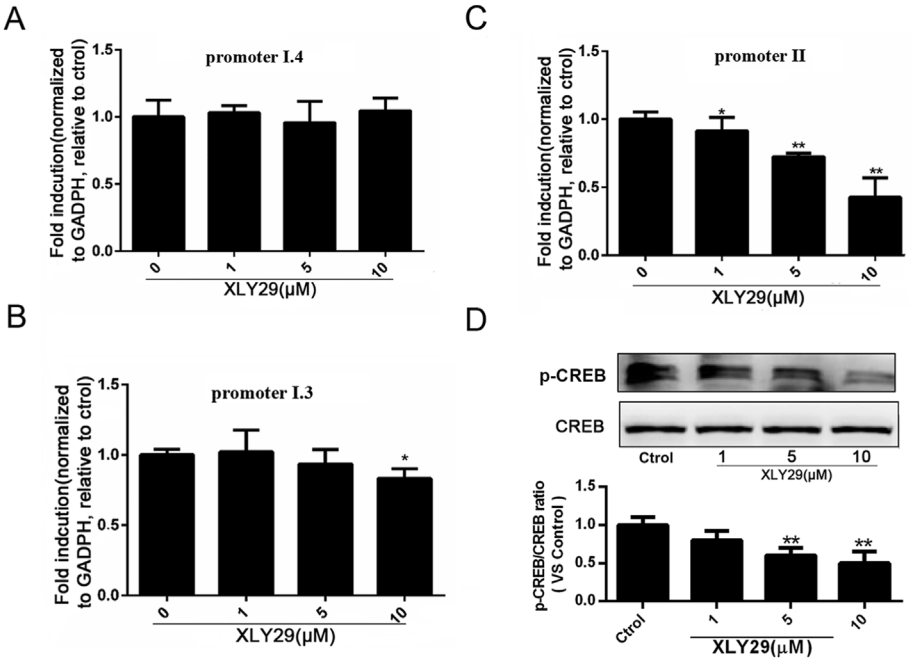

Fig. 6. Effects of XLY29 on aromatase promoter transactivity. KGN cells were treated with the indicated concentrations of the test compounds for 24 h. Aromatase promoter I.4 mRNA (A), promoter I.3 mRNA (B), or promoter II mRNA (C) level was measured in total cellular RNA using qRT-PCR. The results are expressed as fold increase relative to levels in untreated cells. GAPDH was used as an internal control. (D) KGN cells seeded in 6-well plates were treated with the indicated concentrations of XLY29 for 24 h. Cell lysates were immunoblotted with anti-phospho-CREB or anti-CREB antibody. The quantitative results are depicted. (*) $P < 0.05$ and (**) $P < 0.01$ compared with control ($n = 3$).

signaling protein kinases. As shown in Fig. 7A and Fig. S6A, XLY29 inhibited phosphorylation of AKT and p38 in a concentration-dependent manner, but had no effect on phosphorylation of ERK and c-Jun N-terminal kinase (JNK). Furthermore, XLY29 inhibited phosphorylation of AKT and p38 in a time-dependent manner (Fig. 7B and Fig. S6B). To confirm the effect of these signaling kinases on aromatase expression, we treated KGN cells with inhibitors against AKT/PI3K (LY294002), p38 MAPK (SB203580), and ERK (PD98059). As shown in Fig. 7C and Fig. S6C, LY294002 and SB203580 inhibited aromatase expression, whereas PD98059 had no effect on aromatase expression in KGN cells. These results implicated that XLY29 may suppress aromatase expression by inhibiting the AKT and p38 signaling pathways.

3.8. Effect of XLY29 in vivo

We first examined whether XLY29 could be metabolized *in vivo*. After rats were injected intraperitoneally with XLY29, the plasma concentration of XLY29 reached the peak level at 1 h, and then gradually decreased (Fig. S7), indicating that XLY29 could be metabolized *in vivo*. To further examine whether XLY29 can decrease 17β-estradiol level in serum, KM female mice were treated with olive oil, 25 mg/kg letrozole or 50 mg/kg XLY29. Since 17β-estradiol is associated with estrous cycle, we also monitored the estrous cycle. Compared with control, letrozole-treated mice displayed prolonged time in metestrus/diestrus phase, whereas XLY29-treated mice exhibited prolonged time in proestrus phase, indicating that both letrozole and XLY29 disturbed estrous cycle (Fig. 8A–C). Similar with letrozole, XLY29 also significantly decreased the serum 17β-estradiol level compared with control (Fig. 8D), and had no effect on body weight (Fig. S8). Compared with control mice, there were more cystic follicles in the ovaries of both letrozole-treated mice and XLY29-treated mice (Fig. 8 E-G). XLY29 decreased the expression of aromatase in ovary, while letrozole did not (Fig. G–H and Fig. S9). These results indicated that XLY29 can decrease the serum 17β-estradiol level and disturb estrous cycle *in vivo*.

4. Discussion

L. japonicus extracts are widely used in China for the treatment of reproduction system-related disorders implicated in the dysregulation of estrogen biosynthesis; however, the chemical constituents and their mechanism of action in the regulation of estrogen biosynthesis are totally unknown. Among the 46 tested compounds isolated from *L. japonicus*, 10 compounds showed promotive effects on 17β-estradiol biosynthesis, and 7 compounds showed inhibitory effects. Among the 10 compounds showing promotive effects on 17β-estradiol biosynthesis, XLY11, XLY14, XLY23, XLY26, XLY32 and XLY38 were first

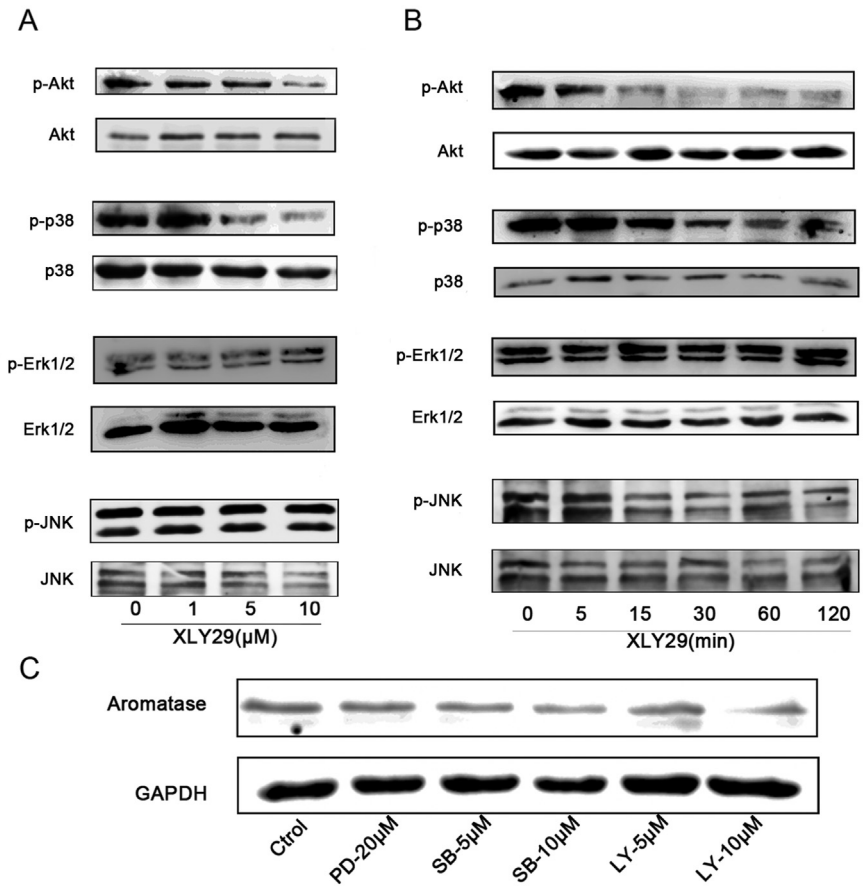

Fig. 7. Effects of XLY29 on protein kinase signaling. (A) KGN cells were treated with the indicated concentrations of XLY29 for 24 h. (B) KGN cells were treated with XLY29 (5 μM) at the indicated time point Cell lysates were immunoblotted with anti-phospho-AKT, anti-AKT, anti-phospho-p38, anti-p38, anti-phospho-ERK, anti-ERK, anti-phospho-JNK, or anti-JNK antibody. (C) KGN cells seeded in 6-well plates were treated with the indicated concentrations of different compounds for 24 h. Cell lysates were immunoblotted with anti-aromatase or anti-GAPDH antibody. Ctrol, DMSO control; PD, PD98059; SB, SB203580; LY, LY294002.

isolated from *L. japnonicus*, whereas, XLY1, XLY3, XLY29, XLY31, XLY35, XLY39, and XLY40 showing inhibitory effects on 17β-estradiol biosynthesis were first isolated from this plant, suggesting these compounds may be potential bioactive components responsible for the pharmacological effects of this plant. *L. japonicus,* containing a minimum of 0.5% hydrochloric stachydrine and 0.05% hydrochloric leonurine, is listed and recognized by the Chinese pharmacopoeia. Here, we found that stachydrine (XLY37) had no effect on estrogen biosynthesis, whereas some other compounds exhibited promotive or inhibitory effects on estrogen biosynthesis, suggesting that other bioactive compounds contained in the formulation of *L. japonicus* should also be considered for the purpose of quality control. It will be valuable to investigate whether other widely used species of motherwort including *L. cardiaca* and *L. sibiricus* contain the compounds to modulate estrogen biosynthesis to exert their functions in women as *L. japonicus* does.

Although many flavonoids have been found to inhibit estrogen biosynthesis through inhibition of aromatase activity, some other studies have reported that certain flavonoids, such as luteolin, hesperetin, and icariin regulate estrogen production by modulating aromatase expression (Li et al., 2011; Lu et al., 2012; Yang et al., 2013). We found that XLY29 exerts its inhibitory effect on KGN cells until 12–36 h, indicating that it modulates aromatase expression at a transcriptional level. This was further supported by the finding that XLY29 inhibited aromatase mRNA and protein expression. However, unlike other flavonoids, XLY29 had no effect on the catalytic activity of aromatase protein. Although luteolin was found to decrease the transcription of aromatase, it also destabilized the aromatase protein (Lu et al., 2012). We found that XLY29 had no effect on aromatase protein stability, suggesting that its mechanism may be different from that of luteolin. In both ovarian granulosa and breast cancer cells, aromatase transcription is primarily controlled by promoter II (Simpson, 2000). XLY29 potently decreased promoter II-driven aromatase expression, highlighting its potential role in the development of tissue-selective AIs for the treatment of breast cancer.

FSH is the primary physiological regulator of aromatase expression in the human ovary. By stimulating cAMP generation, FSH activates the MAPK or PI3K signaling pathway, which phosphorylates and activates CREB to bind to cAMP-responsive element within the aromatase promoter II, and initiates transcription of aromatase (Hunzicker-Dunn and Maizels, 2006). In the present study, we found that XLY29 reduced CREB phosphorylation in a concentration-dependent manner, without any effect on CREB expression, indicating that XLY29 inactivates CREB to suppress the transcription of aromatase in ovarian granulosa cells. We found that XLY29 suppressed the phosphorylation of AKT and p38 in a concentration and time-dependent manner, but had no effect on the phosphorylation of JNK and ERK. These results are consistent with previous findings that inhibition of AKT and p38 can decrease the biosynthesis of estrogen in KGN cells (Guo et al., 2014). XLY29 was previously found to inhibit cAMP phosphodiestrases (PDEs) *in vitro* (Nikaido et al., 1987). However, cAMP PDEs should not be the *bona fide* targets of XLY29 in KGN cells because inhibition of cAMP PDEs by XLY29 would activate the PKA-CREB axis to promote aromatase expression, not to inhibit aromatase expression as observed in this study. Luteolin, an analogue of XLY29, was also found to inhibit aromatase expression in both KGN and MCF-7 breast cancer cells (Li et al., 2011; Lu et al., 2012). Although luteolin has also been reported to inhibit PDEs *in vitro*, we previously found that luteolin may activate cAMP PDEs to decrease cAMP levels in hepatoma cells to sensitize the antiproliferative effect of interferon α/β (Yu et al., 2010; Tai et al., 2014). Therefore, it will be of interest to investigate whether XLY29 activates cAMP-hydrolyzing PDEs to decrease intracellular cAMP levels and

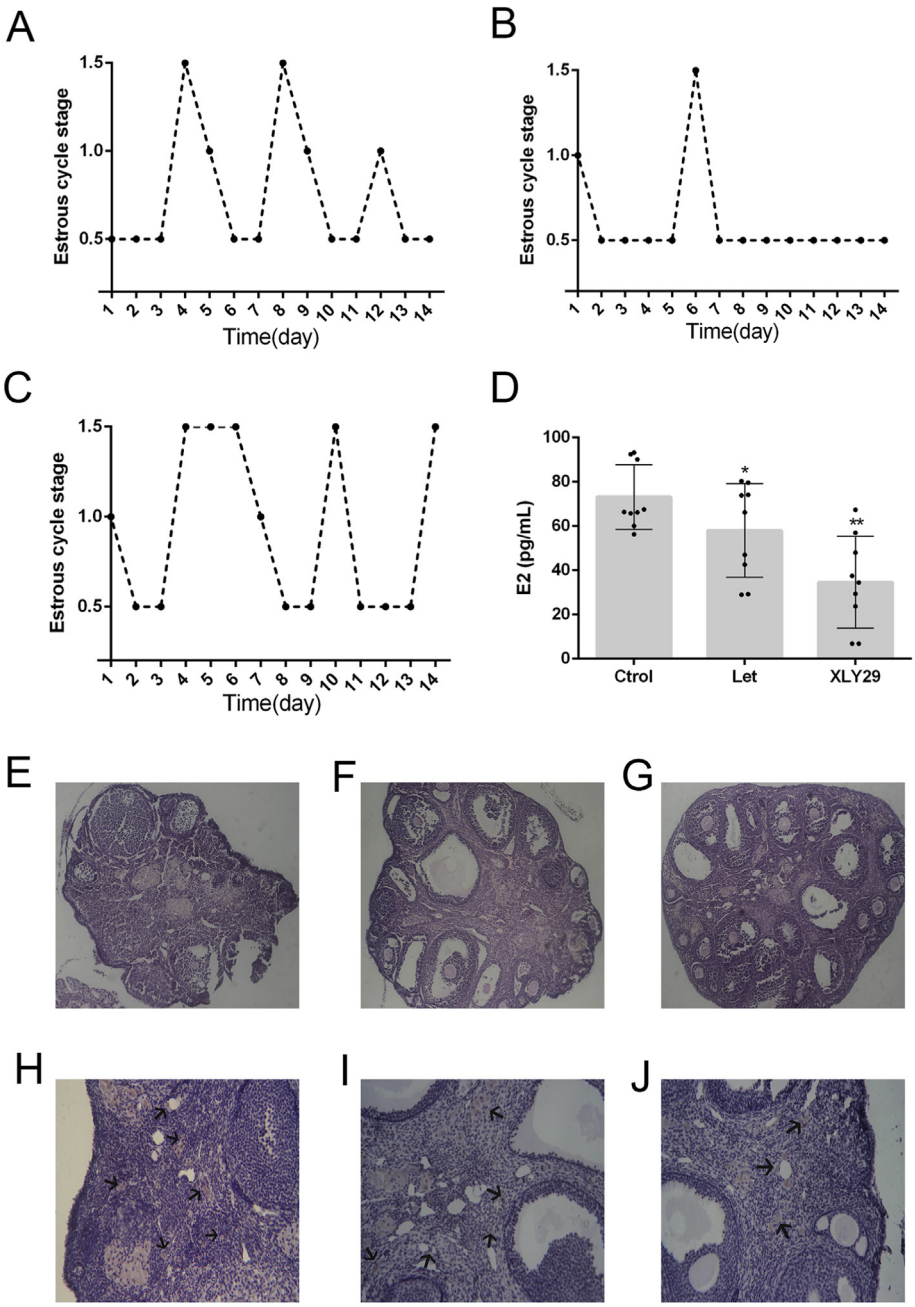

Fig. 8. Effect of XLY29 on normal mice. Estrous cycles of control mice (A), letrozole-treated mice (B) and XLY29-treated mice (C) were monitored by daily vaginal smears. (D) After treatments, whole blood was collected from the orbit. Serum samples were quantified by ELISA. H&E staining of representative control mice ovaries (E), letrozole-treated mice ovaries (F) and XLY29-treated mice ovaries (G). IHC staining of aromatase (arrows) of representative control mice ovaries (H), letrozole-treated mice ovaries (I) and XLY29-treated mice ovaries (J). P, proestrus; E, estrus; M/D, metestrus and diestrus; Ctrol, olive oil control; Let, 25 mg/kg letrozole; XLY29, 50 mg/kg XLY29. (**) P < 0.01compared with control (n = 9).

inhibit PKA-CREB axis for the decreasing of aromatase expression. In addition to CRE sites, the aromatase promoter I.3/II also contains the binding sites for the activator protein 1 (AP-1) family of transcription factors (c-Jun/c-fos/ATF), which can be regulated by p38 MAPK and AKT (Chen et al., 2007, 2009). Thus, XLY29 may decrease aromatase expression by inhibition of p38 MAPK and AKT, which suppresses promoter II activation mediated by the CREB or AP-1 transcription factors.

Several natural products modulate the MAPK signaling pathway to regulate aromatase expression. The soy isoflavone, genistein, promotes aromatase promoter I.3/II activity in hepatoma HepG2 cells by increasing the phosphorylation of ERK and p38 MAPK (Ye et al., 2009). The dietary flavone, luteolin, inhibits aromatase transcription by inhibition of JNK in breast cancer cells (Li et al., 2011). MAPK may regulate aromatase promoters through different mechanisms in a cell-specific manner, allowing the possibility of tissue-selective aromatase modulators. Many natural products including flavonoids have also been found to inhibit p38 MAPK or AKT to exert their anti-inflammatory or anti-cancer effects (García-Lafuente et al., 2009; Sun et al., 2013). Hence, it is of interest to investigate whether these compounds are able to inhibit aromatase expression in the ovary. AIs are the first-line of clinical treatment for breast cancer; however, acquired AI resistance after prolonged endocrine therapy is a hindrance for endocrine-dependent breast cancer treatment (Ma et al., 2015). Abnomal epidermal growth factor receptor signaling and activation of downstream MAPK in breast cancer is associated with patients developing resistance to treatment with either tamoxifen or AIs (Johnston et al., 2005). In the present study, we found that XLY29 inhibits promoter II-driven aromatase expression by suppressing the p38 MAPK or AKT signaling pathway, making XLY29 a novel candidate for overcoming AI resistance in breast cancer.

5. Conclusion

In the present study, we identified 46 natural products with diversified structures isolated from *L. japonicus*. Among them, 10 compounds showed promotive effects on 17β-estradiol biosynthesis and 7 compounds showed inhibitory effects, suggesting that estrogen biosynthesis-promoting or -inhibiting compounds contained in the formulation of *L. japonicus* should also be considered for the purpose of quality control. XLY29 was found to significantly decrease promoter II-driven aromatase expression in human ovarian granulosa-like KGN cells via suppression of the p38 MAPK and PI3K/AKT. Our results not only provide insights into understanding the clinical benefits and side effects of *L. japonicus*, but also provide new clues that some medicinal plants or flavonoids may exert their beneficial health effects by modulating aromatase expression and estrogen biosynthesis. XLY29 warrants further investigation as a new pharmaceutical tool for the prevention and treatment of estrogen-dependent diseases.

CRediT authorship contribution statement

Bao-Wen Du: Formal analysis, Writing - original draft. **Xing-Jie Zhang:** Formal analysis, Writing - original draft. **Nan Shi:** Formal analysis, Writing - original draft. **Ting Peng:** Formal analysis, Writing - original draft. **Jun-Bo Gao:** Formal analysis, Writing - original draft. **Bahtigul Azimova:** Formal analysis, Writing - original draft. **Ruihan Zhang:** Formal analysis, Writing - original draft. **De-Bing Pu:** Formal analysis, Writing - original draft. **Chun Wang:** Formal analysis, Writing - original draft. **Anvar Abduvaliev:** Formal analysis, Writing - original draft. **Alisher Rakhmanov:** Formal analysis, Writing - original draft. **Guo-Lin Zhang:** Formal analysis, Writing - original draft. **Wei-Lie Xiao:** Formal analysis, Writing - original draft. **Fei Wang:** Formal analysis, Writing - original draft.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2020.173154.

References

Balunas, M.J., Kinghorn, A.D., 2010. Natural compounds with aromatase inhibitory activity: an update. Planta Med. 76, 1087–1093.
Bulun, S.E., Sebastian, S., Takayama, K., Suzuki, T., Sasano, H., Shozu, M., 2003. The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. J. Steroid Biochem. Mol. Biol. 86, 219–224.
Chen, D., Reierstad, S., Lin, Z., Lu, M., Brooks, C., Li, N., Innes, J., Bulun, S.E., 2007. Prostaglandin E_2 induces breast cancer related aromatase promoters via activation of p38 and c-Jun NH_2-terminal kinase in adipose fibroblasts. Canc. Res. 67, 8914–8922.
Chen, D., Reierstad, S., Lu, M., Lin, Z., Ishikawa, H., Bulun, S.E., 2009. Regulation of breast cancer-associated aromatase promoters. Canc. Lett. 273, 15–27.
de Boer, H.J., Cotingting, C., 2014. Medicinal plants for women's healthcare in southeast Asia: a meta-analysis of their traditional use, chemical constituents, and pharmacology. J. Ethnopharmacol. 151, 747–767.
García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M.A., Martínez, J.A., 2009. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflamm. Res. 58, 537–552.
Gonzalez-Robayna, I.J., Alliston, T.N., Buse, P., Firestone, G.L., Richards, J.S., 1999. Functional and subcellular changes in the A-kinase-signaling pathway: relation to aromatase and Sgk expression during the transition of granulosa cells to luteal cells. Mol. Endocrinol. 13, 1318–1337.
Guo, J.J., Yuan, Y., Lu, D.F., Du, B.W., Xiong, L., Shi, J.G., Yang, L.J., Liu, W.L., Yuan, X.H., Zhang, G.L., Wang, F., 2014. Two natural products, trans-phytol and (22E)-ergosta-6,9,22-triene-3β,5α,8α-triol, inhibit the biosynthesis of estrogen in human ovarian granulosa cells by aromatase (CYP19). Toxicol. Appl. Pharmacol. 279, 23–32.
Hewitt, S.C., Harrell, J.C., Korach, K.S., 2005. Lessons in estrogen biology from knockout and transgenic animals. Annu. Rev. Physiol. 67, 285–308.
Hunzicker-Dunn, M., Maizels, E.T., 2006. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. Cell. Signal. 18, 1351–1359.
Johnston, S.R., Dowsett, M., 2003. Aromatase inhibitors for breast cancer: lessons from the laboratory. Nat. Rev. Canc. 3, 821–831.
Johnston, S.R., Martin, L.A., Head, J., Smith, I., Dowsett, M., 2005. Aromatase inhibitors: combinations with fulvestrant or signal transduction inhibitors as a strategy to overcome endocrine resistance. J. Steroid Biochem. Mol. Biol. 95, 173–181.
Khan, S.I., Zhao, J., Khan, I.A., Walker, L.A., Dasmahapatra, A.K., 2011. Potential utility of natural products as regulators of breast cancer-associated aromatase promoters. Reprod. Biol. Endocrinol. 9, 91.
Li, F., Ye, L., Lin, S.M., Leung, L.K., 2011. Dietary flavones and flavonones display differential effects on aromatase (CYP19) transcription in the breast cancer cells MCF-7. Mol. Cell. Endocrinol. 344, 51–58.
Li, X.W., Hedge, I., 1994. Lamiaceae. In: In: Wu, Z., Raven, P. (Eds.), Flora of China, vol. 17. Science Press, Beijing, pp. 163.
Liu, Z.K., Wu, D.R., Shi, Y.M., Zeng, T., Liu, S.H., Du, X., et al., 2014. Three new diterpenoids from *Leonurus japonicus*. Chin. Chem. Lett. 25, 677–679.
Lu, D.F., Yang, L.J., Wang, F., Zhang, G.L., 2012. Inhibitory effect of luteolin on estrogen biosynthesis in human ovarian granulosa cells by suppression of aromatase (CYP19). J. Agric. Food Chem. 60, 8411–8418.
Ma, C.X., Reinert, T., Chmielewska, I., Ellis, M.J., 2015. Mechanisms of aromatase inhibitor resistance. Nat. Rev. Canc. 15, 261–275.
Nagasawa, H., Inatomi, H., Suzuki, M., Mori, T., 1992. Further study on the effects of motherwort (*Leonurus sibiricus* L) on preneoplastic and neoplastic mammary gland growth in multiparous GR/A mice. Anticancer Res. 12, 141–143.
Nagasawa, H., Onoyama, T., Suzuki, M., Hibino, A., Segawa, T., Inatomi, H., 1990. Effects of motherwort (*Leonurus sibiricus* L) on preneoplastic and neoplastic mammary gland growth in multiparous GR/A mice. Anticancer Res. 10, 1019–1023.
Nikaido, T., Ohmoto, T., Sankawa, U., 1987. Inhibitors of adenosine 3',5'-cyclic monophosphate phosphodiesterase in *Daphne genkwa* SIEB. et ZUCC. *Chem. Pharm. Bull.* 35, 675–681.
Nishi, Y., Yanase, T., Mu, Y., Oba, K., Ichino, I., Saito, M., et al., 2001. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology 142, 437–445.
Palomba, S., 2015. Aromatase inhibitors for ovulation induction. J. Clin. Endocrinol. Metabol. 100, 1742–1747.
Pu, W.C., Yuan, Y., Lu, D.F., Wang, X., Liu, H.W., Wang, C., et al., 2016. 2-Phenylbenzo[b]furans: synthesis and promoting activity on estrogen biosynthesis. Bioorg. Med. Chem. Lett 26, 5497–5500.
Shang, X., Pan, H., Wang, X., He, H., Li, M., 2014. *Leonurus japonicus* Houtt.: ethnopharmacology, phytochemistry and pharmacology of an important traditional Chinese medicine. J. Ethnopharmacol. 152, 14–32.
Simpson, E.R., 2000. Role of aromatase in sex steroid action. J. Mol. Endocrinol. 25, 149–156.
Smith, I.E., Dowsett, M., 2003. Aromatase inhibitors in breast cancer. N. Engl. J. Med. 348, 2431–2442.
Stocco, C., 2008. Aromatase expression in the ovary: hormonal and molecular regulation. Steroids 73, 473–487.
Subbaramaiah, K., Morris, P.G., Zhou, X.K., Morrow, M., Du, B., Giri, D., et al., 2012. Increased levels of COX-2 and prostaglandin E_2 contribute to elevated aromatase expression in inflamed breast tissue of obese women. Canc. Discov. 2, 356–365.
Sun, H., Wang, Z., Yakisich, J.S., 2013. Natural products targeting autophagy via the PI3K/Akt/mTOR pathway as anticancer agents. Anticancer Agents Med. Chem. 13, 1048–1056.
Tai, Z.F., Lin, Y., He, Y.J., Huang, J.M., Guo, J.J., Yang, L.J., et al., 2014. Luteolin sensitizes the antiproliferative effect of interferon α/β by activation of Janus kinase/signal transducer and activator of transcription pathway signaling through protein kinase A-mediated inhibition of protein tyrosine phosphatase SHP-2 in cancer cells. Cell. Signal. 26, 619–628.
Tao, J., Zhang, P., Liu, G., Yan, H., Bu, X., Ma, Z., et al., 2009. Cytotoxicity of Chinese motherwort (YiMuCao) aqueous ethanol extract is non-apoptotic and estrogen

receptor independent on human breast cancer cells. J. Ethnopharmacol. 122, 234–239.
Yang, L.J., Lu, D.F., Guo, J.J., Meng, X.L., Zhang, G.L., Wang, F., 2013. Icariin from *Epimedium brevicornum* Maxim promotes the biosynthesis of estrogen by aromatase (CYP19). J. Ethnopharmacol. 145, 715–721.
Ye, L., Chan, M.Y., Leung, L.K., 2009. The soy isoflavone genistein induces estrogen synthesis in an extragonadal pathway. Mol. Cell. Endocrinol. 302, 73–80.
Yu, M.C., Chen, J.H., Lai, C.Y., Han, C.Y., Ko, W.C., 2010. Luteolin, a non-selective competitive inhibitor of phosphodiesterases 1-5, displaced [3H]-rolipram from high-affinity rolipram binding sites and reversed xylazine/ketamine-induced anesthesia. Eur. J. Pharmacol. 627, 269–275.
Zhang, R.H., Liu, Z.K., Yang, D.S., Zhang, X.J., Sun, H.D., Xiao, W.L., 2018. Phytochemistry and pharmacology of the genus *Leonurus*: the herb to benefit the mothers and more. Phytochemistry 147, 167–183.