

Differentially expressed genes on the growth of mouse Leydig cells treated with standardised *Eurycoma longifolia* extract

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Abstract. *Eurycoma (E.) longifolia* Jack (Tongkat Ali) is a widely applied medicine that has been reported to boost serum testosterone and increase muscle mass. However, its actual biological targets and effects on an *in vitro* level remain poorly understood. Therefore, the present study aimed to investigate the effects of a standardised *E. longifolia* extract (F2) on the growth and its associated gene expression profile in mouse Leydig cells. F2, even at lower doses, was found to induce a high level of testosterone by ELISA. The level was as high as the levels induced by eurycomanone and formestane in Leydig cells. However, Leydig cells treated with F2 demonstrated reduced viability, which was likely due to the diminished cell population at the G₀/G₁ phase and increased cell population arrested at the S phase in the cell cycle, as assessed by MTT assay and flow cytometry, respectively. Cell viability was revived when the treatment time-point was prolonged to 96 h. Genome-wide gene analysis by reverse transcription-quantitative PCR of F2-treated Leydig cells at 72 h, when the cell growth was not revived, and 96 h, when the cell growth had started to revive, revealed cyclin-dependent kinase-like 2 (CDKL2) to be a potential target in regulating the viability of F2-treated Leydig cells. Functional analysis, as analysed using

GeneMANIA Cytoscape program v.3.6.0 (<https://genemania.org/>), further suggested that CDKL2 could act in concert with Casitas B-lineage lymphoma and sphingosine kinase 1 interactor-A-kinase anchoring protein domain-containing genes to regulate the viability of F2-treated Leydig cells. The findings of the present study provide new insights regarding the potential molecular targets associated with the biological effect of *E. longifolia* extract on cell growth, particularly on the cell cycle, which could aid in enhancing the bioefficacy and reducing the toxicity of this natural product in the future.

Introduction

Plant-based herbal medicines have been extensively applied traditionally for centuries. Recently, attention towards these types of medicine has increased worldwide owing to their nutraceutical value. *Eurycoma (E.) longifolia* Jack is one of the most popular traditional plants found in the tropical rainforests of Southeast Asia, especially in Malaysia and Indonesia. This herb belongs to the Simaroubaceae family that can grow slender up to 15 m high, able to grow in different types of soil (1-4). The plant is known locally as 'Tongkat Ali', 'Pasak Bumi', 'Cay ba binh' and 'Ian-don' in Malaysia, Indonesia, Vietnam and Thailand, respectively (5-7). *E. longifolia* is a promising natural source of biologically active compounds, including quassinoids, mainly those in the 13 β family, 18-dihydroeurycomanol, eurycomanol-2-O- β -D-glucopyranoside, eurycomanol and eurycomanone (6,8,9). Some of the constituents of *E. longifolia* have been previously found to exert antiamebic (10), *in vitro* anticancer or anti-proliferative effects against cancer cells (11) and plasmodial activities (12). A previous study revealed that semi-purified eurycomanone conferred cytotoxic activity towards MCF-7 breast cancer cells, demonstrating the potential anticancer property of this plant (9,13). In addition, *E. longifolia* root extract has also demonstrated significant cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines (14) in other previous studies.

A number of studies have previously demonstrated the efficacy of *E. longifolia* as an effective booster of testosterone and aphrodisiac supplement to improve strength and power during

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Abbreviations: EN, eurycomanone; FM, *formestane*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; PI, propidium iodide; WT, whole transcript; ss, single-stranded; DEGs, differentially expressed genes; CDKL2, cyclin-dependent kinase-like 2; CBL, Casitas B-lineage lymphoma; Sphkap, SPHK1 interactor-AKAP domain-containing; DHEA, dehydroepiandrosterone

Key words: *Eurycoma longifolia*, testosterone, cell viability, cell cycle, gene expression profile, cyclin

sexual activity (15,16). Additionally, *E. longifolia* has been found to either prevent and/or alleviate erectile dysfunction in men (17). The roots and leaves of this plant have long been used to treat a number of diseases (6,7). In addition to fever, intestinal parasites, mouth ulcers and headache (6,18), the roots of *E. longifolia* have been applied as a traditional 'anti-aging' remedy to help older individuals adapt to the reduced energy, mood and libido associated with aging (3,19). The roots of this plant have also been documented to enhance blood flow that function as an herbal supplement for women following childbirth (6,20). By contrast, the leaves of the plant have been used to prevent gum diseases, treat ulcers and sexually-transmitted infections such as syphilis and gonorrhoea (3).

Leydig cells are the primary sites of steroidogenesis in the testis, which serve as the main source of testosterone in this organ (21). Testosterone production in Leydig cells is stimulated by luteinizing hormone, which is secreted by the anterior pituitary gland through the hypothalamic pituitary-gonadal axis (22). Testosterone is the primary male sex hormone, which is crucial for the differentiation of the male urogenital system, spermatogenesis and accumulation of bone and muscle mass in men (23).

The spermatogenic effect of *E. longifolia* is attributed to the presence of quassinoids in the extracts (8,24). Quassinoids are biologically active compounds that contribute to *E. longifolia* as a testosterone production booster, such that extracts enriched with *E. longifolia* quassinoids have been found to exert more potent effects. The effects of *E. longifolia* extracts on steroidogenesis have been previously investigated in animal models (3,25-28). However, despite preliminary findings demonstrating the effect of quassinoid-rich *E. longifolia* on rat interstitial cells (8), the *in vitro* effects of *E. longifolia* extracts in Leydig cells, for example, remain poorly understood. Therefore, the present study aimed to investigate the impact of a standardized *E. longifolia* root extract (F2) on testosterone production, growth and cell cycle progression in mouse Leydig cells. It is hoped that data from the present study will lead to an improved understanding on the mechanism of action mediated by the *E. longifolia* extract so it can be further utilized as a natural product for treating human diseases.

Materials and methods

E. longifolia standardised extract and chemical preparation. A standardised root methanolic extract of *E. longifolia*, F2, and the pure compound, eurycomanone (EN; >96% purity), were received from Professor Chan Kit Lam at the School of Pharmaceutical Science, Universiti Sains Malaysia (Penang, Malaysia). Both the extract and the pure compound were dissolved in double-sterile water (ddH₂O). The aromatase inhibitor, formestane (FM; >98% purity), which was purchased from MedChem Express, was dissolved in DMSO.

TM3 cell culture. TM3 (CRL-1714TM) Leydig cells, purchased from the American Type Culture Collection, were cultured in a 1:1 mixture of Gibco Ham's F12 medium (Invitrogen; Thermo Fisher Scientific, Inc.) and Dulbecco's modified eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 5% (v/v) horse serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 2.5% (v/v) foetal bovine

serum (Invitrogen; Thermo Fisher Scientific, Inc.). Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added to the growth medium to prevent contamination. The culture was incubated in a humidified atmosphere and supplied with 5% CO₂ at 37°C. The Leydig cells were sub-cultured every 2-3 days depending on the confluence of the cell culture. The use of a mouse Leydig cell line may be a limitation of the present study. Human Leydig cells would have been a better cell line for proper verification of the hypothesis.

Measurement of testosterone levels in F2-, EN- and FM-treated Leydig cells using ELISA. TM3 cells were seeded onto 24-well plates at a density of 2.0x10⁴ cells/well. After being cultured at 37°C in an incubator overnight, the cells were treated with 0.1, 1.0 and 10 mg/ml F2 or 0.1, 1.0 and 10 µM EN or FM. Each treatment was carried out in triplicate for four treatment time-points: 24, 48, 72 and 96 h in ≥2 independent experiments, following which the culture supernatant was collected at each treatment time-point. The collected culture supernatants were used for the measurement of testosterone level using a mouse testosterone (T) enzyme-linked immunosorbent assay (ELISA) kit (cat. no. CSB-E05101m; Cusabio Biotech Co., Ltd.), according to the manufacturer's instructions. Briefly, the wells of the microtitre plates were pre-coated with a goat anti-rabbit antibody specific for testosterone. The reactions were then incubated with a secondary horseradish peroxidase-conjugated antibody from the aforementioned kit for the target hormone signal development. A substrate solution was also added to enhance the color development to indicate the presence of the target hormone. The intensity of the signal for each plate was measured at 450 nm by using a MultiskanTM spectrum spectrophotometric plate reader (Thermo Fisher Scientific, Inc.). A standard curve was plotted to estimate the level of testosterone (% relative to control) in each well.

Viability analysis of F2-, EN- and FM-treated Leydig cells using MTT assay. Firstly, 2.5x10³ TM3 cells per well were seeded onto 96-well plates. The cells were cultured overnight in growth medium at 37°C and treated with 0.1, 1.0, and 10 mg/ml F2 and 0.1, 1.0 and 10 µM EN or FM in triplicate for four treatment time-points: 24, 48, 72 and 96 h for ≥2 independent experiments at 37°C. Cell viability was measured by using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At each time-point, 24 µl MTT reagent (2.5 mg/ml) was added to each well before the reactions were incubated for 4 h at room temperature. Subsequently, 100 µl DMSO was added to each well for color development. The color or optical density (OD) was then read at 570 nm by using a MultiskanTM spectrum spectrophotometric plate reader (Thermo Fisher Scientific, Inc.). The cell viability in each well was calculated using the following formula: [(OD of treated sample-OD of blank)/(OD of control-OD of blank)] x100%. The level of cell viability was indicated as % relative to control.

Cell cycle progression analysis of F2-treated Leydig cells using flow cytometry. A density of 6.0x10⁴ TM3 cells/well was seeded onto 6-well plates and incubated at 37°C with 5% CO₂ overnight. On the next day, the cells were treated with F2 at concentrations of 0.1, 1.0 and 10 mg/ml in triplicate at 37°C for

four treatment time-points: 24, 48, 72 and 96 h in ≥ 2 independent experiments. The culture supernatants and the cells were then harvested from each well and transferred to new 15 ml Falcon tubes at each time-point. The tubes were centrifuged at $1,000 \times g$ for 5 min. The supernatant was discarded, and the cell pellet was washed with PBS and centrifuged again at $1,000 \times g$ for 5 min. After discarding the supernatant, $500 \mu\text{l}$ PBS was added to the cell pellets. All these procedures were carried out at room temperature. The number of cells in the cell suspension was then counted and adjusted to 1×10^6 cells, which was suspended in $500 \mu\text{l}$ using 70% cold absolute ethanol. The suspension was mixed gently and stored at 4°C to facilitate ethanol fixation. Before cell cycle analysis, the ethanol-fixed cells were centrifuged at $1,000 \times g$ for 10 min and washed three times by resuspending the cells in PBS at room temperature. The suspension was then centrifuged again at $1,000 \times g$ at room temperature for 10 min and the supernatant was discarded. The cells were stained with $500 \mu\text{l}$ FxCycle™ propidium iodide (PI)/RNase staining solution (Thermo Fisher Scientific, Inc.) at room temperature in the dark for 30 min. The stained samples were then transferred to new sterile flow tubes and maintained on ice until the samples were analysed by flow cytometry using a BD FACSCanto™ II flow cytometer (BD Biosciences). The % of the cell population at each cell cycle phase was then calculated.

mRNA gene expression profile analysis of F2-treated Leydig cells. TM3 cells were seeded onto 24-well plates at a density of 2.0×10^4 cells/well and incubated at 37°C with 5% CO_2 overnight. The next day, cells were treated with $500 \mu\text{l}$ 0.1 mg/ml F2 in triplicate for 72 and 96 h. A total of three wells of treatment solution and treated cells were then harvested and pooled as one sample for RNA extraction. The total RNA extraction was performed by using the RNeasy Mini Kit (QIAGEN Inc.), according to the manufacturer's protocol. The concentration and purity of the extracted total RNA were determined by using a NanoPhotometer® (Implen GmbH), whereas the integrity of the RNA was assessed by 1% (w/v) agarose gel electrophoresis (Bio-Rad Laboratories Inc.). The integrity of the total RNA was confirmed further using a 2100 Bioanalyzer (Agilent Technologies Inc.) coupled with the RNA 6000 Pico LabChip® Kit (cat. no. 5067-1513; Agilent Technologies Inc.). Good quality RNA (showing 2 ribosomal peaks and RNA Integrity Number ≥ 8) was used for steroidogenesis gene expression profiling using Affymetrix GeneChip™ microarray technology (Thermo Fisher Scientific, Inc.). The copy RNA (cRNA) targets for the Affymetrix platform were synthesized, purified, labelled and hybridised, according to the manufacturer's instructions. Briefly, ~ 100 ng extracted total RNA was converted to cDNA and was then amplified to cRNA following the procedures of Affymetrix GeneChip™ Whole Transcript (WT) PLUS Reagent Kit (cat. no. 902280; Thermo Fisher Scientific, Inc.). The cRNA was then purified and quantified before being synthesised into single-stranded (ss)-cDNA using the same kit, according to the manufacturer's protocols. The ss-cDNA was then fragmented and labelled with biotin before being hybridised onto the array strip for GeneChip™ WT Expression Array (Affymetrix; Thermo Fisher Scientific, Inc.). The gene expression profiles were scanned using the Affymetrix GeneArray scanner 3000 7G system (Affymetrix;

Thermo Fisher Scientific, Inc.) at 488 nm and $3\text{-}\mu\text{m}$ resolution. The images of the patterns were analysed using the Affymetrix Microarray Suite software v.5.0 (MAS5). The raw data .txt file obtained from the GeneArray scanner was then loaded onto the GeneSpring Gx software v.12.0 (Agilent Technologies Inc.) for further data processing, including normalization and statistical analysis. The expression ratios for $>22,000$ genes in non-treated and F2-treated Leydig cells at the respective time-points were computed and filtered by comparative analysis of the MAS5 values. The \log_2 values were used to identify differentially expressed genes (DEGs) in F2-treated compared with non-treated Leydig cells. Genes with a fold-change ≥ 2 were identified as statistically significant DEGs.

Identified gene expression validation in F2-treated Leydig cells using reverse transcription-quantitative (RT-q)PCR. Leydig cells were seeded onto 24-well plates at a density of 2.0×10^4 cells/well and incubated at 37°C with 5% CO_2 overnight. The next day, the cells were treated with $500 \mu\text{l}$ either 0.1 or 1.0 mg/ml F2 in triplicate for 72 and 96 h. A total of three wells of treatment solution and treated cells were then harvested and pooled as one sample for RNA extraction using the RNeasy Mini Kit (QIAGEN Inc.), according to manufacturer's protocols. The concentration of the extracted total RNA was determined by using a NanoPhotometer and the integrity of extracted RNA was assessed by using 1% (w/v) agarose gel electrophoresis as aforementioned. Good quality total RNA was reverse-transcribed to cDNA at 60°C using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) and the cDNA was stored at -20°C until it was used to analyse the gene expression by qPCR. All primers used were designed using the Primer Express™ software v.3.0.1 (Thermo Fisher Scientific, Inc.) and are listed in Table I. qPCR was performed by using the Applied Biosystems Power SYBR® Green PCR Master Mix and the 7500 Fast Sequence Detection System (both Thermo Fisher Scientific, Inc.). Each reaction mixture contained $12.5 \mu\text{l}$ of 2X SYBR® Green PCR Master Mix, 20 pmol each of forward and reverse primer and $5 \mu\text{l}$ cDNA (25 ng). A sufficient volume of deionised water was added to the mixture to bring the final reaction volume to $25 \mu\text{l}$. The following thermocycler program was used for the amplification: 50°C for 3 min and 95°C for 5 min for initiation, followed by 35-40 cycles of 95°C for 10 sec and 60°C for 30 sec. Finally, a melting curve for each amplicon was generated with 1°C temperature increments from $72\text{-}95^\circ\text{C}$. All reactions were performed in triplicate and included three non-template reactions as negative controls. The experiment was repeated ≥ 2 times, whereby the mRNA expression levels of the target genes were normalised to that of β -actin in the test sample. The mRNA expression level (relative to control) of the target gene was then calculated using the $2^{-\Delta\Delta C_q}$ method (29).

Functional enrichment analysis. To further study the interaction of identified DEGs at a functional level, Gene Ontology (GO) functional enrichment analysis was performed using GeneMANIA Cytoscape program v.3.6.0 (<https://genemania.org/>). GeneMANIA discovered functionally similar genes using a wealth of genomics and proteomics data from a query gene. The algorithm of the modal was then weighted each

Table I. List of primers used for quantitative PCR analysis.

Gene name	Primer sequence
<i>CDKL3</i>	Forward: 5'-TGTCCCTCCTGTCTGGTGCATT-3' Reverse: 5'-CTACCCGAGAAGACCCTTTTCAG-3'
<i>Cyclin G1</i>	Forward: 5'-AAGGTACAGGCCAAGCATCTTG-3' Reverse: 5'-CCTTTCCTCTTCAGTCGCTTTC-3'
<i>CDKN1A</i>	Forward: 5'-GACCACTGGACCTAGCAATTCAC-3' Reverse: 5'-GTAGGAGTCACCGTCTGTTTACC-3'
<i>Cyclin L1</i>	Forward: 5'-AATAGGCGAAGTTCGATCTGGAA-3' Reverse: 5'-TATGATGTCTTCGAGGGCTTTC-3'
<i>CDKL2</i>	Forward: 5'-CAAATGCCAACTGTCTCCATGA-3' Reverse: 5'-GTTGACATTGCTGCAATCTCTGA-3'
<i>HMOX1</i>	Forward: 5'-GGTGTCCAGAGAAGGCTTTAAGC-3' Reverse: 5'-TGCGCTCTATCTCCTCTTCCA-3'
<i>Cyclin G2</i>	Forward: 5'-GGAGTGTGTTTGTATCCCCAACAA-3' Reverse: 5'-GAACAGCTAAGGATGACCTCGAA-3'
<i>Cyclin B2</i>	Forward: 5'-GTGAAGTCTGGAAGTCATGCA-3' Reverse: 5'-CGATGAACTTGGTACGGTTGTC-3'
<i>CDKN2C</i>	Forward: 5'-AACCATCCCAGTCCTTCTGTCA-3' Reverse: 5'-CCCCTTTCCTTTGCTCCTAATC-3'
β -actin	Forward: 5'-GCTCTGGCTCCTAGCACCAT-3' Reverse: 5'-GCTGATCCACATCTGCTGGAA-3'

CDKL3, cyclin-dependent kinase-like-3; CDKN1A, cyclin-dependent kinase inhibitor 1A; CDKL2, cyclin-dependent kinase-like 2; HMOX1, heme oxygenase 1; CDKN2C, cyclin-dependent kinase inhibitor 2C.

functional genomic dataset, according to its predictive value for the query, whereby a fold change discovery ≤ 0.05 was set as the cut-off criterion for the enrichment analysis (30). A diagram of network weight for CDKL2 was plotted by $-\log_{10}$ (DWeight).

Statistical analysis. All data were presented as the means \pm standard deviation from experimental triplicates, and all statistical analyses were performed using GraphPad Prism 7.05 (GraphPad Software, Inc.). The significant levels of cell viability, hormone and target gene expression were analysed by using one-way ANOVA with the Tukey's post hoc test. $P < 0.005$ was considered to indicate a statistically significant difference.

Results

Comparison of testosterone production in F2-, EN- and FM-treated Leydig cells. Testosterone levels in culture supernatants of F2- and EN-treated TM3 cells, as measured by ELISA, were first inhibited 24 h after treatment. Following that, the levels were induced with increasing treatment time-points compared with those of control (Fig. 1A and B), indicating the ability of F2 to stimulate testosterone production by these cells. The experiments further validated the highest activity of F2 on hormone production by TM3 cells 96 h after treatment. FM, a known aromatase inhibitor, also demonstrated a similar effect and induced high levels of testosterone production in TM3 cells but only ≥ 72 h after treatment with the highest

concentration of FM (10 μ M) tested (Fig. 1C). Therefore, these results suggest that F2 and EN may exert similar effects as aromatase inhibitors in regulating testosterone synthesis and production. In addition, it should also be noted that high levels of testosterone production ($\sim 250\%$ increase) were also observed in F2-treated TM3 cells even at lower concentrations (0.1 and 1.0 mg/ml; Fig. 1A). Therefore, F2 may be more potent at stimulating testosterone production compared with EN and FM in Leydig cells.

Reduced cell viability of F2-, EN- and FM-treated Leydig cells compared with control cells. According to the results from MTT assay (Figs. 2-4), a significant reduction in the viability of F2-, EN- and FM-treated TM3 cells was observed compared with that of their corresponding controls in a dose-dependent manner. Treatment of Leydig cells with F2 demonstrated a significant reduction in cell viability at all concentrations and treatment time-points tested (Fig. 2). The viability of TM3 cells treated with lower concentrations of F2, including 0.1, 0.5 and 1.0 mg/ml, were able to revive 96 h following treatment, although the viability of the treated cells remained low at this treatment time-point (Fig. 2D). Notably, a less potent inhibitory effect on cell viability was observed for EN, whereby cell treated with EN were viable throughout all experimental time-points tested (Fig. 3). A gradual increment of cell viability inhibition on TM3 was only observed when higher concentrations of EN, including 1, 5 and 10 μ M were used (Fig. 3). In the FM-treated cells, a stronger growth inhibitory effect was observed at earlier

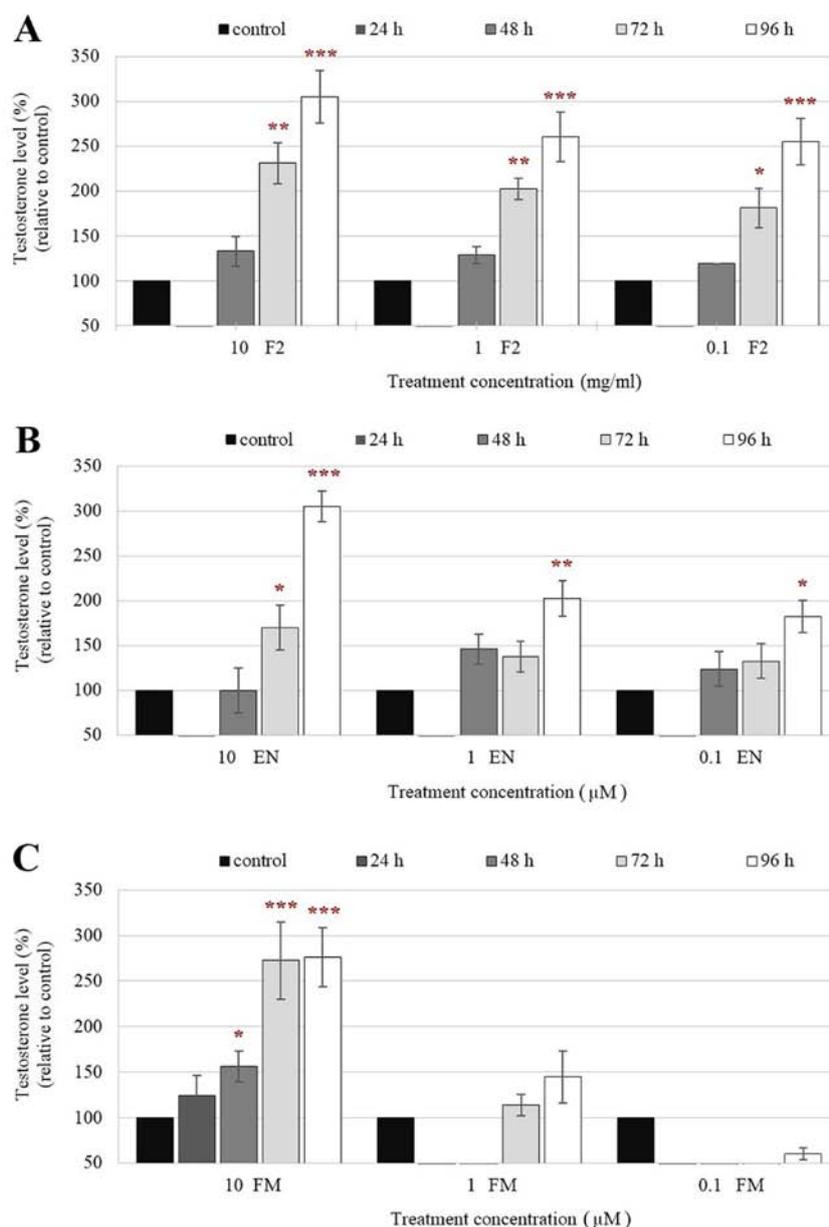


Figure 1. Level of testosterone in the culture supernatants of Leydig cells treated with different concentrations of F2, FM and EN. Testosterone production by TM3 cells was measured following treatment with (A) F2, (B) EN and (C) FM. Data are expressed as means \pm SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 indicate a significant induction of testosterone level at specific time-points vs. the level at 0 h (control). EN, eurycomanone; FM, formestane; F2, *Eurycoma longifolia* extract.

treatment time-points compared with that observed in EN treated cells (Fig. 4). Notably, the FM-treated cells remained viable at 96 h, although a dose-dependent inhibition of cell viability was also observed at higher concentrations (5 and 10 μ M) of FM treatment (Fig. 4D). The revival of the treated cells could be attributed to the reduction in the drug effect with prolonged treatment duration. Therefore, F2, EN and FM are suitable for long term treatments of Leydig cells at low concentrations.

S-phase cell cycle arrest in F2-treated Leydig cells. Cell cycle analysis of TM3 cells by flow cytometry (Fig. 5A) demonstrated that the percentage of cells at the G₀/G₁ phase was significantly reduced after treatment with 0.1, 1.0 and 10 mg/ml compared with that in the untreated cells (control)

24 h after treatment (Fig. 5B). The reduced percentage of F2-treated TM3 cells at the G₀/G₁ phase was accompanied by an accumulation in the percentage of cells at the S phase (Fig. 5B). This phenomenon suggests that the growth of F2-treated cells was affected by cell cycle arrest in the S phase. This observation persisted 48 h following treatment, particularly in the cells treated with higher concentrations of F2 (1.0 and 10 mg/ml; Fig. 5C). However, the percentage of cells in the G₀/G₁ phase appeared to be revived 48 h after treatment with 0.1 mg/ml F2 (Fig. 5C). The opposite trend was observed in the percentage of cells in the S phase (Fig. 5C). Cells treated with higher concentrations of F2 were only revived 72 h following treatment (Fig. 5D); the number of cells in G₀/G₁ cell cycle phase 72 h following treatment was higher compared with the number of cells in the same phase

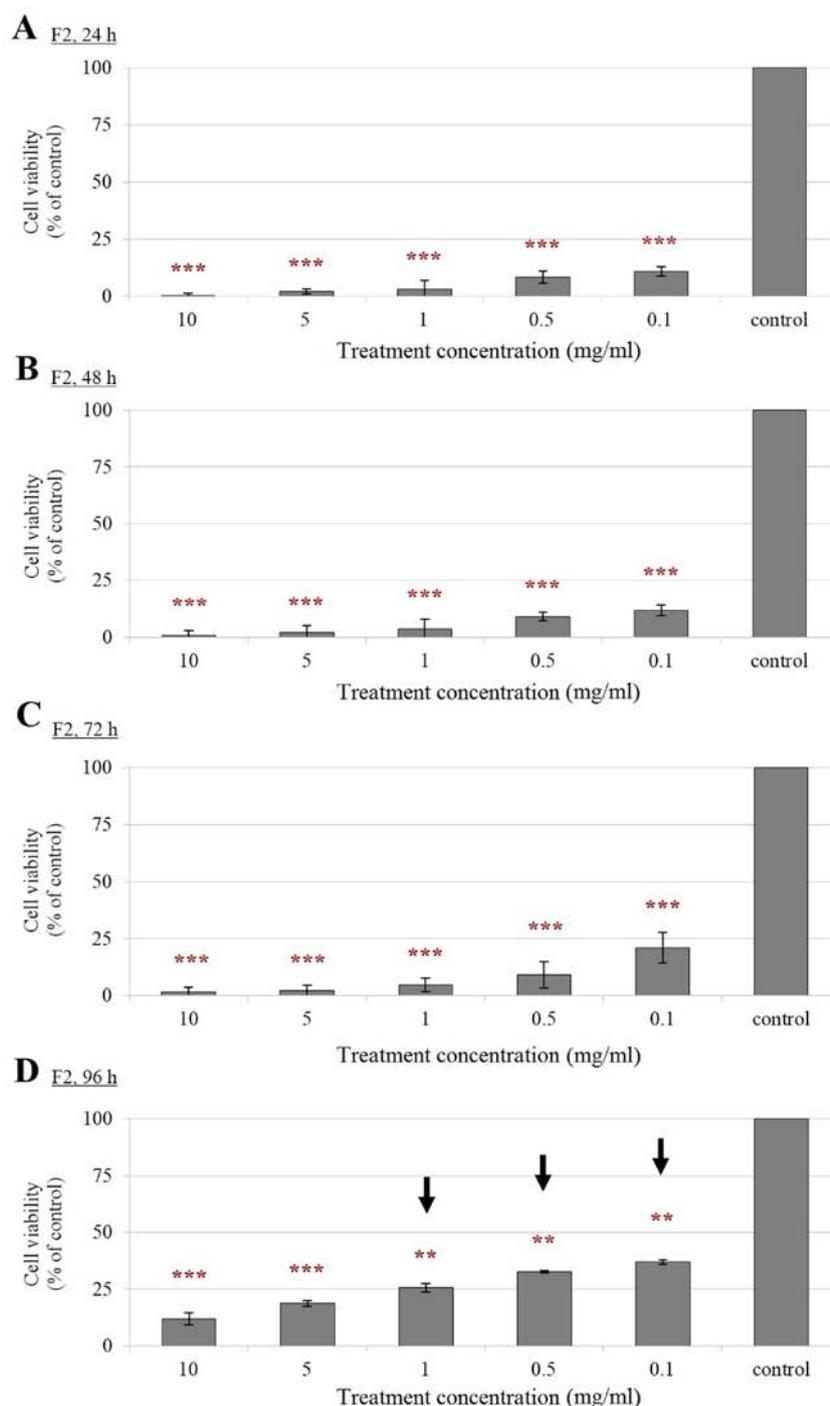


Figure 2. Effects of F2 treatment on Leydig cell viability. Viability of TM3 cells was measured (A) 24, (B) 48, (C) 72 and (D) 96 h following treatment with different concentrations of F2. Data are expressed as means \pm SD (n=4). **P<0.01 and ***P<0.001 indicate a significant reduction of cell viability in treated cells vs. control. Arrows (\rightarrow) indicate that the cells were significantly revived. EN, eurycomanone; F2, *Eurycoma longifolia* extract.

48 h following treatment (Fig. 5C). Following 96 h of F2 treatment, reductions in the proportion of TM3 cells in the G₀/G₁ phases treated with 0.1 mg/ml F2 observed at 24, 48 and 72 h time-points were reversed (Fig. 5E). Likewise, increments in the proportion of TM3 cells at the S phase treated with F2 observed at 24, 48 and 72 h were also reversed after 96 h, particularly in cells treated with 0.1 mg/ml F2 (Fig. 5E). The percentages of treated cells at the G₂ phase post F2 treatment demonstrated no difference compared with those of control following F2 treatment for 24-96 h (Fig. 5).

Functional level of identified DEGs in F2-treated Leydig cells. Microarray analysis revealed 237 and 324 genes to be up- and downregulated, respectively, in Leydig cells 72 h following F2 treatment (Fig. 6A). However, the number of genes that were found to be up- and downregulated in Leydig cells was reduced to 192 and 212, respectively, 96 h following F2 treatment (Fig. 6A). These DEGs could indicate the mechanism on the revival of F2-treated Leydig cells and should be studied in depth to improve the understanding and safety of F2 consumption. A scatter plot was generated to assess the variations in

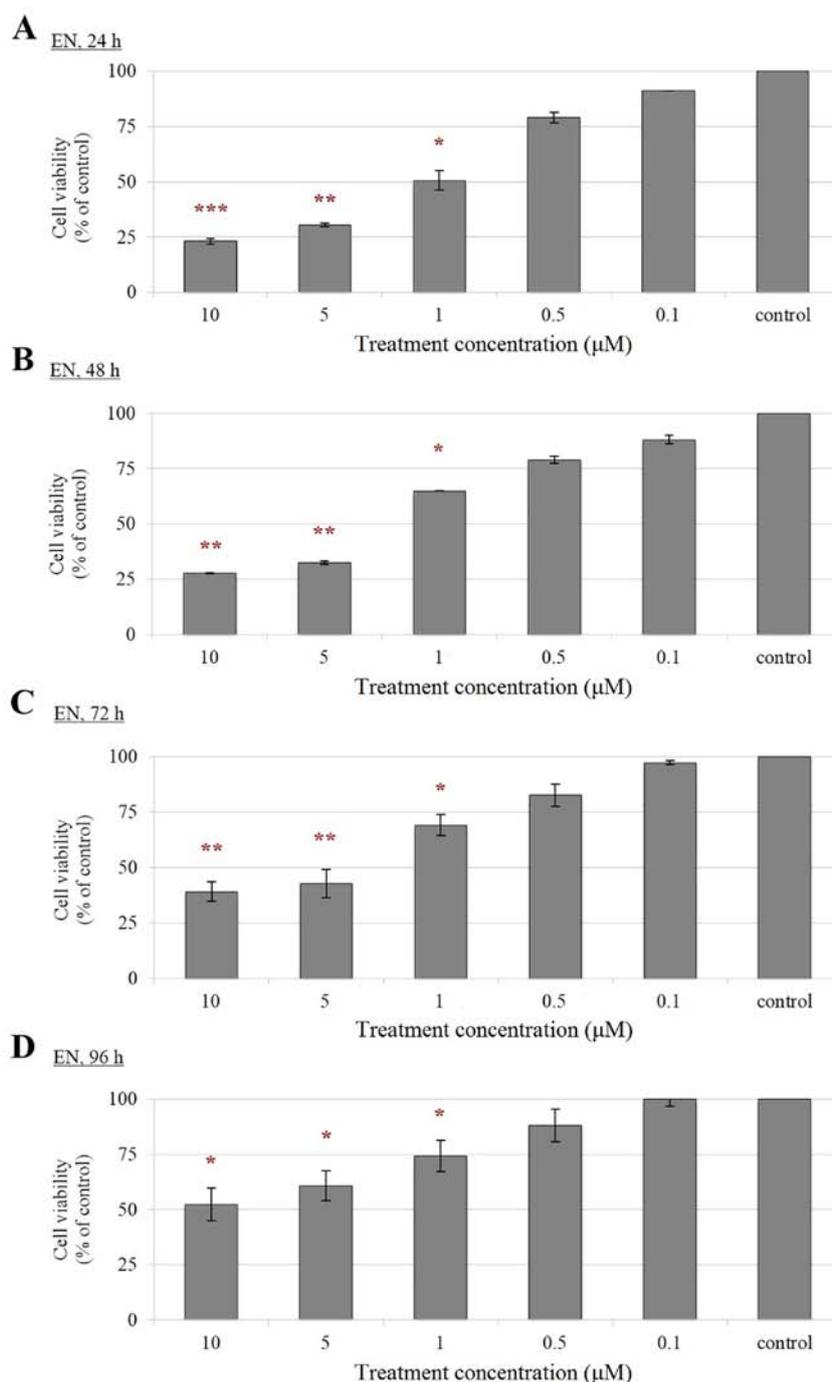


Figure 3. Effects of EN treatment on Leydig cell viability. Viability of TM3 cells was measured (A) 24, (B) 48, (C) 72 and (D) 96 h following treatment with different concentrations of EN. Data are expressed as means \pm SD (n=4). *P<0.05, **P<0.01 and ***P<0.001 indicate a significant reduction of cell viability in treated cells vs. control. EN, eurycomanone.

DEGs in the cyclin family, where its related members among non-treated (control) and F2-treated Leydig cells 72 and 96 h following treatment with F2 were compared (Fig. 6B). The DEGs in F2-treated Leydig cells were clearly observed in the plots, whereby the top 9 cyclin-related genes that demonstrated >2-fold up- and downregulated expression in F2-treated Leydig cells for 72 and 96 h were selected. Gene ontology of the DEGs revealed the role of the selected genes encoding the cyclin family of proteins and cyclin-dependent kinases (Table II). The selected genes, including cyclin-dependent kinase-like-3, cyclin G1, cyclin-dependent kinase inhibitor 1A (CDKN1A),

cyclin L1, cyclin-dependent kinase-like 2 (CDKL2), heme oxygenase 1, cyclin G2, cyclin B2 and cyclin-dependent kinase inhibitor 2C were then measured using RT-qPCR. The analysis revealed consistent expression of all 9 genes in Leydig cells treated with 0.1 mg/ml F2 at all treatment time-points (Fig. 7). However, when 1.0 mg/ml F2 was used for treatment, a significant reduction of CDKL2 expression was observed in Leydig cells treated for 72 and 96 h (Fig. 7E). The CDKL2 expression was reduced to 0.708 (P<0.05) and 0.743 (P<0.05) fold-change in Leydig cells treated with 1.0 mg/ml F2 for 72 and 96 h, respectively, compared to the CDKL2

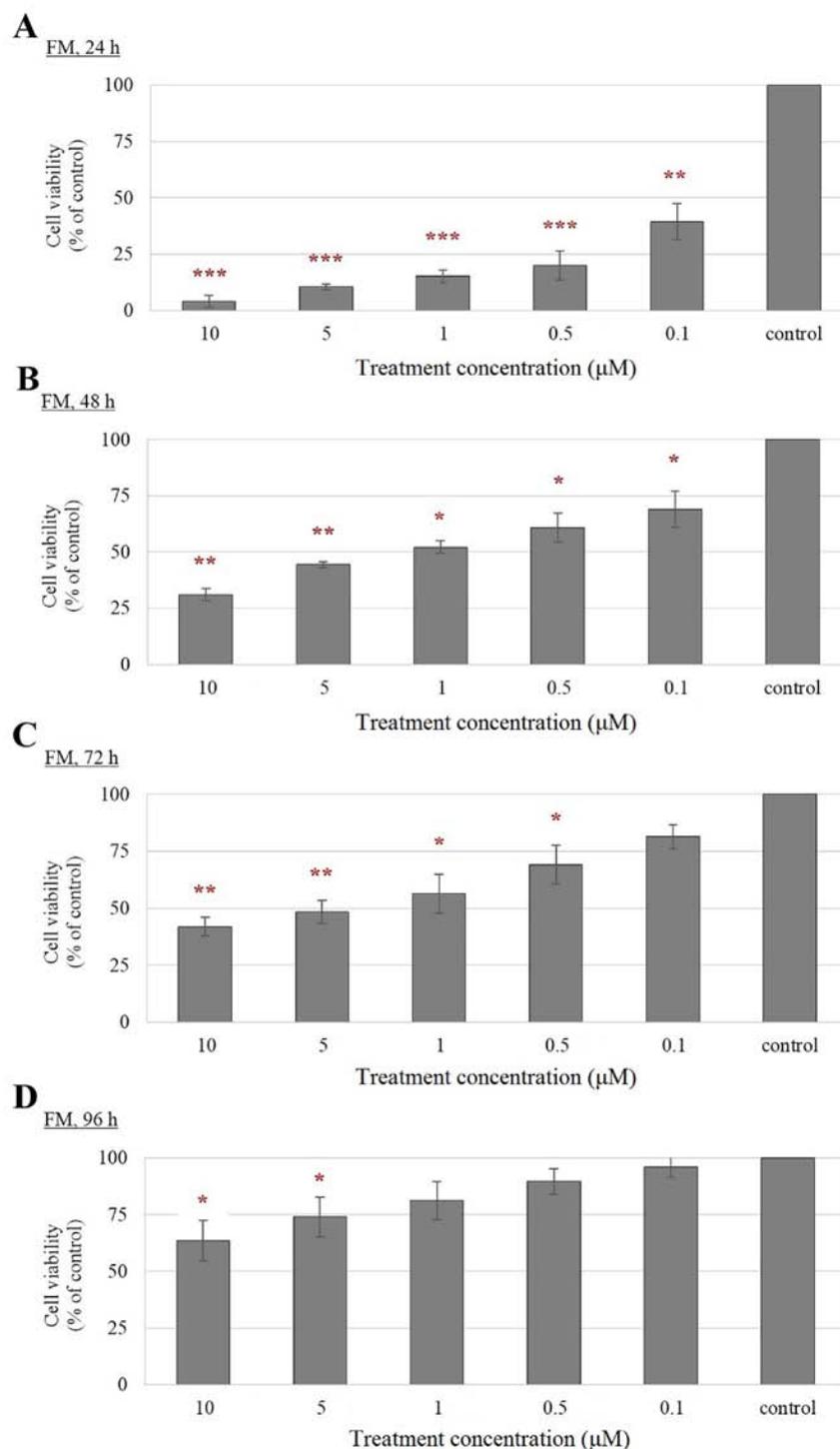


Figure 4. Effects of EN treatment on Leydig cell viability. Viability of TM3 cells was measured (A) 24, (B) 48, (C) 72 and (D) 96 h following treatment with different concentrations of FM. Data are expressed as means \pm SD (n=4). *P<0.05, **P<0.01 and ***P<0.001 indicate a significant reduction of cell viability in treated cells vs. control. FM, formestane.

expression in 1.0 mg/ml F2-treated Leydig cells for 24 h (1.000 fold-change). Comparison of the CDKL2 expression in the Leydig cells treated with 1.0 mg/ml F2 (0.708 fold change; P<0.05) than that with 0.1 mg/ml F2 (0.991 fold-change) for 72 h also showed significant reduction of the CDKL2 expression. Gene-gene interaction studies demonstrated that CDKL2 closely interacted with Casitas B-lineage lymphoma (CBL) and sphingosine kinase 1-interactor-A-kinase adaptor protein domain-containing (Sphkap), which may also serve important

roles in the mechanism and action of cell revival in F2-treated Leydig cells (Fig. 8).

Discussion

The importance of exploiting naturally occurring medicines from herbal plants has been growing around the world. An increasing number of studies are recognising the use of herbal medicine for therapy due to fewer side effects,

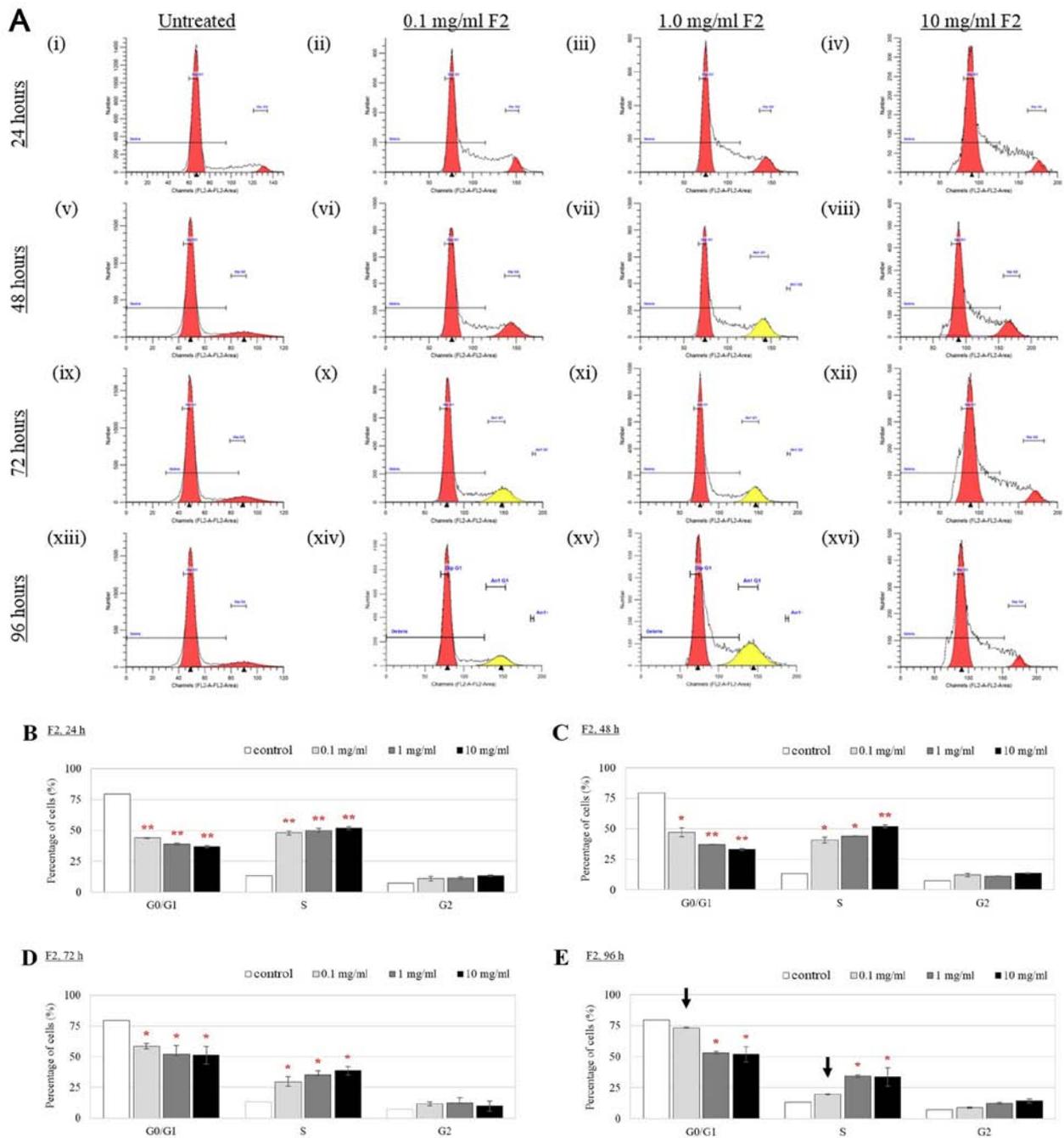


Figure 5. Cell cycle distribution of Leydig cells after F2 treatment. (A) Histograms of cell cycle distribution. (i) Untreated Leydig cells (control), Leydig cells treated with (ii) 0.1 mg/ml F2, (iii) 1.0 mg/ml F2, and (iv) 10 mg/ml F2 for 24 h; (v) Untreated Leydig cells (control), Leydig cells treated with (vi) 0.1 mg/ml F2, (vii) 1.0 mg/ml F2, and (viii) 10 mg/ml F2 for 48 h; (ix) Untreated Leydig cells (control), Leydig cells treated with (x) 0.1 mg/ml F2, (xi) 1.0 mg/ml F2, and (xii) 10 mg/ml F2 for 72 h; (xiii) Untreated Leydig cells (control), Leydig cells treated with (xiv) 0.1 mg/ml F2, (xv) 1.0 mg/ml F2, and (xvi) 10 mg/ml F2 for 96 h. Quantification of cell cycle distribution (B) 24, (C) 48, (D) 72 and (E) 96 h after F2 treatment. Data are expressed as means \pm SD (n=3). *P<0.05 and **P<0.01 indicate a significant change of cell cycle phase in treated cells vs. control at specific time-points. Arrows (\rightarrow) indicate the cells were significantly revived. F2, *Eurycoma longifolia* extract.

environmentally-friendly and increased cost-effectiveness (1,31,32). Previous studies by Low *et al* (8,26) revealed that quassinoid (EN) found in *E. longifolia* increased testosterone levels in male rats testicular Leydig cell-rich interstitial cells. However, the scientific evidence for the mechanism behind this phenomenon remains unknown. The present study demonstrated that Leydig cells treated with F2, EN and FM exhibited higher levels of testosterone in the culture supernatants. F2 acted as a potential testosterone booster because

induction could also be achieved at lower concentrations (0.1 mg/ml) according to results in the *in vitro* assays. Although F2 treatment did not affect the accumulated testosterone level, treatment of Leydig cells with as little as 0.1 mg/ml F2 reduced the viability of the treated cells. A previous study revealed that the treatment of a rat cell suspension at doses 0.1-10 μ M EN demonstrated non-cytotoxicity to the cells; >95% cell viability remained (26). The cell suspension contained ~40% Leydig cells with a viability of >97%. However, the effect of

Table II. Top nine selected genes showing >2-fold up- and downregulation by microarray analysis. The Leydig cells were treated with 0.1 mg/ml F2 for 72 and 96 h.

No.	Gene name	72 h (fold)	96 h (fold)	Gene function
1	Cyclin-dependent kinase like-3	2.204	1.959	A member of the cyclin family that encodes a CMGC-type serine/threonine protein kinase for an unknown function.
2	Cyclin G1	2.189	2.114	A transcriptional target of p53 and is induced by DNA damage in a p53 dependent manner.
3	Cyclin-dependent kinase inhibitor 1A	3.531	3.249	Encodes a potent cyclin-dependent kinase inhibitor that binds to and inhibits the activity of cyclin-cyclin-dependent kinase 2 or -cyclin-dependent kinase4 complexes. A regulator of cell cycle progression at G ₁ .
4	Cyclin L1	2.056	1.959	A regulatory subunit of CDK11 and involved in splicing process for mRNA production.
5	Cyclin-dependent kinase-like 2	2.085	1.647	A member of a large family of CDC2-related serine/threonine protein kinases.
6	Heme oxygenase 1	3.758	3.424	An enzyme that catalyzes the degradation of heme to produce biliverdin, ferrous iron and carbon monoxide.
7	Cyclin G2	-2.479	-2.235	Contains a C-terminal PEST protein destabilization motif, suggesting that their expression is tightly regulated through the cell cycle.
8	Cyclin B2	-2.219	-1.424	A member of the cyclin family that regulate PLK1 activity at G ₂ /M Transition.
9	Cyclin-dependent kinase inhibitor 2C	-1.866	-2.189	A protein coding gene for the member of the INK4 family that contains 5 ankyrin repeats.

F2, *Eurycoma longifolia* extract; CDK, cyclin-dependent kinase; CDC2, cyclin-dependent kinase 1; polo-like kinase 1; INK4, cyclin-dependent kinase inhibitor 2A.

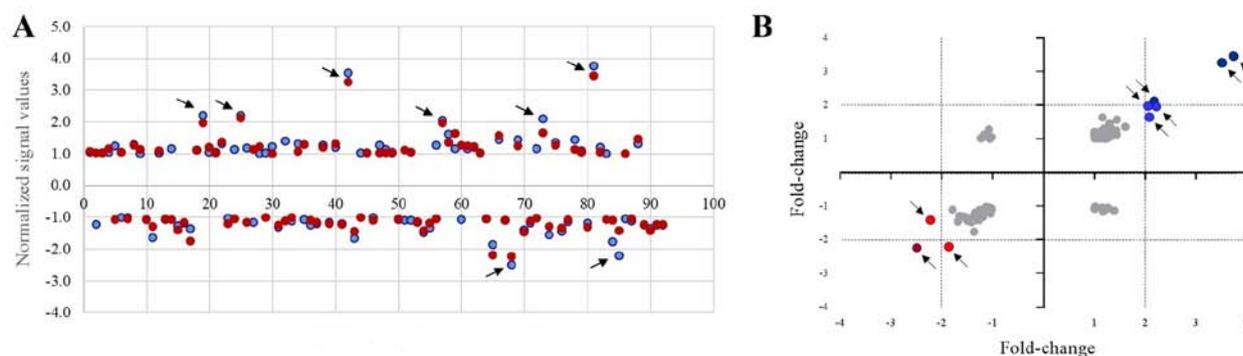


Figure 6. Analysis of differentially expressed genes in Leydig cells following F2 treatment. (A) One-dimensional and (B) two-dimensional scatter plots for differentially expressed genes in F2-treated Leydig cells. The cells were treated with 0.1 mg/ml F2 for 72 and 96 h. The x-axis presents the gene distribution, and the y-axis presents the average normalized signal values of each gene in treated and non-treated cells (control). The blue and red dots indicate the normalized gene signal values in F2-treated cells compared with controls after 72 and 96 h, respectively. The deeper shades of blue or red dots represent higher magnitudes of differential expression. Arrows (→) indicate significant up- or downregulated genes. F2, *Eurycoma longifolia* extract.

F2 on the viability of the cell suspension was not mentioned in this previous study (26). The present study revealed that high viability (>95%) of EN-treated cells was only observed when lower concentrations of EN (<0.5 μ M) were used. By contrast, a 50% reduction in cell viability was found when higher concentrations of EN were used. The current formulation of F2 contains 7.46% EN, which may be a factor to consider in future formulations of F2, which should contain \leq 0.5 μ M EN to reduce the inhibitory effect of F2 on Leydig cells (33). The reduction of cell viability could be attributed to

the biologically active components in F2 (13,21-dihydroeurycomanone, 13 α (21)-epoxyeurycomanone or eurycomanol) that have been previously found to inhibit cell replication, division and proliferation, in turn terminating the growth of the treated cells.

Cell cycle is a continuously organised process during which replication and division of a cell occurs. The primary principle of cell cycle assays is to quantify the cellular DNA content at each phase of the cycle (34). PI, which binds to the DNA of the cell and produces a fluorescent signal, has been

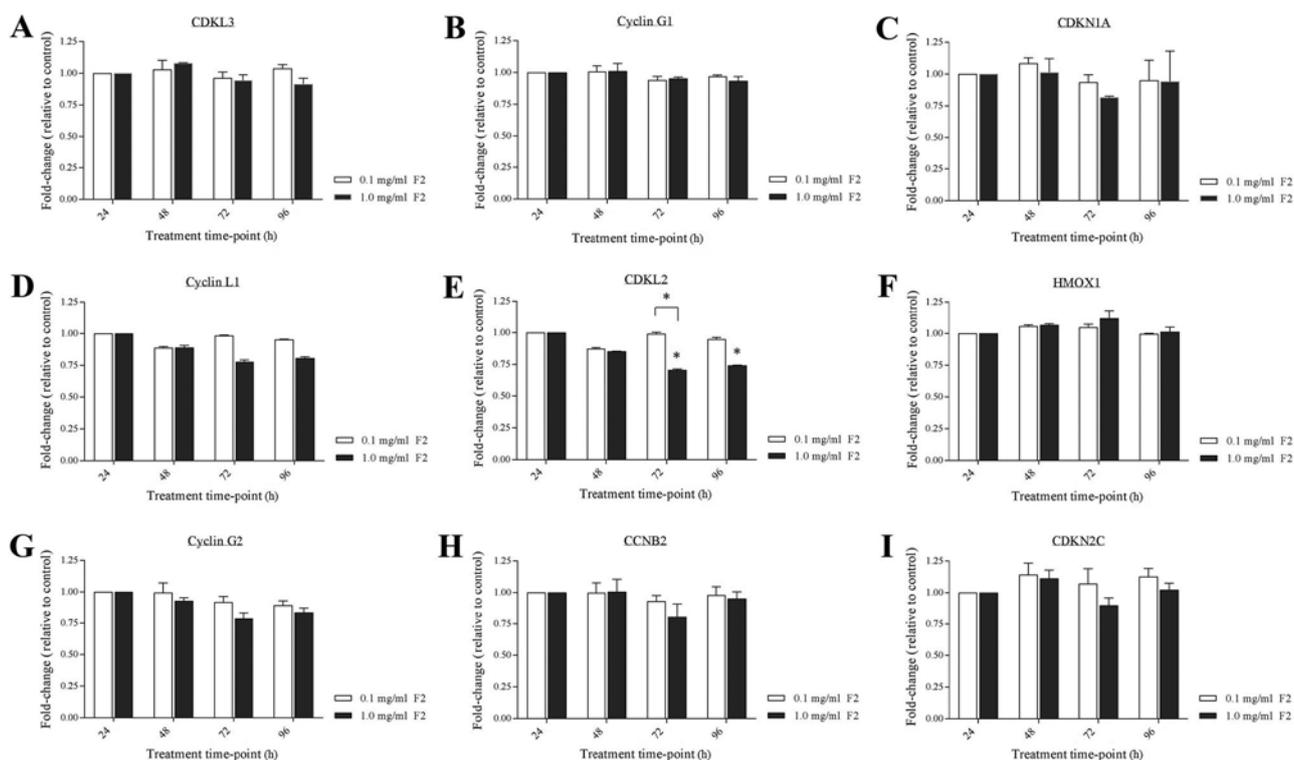


Figure 7. Gene expression validation in F2-treated Leydig cells. (A) CDKL3, (B) Cyclin G1, (C) CDKN1A, (D) Cyclin L1, (E) CDKL2, (F) HMOX1, (G) Cyclin G2, (H) CCNB2 and (I) CDKN2C mRNA expression in TM3 cells were measured after F2 treatment. Data are expressed as means \pm SD (n=3). *P<0.05 indicates a significant fold-change of Leydig cells treated with 1.0 mg/ml F2 after specific time-point vs. the same treated cells after 24 h or vs. the cells treated with 0.1 mg/ml F2 after the same time-point. CDKL3, cyclin-dependent kinase-like-3; CDKN1A, cyclin-dependent kinase inhibitor 1A; CDKL2, cyclin-dependent kinase-like 2; HMOX1, heme oxygenase 1; CCNB2, cyclin B2; CDKN2C, cyclin-dependent kinase inhibitor 2C.

used extensively for cell cycle analyses (34). The cell cycle consists of two major phases; interphase (90%) and mitosis (10%) (34). The interphase can be divided further into another three sub-phases, which are G_0/G_1 , S and G_2 , and the mitotic phase, which is broken down further into mitosis and cytokinesis (35). The present study revealed that Leydig cells treated with F2 demonstrated reductions in cell viability by reducing the cell population in the G_0/G_1 phase, which could have been caused by cell cycle arrest at the S phase. However, cell viability was revived, especially following 96 h treatment with F2, which may be due to changes in the number of cells at the G_0/G_1 and S phases. Following 96 h treatment with 0.1 mg/ml F2, the proportions of Leydig cells at the G_0/G_1 and S phases were not found to be significantly different compared with those of the control cells. The cell revival may also be attributed to the reduced drug effect and adaptation of cells to F2 treatment. Therefore, further identification of the potential molecular target(s) that regulate this mechanism is warranted.

A number of studies have previously demonstrated cell cycle arrest at the S phase upon treatment of Leydig cells with different compounds (36-38). A study recently investigated the effects of calretinin on the testicular Leydig cell line, MLTC-1, whereby cell cycle analysis demonstrated a significantly high cell number at the S phase, suggesting that the reduction in cell viability was due to S-phase cell cycle arrest (36). Similarly, a study conducted a cell cycle test by using primary Leydig cells treated with different concentrations of dehydroepiandrosterone (DHEA) and discovered that after 48 h 50 μ M DHEA treatment, the cell number at S phase was significantly

increased following a reduction in the cell number at the G_2 phase compared to that in the control group (37). These observations indicate that cell cycle arrest in S phase inhibited the growth of Leydig cells. To the best of our knowledge, only two articles have reported the effect of *E. longifolia* extracts on the cell cycle progression. Tong *et al* (38) previously reported that a standardised *E. longifolia* extract, SQ40, could inhibit LNCaP cell development by suppressing cell growth via G_0/G_1 phase arrest accompanied by downregulation of cyclin dependant kinase (CDK)2, CDK4, Cyclin D1 and Cyclin D3 expression and the upregulation in the protein level of p21CDKN1A. By contrast, Al-Salahi *et al* (39) demonstrated that TAF273 from the *E. longifolia* root methanolic extract exerted a potent cytotoxic effect on K562 cells via cell cycle arrest at the G_1 and S phases. In the present study, the ability of F2 to regain the cell population at the G_0/G_1 phase could be attributed to the revival of the F2-treated Leydig cells following 96 h of treatment. Therefore, it is reasonable to suggest that the G_0/G_1 and S phases are the critical factors to explore in improving the viability of F2-treated Leydig cells.

At the molecular level, CDKL2 expression was found to be significantly affected in Leydig cells treated with 1.0 mg/ml F2 for 72 and 96 h compared with that of respective controls, implying the essential role of CDKL2 in regulating the viability of F2-treated Leydig cells. However, the detection of CDKL2 expression does not clearly define the mechanisms that were being studied. The CDKL2 expression profiles in Leydig cells treated with 0.1 and 1.0 mg/ml F2 for 72 and 96 h did not clearly reveal the mechanisms of S-phase cell cycle arrest and reduced cell

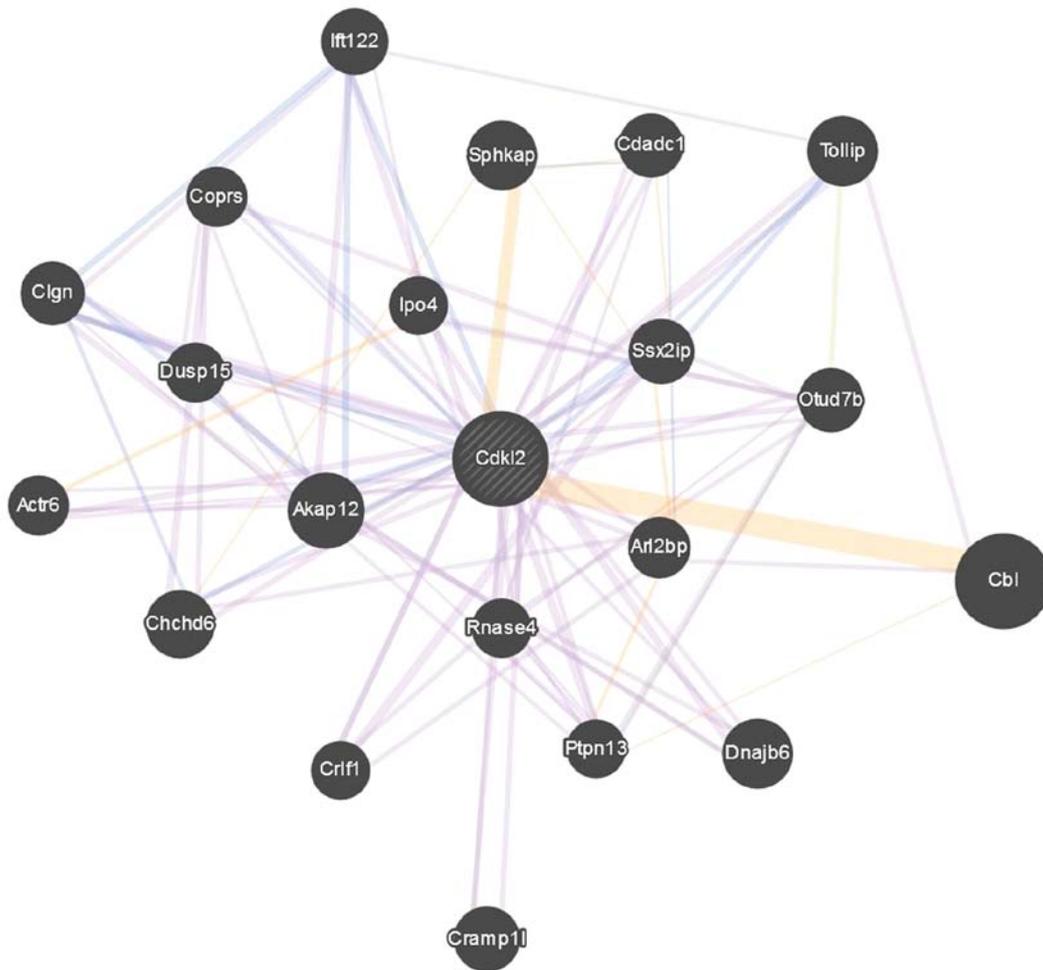


Figure 8. Prediction of potential genes associated with CDKL2 using the GeneMANIA Cytoscape program v.3.6.0 (<https://genemania.org/>). The genes are shown in circles, and the lines reflect the interrelatedness of genes. The relevance detection algorithms assign the circle size and the line thickness in each individual gene network. Orange, purple, blue and grey lines indicate prediction, co-expression, co-localization and others, respectively. CDKL2, cyclin-dependent kinase-like 2.

viability in the cells. Leydig cells treated with ≤ 1.0 mg/ml F2 for 72 h were not revived compared with those treated with the same concentrations of F2 for 96 h, which started to revive. In addition, the populations of Leydig cells treated with 0.1 mg/ml F2 for 96 h in G_0/G_1 and S phases of the cell cycle were not significantly different compared with those of the respective controls, indicative of cell revival. Compared with the populations of Leydig cells treated with 1.0 mg/ml F2 for 96 h at the G_0/G_1 and S phases of the cell cycle, there was a significant difference compared with those of the respective controls. CDKL2 is a cdc2-related serine/threonine protein kinase that is postnatally expressed in various brain regions, including the cerebral cortex, entorhinal cortex, hippocampus, amygdala and dorsal thalamus (40). The upregulation of CDKL2 in these regions suggests that it has a role in cognition and emotion. Although studies have elucidated the physiological role of CDKL2 in mice models lacking *Cdkl2*, detailed studies on *Cdkl2* remain rare. CDKL2 may also be involved in gastric cancer progression, where a previous study exploring the clinical effect of CDKL2 on gastric cancer indicated that the loss of CDKL2 was positively associated with several clinical and pathological characteristics (40-42). Patients with low CDKL2 expression levels exhibited significantly worse disease-free and overall survival rates compared with those with

high CDKL2 expression levels (41). Cellular studies revealed that CDKL2 overexpression impaired the proliferation and invasion of gastric cancer cells (41); therefore, the loss of CDKL2 may serve as a biomarker for predicting the outcome in patients with gastric cancer and is a potential therapeutic target for gastric cancer treatment. To the best of our knowledge, only one study has previously demonstrated that the expression of CDKL2 was upregulated in human breast cancer tissues and cells compared with that in normal breast tissues and cells (43).

CDKL2 is similar to another member of the cyclin-dependent kinase family, CDKL1, and is presumably derived from an early vertebrate duplication (41). Reanalysis of genome-wide association studies suggested that CDKL2 may contribute to breast cancer, where human mammary gland epithelial cells expressing CDKL2 demonstrated increased epithelial-mesenchymal transition and stem cell properties, which were obtained from the activation of a positive feedback loop comprising of Zinc finger E-box-binding homeobox 1, E-cadherin and β -catenin (43). In addition, CDKL2 promoted xenograft proliferation and metastasis *in vivo* (41,43). In particular, CDKL2 expression was found to be upregulated in mesenchymal breast cancer cells compared with that in epithelial cells, where its upregulation is negatively associated with

disease-free survival (41,43). The present study demonstrated that the reduced growth of Leydig cells following treatment with high concentrations of F2 may be due to the downregulation of CDKL2 expression. Therefore, further investigation on the regulation of this specific molecular target and its associated targets, such as CBL and Sphkap, which may act in concert with CDKL2 in F2-treated Leydig cells, is warranted.

In conclusion, the present study demonstrated the elevation of testosterone production in Leydig cells treated with the standardised *E. longifolia* extract, F2. However, this phenomenon was accompanied by the reduced viability of treated cells. Also, the present study revealed CDKL2 as an important molecular target for the regulation of these F2-dependent mechanisms. However, further studies are warranted to improve understanding of the growth inhibitory effect caused by F2 treatment in Leydig cells.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HS and CL contributed to the conception of the present study. LY, KY and CL made substantial contributions to the design of the present study. NA and LK performed the experiments under technical support provided by GP, HS and CL. NA, GP and KY interpreted the results, drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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