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***Full Paper***

# Apoptotic induction of sub-G1 cells accumulation and cell-cycle-related protein down-regulation in cancer cell lines by *Hyptis suaveolens* (L.) leaf extract

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**Abstract:** The effects of *Hyptis suaveolens* leaf ethanolic extract (HLEE) on cell proliferation of four human cancer cell lines, namely Jurkat, MCF-7, HepG2 and PC-3, were observed. The effects of HLEE on apoptotic cells and cell cycle distribution were analysed and the expression of cell cycle regulatory proteins was assessed. HLEE shows an anti-proliferative effect against human cancer cells with a more pronounced activity in Jurkat cells than MCF-7, HepG2 and PC-3 cells. In addition, HLEE also induces cell death in early and late apoptosis, where the dead cells accumulate in the Sub-G1 phase. In addition, HLEE decreases the expression of cyclin D1, cyclin E, CDK2 and CDK4 in a time-dependent manner. The results indicate that HLEE possesses anti-cancer activity in Jurkat cells, induces cell accumulation in Sub-G1, and down-regulates cyclin D1/CDK4 and cyclin E/CDK2 complexes. Thus, *H. suaveolens* could be a promising phytotherapeutic inhibitor of T lymphocyte leukemia.

**Keywords:** *Hyptis suaveolens*, apoptosis, cell cycle, cancer cells, Jurkat cell

## INTRODUCTION

Recently, there has been a significant increase in the search for new effective chemotherapeutic anticancer agents, especially those of natural origin with relatively low toxicity. Phytochemicals are known to prevent carcinogenesis by inducing antioxidant enzymes and blocking the development of carcinogenic cells by inducing apoptosis and cell cycle arrest [1]. *Hyptis suaveolens* (Linn.), belonging to the Lamiaceae family, is one of the most important traditional medicinal plants. It is a weed with a mint aroma and is distributed mostly in tropical and subtropical

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regions. It has been used in traditional medicine to treat various diseases such as gastrointestinal disorders, respiratory tract infections, colds, pain, fever, cramps and skin diseases [2]. Ethanolic extracts from leaves of *H. suaveolens* show the presence of alkaloids, glycoside, saponin, tannins, flavonoids and triterpenoids as major active constituents [3, 4]. The major volatile compositions of *H. sauveolens* are 1,8-cineole, caryophyllene, sabinene, limonene, biclyclogermacrene, phellandrene, spathulenol and eucalyptol [5, 6, 7]. Moreover, the plant has been reported to possess antioxidant, antimicrobial [8, 9, 10], anti-nociceptive [11, 12], antidiarrheal [3], anti-hyperglycemic [13] and anti-inflammatory activities [2]. The plant also possesses anti-proliferative and anticancer activities [7, 14, 15].

Cancer is a very complex and important disorder that is one of the leading causes of death worldwide. The most crucial characteristic of tumor cells is the uncontrollable cell growth due to changes in a variety of molecules in the regulation pathways of the cell cycle [16]. Mitogenic signals induce the expression and binding of cyclin D1 to CDK4/CDK6 in G1 phase of the cycle. Then cyclin E/CDK2 is activated in the late G1 phase and cyclin A/CDK2 complex is formed in the S phase, introducing the proteins involved in DNA replication. Cyclin A/CDK1 complex is necessary for the initiation of prophase and finally cyclin B/CDK1 complex takes part in and completes the mitosis [17]. Our previous research study has illustrated the cytotoxic and apoptotic properties of *H. suaveolens* extract against Jurkat cells. *H. suaveolens* induces nuclear blebbing, DNA fragmentation and up-regulates Caspase-9, Bcl-2 and Bax expressions [18]. However, the effects of *H. suaveolens* on cell cycle progression in Jurkat cells have not been investigated. Therefore, the present study examines the effects of *H. suaveolens* leaf ethanolic extract (HLEE) on the cell proliferation of other human cancer cell lines, viz. MCF-7, HepG2 and PC-3, compared to the Jurkat cell. Further assessment of the effects of HLEE on apoptotic induction and cell cycle progression in Jurkat cells is also conducted.

## MATERIALS AND METHODS

### Chemicals and Reagents

Alamar blue was purchased from Invitrogen (USA). High glucose DMEM medium, RPMI-1640 medium, Hepes, penicillin-streptomycin and fetal bovine serum were purchased from Gibco (USA). Propidium iodide and SeeBlue® Plus 2 pre-stained standard were purchased from Invitrogen (USA). Super Signal West Pico chemilluminesence substrate was obtained from Thermoscientific (USA). Bovine serum albumin (BSA) fraction V and TEMED were from BDH chemicals (England). Protease inhibitor cocktail was obtained from Roche (Germany). Antibodies were obtained from Santa-Cruz Biotechnology (USA). Nitrocellulose membrane was obtained from Amersham Biosciences (Austria). All other reagents were purchased from Sigma-Aldrich Co.

### Preparation of Plant Extracts

*H. suaveolens* was collected near Suranaree University of Technology, Nakhon Ratchasima, Thailand. The plant was identified by Professor Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University, Thailand. The leaves were washed, sun dried for 3 days, and ground into fine powder by an electric blender. Fifty grams of the powder were extracted in 500 mL of 70% ethanol (w/v) by Soxhlet extraction for 24 hr. The extract was filtered, concentrated by a rotary evaporator (Buchi, Switzerland), lyophilised (Freeze-zone 12 plus, Labconco Corporation, USA) to

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give a crude powder of HLEE and stored at -20°C until use. The powder extract was dissolved in DMSO and diluted in a culture medium containing 0.2% (v/v) DMSO when used in all experiments.

**Cell Culture**

MCF-7 (human breast cancer cell line), HepG2 (human hepatocellular carcinoma cell line) and PC-3 (human prostate cancer cell line) were obtained from American Type Culture Collection. Human T lymphocyte leukemia cell line, Jurkat E6.1, was purchased from Cell Line Services (Germany). HepG2 and MCF-7 cells were cultured in high glucose DMEM medium. Jurkat and PC-3 were cultured in RPMI-1640 medium. All culture media were supplemented with 100 U/mL penicillin-streptomycin and 10% fetal bovine serum. The cells were cultured under a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C.

**Cell Viability**

Cell viability was determined by alamar blue assay [19] with slight modification. Jurkat ($2.5x10^4$ cells/well), MCF-7, HepG2 ($2x10^4$ cells/well) and PC-3 cells ($1.5x10^4$ cells/well) were plated in a 96-well black clear bottom plate (Costar, Corning Incorporated, USA) for 18 hr and then treated with different concentrations of HLEE at a final volume of 100 µL/well. After incubation for 24 and 48 hr, 10 µL of alamar blue solution was added and the samples further incubated for 4 hr. The fluorescence of the assay solution was measured at an excitation wavelength of 540 nm and an emission wavelength of 590 nm in a spectrofluorometer (Spectra MAX Gemini EM, Molecular Devices, USA). The percentage of cell viability was plotted against concentration of extract and the value of half maximal inhibitory concentration of the extract ($IC_{50}$) was calculated.

**Annexin V-PI Staining**

The Annexin V-PI staining was used to evaluate early and late apoptotic cells. Jurkat cells ($1x10^6$ cells) were treated with 400, 600 and 800 µg/mL HLEE for 24 hr. In separate experiments, the cells were incubated with 600 µg/mL HLEE for 0, 6, 12 and 24 hr. The cells were collected by centrifugation at 400×*g* for 5 min., washed with phosphate buffer saline (PBS) and stained with Annexin V-FITC Apoptosis detection kit (EXBIO, Czech Republic). Briefly, $5×10^5$ cells in 100 µL binding buffer (1X) were incubated with 5 µL Annexin V-FITC and 5µL of 100 µg/mL propidium iodide in 1X binding buffer for 15 min. in the dark at room temperature. Then 400 µL binding buffer was added and the mixture analysed by a flow cytometer (Becton Dickinson Biosciences, USA).

**Cell Cycle Analysis**

Cell cycle progression was detected by flow cytometer, following the methodology of Chen et al. [20] with slight modification. Jurkat cells ($1x10^6$ cells) were incubated with different concentrations of HLEE for 0, 6, 12 and 24 hr, harvested by centrifugation at 400×*g*, washed twice with PBS and resuspended in 500 µL PBS. The cells were fixed by adding 4 mL 70% ethanol under gentle shaking and kept at -20ºC overnight. The fixed cells were harvested, washed twice with 0.1% BSA in PBS and incubated for 30 min. at 37ºC with 3.8 mM sodium citrate pH 8.45 (500 µL) containing a final concentration of 100 µg/mL RNase A and 0.5% Triton X-100. The cells were then stained with 50 µg/mL propidium iodide (in deionised water) in the dark at room temperature

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and analysed by a flow cytometer (Becton Dickinson Biosciences, USA) using Cell Quest Pro software.

### SDS-PAGE Gel Electrophoresis and Western Blotting

Jurkat cells ($4x10^6$ cells) were treated with 400 and 600 µg/mL of HLEE for 0, 1.5, 3 and 6 hr, harvested, washed twice with PBS and then lysed with a lysis buffer (50 mM Tris pH 8, 150 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail) for 30 min. on ice. The cell lysate was collected by centrifugation at 13,000 rpm (CT15RT versatile refrigerated centrifuge, Techcomp, Hong Kong) at 4°C for 30 min. The lysate protein content was determined by Bradford assay. The lysate proteins (40 µg) were separated by 12% SDS-PAGE gel at constant voltage of 120 V for 120 min. and transferred onto nitrocellulose membrane by electroblotting at 400 mA at 4°C for 4 hr. The non-specific binding sites were blocked by incubating the membrane with 3% BSA (for cyclin D1) and 5% BSA (for cyclin E, CDK2, CDK4 and β-actin) in 0.1% (v/v) Tween-20 in Tris-buffered saline (TBST) for 1 hr at room temperature. The membrane was washed with Tris-buffered saline and incubated with primary antibodies against anti-mouse cyclin D1, cyclin E, CDK2, CDK4 (1: 1000 dilution with 3% BSA in TBST, 3 hr) and actin (1:1000 dilution with 5% BSA in TBST, 3 hr) at room temperature. Then the membrane was washed thrice with TBST and incubated with a diluted secondary goat anti-mouse IgG-horseradish peroxidase-conjugated antibody (1:10000 dilution with 3% BSA in TBST) for 1 hr at room temperature. The blotted membrane was washed thrice with TBST and twice with Tri-buffered saline, incubated in a Super Signal West Pico chemiluminescent substrate (Thermo Scientific, USA) for 5 min. and exposed to film using X-ray film cassette. The relative expression of proteins was densitometrically quantified by using the imageJ software and calculated according to the reference band of β-actin.

### Statistical Analysis

The data were analysed by ANOVA followed by Tukey's honestly significant difference post hoc test to determine significant differences between groups at $p<0.05$.

## RESULTS AND DISCUSSION

### Effects of HLEE on Cell Proliferation

When cancer cell lines were incubated with different concentrations of HLEE for 24 and 48 hr, HLEE decreased the proliferation of four different human cancer cells in a concentration-dependent manner as shown in Figure 1. All cancer cell lines exhibit significant differences ($p<0.05$) in HLEE cytotoxicity in the order of Jurkat > MCF-7 > HepG2 > PC-3. In addition, HLEE was found to be less toxic to normal peripheral blood mononuclear cells (PBMCs) [18]. The highest anti-proliferative effect is observed on Jurkat cells (Table 1).

The result is in accordance with the study of Gurunagarajan and Pemaiah [14], who found that the ethanolic extracts of *H. suaveolens* showed potent cytotoxicity against Ehrlich Ascites carcinoma by activating the apoptotic pathway. *H. suaveolens* essential oil showed strong cytotoxicity to breast cancer cells (MCF-7) [7], prostate cancer cells (LNCaP) and cervical cancer cells (HeLa) [21]. Based on this potent anti-proliferative effect of HLEE on Jurkat cells, its mechanisms of actions associated with apoptotic induction and cell cycle progression were further investigated.

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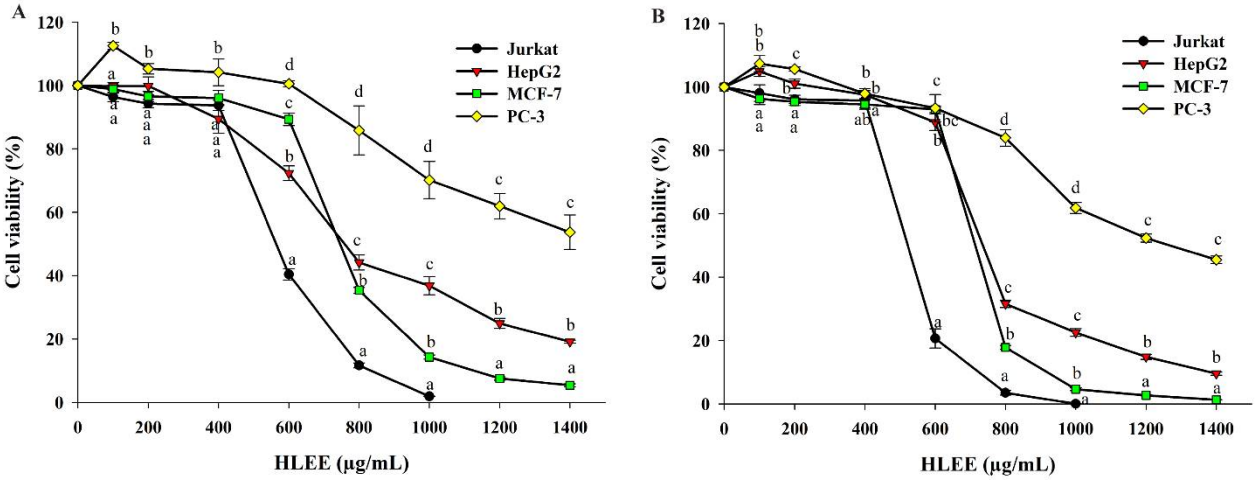


**Figure 1.** Proliferation of Jurkat, HepG2, MCF-7 and PC-3 cells after incubation with different concentrations of HLEE for 24 hr (A) and 48 hr (B). Data are mean ± S.D. (n = 6). Lines vertically marked with different letters represent statistical difference ($p<0.05$).

**Table 1.** Comparison of antiproliferative efficacy of HLEE on different human cancer cell lines at 24 and 48 hr

| Cell line | $IC_{50}$ (µg/mL), 24 hr | $IC_{50}$ (µg/mL), 48 hr |
|---|---|---|
| Jurkat | 559.00±5.99[a] | 519.43±3.57[a] |
| MCF-7 | 763.12±9.51[b] | 707.45±7.84[b] |
| HepG2 | 866.06±16.44[b] | 798.28±20.23[c] |
| PC-3 | 1,636.16±152.90[c] | 1,381.99±43.85[d] |

Note: Different letters within the same column indicate significant difference ($p<0.05$).

## Apoptosis Induction of HLEE on Jurkat Cells

In order to further investigate the apoptotic induction of HLEE on Jurkat cells, the cells were stained with annexin V-FITC/PI and analysed by flow cytometry. A key step in the early stages of apoptosis is the translocation of phosphatidylserine to the outer leaflet of the cell membrane. Annexin V selectively binds to phosphatidylserine and helps to identify cells undergoing apoptosis. HLEE treatment increases the percentage of early and late apoptotic cells. Increasing HLEE concentration induces a higher rate of apoptosis compared to control, as shown by the cytogram of apoptosis (Figure 2). HLEE shows a dose-dependent effect on apoptotic cell population (early + late apoptosis, Q4+Q2). The percentage of apoptotic cells increases by 6.21%, 32%, 73.97% and 93.27% when the cell are treated with HLEE at 0, 400, 600 and 800 µg/mL respectively (Figure 2, upper row). Similarly, the percentage of apoptotic cells increases with time at 4.20%, 17.55%, 25.28% and 74% after incubation with 600 µg/mL of HLEE for 0, 6, 12 and 24 hr respectively (Figure 2, lower row). Flow cytometric analysis shows that HLEE induces cell death through early and late apoptosis in both a dose- and time-dependent manner.

Previously, Musika and Indrapichate [18] reported that *H. suaveolens* induced apoptotic cell death in Jurkat cells as indicated by chromatin condensation and DNA fragmentation, and apoptotic

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proteins were increased at both the Caspasse-9 and Bax levels while there was a decrease at the Bcl-2 level.

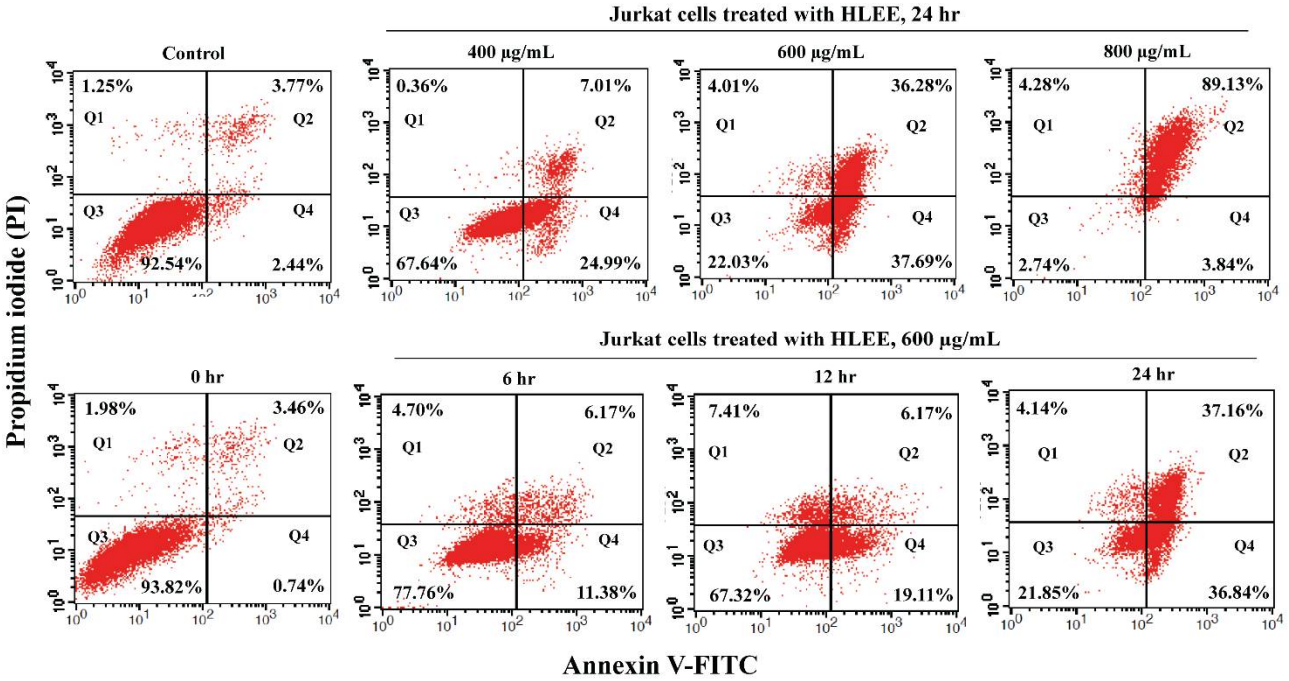


**Figure 2.** Cytogram of annexin V binding and propidium iodide uptake in Jurkat cells after treatment with 400, 600 and 800 µg/mL HLEE for 24 hr, and with 600 µg/mL HLEE for 0, 6, 12 and 24 hr. X-axis represents FL1-H (log) with green fluorochrome for annexin V labelling, and Y-axis represents FL3-H (log) with red fluorochrome for propidium iodide labelling. Q represents quadrant in cytogram: Q1=necrotic cells, Q2=late apoptosis, Q3=live cells, Q4=early apoptosis. Percentages of Jurkat cells in cytogram indicate single or double positive for annexin V and propidium iodide. Data shown are representatives of three independent experiments.

## Effects of HLEE on Cell Cycle Progression

The effects of HLEE on cell cycle progression of Jurkat cells were determined by flow cytometry (Figure 3). HLEE decreases the percentage of cell population in G0/G1, S and G2/M phases, whereas in Sub-$G_1$ phase this percentage increases in a dose-dependent manner (Figure 3A). The cells treated with 400 µg/mL HLEE significantly increase in S phase (20.09%) compared to control (18.75%). In addition, the accumulation of Sub-G1 cell population is significant in both concentration- and time-dependent manner (Figures 3A, 3B). The accumulation of cell population in the Sub-$G_1$ phase represents the appearance of apoptotic cells, which corresponds to the increase in the number of apoptotic cells by flow cytometric analysis in Figure 2.

HLEE at 600 µg/mL for 6, 12 and 24 hr decreases the cell population in G0/G1, S and G2/M phases compared to control groups (Figure 3C). These results indicate that growth inhibition and apoptosis induction by HLEE on Jurkat cells are associated with the induction of S phase arrest at lower concentration (400 µg/mL) and the accumulation of cell population in the Sub-G1 phase in the cell cycle at higher concentration (600 µg/mL). This agrees with the result that the essential oil of *H. suaveolens* showed antiproliferative activity with G0/G1 arrest on LNCaP and HeLa cells [21].

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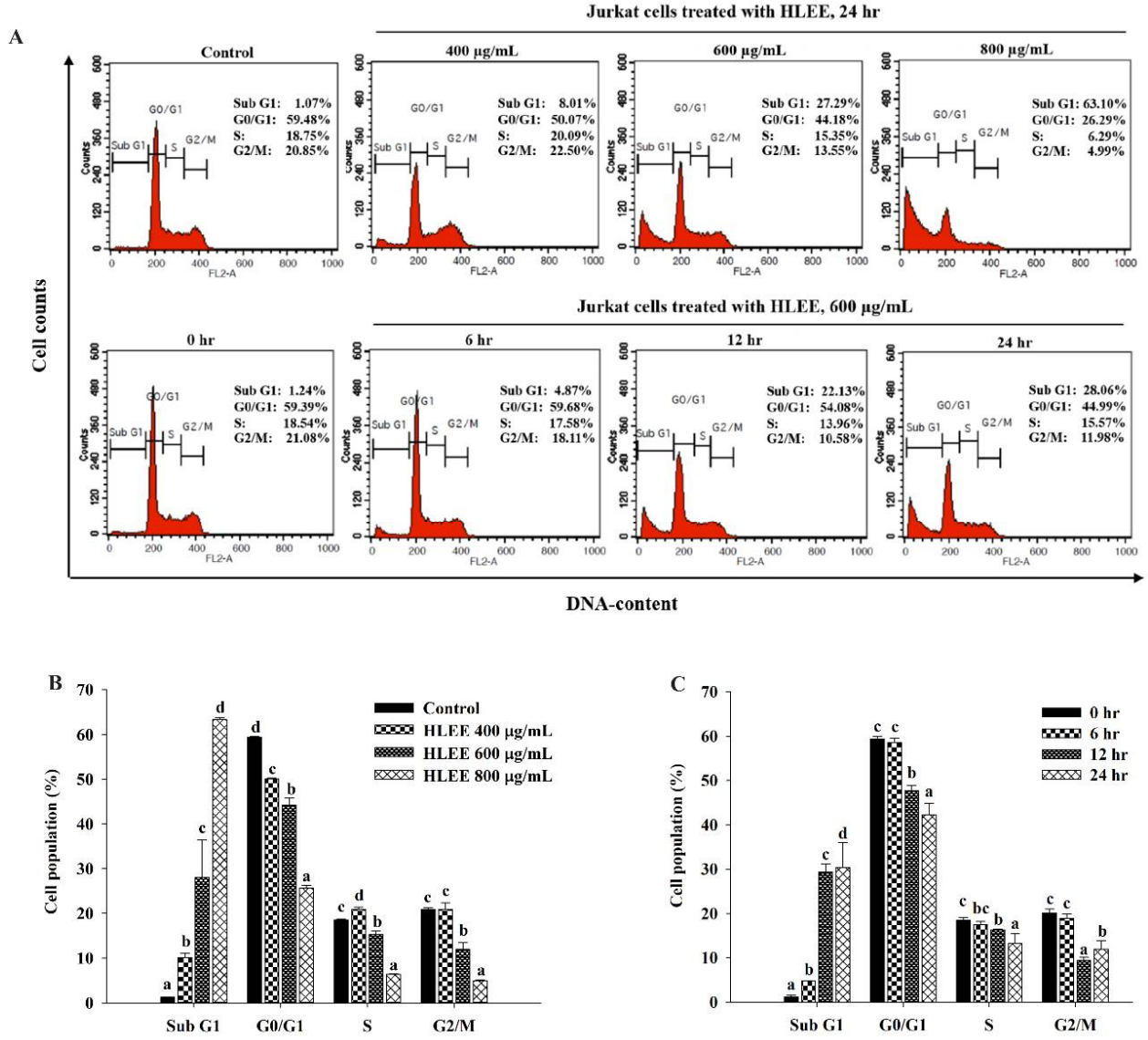


**Figure 3.** Effects of HLEE on cell cycle of Jurkat cells: (A) Cells treated with different concentrations of HLEE (upper panel) for 24 hr and cells incubated with 600 µg/mL HLEE (lower panel) for 0, 6, 12 and 24 hr; (B) Percentage of cells in sub-G1, G0/G1, S and G2/M phases of cell cycle after treatment with HLEE at 400, 600 and 800 µg/mL for 24 hr; (C) Percentage of cell population in cell cycle after treatment with HLEE at 600 µg/mL for 0, 6, 12 and 24 hr.

Bars marked with different letters within the group indicate significant difference at $p<0.05$. Percentages of cells in G0/G1, S and G2/M phases are indicated. The sub-G1 represents the apoptotic population. Data shown are representatives of three independent experiments.

## Effects of HLEE on Cell Cycle Regulatory Protein Expression

To ascertain that the molecular mechanisms of HLEE can induce cell cycle arrest, its effects on regulatory proteins in the cycle were further analysed by Western blot. As shown in Figure 4, HLEE decreases the expression of cyclinD1, cyclinA, CDK4 and CDK2 in Jurkat cells in a concentration-dependent manner. HLEE treatments at 600 µg/mL for 1.5, 3 and 6 hr intensively down-regulate the expression of cyclinD, CDK4, cyclinE and CDK2. Prolonged treatment of HLEE at 400 µg/mL slightly alters the expressions of cyclinD1, CDK4, cyclinE and CDK2 in Jurkat cells. Thus, the anti-proliferative effects of HLEE could be associated with the down-regulation of

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cyclinD1/CDK4 and cyclinE/CDK2 complexes, causing accumulation of cells in Sub-G1 afterwards (Figure 3A, lower panel). Thus, cyclinE/CDK2 complexes in Jurkat cells decrease after treatment with HLEE. The accumulation of Jurkat cells in Sub-G1 after incubating with HLEE causes cell apoptosis (Figure 3A). This may be due to p53-mediated growth inhibition, which activates $p21^{Cip1/WAF1}$, a CDK inhibitor, resulting in the inhibition of CDK2:cyclinE activity during all phases of the cell cycle, while $p16^{INK4a}$ is a selective inhibitor for the CDK4/6:cyclinD complex [22, 23]. As a result, fewer cells enter the cell cycle. These findings suggest that the HLEE-mediated Sub-G1 cell cycle arrest is associated with the decrease in expression for the corresponding cyclins and CDKs in Jurkat cells.

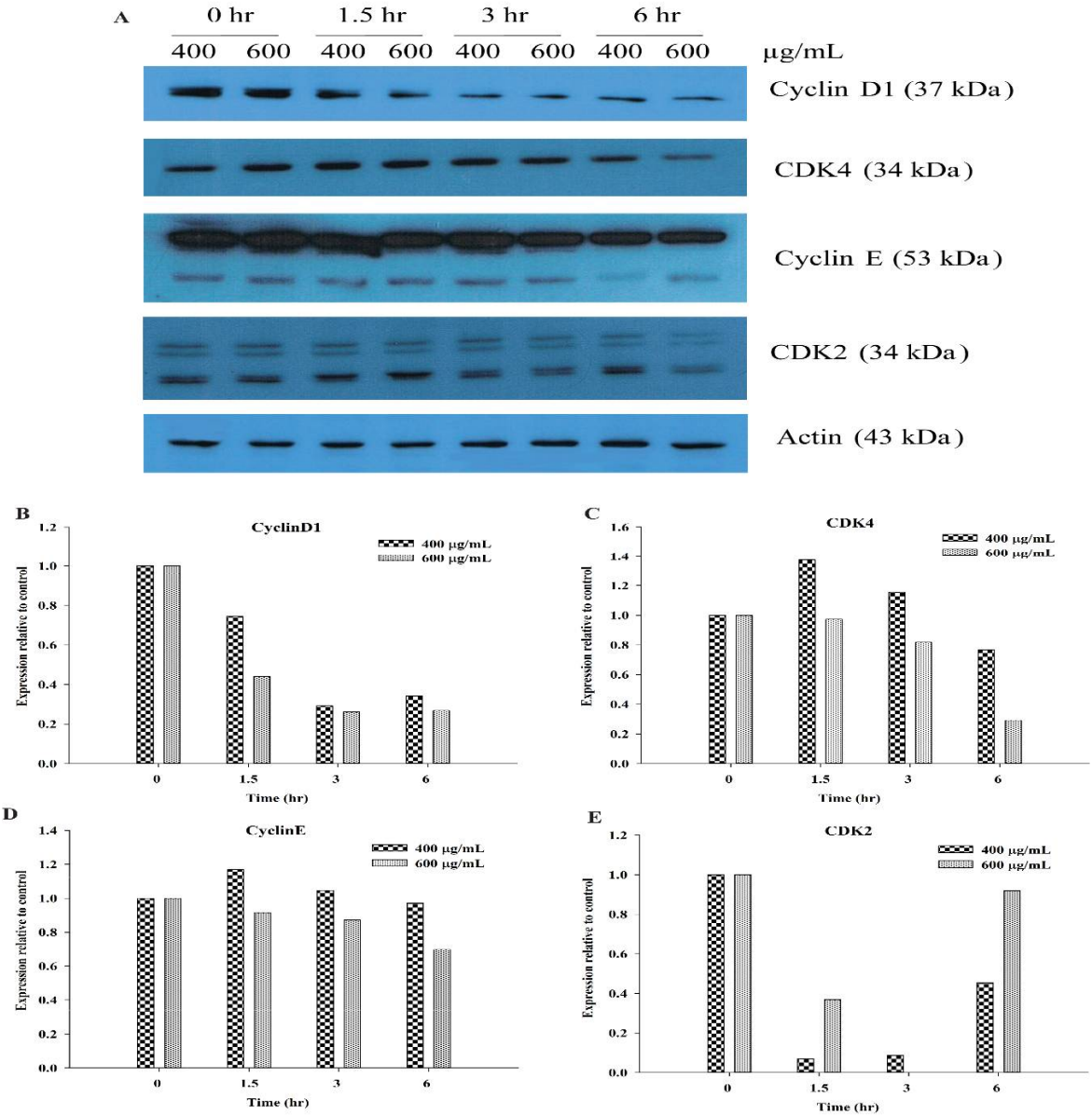


**Figure 4.** (A) Western blot analysis of effects of HLEE (400 and 600 µg/mL for 0, 1.5, 3 and 6 hr) on expression of cyclin D1, CDK4, cyclin E and CDK2 in Jurkat cells. The cell lysate (40 µg) was separated by 12% SDS-PAGE and immunoblotted; (B-E) Cyclin D1, CDK4, Cyclin E and CDK2 expression respectively. β-Actin was used as protein loading control. The data shown represents two independent experiments.

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## CONCLUSIONS

HLEE exhibits the highest selective cytotoxicity against Jurkat cells, followed by MCF-7, HepG2 and PC-3. Importantly, HLEE also induces apoptosis by increasing the cell population of the Sub-G1 phase, i.e. cells entering apoptosis. In addition, HLEE alters cell cycle regulation-related proteins by decreasing the expression of cyclin D1, CDK4, cyclin E and CDK2. These findings indicate that *H. suaveolens* is a good candidate for being further developed into a chemotherapeutic agent acting against T lymphocyte leukemia.

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