

ANTI-PROLIFERATIVE AND APOPTOSIS INDUCTION OF *HYPTIS SUAVEOLENS* (L.) SEED EXTRACTS ON JURKAT CELLS

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Abstract

Hyptis suaveolens seed ethanolic extract (HSEE) were investigated for their antioxidant, anti-proliferative, and apoptosis-inducing activities against Jurkat cells. The total phenolic and total flavonoid contents, as well as the antioxidant properties of HSEE were evaluated using the colorimetric method. Cell proliferation was assessed by alamar blue assay. Nuclear morphology changes were detected by Hoechst 33258 staining, while the DNA laddering pattern was assessed using agarose gel electrophoresis. The cell cycle distribution was assessed using flow cytometry, whereas the apoptosis-related protein changes were investigated by Western blotting analysis. HSEE exhibited radical scavenging activity in various radical systems (FRAP, DPPH, and TBARS). As indicated by DNA fragmentation and cell accumulation in the sub-G1 phase, HSEE displayed anti-proliferation and induced apoptosis in Jurkat cells. In addition, HSEE caused cell cycle arrest in Jurkat cells at the G2/M phase and triggered apoptosis through caspase-dependent pathways. After exposure to HSEE for 24 h, the level of Bcl-2 in Jurkat cells dramatically decreased, while the levels of caspase-9 and Bax significantly increased. Overall, these findings revealed that the anti-proliferative effects of HSEE involved the induction of G2/M phase arrest and the intrinsic apoptotic pathway in Jurkat cell lines.

Keywords: *Hyptis suaveolens*; apoptosis; Jurkat cells; DNA fragmentation

Introduction

Cancer is one of the main causes of death globally, characterized by a group of disorders involving abnormal cell growth and uncontrolled cell proliferation. Numerous plant-based anti-cancer medications have been shown to induce apoptosis in cancerous cells (Sittisart *et al.*, 2019). Apoptosis is a type of programmed cell death (PCD) that

regulates cell numbers and eliminates damaged cells. As a result, apoptosis is a promising target for anticancer therapy. Natural agents hold great promise for chemopreventive use due to their ability to reduce cancer growth while minimizing adverse effects. *Hyptis suaveolens* (L.) is a Lamiaceae plant found in tropical areas around the world. It is an

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aromatic shrub-like plant with small blue or purple flowers. Flowering and fruiting occur between October and January, and the plant's seeds swell when immersed in water and look like flour balls. Phenols, flavonoids, essential oils, terpenoids, tannins, saponins, alkaloids, and sterols have been found in *H. suaveolens* (Mishra *et al.*, 2021). According to the analysis by Poonkodi *et al.* (2017), the essential oil of *H. suaveolens* contains several terpenoids that are medicinally important. These terpenoids include sabinene, trans-caryophyllene, E-spathulenol, rimuene, 1,8-cineole, beta-elemene, eucalyptol, bergamotol, Z-alpha-trans, alpha-selinene, caryophyllene oxide, and alpha-humulene. In addition, *H. suaveolens* seed oil revealed a predominant presence of unsaturated fatty acids, particularly linoleic and oleic acids. In contrast, the oil contained lower levels of saturated fatty acids, including palmitic, stearic, and heptacosanoic acids (Bachheti *et al.*, 2015). The extracts from *H. suaveolens* are a source of pharmacologically significant secondary metabolites that possess antioxidant, as well as neuroprotective properties, antimicrobial and anti-proliferative effects (Nantitanon *et al.*, 2007; Ghaffari *et al.*, 2014; Oscar *et al.*, 2020; Akharaiyi *et al.*, 2023). Furthermore, it has been documented that *H. suaveolens* possesses anti-inflammatory (Shenoy and Shirwaekar, 2002), anti-hyperglycemic (Mishra *et al.*, 2011), anticancer (Gurunagarajanand and Pemaiah, 2011) and apoptosis-inducing activities (Musika and Indrapichate, 2014). In addition, essential oils derived from *H. suaveolens* have demonstrated beneficial biological activities, including antioxidant (Aguale *et al.*, 2023) and antimicrobial properties (Wu *et al.*, 2019). The seed of *H. suaveolens* is rich in dicaffeoylquinic acids, which is beneficial for gout patients (Hsu *et al.*, 2019). Chemical studies have shown that the major components of the *H. suaveolens* seed are protein and neutral poly- and oligosaccharides (Bojórquez-Velázquez *et al.*, 2016; Mueller *et al.*, 2016). In addition, linoleic acid and unsaturated fatty acids are abundant in *H. suaveolens* seed oil (Bachheti *et al.*, 2015). The ethanolic extract of *H. suaveolens* seeds possesses analgesic, antidiarrheal and cytotoxic activities (Islam *et al.*, 2010). Our previous report on the ethanolic extract of *H. suaveolens* leaf demonstrated its ability to induce apoptosis in cancer cell lines, as indicated by the accumulation of sub-G1 cells and downregulation of cell division cycle-associated proteins (Musika *et al.*, 2021). However, the effects of *H. suaveolens* seed extracts on Jurkat cells have not yet been evaluated.

Therefore, the present research evaluated the antioxidant activity, anti-proliferative and

apoptosis-inducing activities of *H. suaveolens* seed ethanolic extracts against human T cell leukemia Jurkat cells.

Materials and Methods

Preparations of HSEE

The *H. suaveolens* seeds were collected in January 2015 from Suranaree University of Technology, Nakhon Ratchasima, Thailand. After removing the seed mucilage, the seeds were dried in a hot air oven at 60°C for a day. The dried seeds were finely ground and extracted using a soxhlation method in 70% ethanol at a ratio of 1:10 (w/v) for 24 h. The extract was filtered, concentrated, and lyophilized. *H. suaveolens* seeds ethanol extracts (HSEE) were kept at -20°C until analysis.

Total Phenolic Contents of HSEE

The quantification of the total phenolic contents (TPC) of the HSEE was performed using the folin-ciocalteu method (Matthaus, 2002). After adding 0.1 mL of HSEE into 2% Na₂CO₃ (2 mL) for 2 min, the mixture was then incubated with folin-ciocalteu reagent (0.1 mL) for 30 min. The absorbance was read at 750 nm using spectrophotometer (CE1011, Cecil Instruments, Cambridge, England). The TPC value was reported as mg gallic acid eq./g sample.

Total Flavonoid Contents of HSEE

An aluminum chloride colorimetric assay was used to evaluate the total flavonoid contents (TFC) of the HSEE (Liu *et al.*, 2002). The mixtures of HSEE (0.25 mL) and deionized water (1.25 mL) were incubated with 5% NaNO₂ (0.075 mL) for 6 min. Next, 0.1 mL of 10% AlCl₃·6H₂O was added and incubated for 5 min. Subsequently, 0.5 mL of 1 M NaOH was added to the mixture. To make the final volume of 2.5 mL, 0.275 mL of deionized water was added, and an absorbance measurement was taken at 510 nm. The TFC value was reported as mg catechin eq./g sample.

Ferric Reducing Ability Power (FRAP)

The capacity for reducing ferric ions was assessed using the method outlined by Benzie and Strain (1996). A mixture containing 50 µL of HSEE and 1.5 mL of freshly prepared FRAP reagent was incubated for 10 min. The measurement of absorbance was taken at 593 nm, and the FRAP values were reported as µmol FeSO₄·7H₂O eq./mg sample.

DPPH Radical Scavenging Activity

Free radical scavenging capacity of the HSEE was estimated following the procedure of Sanchez-Moreno *et al.* (1999). A DPPH[•] solution (40 mg/L) was prepared using methanol as the solvent. The experiment began by mixing HSEE (50 μ L) with DPPH[•] solution (1.95 mL) and thereafter incubating it in the dark for 45 min. The measurement of absorbance was done at a wavelength of 515 nm. Standard controls for the experiment were L-ascorbic acid and catechin. The DPPH radical scavenging activity was reported as the percentage of inhibition and calculated using the following equation:

$$\text{Inhibition (\%)} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (1)$$

The IC₅₀ values of the extract for scavenging the DPPH radical were calculated using a linear regression equation derived from plotting the percentage of inhibition against different concentrations of the HSEE.

Lipid Peroxidation Inhibition

Lipid peroxidation (LPO) was evaluated by phosphatidylcholine liposomes (Gonzalez-Paramas *et al.*, 2004). Different concentrations of HSEE were added into a reaction mixture containing 1 mg/mL of phosphatidylcholine liposome, 150 mM KCl, and 0.2 mM FeCl₃. The peroxidation reaction was initiated by adding 0.05 mM sodium L-ascorbate into a final volume of 0.4 mL and then incubated at 37°C for 60 min. The reaction was terminated by adding 20% trichloroacetic acid (0.4 mL), 0.25 N HCl, 0.01% butylated hydroxytoluene (BHT), and 0.8% thiobarbituric acid (0.4 mL) and further incubated at 80°C for 30 min. The mixture was then allowed to cool and centrifuged at 5,000 rpm for 10 min. The absorbance was measured at 535 nm, and the following formula was used to calculate the percentage of LPO inhibition:

$$\text{LPO inhibition (\%)} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \quad (2)$$

The IC₅₀ values of the extract were calculated using a linear regression equation derived from plotting the percentage of LPO inhibition against different concentrations of the HSEE.

Cell Culture

The Jurkat E6-1 cell line, derived from human T-cell leukemia (Korean Cell Line Bank, Seoul, Korea), was cultured in RPMI 1640 medium. The culture medium was supplemented with 1 mM

sodium pyruvate, 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. The Jurkat cells were incubated in 5% CO₂ at 37°C throughout the experimental period.

Cell Viability

Jurkat cells, at a density of 2.5×10^4 cells per well, were cultured and co-incubated with different concentrations of the HSEE in a 96-well plate for 24 h. Following this incubation period, 10 μ L of alamar blue was added to each well, and the plates were further incubated for an additional 4 h. A spectrofluorometer was then utilized to measure the fluorescence intensity at excitation/emission wavelengths of 570/590 nm. The following formula was used to determine the percentage of cell viability:

$$\text{Cell viability (\%)} = \left[\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right] \times 100 \quad (3)$$

The IC₅₀ values of the extract were calculated using a linear regression equation derived from plotting the percentage of viable cells against different concentrations of the HSEE.

Nuclear Morphology Analysis

The nuclear morphology of apoptotic cells was assessed using the methodology outlined by Chen *et al.* (2008). Jurkat cells, at a density of 2.5×10^4 cell/well, were exposed to HSEE with different concentrations in a 96-well plate and then kept in an incubator for 24 h. After washing the cells twice with PBS, 200 μ L of 4% p-formaldehyde was added to fix the cells for 20 min. The cells were then washed and stained with 10 μ g/mL of 100 μ L Hoechst 33258 (Invitrogen, California, USA) in the dark at 37°C for 30 min. After this treatment, the cells were washed, examined, and photographed using an inverted fluorescence microscope (Olympus IX51, Olympus corporation, Japan).

DNA Fragmentation Analysis

Jurkat cells (1×10^6 cells/well) were co-cultured with different concentrations of HSEE in a 6-well plate for 24 h. The cells were then harvested and lysed with 180 μ L of lysis buffer and 10% SDS (20 μ L). After lysis, the cells were further incubated at a final concentration of 100 μ g/mL Proteinase K for 4 h at 56°C. An equal volume of a mixture of phenol, chloroform, and isoamyl (25:24:1) solution were used to extract DNA twice, and then the upper layer was collected. After addition of 3 M sodium acetate into the upper layer at a ratio of 10:1 (v/v), the DNA was precipitated by adding 2.5 volumes of absolute ethanol and then kept chilled at -20°C overnight. The DNA pellet was collected following

a 30 min centrifugation at 12,000 rpm at 4°C. The DNA was then washed twice with cold 70% ethanol, dried, and finally resuspended in Tris-EDTA buffer (TE buffer). To eliminate RNA, 5 µg DNA sample in TE buffer was incubated with 100 µg/mL of RNase A solution and then further incubated at 37°C for 30 min. The DNA samples were mixed with loading dye in a 1:5 (v/v) ratio before being loaded in 1.5% agarose gel using 70 volts for 90 min. After applying 0.5 µg/mL ethidium bromide stain, the DNA pattern was visualized and photographed under gel documentation system (Wealtec Corp., Sparks, USA).

Cell Cycle Progression Analysis

Jurkat cells (1×10^6 cells/well) were co-cultured with different concentrations of HSEE in a 6-well plate for 24 h. The cells were collected and fixed with 4 mL of 70% ethanol and kept overnight at -20°C. The fixed cells were washed with 0.1% BSA in PBS, followed by incubation with RNase A (100 µg/mL) diluted in a mixture of 0.5% Triton X-100 and 3.8 mM sodium citrate (pH 8.45) at 37°C for 30 min. The cells were stained with propidium iodide (Thermoscientific, Lillinos, USA) at a concentration of 50 µg/mL at room temperature in the dark before being subjected to flow cytometry (BD FACSCalibur, BD Biosciences, USA).

Western Blotting Analysis

Jurkat cells (4×10^6 cells) were cultured and treated with different concentrations of HSEE for 12 and 24 h, and the expression of Bax, caspase-9, and Bcl-2 protein was detected (Musika *et al.*, 2021). Following incubation, the cells were collected, washed, and lysed on ice for 30 min using 180 µL of lysis buffer containing a protease inhibitor cocktail. After centrifugation at 13,000 rpm at 4°C for 30 min, the cell lysate was collected and the protein content was evaluated using the Bradford assay (Bradford, 1976). The lysate proteins (40 µg) were separated using a 10% SDS-PAGE gel at 120 volts for 120 min and the protein was transferred onto a nitrocellulose membrane with a constant current of 400 mA at 4°C for 4 h. To block the non-specific protein, membranes were incubated with 3% for caspase-9 detection and 5% BSA for the detection of Bcl-2, Bax, and β-actin. The membrane was washed and incubated for 1 h at room temperature with primary antibodies against anti-mouse Bcl-2, Bax, and β-actin (Santa-Cruz Biotechnology, California, USA) at a 1:1000 dilution in 5% BSA in TBST and Caspase 9 at a 1:500 dilution with 1% BSA in TBST. The membrane was washed and exposed to the secondary antibody, goat anti-mouse IgG-

horseradish peroxidase-conjugated antibody at room temperature for 1 h (at either a 1:5,000 or 1:10,000 dilution with 3% BSA). The membrane was washed, detected using a Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, USA) for 5 min, and then exposed to X-ray films. The β-actin was used as a housekeeping protein.

Statistical Analysis

The data were examined using One-way ANOVA, and any statistically significant variations between the groups at $p < 0.05$ were determined using the Duncan's Multiple Range Test using SPSS program. The results were presented as mean $\pm$ SD.

Results and Discussion

Total Phenolic, Total Flavonoid Contents and Antioxidant Activities of HSEE

The total phenolic and total flavonoid contents were 135.92 ± 2.17 mg gallic acid eq./g sample and 86.28 ± 0.67 mg catechin eq./g sample, respectively. Akharaiyi *et al.* (2023) reported that the ethanolic extract of *H. suaveolens* contained 3.20 ± 0.20 mg/g of phenols and 24.00 ± 0.02 mg/g of flavonoids. The total phenolic content ranged from 253.68-336.50 mg GAE/g for the extracts (Oscar *et al.*, 2020). Preliminary phytochemical screening of *H. suaveolens* seed extraction showed the presence of flavonoids, alkaloids, glycoside, tannins, and saponins (Islam *et al.*, 2010). In addition, *H. suaveolens* leaf is a rich source of medicinally significant phytochemicals, including essential oils, tannins, saponins, phenols, flavonoids, terpenoids, alkaloids, and sterols (Oscar *et al.*, 2020; Mishra *et al.*, 2021). These bioactive compounds contribute to its wide-ranging therapeutic applications. Mishra *et al.* (2021) reported that the Lamiaceae family is rich in various polyphenolic compounds such as caffeic acid, gentisic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, protocatechuic acid, ferulic acid, chicoric acid, and caftaric acid. *H. suaveolens* is notable for its abundance of flavonoids, including gallic acid, ferulic acid, quercetin, chlorogenic acid, rutin, apigenin, catechin, naringenin, hesperidin, and luteolin.

The antioxidant property of HSEE is presented in Table 1. The ferric reducing activity of HSEE was 2.01 ± 0.35 µM FeSO₄ eq./mg sample. In addition, HSEE showed radical scavenging activity against DPPH and TBARS radicals with IC₅₀ values of 32.85 ± 0.05 and 39.77 ± 1.78 µg/mL, respectively. Although HSEE showed antioxidant activity, it was lower compared to that of the standard controls, catechin, and ascorbic acid. In an *in vitro*

antioxidant assessment, the ethanolic extract of *H. suaveolens* leaves demonstrated significant free radical scavenging activity, with ferric ion reduction and hydroxyl radical scavenging capacities of 1.57 ± 1.40 , 1.69 ± 0.18 , and 0.43 ± 0.50 mg ascorbic acid/g, respectively (Akharaiyi *et al.*, 2023). Additionally, antioxidant activity, as evaluated using the DPPH method, showed an IC_{50} range of 2.73-3.58 mg/mL (Oscar *et al.*, 2020). Phenolic compounds exhibit antioxidant properties due to their redox potential, acting as reducing agents, free radical scavengers, hydrogen donors, metal chelators, and inhibitors of lipid peroxidation. Similarly, flavonoids contribute to antioxidant defense by scavenging free radicals, chelating metal ions like iron and copper, and inhibiting enzymes involved in ROS production. Additionally, flavonoids help protect lipids from oxidative damage caused by free radicals (Wang *et al.*, 2018). The antioxidant capacity of HSEE may be due to the redox properties of its phytochemical constituents, including phenolic compounds and flavonoids. Previous studies have shown a correlation between antioxidant activity and total phenolic and flavonoid content (Muflihah *et al.*, 2021)

Table 1. Antioxidant activity of HSEE against FRAP, DPPH, and TBARS assay

Sample	FRAP (μM FeSO ₄ eq./mg sample)	DPPH IC ₅₀ ($\mu g/mL$)	TBARS IC ₅₀ ($\mu g/mL$)
HSEE	2.01 ± 0.35^c	32.85 ± 0.05^c	39.77 ± 1.78^b
Catechin	16.26 ± 0.65^b	2.67 ± 0.06^a	17.92 ± 0.18^a
Ascorbic acid	19.31 ± 0.22^a	4.22 ± 0.01^b	NP

Within the same column, different letters (^{a,b,c}) exhibit significant differences ($p < 0.05$). NP=Not performed.

Nuclear Morphology and DNA Fragmentation of HSEE

Apoptosis, or programmed cell death, is characterized morphologically by membrane blebbing, cell shrinkage, and DNA fragmentation (Saraste and Pulkki, 2000). To determine the growth inhibitory effects of HSEE on the induction of apoptosis, the morphological changes in Jurkat cells were investigated using Hoechst 33258 staining. The morphological changes in apoptotic cells after 24 h of treatment with different

concentrations of HSEE are shown in Figure 1a. The yellow arrows indicate the morphological hallmarks of apoptosis, which include DNA fragmentation, chromatin condensation, and cell shrinkage. No evidence of hallmark of apoptosis was observed in the untreated group. The HSEE-treated cells had smaller nuclei and condensed staining compared to the untreated control cells. Cell shrinkage, DNA fragmentation, and chromatin condensation were all observed, which suggests that HSEE could induce apoptosis in Jurkat cells. Furthermore, DNA fragmentation analysis was further carried out to confirm that HSEE could induce apoptosis. Jurkat cells showed signs of apoptotic DNA fragmentation after 24 h of treatment with various concentrations of HSEE (Figure 1b). The DNA laddering pattern observed in the HSEE-treated cells exhibited the characteristic appearance of multiple fragmented bands, which is typical of apoptosis-induced DNA fragmentation. These bands were sharper and more distinct, indicating the presence of fragmented DNA, whereas no such bands were observed in the control group, where the DNA remained intact. The differences in band thickness or clarity between the treated and control cells highlight the apoptotic effect of HSEE, confirming that the treatment induced DNA fragmentation while the control cells showed no signs of apoptosis (Figure 1b). DNA ladders were found in Jurkat cells at a concentration of 2 mg/mL HSEE, while the concentrations of 2.5 and 3 mg/mL HSEE induced an extensive DNA fragmentation. These results demonstrated that apoptosis in Jurkat cells was induced by HSEE in a dose-dependent manner, as demonstrated by chromatin condensation and DNA ladder. Numerous studies on plant extracts rich in bioactive compounds, such as flavonoids and phenolics, have demonstrated similar apoptotic effects, often leading to nuclear condensation and fragmentation in cancer cell lines. *Wrightia arborea* leaf extract demonstrated cytotoxic effects on K562 leukemic cells by inducing apoptosis, as evidenced by apoptotic body formation, mitochondrial membrane depolarization, and DNA fragmentation in the form of DNA laddering (Lakshmipriya *et al.*, 2018). Ethanolic extract of *Derris elliptica* leaves induced chromatin condensation, apoptotic bodies and DNA fragmentation in HepG2, Jurkat, and MCF-7 cell lines (Sittisart *et al.*, 2019).

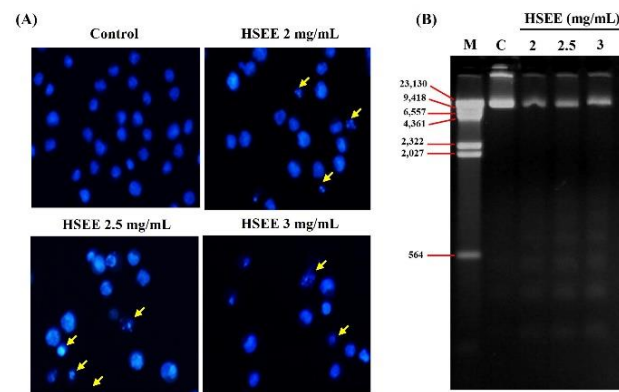


Figure 1. Nuclear morphology and DNA ladder in Jurkat cells after 24 h of treatment with HSEE at indicated concentrations. (a) Apoptosis induction in Jurkat cells observed under fluorescence microscope (Hoechst 33258 staining, 400x). Apoptotic bodies and chromatin condensation are indicated by yellow arrows. (b) DNA fragmentation in Jurkat cells was determined by 1.5% agarose gel electrophoresis. C=control; M=marker

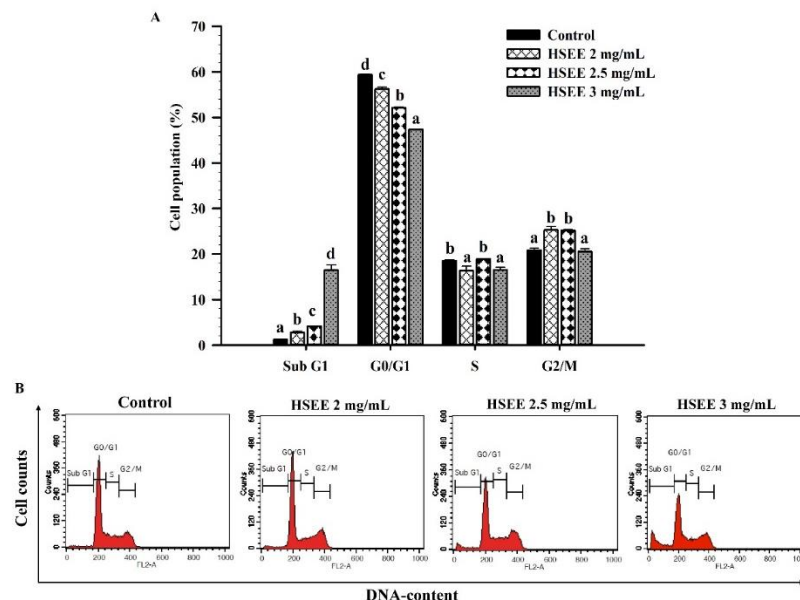


Figure 2. Cell cycle progression in Jurkat cells after 24 h of treatment with HSEE at concentrations of 2, 2.5, and 3 mg/mL. (a) The percentage of cells population in sub-G1, G0/G1, S, and G2/M phases at indicated concentrations; (b) Jurkat cells after the treatment with HSEE at indicated concentrations were analyzed by flow cytometer. Bars within the group with different letters indicate significant differences at $p < 0.05$

Cell Cycle Progression of HSEE

The development of cancerous cells is caused by the disruption of normal cell cycle control, which leads to unregulated proliferation, one of the hallmarks associated with cancer (Hanahan and Weinberg, 2011). When cellular damage cannot be effectively repaired, checkpoint signals may potentially activate pathways that lead to programmed cell death (Pietenpol and Stewart, 2002). Several investigations have revealed that

certain anti-cancer drugs activate the cell cycle arrest checkpoint, leading the cells to undergo apoptosis (Park *et al.*, 2019; Sun *et al.*, 2019). The cell cycle distribution profile was evaluated in order to investigate the mechanism underlying the anti-proliferative property of HSEE in Jurkat cells (Figure 2a and Figure 2b). The results showed that HSEE at concentrations of 2 and 2.5 mg/mL increased the frequency of arrested cells at the G2/M phase and also decreased the population of

cells in the G0/G1 phases. In addition, an increase in the concentration of HSEE was accompanied by a significant increase in the cells in the apoptotic Sub-G1 phase. Flavonoids from *Tephrosia kirilowii* (Turcz.) Holub were found to inhibit cell proliferation in various human cancer cell lines, causing G2/M phase arrest, and inducing both apoptosis and autophagy in human breast cancer cells (Zhang *et al.*, 2018). Similarly, *Wrightia arborea* extract increased the sub-G1 cell population, signaling apoptosis, while also causing DNA damage, disrupting G2/M phase protein expression, and inducing cell cycle arrest and strong G2 phase abrogation (Lakshmipriya *et al.*, 2018). The cell cycle arrest at the G2/M phase indicates potential difficulties in repairing intracellular DNA damage (Lezaja and Altmeyer, 2018). Therefore, HSEE induced DNA damage, leading to Jurkat cell arrest in the G2/M phase and inhibition of cellular entry into mitotic phase. According to the findings, HSEE significantly decreased the cell cycle progression, causing cell cycle arrest at the G2/M phase, and leading Jurkat cells to undergo apoptosis. Growing evidence suggests that the ablation of G2 checkpoint function enhances susceptibility to several anticancer drugs.

Effect of HSEE on Caspase-9, Bax, and Bcl-2 Protein Expression

Apoptotic pathways can be activated by many stimuli. These stimuli cause cell death via intrinsic and extrinsic mechanisms that interact at various levels. The extrinsic pathway is mediated by caspase-8, whereas the intrinsic pathway can be initiated through caspase-9 (Wu *et al.*, 2016). To ascertain whether the altered expression of caspase-9, Bax and Bcl-2 contributes to HSEE-induced apoptosis, Western blot analysis was carried out to assess their levels. After a 24 h exposure, HSEE at 2.5 mg/mL treatment caused an increase in caspase-9 level. In addition, Bcl-2 expression began to decrease in Jurkat cells treated with HSEE after 12 and 24 h, while Bax expression increased. (Figure 3). These findings suggest that HSEE activated caspase-9, thereby promoting apoptosis in Jurkat cells through the intrinsic pathway. The relationship between Bax, Bcl-2, and caspase-9 in this pathway is well-established. Bax, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic protein, regulate mitochondrial membrane permeability. An increase in Bax expression and a decrease in Bcl-2 expression alter mitochondrial outer membrane permeabilization (MOMP), leading to cytochrome c release and activation of caspase-9 via the apoptosome complex.

This cascade is key to triggering the intrinsic apoptosis pathway (Qian *et al.*, 2022). The decline in Bcl-2 levels demonstrates that HSEE effectively inhibited the expression of anti-apoptotic protein, thereby facilitating intrinsic apoptosis. Notably, Bcl-2 is typically expressed at higher levels in tumor cells than in normal cells, making normal cells less susceptible to inhibitors targeting Bcl-2 (Buja, 2021). Consequently, targeting the anti-apoptotic proteins of the Bcl-2 family may help overcome chemotherapy resistance while minimizing adverse effects on normal cells (Kang and Reynolds, 2009). Various plant extracts play a significant role in modulating apoptotic pathways by regulating Bax and Bcl-2 proteins. Treatment with *Dioscorea* extract has been shown to increase Bax expression while decreasing Bcl-2 expression (Doulaby *et al.*, 2023). Similarly, *Croton tiglium* extract inhibited the proliferation of A549 cells and promoted apoptosis through the Bax/Bcl-2 pathways (Li *et al.*, 2016). Groenlandicine, derived from *Coptidis rhizoma*, enhanced tumor-killing activity in cisplatin-resistant osteosarcoma cells by activating Bax and cleaved caspases-3 and -9 while inhibiting the Bcl-2 pathway (Zhao *et al.*, 2024). These findings demonstrate that the upregulation of caspase-9, along with increased Bax expression and decreased Bcl-2 expression, suggests that HSEE activated caspase-9 both directly and indirectly. The direct rise in caspase-9 further implies that HSEE may have had an immediate effect on its activation, highlighting its role in promoting apoptosis through direct caspase-9 activation and mitochondrial modulation.

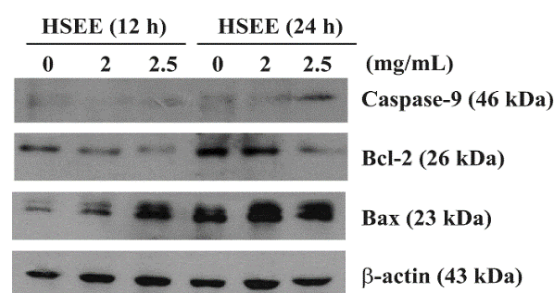


Figure 3. Western blot analysis of HSEE on caspase-9, Bcl-2, and Bax protein expression in Jurkat cells at indicated concentrations for 12 and 24 h. Following 12% SDS-PAGE separation of equal proteins from cell lysates (40 µg), antibodies against caspase-9, Bcl-2, and Bax were immunoblotted. The control for protein loading was β-actin

Conclusions

This study demonstrates that HSEE could effectively induce apoptosis and G2/M cell cycle arrest in Jurkat cells. Apoptosis induction in Jurkat cells was partially associated with DNA fragmentation, nuclear condensation, and cell accumulation in sub-G1 phase. In addition, HSEE treatment substantially increased Bax and caspase-9 levels, while Bcl-2 levels decreased. Therefore, *H. suaveolens* seed is a naturally occurring anti-cancer agent capable of inducing apoptosis in Jurkat cells.

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References

- Aguele, F.O., Oke, E.O., Abam, F.I., Nnabodo, D., and Agbana, A.S. (2023). Biological and antioxidant activities, extraction methodology and prospects of essential oil from *Hyptis suaveolens* (L.): A review. *Cleaner Engineering and Technology*, 17:100685. <https://doi.org/10.1016/j.clet.2023.100685>
- Akharaiyi, F.C., Ehis-Eriakha, C.B., Olagbemide, P.T., and Igbudu, F.H. (2023). *Hyptis suaveolens* L. leaf extracts in traditional health care systems. *Foods and Raw Materials*, 11(2):293-299. <https://doi.org/10.21603/2308-4057-2023-2-577>
- Bachheti, R.K., Rai, I., Joshi, A., and Satyan, R.S. (2015). Chemical composition and antimicrobial activity of *Hyptis suaveolens* poit. seed oil from Uttarakhand State, India. *Oriental Pharmacy and Experimental Medicine*, 15(2):141-146. <https://doi.org/10.1007/s13596-015-0184-8>
- Benzie, I.F.F., and Strain, J.J. (1996). The ferric reducing ability of plasma as a measure of antioxidant power: The FRAP assay. *Analytical Biochemistry*, 239(1):70-76. <https://doi.org/10.1006/abio.1996.0292>
- Bojórquez-Velázquez, E., Lino-López, G.J., Huerta-Ocampo, J.A., Barrera-Pacheco, A., Barba de la Rosa, A.P., Moreno, A., and Osuna-Castro, J.A. (2016). Purification and biochemical characterization of 11S globulin from chan (*Hyptis suaveolens* L. poit) seeds. *Food Chemistry*, 192:203-211. <https://doi.org/10.1016/j.foodchem.2015.06.099>
- Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2):248-254. <https://doi.org/10.1006/abio.1976.9999>
- Buja, L.M. (2021). The cell theory and cellular pathology: Discovery, refinements, and applications fundamental to advances in biology and medicine. *Experimental and Molecular Pathology*, 121:104660. <https://doi.org/10.1016/j.yexmp.2021.104660>
- Chen, Z., Wu, Q., Chen, Y., and He, J. (2008). Effects of betulinic acid on proliferation and apoptosis in Jurkat cells and its in vitro mechanism. *Journal of Huazhong University of Science and Technology [Medical Sciences]*, 28(6):634-638. <https://doi.org/10.1007/s11596-008-0604-9>
- Doulaby, F.B., Kavousi, M. and Jamshidian, F. (2023). Effect of Dioscorea extract on Bax and Bcl-2 gene expression in MCF-7 and HFF cell lines. *Egyptian Journal of Medical Human Genetics*, 24:70. <https://doi.org/10.1186/s43042-023-00450-w>
- Ghaffari, H., Ghassam, B.J., Chandra Nayaka, S., Ramchandra Kini, K., and Prakash, H.S. (2014). Antioxidant and neuroprotective activities of *Hyptis suaveolens* (L.) poit. against oxidative stress-induced neurotoxicity. *Cellular and Molecular Neurobiology*, 34(3):323-331. <https://doi.org/10.1007/s10571-013-0016-7>
- Gonzales-Paramás, A.M., Esteban-Ruano, S., Santos-Buelga, C., de Pascual-Teresa, S., and Rivas-Gonzalo, J.C. (2004). Flavanol content and antioxidant activity in winery byproducts. *Journal of Agricultural and Food Chemistry*, 52(2):234-238. <https://doi.org/10.1021/jf0348727>
- Gurunagarajan, S. and Pemaiah, B. (2011). Comparative studies on cytotoxic effect of *Hyptis suaveolens* poit. and *Leonotis nepeatefolia* R. Br. against EAC cell lines. *Journal of Pharmacy Research*, 4(4):1222-1224.
- Hanahan, D. and Weinberg, R.A. (2011). Hallmarks of cancer: The next generation. *Cell*, 144(5):646-674. <https://doi.org/10.1016/j.cell.2011.02.013>
- Hsu, F.C., Tsai, S.F., and Lee, S.S. (2019). Chemical investigation of *Hyptis suaveolens* seed, a potential antihyperuricemic nutraceutical, with assistance of HPLC-SPE-NMR. *Journal of Food and Drug Analysis*, 27(4):897-905. <https://doi.org/10.1016/j.jfda.2019.05.006>
- Islam, M.A., Parvin, S., Rahman, M.M., Ahmed, M.I., Biswas, N.N., Karmakar, U.K., and Naher, K. (2010). Phytochemical and biological investigation of seeds of *Hyptis suaveolens*. *KU Studies*, 10:185-190.
- Kang, M.H. and Reynolds, C.P. (2009). Bcl-2 inhibitors: Targeting mitochondrial apoptotic pathways in cancer therapy. *Clinical Cancer Research*, 15(4):1,126-1,132. <https://doi.org/10.1158/1078-0432.CCR-08-0144>
- Lakshmi Priya, T., Soumya, T., Jayasree, P.R., and Manish Kumar, P.R. (2018). Selective induction of DNA damage, G2 abrogation, and mitochondrial apoptosis by leaf extract of traditional medicinal plant *Wrightia arborea* in K562 cells. *Protoplasma*, 255:203-216. <https://doi.org/10.1007/s00709-017-1137-5>
- Lezaja, A. and Altmeyer, M. (2018). Inherited DNA lesions determine G1 duration in the next cell cycle. *Cell Cycle*, 17(1):24-32. <https://doi.org/10.1080/15384101.2017.1383578>
- Li, C., Wu, X., Sun, R., Zhao, P., Liu, F., and Zhang, C. (2016). *Croton tiglium* extract induces apoptosis via Bax/Bcl-2 pathways in human lung cancer A549 cells. *Asian Pacific Journal of Cancer Prevention*, 17(11):4,893-4,898.
- Liu, M., Li, X., Weber, C., Lee, C.Y., Brown, J., and Liu, R. (2002). Antioxidant and antioxidative activities of raspberries. *Journal of Agricultural and Food Chemistry*, 50(10):2,926-2,930. <https://doi.org/10.1021/jf0111209>
- Matthaus, B. (2002). Antioxidant activity of extract obtained from residues of different oil seeds. *Journal of Agricultural and Food Chemistry*, 50(12):3,444-3,452. <https://doi.org/10.1021/jf011440s>

- Mishra, B.S., Verma, A., Mukerjee, A., and Vijaykumar, M. (2011). Anti-hyperglycemic activity of leaves extract of *Hyptis suaveolens* L. Poit in Streptozotocin-induced diabetic rats. *Asian Pacific Journal of Tropical Medicine*, 4(9):689-693. [https://doi.org/10.1016/S1995-7645\(11\)60175-2](https://doi.org/10.1016/S1995-7645(11)60175-2)
- Mishra, P., Sohrab, S., and Mishra, S.K. (2021). A review on the phytochemical and pharmacological properties of *Hyptis suaveolens* (L.) Poit. *Future Journal of Pharmaceutical Sciences*, 7(1):65. <https://doi.org/10.1186/s43094-021-00219-1>
- Mueller, M., Cavarkapa, A., Unger, F.M., Viernstein, H., and Praznik, W. (2016). Prebiotic potential of neutral oligo- and polysaccharides from seed mucilage of *Hyptis suaveolens*. *Food Chemistry*, 221(15):508-514. <https://doi.org/10.1016/j.foodchem.2016.10.075>
- Muflihah, Y.M., Gollavelli, G., and Ling, Y.C. (2021). Correlation study of antioxidant activity with phenolic and flavonoid compounds in 12 Indonesian indigenous herbs. *Antioxidants*, 10(10):1530. <https://doi.org/10.3390/antiox10101530>
- Musika, S., and Indrapichate, K. (2014). Cytotoxicity and apoptotic induction of mintweed (*Hyptis suaveolens* L. Poit) leaf extracts on human T-leukemia cell line, Jurkat cells. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3(3):304-317.
- Musika, S., Woraratphoka, J., and Indrapichate, K. (2021). Apoptotic induction of sub-G1 cells accumulation and cell-cycle-related protein down-regulation in cancer cell lines by *Hyptis suaveolens* (L.) leaf extract. *Maejo International Journal of Science and Technology*, 15(2):199-208.
- Nantitanon, W., Chowwanapoonpohn, S., and Okongi, S. (2007). Antioxidant and antimicrobial activities of *Hyptis suaveolens* essential oil. *Scientia Pharmaceutica*, 75(1):35-46. <https://doi.org/10.3797/scipharm.2007.75.35>
- Oscar, S.A., Antonio, C.N., Marina, G.V., Elsa, R.S., and Gabriel, V.A. (2020). Phytochemical screening, antioxidant activity, and in vitro biological evaluation of leave extracts of *Hyptis suaveolens* (L.) from south of Mexico. *South African Journal of Botany*, 128:62-66. <https://doi.org/10.1016/j.sajb.2019.10.016>
- Park, C., Cha, H.J., Lee, H., Hwang-Bo, H., Ji, S.Y., Kim, M.Y., and Choi, Y.H. (2019). Induction of G2/M cell cycle arrest and apoptosis by genistein in human bladder cancer T24 cells through inhibition of the ROS-dependent PI3k/Akt signal transduction pathway. *Antioxidants*, 8(9):327. <https://doi.org/10.3390/antiox8090327>
- Pietenpol, J.A., and Stewart, Z.A. (2002). Cell cycle checkpoint signaling: Cell cycle arrest versus apoptosis. *Toxicology*, 181:475-481. [https://doi.org/10.1016/S0300-483X\(02\)00460-2](https://doi.org/10.1016/S0300-483X(02)00460-2)
- Poonkodi, K., Karthika, J., Tamilselvi, V., Anitha, R., and Vasanthamani, S. (2017). Chemical composition of essential oil of *Hyptis suaveolens* (L.) Poit and its in vitro anticancer activity. *Journal of Pharmaceutical Research*, 11(5):410-413.
- Qian, S., Wei, Z., Yang, W., Huang, J., Yang, Y., and Wang, J. (2022). The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. *Frontiers in Oncology*, 12:985363. <https://doi.org/10.3389/fonc.2022.985363>
- Sanchez-Moreno, C., Larrauri, J.A., and Saura-Calixto, F. (1999). Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. *Food Research International*, 32(6):407-412. [https://doi.org/10.1016/S0963-9969\(99\)00097-6](https://doi.org/10.1016/S0963-9969(99)00097-6)
- Saraste, A. and Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. *Cardiovascular Research*, 45(3):528-537. [https://doi.org/10.1016/S0008-6363\(99\)00384-3](https://doi.org/10.1016/S0008-6363(99)00384-3)
- Shenoy, R. and Shirwaekar, A. (2002). Anti-inflammatory and free radical scavenging studies of *Hyptis suaveolens* (Labiatae). *Indian Drugs*, 39:574-577.
- Sittisart, P., Piakaew, N., Chuea-Nongthong, C., and Dunkhunthod, B. (2019). Anti-proliferative and apoptosis-inducing activities of *Derris elliptica* (Roxb.) Benth. leaf extract on three human cancer cell lines. *Maejo International Journal of Science and Technology*, 13(1):62-71.
- Sun, L., Ain, Q.U., Gao, Y.S., Khan, G.J., Yuan, S.T., and Roy, D. (2019). Effect of *Marsdenia tenacissima* extract on G2/M cell cycle arrest by upregulating 14-3-3 σ and downregulating c-myc in vitro and in vivo. *Chinese Herbal Medicines*, 11(2):169-176. <https://doi.org/10.1016/j.chmed.2019.03.002>
- Wang, T., Qing, L., and Bi, K. (2018). Bioactive flavonoids in medicinal plants: Structure, activity, and biological fate. *Asian Journal of Pharmaceutical Sciences*, 13(1):12-23. <https://doi.org/10.1016/j.ajps.2017.08.004>
- Wu, K. G., Lin, Y.H., Chai, X.H., Duan, X.J., Zhao, X.X., and Chun, C. (2019). Mechanisms of vapor-phase antibacterial action of essential oil from *Cinnamomum camphora* var. *linaloofera* Fujita against *Escherichia coli*. *Food Science and Nutrition*, 7:2546-2555. <https://doi.org/10.1002/fsn3.1104>
- Wu, Y., Zhao, D., Zhuang, J., Zhang, F., and Xu, C. (2016). Caspase-8 and caspase-9 functioned differently at different stages of the cyclic stretch-induced apoptosis in human periodontal ligament cells. *PLoS One*, 11(12):e0168268. <https://doi.org/10.1371/journal.pone.0168268>
- Zhang, H.W., Hu, J.J., Fu, R.Q., Liu, X., Zhang, Y.H., Li, J., Liu, L., Li, Y.N., Deng, Q., Luo, Q.S., Ouyang, Q., and Gao, N. (2018). Flavonoids inhibit cell proliferation and induce apoptosis and autophagy through downregulation of PI3K γ -mediated PI3K/AKT/mTOR/p70S6K/ULK signaling pathway in human breast cancer cells. *Scientific Reports*, 8:11255. <https://doi.org/10.1038/s41598-018-29308-7>
- Zhao, Z., Wu, Q., Xu, Y., Qin, Y., Pan, R., Meng, Q., and Li, S. (2024). Groenlandicine enhances cisplatin sensitivity in cisplatin-resistant osteosarcoma cells through the BAX/Bcl-2/Caspase-9/Caspase-3 pathway. *Journal of Bone Oncology*, 48:100631. <https://doi.org/10.1016/j.jbo.2024.100631>