CYTOTOXIC EFFECTS OF THE ETHYL ACETATE FRACTION FROM *SCHIMA SUPERBA* STEM BARK IN KG-1 LEUKEMIA CELLS

TÁC DỤNG GÂY ĐỘC TẾ BÀO CỦA PHÂN ĐOẠN ETHYL ACETATE TỪ VỎ THÂN CÂY *SCHIMA SUPERBA* TRÊN TẾ BÀO BẠCH CẦU KG-1

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Abstract - This study aimed to evaluate the cytotoxic activity of different fractions of *Schima superba* stem bark on human leukemia cells. Among the tested fractions, the ethyl acetate fraction (EA) showed the strongest inhibitory effect on cell viability. Flow cytometric analysis revealed that EA treatment induced accumulation of KG-1 cells in the G0/G1 phase, accompanied by a reduction in S and G2/M populations, indicating arrest at the G1/S checkpoint. An increased sub-G1 fraction was also observed, suggesting apoptosis. In addition, EA elevated caspase-3 activity in a time- and concentration-dependent manner. These findings indicate that the cytotoxic effect of the EA fraction of *Schima superba* is associated with G1/S cell cycle arrest and caspase-dependent apoptosis, supporting further phytochemical and mechanistic investigations of its anticancer activity.

Key words - *Schima superba;* stem bark; human leukemia cells; anticancer; apoptosis

Tóm tắt - Nghiên cứu này đánh giá hoạt tính gây độc tế bào của các phân đoạn chiết xuất từ vỏ thân cây Vối răng cưa *Schima superba* trên các dòng tế bào bạch cầu người. Trong các phân đoạn khảo sát, phân đoạn ethyl acetate (EA) thể hiện hoạt tính mạnh nhất trong việc ức chế khả năng sống của tế bào. Phân tích tế bào dòng chảy cho thấy EA gây bắt giữ chu kỳ tế bào ở pha G0/G1, làm giảm tỷ lệ tế bào ở các pha S và G2/M, đồng thời làm tăng quần thể sub-G1, cho thấy sự khởi phát quá trình chết theo chương trình (apoptosis). Bên cạnh đó, EA làm tăng hoạt tính caspase-3 theo cả thời gian và nồng độ. Các kết quả này cho thấy tác dụng gây độc tế bào của phân đoạn EA liên quan đến việc ngừng chu kỳ tế bào tại điểm kiểm soát G1/S và kích hoạt apoptosis phụ thuộc caspase, cung cấp cơ sở cho các nghiên cứu tiếp theo về thành phần hóa thực vật và tiềm năng phát triển tác nhân kháng ung thư từ *Schima superba*.

Từ khóa - Vối thuốc; vỏ thân cây; tế bào bạch cầu người; chống ung thư; quá trình chết theo chương trình

1. Introduction

Schima superba Gardn. et Champ., commonly known as "Vối răng cưa" in Vietnamese, is an evergreen tree species of the family Theaceae. It is widely distributed throughout tropical and subtropical regions of Asia, particularly in southern China, northern Vietnam, and other parts of Southeast Asia, where it commonly occurs in evergreen broad-leaved forests at elevations ranging from 100 to 1,200 m [1]. Morphologically, *S. superba* is a medium- to large-sized tree, typically reaching 15-30 m in height with a trunk diameter of up to 1 m [1, 2]. In traditional Chinese medicine, *S. superba* has been used for heat-clearing, detoxification, and the treatment of furuncles, and has also been applied as an insecticidal agent [1]. The root and stem bark are traditionally employed to dispel wind-dampness, relieve pain, induce emesis, and promote diuresis for edema management [2].

From a phytochemical perspective, *S. superba* represents a rich source of bioactive secondary metabolites, notably polyphenols, flavonoids, and triterpenoid saponins [3]. Numerous oleanane-type saponins, including schisusaponins A-H and related derivatives, have been isolated from the root bark, with their structures elucidated using advanced spectroscopic techniques. Several triterpenoid saponins have demonstrated cytotoxic activity against B16 melanoma cells, highlighting their potential as lead compounds for anticancer drug development [3]. In addition, extracts from the stem bark of *S. superba* have been reported to exhibit antifungal activity against *Candida albicans*, which correlates with their high total polyphenol and saponin contents (256.6 ± 5.1 µg/mg and 357.8 ± 31.5 µg/mg, respectively). Among these constituents, sasanquasaponin III was identified as a major contributor to the observed antifungal activity [4]. Lignans isolated from the stem of *S. superba* showed cell growth inhibitory activity against HeLa, CNE, HepG-2, and HEp-2 cell lines [5]. Triterpenoid saponins were found in the leaves of *S. superba* [6]. Beyond its medicinal relevance, *S. superba* has also attracted attention from ecological and forestry perspectives [7].

Given the reported diversity of phytochemical constituents and biological activities of *Schima superba*,

further investigation of its cytotoxic potential remains of interest. Although several studies have described certain pharmacological properties of this species, systematic evaluation of the cytotoxic activity of its extracts and solvent fractions, particularly against leukemia cells, is still limited [8]. In particular, information regarding their effects on the KG-1 leukemia cell line has not been clearly documented. Therefore, the present study aimed to evaluate the cytotoxic activity of different fractions obtained from *S. superba* stem bark and to provide experimental evidence that may contribute to understanding its potential as a natural source of anticancer agents.

2. Materials and Methods

2.1. Plant material and extraction

Stem bark of *Schima superba* was collected from the mountainous region of Lao Bao District, Quang Tri Province, Vietnam in October 2024. The plant specimen was identified by Dr. Tuan Anh Le of the Vietnam Academy of Science and Technology (VAST). The collected samples are currently stored in the Laboratory of the Department of Pharmacognosy and Drug Control, Faculty of Pharmacy, The University of Danang - School of Medicine and Pharmacy. The dried material was extracted using a combination of solid-liquid maceration and liquid-liquid partitioning. Briefly, dried stem bark (0.5 kg) was macerated in 96% ethanol (2 L) at room temperature for 7 days. The extraction was repeated three times. After each cycle, the extract was filtered, and the filtrates were pooled. The combined ethanolic extracts were concentrated under reduced pressure using a rotary evaporator to obtain the crude ethanolic extract. The crude extract (16.5 g) was re-dissolved in distilled water (2 L) and sequentially partitioned with *n*-hexane (2 L, 3 times), dichloromethane (2 L, 3 times), ethyl acetate (2 L, 3 times), and water residue. The organic layers were collected separately and evaporated under reduced pressure to yield the corresponding n-hexane (2.75 g), dichloromethane (4.82 g), ethyl acetate (4.3 g), and water residue fractions.

2.2. Chemicals and equipment

Organic solvents used for extraction included ethanol, n-hexane, dichloromethane, and ethyl acetate (analytical grade). Reagents for biological assays included RPMI, DMEM, PBS, and FBS (Gibco, USA), dimethyl sulfoxide (DMSO, Sigma, USA); trichloroacetic acid (TCA), Tris base, EDTA, Triton X-100, HEPES, dithiothreitol (DTT), sucrose, CHAPS, bovine serum albumin (BSA); propidium iodide (PI), Annexin-V-FLUOS (BD Biosciences), protease inhibitor cocktail (Roche), and the Bio-Rad DC Protein Assay kit (Bio-Rad, USA). Cell culture was performed in DMEM or MEM supplemented with L-glutamine, sodium pyruvate, $NaHCO_3$, penicillin/ streptomycin, and 10% FBS. Laboratory equipment included a CO_2 incubator, biosafety cabinet, centrifuge, inverted phase-contrast microscope, microplate reader (BioTek), fluorescence spectrophotometer (Twinkle LB970), flow cytometer (BD Accuri C6, FACSCalibur), -80°C freezer, liquid nitrogen storage system, analytical balance, and standard cell culture plasticware.

2.3. Cell lines

The human leukemia cell lines HL-60 and KG-1, together with the human embryonic kidney cell line HEK293, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.4. In vitro cytotoxicity assay

Cell proliferation was evaluated using a commercially available Dojindo Cell Counting Kit following a modified protocol. Briefly, viable cancer cells were suspended in growth medium and seeded into 96-well microplates at a density of 1×10^4 cells per well in a total volume of 95 µL. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 to allow cell growth and stabilization. The test samples were initially dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted with culture medium to obtain working concentrations ranging from 1 to 500 µg/mL. Each concentration was tested in triplicate. The final concentration of DMSO in all wells, including the control group, was maintained below 0.1%. After pre-incubation period of approximately 4 h, 5 µL of the prepared sample solutions were added to the wells containing cells. Control wells received an equivalent volume of culture medium containing 0.1% DMSO. The plates were then returned to the incubator and maintained for 48 h. Following the incubation period, 10 µL of Dojindo reagent was added to each well. The plates were further incubated for 4 h to allow color development [9]. The absorbance of each well was then measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was calculated relative to the vehicle-treated control, and the IC_{50} value was defined as the concentration of the tested fractions and positive control that reduced the absorbance by 50% compared with the blank group [9,10].

2.5. Caspase-3 activity assay

Caspase-3 activation was determined using a fluorometric assay based on cleavage of the Ac-DEVD-AFC substrate. KG-1 cells (1×10^6 cells/well) were treated with EA (10, 20, and 40 µg/mL) for 12, 24, and 48 h. Cells were harvested, washed with PBS, and lysed. Reaction buffer and fluorogenic substrate were added, and samples were incubated at 37°C for 1 h. Fluorescence was measured at 370 nm (excitation) and 505 nm (emission) [10]. Caspase-3 activity was expressed as the fold increase relative to untreated control cells. Data were analyzed using GraphPad Prism.

2.6. Flow cytometric analysis

Cell cycle distribution was analyzed by flow cytometry based on DNA content measurement. KG-1 cells were treated with the EA fraction (20 µg/mL) for 24 h, harvested, washed, and fixed in 70% ethanol at 4°C overnight. After washing, cells were treated with RNase A and stained with propidium iodide (PI). DNA content was analyzed using a BD Accuri C6 flow cytometer. The percentages of cells in G0/G1, S, and G2/M phases were determined using FlowJo software. Changes in cell-cycle distribution were compared between treated and control groups to evaluate cell cycle arrest [11].

2.7. Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's test was performed on pre-validated data. Additionally, statistical analysis was conducted using Prism (GraphPad Software, San Diego, CA, USA) with a t-test for comparisons between two groups or ANOVA followed by Bonferroni post-hoc analysis for multiple group comparisons and correlation analysis. Data were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Cytotoxic activity screening

Table 1. *Cytotoxic activity of fractions from S. superba*

Fractions	Cells, IC_{50} (μg/mL)[a]		
	HL-60	KG-1	HEK293
Hx	> 300	> 300	> 300
MD	89.5 ± 6.4	52.7 ± 5.3	> 300
EA	76.8 ± 4.3	22.5 ± 3.1	> 300
Water	112.5 ± 8.6	48.5 ± 6.4	> 300
Camptothecin[b]	1.8 ± 0.3	0.5 ± 0.1	-

[a] *IC_{50} values were calculated from three independent experiments and are expressed in μg/mL.*

[b] *Camptothecin was used as a positive control, and its IC_{50} values are expressed in μM. Data are presented as mean ± SD within acceptable experimental variation. (–): no test.*

Following extraction, four fractions were obtained from the stem bark of *S. superba*, including the *n*-hexane, dichloromethane, ethyl acetate, and the remaining aqueous fraction (Table 1). These fractions were prepared at concentrations ranging from 1 to 500 μg/mL and applied to cultured HL-60 and KG-1 leukemia cells, and the non-cancerous HEK293 cell line (immortalized human embryonic kidney cells). In Table 1, the *n*-hexane (Hx) fraction did not exhibit cytotoxic activity in any of the tested cell lines (IC_{50} > 300 μg/mL), suggesting that non-polar constituents are unlikely to contribute substantially to the observed anticancer effects. In contrast, the dichloromethane (MD), ethyl acetate (EA), and aqueous fractions showed varying degrees of growth inhibition in both leukemia cell lines. In HL-60 cells, the EA fraction displayed the lowest IC_{50} value (76.8 ± 4.3 μg/mL), followed by the MD fraction (89.5 ± 6.4 μg/mL) and the aqueous fraction (112.5 ± 8.6 μg/mL). However, these values indicate moderate cytotoxic activity according to the NCI criteria for crude extracts. KG-1 cells exhibited greater sensitivity to the tested fractions. The EA fraction demonstrated the strongest activity (IC_{50} = 22.5 ± 3.1 μg/mL), while the aqueous (48.5 ± 6.4 μg/mL) and MD fractions (52.7 ± 5.3 μg/mL) also showed measurable inhibitory effects. These findings suggest differential responsiveness between the two leukemia cell lines and indicate that KG-1 cells are more susceptible to the bioactive constituents present in the semi-polar and polar fractions. Importantly, none of the active fractions exhibited significant cytotoxicity toward HEK293 cells (IC_{50} > 300 μg/mL), indicating a degree of selectivity toward malignant cells under the experimental conditions. Camptothecin, used as a positive control, showed potent cytotoxicity in both HL-60 (IC_{50} = 1.8 ± 0.3 μM) and KG-1 cells (0.5 ± 0.1 μM), confirming the validity of the assay system. Consequently, among the tested fractions, EA fraction demonstrated the most potent cytotoxic activity, particularly against KG-1 cells, and may warrant further mechanistic investigation.

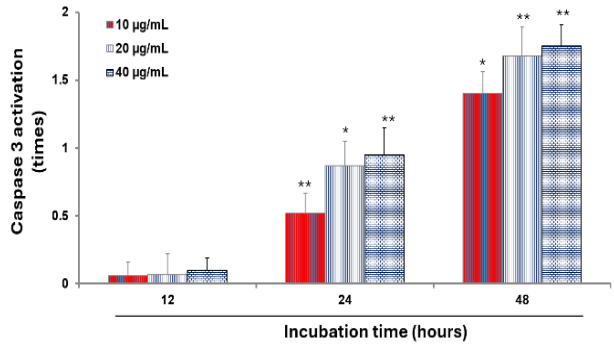

Figure 1. *Caspase-3 activation in KG-1 cells. KG-1 cells were treated with EA fractions (10, 20, and 40 μg/mL) for 12-48 h. The control group was treated with 0.1% DMSO. Data are expressed as the mean ± standard deviation (SD) of three independent experiments (*p < 0.05; **p < 0.01)*

3.2. Caspase-3 activation analysis

In the next study, the EA fraction was selected for further investigation of caspase-3 activation in KG-1 cells. The results in Figure 1 indicate that the EA fraction increased caspase-3 activity in a time- and concentration-dependent manner. At 12–24 h, caspase-3 activity remained low or showed only marginal changes. A more apparent increase was observed after 48 h, reaching approximately 1.4-1.7-fold relative to the control. This finding suggests that caspase-3 activation was limited during the early exposure period. However, caspase-3 activity further increased across all concentrations at 48 h. The 10 μg/mL treatment resulted in approximately 1.4-fold activation, whereas 20 and 40 μg/mL produced increases of approximately 1.6-1.7-fold compared with the control, with statistical significance. The progressive elevation in enzyme activity over time and with increasing concentration supports a concentration- and time-dependent effect of the EA fraction. These findings suggest that the cytotoxic activity of the EA may be associated with the induction of apoptosis via caspase-3 activation, providing mechanistic evidence consistent with its observed antiproliferative effects.

3.3. Cell cycle arrest results

Cell cycle distribution of KG-1 leukemia cells was analyzed by propidium iodide staining and flow cytometry after 24 h of incubation. In the control group, the majority of cells were distributed in the G0/G1 phase (47.05%), followed by the S phase (21.60%) and G2/M phase (23.10%), while the sub-G1 population accounted for 1.85% (Figure 2A). This distribution is consistent with a normally proliferating leukemia cell population under standard culture conditions, with only a limited proportion of cells undergoing spontaneous cell death. These values were used as a baseline for comparison with treated cells.

Treatment of KG-1 cells with the EA fraction (20 µg/mL) for 24 h resulted in moderate alterations in cell cycle distribution (Figure 2B). The proportion of cells in the G0/G1 phase increased from 47.05% to 50.40%, whereas slight reductions were observed in the S and G2/M populations. In parallel, the sub-G1 fraction increased from 1.85% to 3.30%. These changes suggest that the EA fraction affects cell cycle progression primarily at the G1/S transition, leading to partial accumulation of cells in the G0/G1 phase and a reduction in the proportion of cells entering DNA synthesis and mitosis. No apparent accumulation was observed in the G2/M phase, indicating that the effect was more closely associated with early-stage cell cycle regulation rather than mitotic arrest. The G1/S checkpoint is regulated by cyclin-cyclin-dependent kinase (CDK) complexes, including cyclin D/CDK4/6 and cyclin E/CDK2, together with cyclin-dependent kinase inhibitors that modulate cell cycle progression under conditions of cellular stress or DNA damage [12-14]. Although these regulatory proteins were not examined in the present study, the observed G0/G1 accumulation is consistent with cell cycle responses previously reported for several plant-derived compounds [14, 15]. In addition to changes in cell cycle distribution, the increase in the sub-G1 population suggests the presence of cells with reduced DNA content, a feature commonly associated with apoptotic DNA fragmentation [16]. This observation is in agreement with the caspase-3 activation assay, in which increased enzyme activity became more evident after prolonged exposure to the EA fraction.

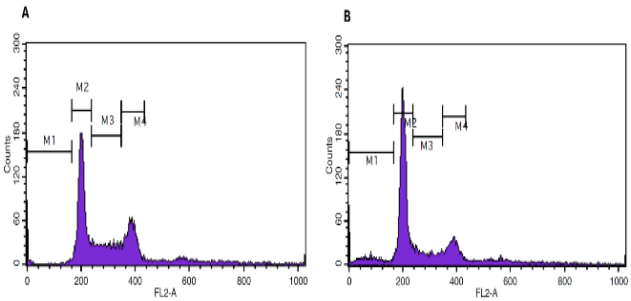

Figure 2. *Cell cycle distribution analysis of KG-1 cells. (A) Vehicle control group (treated with 0.1% DMSO) and (B) KG-1 cells treated with the EA fraction (20 µg/mL) for 24 h. Cells were harvested and stained with propidium iodide (PI) for 30 min at 37 °C before flow cytometric analysis to determine the percentage of cells in each phase*

Although the proportion of sub-G1 cells was relatively limited, this observation was consistent with the results obtained from the caspase-3 activation assay. Caspase-3 activity increased in both a concentration- and time-dependent manner following EA treatment, with higher activity detected after 24 and 48 h. Caspase-3 functions as a key executioner enzyme in the caspase cascade and mediates cleavage of multiple cellular substrates during apoptosis [17, 18]. Activation of caspase-3 therefore provides biochemical evidence supporting the occurrence of programmed cell death in EA-treated KG-1 cells. Similar interactions between cell cycle arrest and apoptosis have been widely reported as a common mechanism underlying the antiproliferative effects of many natural compounds [18, 19]. The present findings provide preliminary evidence that the cytotoxic activity of the EA fraction may involve modulation of cell cycle progression and apoptosis-related pathways, supporting further studies to identify its bioactive constituents and underlying molecular mechanisms.

Despite these findings, some limitations should be considered. The cytotoxic activity of the EA fraction was evaluated primarily in a single leukemia cell model, and additional hematological and solid tumor cell lines are required to confirm the broader antiproliferative potential of the extract. Although cell cycle modulation and caspase-3 activation were observed, the underlying molecular mechanisms were not comprehensively investigated, and key regulatory proteins involved in G1/S progression and apoptosis were not directly examined. In addition, the bioactive constituents and the *in vivo* efficacy and safety evaluations are necessary to further assess the pharmacological relevance and therapeutic potential of the extract.

4. Conclusion

In conclusion, the present study demonstrates that extracts of *Schima superba* exhibit cytotoxic activity against KG-1 leukemia cells, with the EA fraction showing the most pronounced effect among the tested fractions. The EA fraction displayed a significant inhibitory effect on cell viability indicating measurable cytotoxic potency against KG-1 cells. Flow cytometric analysis revealed that EA treatment induced accumulation of cells in the G0/G1 phase, accompanied by a reduction in S and G2/M phase populations, suggesting disruption of cell cycle progression at the G1/S checkpoint. An increase in the sub-G1 fraction further indicated the occurrence of DNA fragmentation. Consistently, EA enhanced caspase-3 activity in a time- and concentration-dependent manner, supporting the involvement of caspase-dependent apoptosis. Collectively, these findings suggest that EA fraction induced a moderate increase in the G0/G1 population and sub-G1 fraction, suggesting possible modulation of cell-cycle progression and apoptosis-related events.

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