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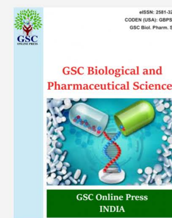

(Research Article)

Therapeutic potential of doxorubicin and *Evodia suaveolens* leaves extract in targeting cell cycle arrest and proliferation on acute myeloid leukemia *in vitro*

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Abstract

Acute myeloid leukemia (AML) is a hematopoietic malignancy characterized by uncontrolled proliferation of myeloid progenitor cells in the bone marrow. The standard chemotherapy in treating AML cases is anthracycline, including doxorubicin; however, it could cause severe side effects if administered continuously at high doses, making it less tolerable for some patients. *Evodia suaveolens*, a herbal medicine, is known to contain active compounds and anti-cancer activities, which could inhibit cancer cell proliferation. This study aims to determine the combined effect of doxorubicin and *Evodia suaveolens* on inhibiting the G2/M cell cycle phase and proliferation in AML cells. This experimental study used HL-60 cells which were divided into six treatment groups: K- (control), K+ (doxorubicin 0.2 µg/mL), D1-D3 (combination of doxorubicin 0.2 µg/mL and *Evodia suaveolens* leaf extract with concentrations of 0.2, 0.4, 0.8 mg/mL) and D4 (*Evodia suaveolens* leaf extract 0.8 mg/mL). The findings indicated that the G2/M phase of the cell cycle was most effectively inhibited at the D3 dose (doxorubicin at 0.2 µg/mL combined with *Evodia suaveolens* leaf extract at 0.8 mg/mL), which also significantly reduced cell proliferation. Consequently, this study concludes that the combination of doxorubicin and *Evodia suaveolens* effectively inhibits the G2/M cell cycle phase and proliferation in AML cells.

Keywords: Anticancer; *Evodia suaveolens*; Acute myeloid leukemia; HL-60; Cell cycle; Proliferation

1. Introduction

Leukemia arises from the excessive growth of abnormal leukocytes in the bone marrow, often due to various factors including genetic aberrations. These neoplastic changes halt the maturation of stem cells during the hematopoietic stages, leading to uncontrolled proliferation of immature leukocytes [1]. Excessive leukocytes would disrupt the functioning of other blood cells, causing various deleterious clinical manifestations [2].

Acute myeloid leukemia (AML) is distinguished by the abnormal proliferation of myeloid progenitor cells in the bone marrow. Mutations, commonly observed in AML cases, can alter the cell cycle by affecting various regulatory proteins, leading to excessive proliferation of cells and immortalization by activating mitogenic signals relentlessly [3]. According to Global Cancer Statistics (GLOBOCAN), leukemia ranks as the ninth most prevalent cancer in Indonesia, with AML

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being the most common type found in adults [4]. The prognosis for AML varies and is influenced by factors such as age, comorbidities, and patient-specific cytogenetics [5].

The five-year survival rate of AML patients is only about 30%, categorizing it as having a dismal prognosis [6]. The standard treatment regimen typically involves a combination of chemotherapy drugs, such as cytarabine, anthracycline (such as daunorubicin, doxorubicin), and etoposide with or without radiotherapy [7]. However, prognosis remains poor in patients over 65 years old, who are also the group predominantly affected by AML. This is primarily due to the prevalence of comorbidities in this age group and the low tolerability to the chemotherapeutics agent [8].

One of the indigenous Indonesian plants with potential anticancer properties is *Evodia suaveolens* The content of secondary metabolites in these plants exhibits anticancer activity by inhibiting proliferation, invasion, and metastasis and inducing apoptosis in various types of tumor cells [9]. However, there has been no research exploring the combination of standard therapy and administration of *Evodia suaveolens* leaf extract in treating leukemia cells. Therefore, the purpose of this study is to investigate the effects of combining doxorubicin and *Evodia suaveolens* leaf extract on cell cycle inhibition and proliferation in AML cells.

2. Material and methods

2.1. Materials and reagents

The materials and reagents utilized in this study included *Evodia suaveolens* leaves, 96% ethanol, HL-60 cell line (CCL-240, ATCC), phosphate-buffered saline (PBS), RPMI 1640 media, CO_2, penicillin, streptomycin, doxorubicin, 96% ethanol extract of *Evodia suaveolens* leaves, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution, dimethyl sulfoxide (DMSO) solution, RNase, and propidium iodide (PI) solutions.

2.2. Methods

2.2.1. Ethanolic extract preparation of Evodia suaveolens

Evodia suaveolens leaves were harvested and washed, aerated for three days, and then oven-dried for two to three days at 45 °C. The dried leaves were pulverized into a powder using a blender and sieved with a 40-mesh sieve. Approximately, 1000 grams of *Evodia suaveolens* leaf simplisia was dissolved in 3 liters of 96 % ethanol, ensuring all samples were submerged and soaked for 72 hours. The maceration product was filtered using a tea strainer to separate the filtrate from the residue. The filtrate, which was a dark green color, was then concentrated to a thick ethanol extract (paste) using a rotary evaporator at 40 °C.

2.2.2. HL-60 cell culture and treatment

The human acute myeloid leukemia (HL-60) cell line was aseptically cultured in RPMI 1640 media supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, incubated at 37 °C in a 5 % CO_2 atmosphere. Culturing continued until the cells reached confluence.

Confluent HL-60 cells were counted using trypan blue and seeded at densities ranging from 5×10^5–1×10^6 cells per well plate. Treatment groups were as follows: K- (no treatment), K+ (doxorubicin (DOX) 0.2 μg/mL), D1-D3 (combinations of DOX 0.2 μg/mL with *Evodia suaveolens* leaf extract at 0.2, 0.4, and 0.8 mg/mL, respectively), and D4 (*Evodia suaveolens* leaf extract 0.8 mg/mL only).

2.2.3. Cell cycle detection with flowcytometry

Treated HL-60 cells were seeded in 24-well plates and incubated for 24 hours. After incubation, the cells were then harvested and each placed in a 1.5 ml eppendorf tube, then centrifuged at 1500 rpm for 5 minutes at 4 °C. The supernatant was discarded, and the cells were fixed with 300 μL of cold 70 % ethanol for one hour on ice. After washing with 800 μL of cold PBS and re-centrifuging at 3000 rpm for 3 minutes at 4 °C, PBS was discarded, and 40 μL of RNase was added and incubated for 40 minutes. Following another wash and centrifuge, 500 μL of 25 μg/mL PI solution was added, and the cells were incubated in the dark at 4 °C for 30-60 minutes before analysis with a flow cytometer.

2.2.4. Cell proliferation analysis

HL-60 cells seeded in 96-well plates were incubated for 24 and 48 hours for proliferation assays with MTT. Post incubation, cells were observed under an inverted microscope. MTT solution at a concentration of 100 μg was added, homogenized, and incubated for 4 hours. Formazan crystal formation in the well plates was observed. Cells were

homogenized using a micropipette, transferred to 1.5 mL Eppendorf tubes, and centrifuged at 1500 rpm for 5 minutes at room temperature. The supernatant was discarded, 80 µL of DMSO added, and incubated for 30 minutes at 37 °C. Following final centrifugation at 1500 rpm for 5 minutes RT, the supernatant was transferred to a 96-well plate for absorbance reading at 630 nm, and the cell growth percentage was calculated with the following formula.

$$Cell\,Viability\;(\%) = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100\%$$

2.3. Statistical analysis

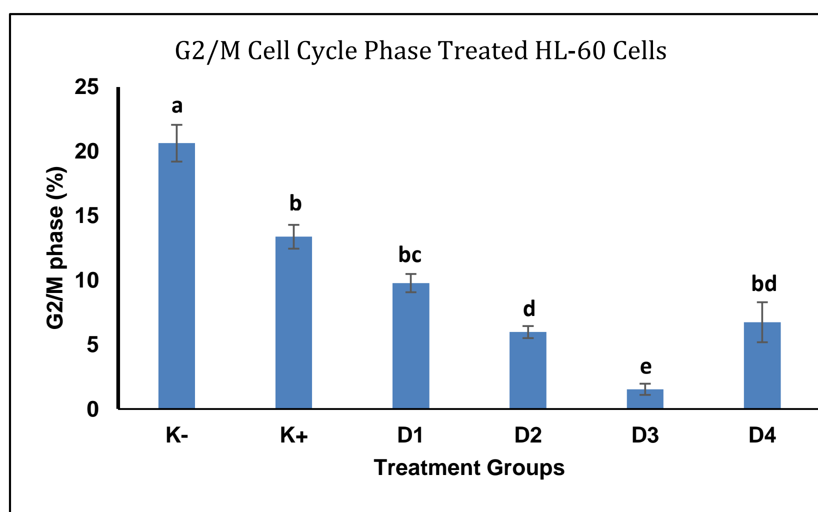
Data are reported as the mean ± standard deviation (SD) and were analyzed using one-way analysis of variance (ANOVA) and two-way ANOVA followed by the post-hoc Tukey's HSD test, utilizing IBM SPSS software version 25.0. A p-value of less than 0.05 was considered to indicate statistically significant differences.

3. Results and discussion

3.1. *Evodia suaveolens* leaf extract induced cell cycle arrest in Acute Myeloid Leukemia cell

Cell cycle analysis in this study was conducted using flow cytometry to obtain quantitative data. Each group was tested in triplicate. There were significant differences among the six treatment groups ($p < 0.001$, $p < 0.05$). The post-hoc tests revealed a significant difference in the percentage of cells in the G2/M phase between the control group (K-) and each treatment group, as denoted by different letter notations. Specifically, the D3 (combinations of DOX 0.2 µg/mL with *Evodia suaveolens* extract at 0.8 mg/mL) group exhibited a significantly different response compared to other treatment groups. However, no significant differences were observed in the percentage of G2/M phase between the positive control group receiving standard therapy (DOX, K+) and the D1 and D4 groups, as well as between the D2 and D4 groups.

According to the results presented in **Figure 1**, the highest percentage of cells in the G2/M phase was observed in the negative control group (K-), which consisted of untreated HL-60 cells. Conversely, the lowest percentage of G2/M phase cells was found in group D3, where HL-60 cells received a combination of standard therapy with doxorubicin and the highest dose of *Evodia suaveolens* extract. The results for standard therapy with doxorubicin were lower than those of the negative control (K-) but higher than those observed in group D3.



Note: The G2/M cell cycle phase of HL-60 cells was analysed with flow cytometry after the 24-hour incubation period, each tested in triplicate. K- (without treatment), K+ (doxorubicin 0.2 µg/Ml [DOX] only), D1 (DOX + 0.2 mg/mL *Evodia suaveolens* leaf extract), D2 (DOX + 0.4 mg/mL *Evodia suaveolens* leaf extract), D3 (DOX + 0.8 mg/mL *Evodia suaveolens* leaf extract) dan D4 (0.8 mg/mL *Evodia suaveolens* leaf extract only). Vertical lines indicated standard deviation (SD). Different letter or alphabetical notations indicated a statistically significant difference ($p < 0.05$) between treatment groups.

Figure 1 G2/M cell cycle phase in HL-60 cells

The cell cycle encompasses a series of events that occur within a cell, leading to its division and the formation of two daughter cells. In eukaryotic cells, this cycle typically consists of four phases: G1 (Growth 1), S (DNA synthesis), G2 (Growth 2), and M (Mitosis) phase [10]. This study focused on the G2/M phase, specifically on inhibiting the transition from the G2 phase to the dividing M phase. In this present study, it could be observed that the cell cycle was blocked at

the G2/M stage after the combination treatment of doxorubicin and *Evodia suaveolens* extract, with the effect increasing in a dose-dependent manner. This result was in concordance with the inhibition of cell proliferation that would be explained next.

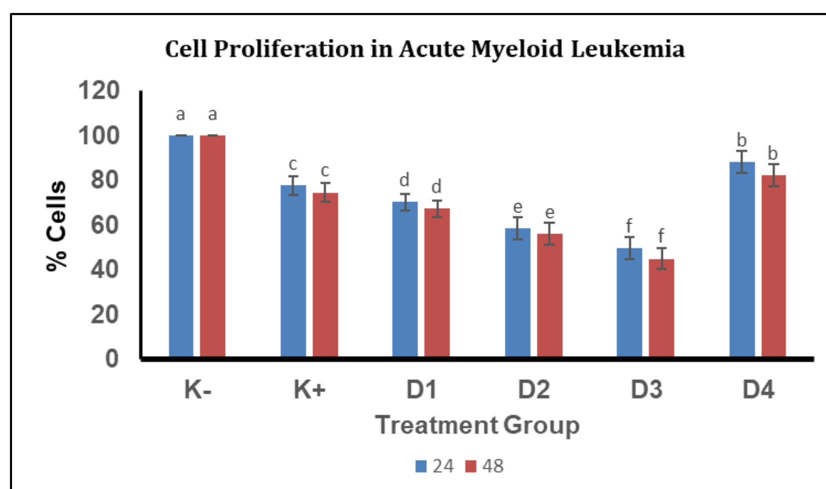
The cell cycle involves a series of processes that engage numerous proteins. Each phase features checkpoints that ensure the requirements are met to progress to the next phase [11]. Among the proteins influencing the G2/M phase are Cyclin B1, CDK1 (Cyclin Dependent Kinase 1) [12], and MAP4 (Microtubule-Associated Protein 4). Cyclin B1 and CDK1 binds together to halt cell division in the G2 phase before initiating the transition to the M phase. Once in the M phase, Cyclin B1 and CDK1 begin to degrade, and inhibiting their binding can cause cell cycle arrest at the G2/M phase [13].

Additionally, the effect of G2/M blocking might be attributable to other regulatory protein of cell cycle, such as MAP4. Previous study showed that MAP4 (*microtubule-associated protein 4*) played a crucial role in G2/M cycle arrest by stabilizing microtubules essential for cell division, making it impossible for mitotic spindle disruption to occur; thus, the cell cycle halt at the metaphase-anaphase transition [14]. Disruption of MAP4 function will cause abnormalities in cell division by halting the cell cycle and eventually inhibiting proliferation. As expected, in cancer research, targeting MAP4 to inhibit its function can restrict cancer cell growth by disrupting microtubule dynamics [15,16]. These interesting notions of regulatory protein in cell cycle in regards of *Evodia suaveolens* anticancer activity would warrant further investigation.

3.2. *Evodia suaveolens* leaf extract inhibited Acute Myeloid Leukemia cell proliferation

The results of the cell proliferation study with MTT assay indicated that the combination of doxorubicin and *Evodia suaveolens* leaf extract led to a progressive decrease in cell proliferation with each increasing time of incubation. This trend was found to be similar to the cell cycle result previously explained. Statistical analysis using two-way ANOVA revealed significant differences across various doses of *Evodia suaveolens* extract and incubation times ($p<0.001$). Additionally, there was a significant interaction between incubation time and extract dose ($p=0.012$). A multiple correlation test confirmed this interaction, demonstrating a moderate correlation ($R = 0.505$) with an F-value of 0.001, indicating statistical significance ($F < 0.05$).

Cell proliferation measurements, as illustrated in **Figure 2,** showed the highest proliferation in the negative control group (K-). The most substantial inhibition of proliferation occurred in D3, which used a combination of doxorubicin and the highest *Evodia suaveolens* extract dose of 0.8 mg/ml. Standard therapy with doxorubicin inhibited proliferation more significantly than the negative control (K-), but to a lesser extent than the combination treatment with *Evodia suaveolens* leaf extract.



Note: Proliferation of HL-60 cells was measured with MTT assay after 24 and 48-hour incubation periods, each tested in triplicate. K- (without treatment), K+ (doxorubicin 0.2 µg/Ml [DOX] only), D1 (DOX + 0.2 mg/mL Evodia suaveolens leaf extract), D2 (DOX + 0.4 mg/mL *Evodia suaveolens* leaf extract), D3 (DOX + 0.8 mg/mL *Evodia suaveolens* leaf extract) dan D4 (0.8 mg/mL *Evodia suaveolens* leaf extract only). Vertical lines indicated standard deviation (SD). Different letter or alphabetical notations indicated a statistically significant difference ($p < 0.05$) between treatment groups.

Figure 2 Proliferation results of HL-60 cells

As **Figure 2** depicted, there was a trend of decreasing cell proliferation with increasing doses of *Evodia suaveolens* leaf extract and prolonged incubation times. Similar to the cell cycle results, this trend suggests that arresting the cell cycle

in cancer cells would ultimately halt their proliferation. This is a potential therapeutic target because cancer cells will continue to divide abnormally, so stopping proliferation is needed as one of the therapeutic targets [17,18]. This study shows that the combination of doxorubicin and *Evodia suaveolens* extract has the potential to be a complementary therapy in the treatment of AML.

Previous studies on *Evodia suaveolens* leaf, showed that this indigenous plant of Indonesia possesses multiple active compounds with anticancer properties, including evodiamine, linalool, lanosterol, and obtusifoliol [19,20]. These compounds have been extensively researched for their anticancer, antiproliferative, and apoptosis-inducing activities [21]. They act by binding to specific proteins to inhibit the abnormal growth occurring in cancer cells [9,22].

In addition to the combination of doxorubicin and *Evodia suaveolens* extract, treatment was also administered using doxorubicin alone and *Evodia suaveolens* extract alone. The results indicated that single therapy (either doxorubicin or extract alone) alone had an effect in inhibiting both cell cycle and proliferation. This suggests that doxorubicin and *Evodia suaveolens* extract may synergistically inhibit the growth of acute myeloid leukemia cells.

Limitations of the study

This present study is the first to investigate the antileukemic effects of *Evodia suaveolens* leaf extract in AML. However, it has several limitations. The study was conducted using an *in vitro* approach on a single cell line, which may not adequately represent the heterogeneous cytogenetic landscape of AML. Furthermore, additional research is encouraged to evaluate the specific inhibition markers that contribute to cell cycle arrest and proliferation of AML cells, which may not have been covered in this study.

4. Conclusion

Combination therapy of doxorubicin and *Evodia suaveolens* leaf extract demonstrated significant efficacy in halting the cell cycle and causing inhibition of cell proliferation. This finding suggests that *Evodia suaveolens* leaf extract could be a potential candidate for anticancer therapy in AML. Future research should focus on investigating additional proteins or molecular pathways that may influence the interruption of the cell cycle and proliferation.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflicts of interest related to the publication of this study.

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