

Original Article

Anti-leukemic effects of *Vernonia amygdalina* extract

Efeitos antileucêmicos do extrato de *Vernonia amygdalina*

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Abstract

This study aimed to investigate the impact of *Vernonia amygdalina* leaf extract on FLT3 regulation. *V. amygdalina* was extracted with 96% ethanol (VAE-96), and its cytotoxicity against FLT3- cell lines (MOLM-13 and MV-4-11) was evaluated. The results indicated that VAE-96 induced apoptosis in these cells and inhibited the phosphorylation of AKT, MAPK, and FLT3. Additionally, VAE-96 substantially diminished the activity of the *FLT3* promoter and the expression of *FLT3* mRNA. The extract was found to contain alkaloids, saponin, reduced sugar compounds, and polyphenols, including tannins and flavonoids, as per the phytoconstituents analysis. The potential of alkaloid fractions on MOLM-13 cells was indicated by the robust cytotoxic effect of the alkaloid fractions, which resulted in over 50% cell mortality at 30 µg/ml. Our results suggest that VAE-96 may be a beneficial agent for the prevention and treatment of AML with FLT3-ITD mutation.

Keywords: *V. amygdalina*, FLT3, leukaemia, apoptosis.

Resumo

Este estudo teve como objetivo investigar o impacto do extrato da folha de *Vernonia amygdalina* na regulação do FLT3. *V. amygdalina* foi extraída com etanol 96% (VAE-96) e sua citotoxicidade contra linhagens celulares FLT3 (MOLM-13 e MV-4-11) foi avaliada. Os resultados indicaram que o VAE-96 induziu apoptose nestas células e inibiu a fosforilação de AKT, MAPK e FLT3. Além disso, o VAE-96 diminuiu substancialmente a atividade do promotor *FLT3* e a expressão do mRNA do *FLT3*. Descobriu-se que o extrato contém alcaloides, saponinas, compostos de açúcar reduzido e polifenóis, incluindo taninos e flavonoides, conforme análise de fitoconstituintes. O potencial das frações alcaloides nas células MOLM-13 foi indicado pelo intenso efeito citotóxico das frações alcaloides, que resultou em mais de 50% de mortalidade celular a 30 µg/ml. Nossos resultados sugerem que o VAE-96 pode ser um agente benéfico para a prevenção e tratamento da LMA com mutação FLT3-ITD.

Palavras-chave: *V. amygdalina*, FLT3, leucemia, apoptose.

1. Introduction

As a member of subclass III of the receptor tyrosine kinase family, FLT3 (FMS-like tyrosine kinase 3) is essential for myeloid cell proliferation, differentiation, and apoptosis (Abu-Duhier et al., 2001). This gene's mutations can result in uncontrolled cell growth and division, causing the onset of acute myeloid leukaemia (AML) (Stirewalt et al., 2001). Compared to AML without the FLT3 mutation, AML with mutagenesis is associated with a poor prognosis and a higher risk of relapse (Ravandi et al., 2010). FLT3 mutations can be further classified into internal tandem duplication (ITD) and point mutations in the domain of tyrosine kinase (TKD). FLT3-ITD mutation patients typically have a poorer prognosis than FLT3-TKD mutation patients (Stirewalt et al., 2001). However, as of 2022, the World Health Organisation clarified that *FLT3-ITD*-associated AML is classified as

intermediate-risk, regardless of the *NPM1* mutation status (Chaer et al., 2023; Levis et al., 2011; Ravandi et al., 2010).

Treatment for AML with the *FLT3* mutation often involves intensive chemotherapy to achieve remission, followed by consolidation therapy, which can include stem cell transplantation. Moreover, patients with the *FLT3-ITD* mutation have a higher risk of relapse, even with intensive chemotherapy and stem cell transplantation (Kiyoi et al., 2005). Therefore, targeted therapies have been developed to specifically target the *FLT3* mutation in AML cells. More than twenty types of FLT3 inhibitors have been identified and studied, with some currently undergoing clinical trials and applications. Midostaurin, sorafenib, lestaurtinib, sunitinib, tandutinib, AC220, ABT-869, AKN-032, KW-2449, crenolanib, gilteritinib, and

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Received: May 31, 2024 – Accepted: August 15, 2024



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quizartinib are prominent inhibitors, with midostaurin being the first FDA-approved drug for the treatment of AML (Auclair et al., 2007; Cortes et al., 2019; Kelly et al., 2002; Kindler et al., 2010; Lancet, 2015; O'Farrell et al., 2003; Perl et al., 2019; Pratz et al., 2009; Shankar et al., 2007; Wiernik, 2010). Despite a significant remission following treatment, the incidence of AML relapse remains high (Gebbru and Wang, 2020). Statistics indicate that no more than thirty per cent of patients can survive five years (Gebbru and Wang, 2020).

However, the treatment mentioned above has various undesirable side effects and comes at a high expense to the patient (Crossnohere et al., 2019). Regardless of the impact of the recovery, patients in impoverished and developing countries frequently cannot afford to pay for these medicines in the long term. Instead, to ease the symptoms produced by the progressing disease, patients continually seek out traditional folk medicines that are said to be helpful. Recent studies have examined the potential of specific conventional herbal remedies, including acupuncture, traditional Chinese medicine, green tea catechins, extract from *Euphorbia formosana*, avocatin B, and feverfew isolated from *Tanacetum parthenium*, to alleviate symptoms associated with AML and supplement standard medical treatments (Fleischer et al., 2017; Hsieh et al., 2013; Ly et al., 2013; Tcheng et al., 2017; Yang et al., 2017).

V. amygdalina belongs to the *Vernonia* genus, which is widely used in food and medicine and has over a thousand species (Toyang and Verpoorte, 2013). More than a hundred members of this genus have been identified as being used in curing human diseases (Toyang and Verpoorte, 2013). Many members of the *Vernonia* genus have been shown to have the ability to kill cancer cells, including *Vernonia cinerea*'s activity against HT29 and HepG2 cell lines and *Vernonia condensata*'s inhibitory effect on K562, MCF7, Reh, and Nalm6 (Khay et al., 2012; Thomas et al., 2016). Due to its bitter taste, *V. amygdalina*, also widely recognized as a "bitter leaf" and it is also one of the most researched species in its genus (Oyeyemi et al., 2017). *V. amygdalina* is a perennial plant with a height of 1 m and 6 m, dark green leaves, and rough bark, growing mainly in the tropics (Oyeyemi et al., 2017). *V. amygdalina* is used for many purposes, such as insecticidal, timber, food, fodder, medicinal, etc.. More than 13 bioactive compounds have been found in *V. amygdalina*, and many reports have determined their effects on anti-oxidation and anti-cancer (Oyeyemi et al., 2017). The extract of *V. amygdalina* was reported to have activity against breast cancer cell lines (MCF-7 (IC₅₀ = 50.36 µg/ml), 4T1 (IC₅₀ = 25.04 ± 0.36 µg/ml), T47D (IC₅₀ = 59.19 ± 0.55 µg/ml)) (Wong et al., 2013); brain cancer cell line (U-87 (IC₅₀ = 18.80 ± 1.11 µg/ml)) (Mohd et al., 2016); prostate cancer cell lines (PC-3 (IC₅₀ = 196.60 µg/ml), DU145 (IC₅₀ = 40.10 ± 4.30 µg/ml)) and human myeloid leukaemia cell line (HL-60 (IC₅₀ = 5.58 g/ml)) (Iweala et al., 2015). Although *V. amygdalina* greatly impacted leukaemia, the related studies are still limited.

In our previous paper, we demonstrated that the ethanol extract of *Vernonia amygdalina* (VAE-96) inhibited the growth of AML cells in a dose and time-dependent manner (Nguyen et al., 2022). In this study, we will continue

demonstrating how this extract kills cells using a model of leukaemia cells with FLT3-ITD abnormalities.

2. Materials and Methods

2.1. Plant material

Fresh *V. amygdalina* Del (VA) leaves were collected in September 2017 in Vung Tau Province, Vietnam. Dr. Tuan Le Anh Dang (the University of Science, Vietnam National University in Ho Chi Minh City) made the botanical identification, and a voucher specimen (PHH0004908) was placed in the herbarium of this division (Supplementary Material Fig. S1).

2.2. Crude extract preparation

Fresh VA leaves (10 kg) were washed and air-dried. The ground powder (2 kg) was macerated with 96% ethanol (VAE-96) at room temperature. The chlorophyll was removed from the extract by passing the extract through activated charcoal. Following filtration, the solvent evaporated under reduced pressure until it was scorched; this produced the crude extracts (about 600 g of each). The stock solutions of VAE-96 (200 mg/mL in Dimethyl sulfoxide (DMSO)) were obtained and kept at -20°C until used.

2.3. Total alkaloid and its fractions preparation

The crude VAE-96 (600 g) was extracted with 6 L of 5% tartaric acid under agitation for 1 hour. The acid solution was filtered and adjusted to a pH of 9-10 with 100% (w/w) ammonium hydroxide solution before being partitioned with 50 mL of chloroform until it was negative for Dragendorff's reagent. The total alkaloid fraction (8 g; the efficiency was about 1.3%) was obtained by drying the organic phase with anhydrous sodium sulfate and then concentrating it. The total alkaloids were subjected to liquid-liquid extraction by dissolving in a series of solvent systems, including n-hexane, n-hexane: ethyl acetate (EtOAc), EtOAc: methanol (MeOH), and MeOH (Supplementary Material Fig. S2) to yield nine fractions after thin layer chromatography checking, and these fractions were named VA-1 to VA-9.

2.4. Phytochemical screening

Using Ciulei's method, secondary metabolism compounds were identified (Ciulei et al., 1993). A folin-ciocalteu reagent was used to determine the total polyphenol content (TPC) of VA extracts (Ainsworth and Gillespie, 2007) in accordance with a slightly modified Nunzia et al. (Cicco and Lattanzio, 2011). Briefly, 200 µL extract (20 mg/mL) was mixed with 200 µL of folin-ciocalteu reagent (100%). After 5 minutes at room temperature, 1600 µL of 5% sodium carbonate solution (w/v) were blended. The reaction solution was mixed and incubated at 40°C (20 minutes) before being put into 96-well plates. The results were recorded at an absorbance of 765 nm. The range of 0 to 500 µg/mL of gallic acid was utilized as the standard curve. The amount of TPC present in the

crude extract is denoted in milligrams per gram of gallic acid equivalent (GAE).

2.5. Cell lines and culture conditions

The following cells were used in the cytotoxic study: MOLM-13 cells have FLT3-ITD (a duplication of 21 bp corresponding to codons Phe594-Asp600) on the one allele (Matsuo et al., 1997; Quentmeier et al., 2003; Taketani et al., 2004), and MV4-11 cells harbor a homozygous genotype of FLT3-ITD (Quentmeier et al., 2003; Taketani et al., 2004). MOLM-13 and MV4-11 cells were cultured in the Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich); this medium was supplemented with heat-inactivated fetal bovine serum (10%; Thermofisher Scientific), penicillin (100 IU/mL; Sigma-Aldrich), and streptomycin (0.1 mg/mL; Sigma-Aldrich), and incubated at 37 degrees Celsius with 5% carbon dioxide. The input cell density was set at 10^5 cells/mL.

The 293T cells were used for the luciferase assay. Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 1% sodium pyruvate, 10% fetal bovine serum, and 1% L-glutamine was used to cultivate the cells at 37°C in a humidified incubator containing 5% carbon dioxide.

2.6. Flow cytometry analysis

The MOLM-13 cells were treated with 50 µg/mL VAE for 16 hours, and subsequently, the apoptotic cells were analyzed using 7-aminoactinomycin (7-AAD) (BD PharMingen) and the FACS Calibur instrument. The data obtained was analyzed using FlowJo software provided by Tree Star.

2.7. Western blot analysis

The MOLM-13 and MV4-11 cells were plated at a density of 1×10^5 cells/mL on a 10-cm dish with varying concentrations of VAE-96 (12.5, 25, or 50 µg/mL) or Epigallocatechin gallate (EGCG) (60 µM as positive control). After 8 or 16 h incubation with reagents, the cells were collected and washed twice with PBS (-) (TBR Technology Corporation). A protein lysis buffer (Chaer et al., 2023) was used to lyse the cells in an ice-cold environment. The cell lysates were collected by centrifuging at a speed of 15,000 times the force of gravity for 10 minutes at a temperature of 4°C. 20 µg of protein samples were loaded into wells, separated using polyacrylamide gel electrophoresis (12.5%), and transferred onto a Hybond-P membrane (Amersham; Cytiva) through electroblotting. Afterwards, a 5% skim milk buffer obstructed the membrane for 1 hour at ambient temperature. Antibodies were employed to probe the membrane after washing and antibody binding was detected using enhanced chemiluminescence ECL (Amersham; Cytiva). Flt-3/Flk-2 (S-18, sc-480), ERK1 (sc-93), total Akt (sc-1618) (1:500; Santa Cruz Biotechnology, Inc.); phospho-FLT3 (Tyr591), phospho-p44/42 Map kinase (Thr202/Tyr204), phospho-Akt (Ser473), caspase-3 (9662), caspase-9 (C9), XIAP (1: 1,000; Cell Signaling Technology, Inc.); anti-actin (1: 1,000; cat. no. A2066; Sigma-Aldrich; Merck KGaA), and anti-PARP (1: 1,000; cat. no. 016-16831; FUJIFILM

Wako Pure Chemical Corporation) were used as primary antibodies. The secondary antibodies were incubated at 4 degrees Celsius for 24 hours or at room temperature for 1 hour. After two 15-minute washes, the membranes were incubated at room temperature with a secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hour. This secondary antibody was either anti-mouse IgG HRP (cat. no. sc-2031) or anti-rabbit IgG HRP (1:1,000). (cat. no. sc-2317; both from Santa Cruz Biotechnology, Inc.)

2.8. Semiquantitative reverse transcription-PCR

Total RNA was extracted from MOLM-13, MV4-11 cells treated with or without VAE, and EGCG (60 µM) was used as a positive control, using Sepasol (Nakalai). SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers were used to synthesize first-strand cDNA from 1 µg of total RNA. The FLT3.1675F (5'-GACAACATCTCATTCTATGCAAC-3') and FLT3.18R1 (5'-TCTGAACCTCTTGAACCA-3') primers were used to amplify a region of FLT3 that spans exons 14 and 15. The thermal cycling profile consisted of an initial denaturation at 94°C for two minutes, followed by 40 cycles of denaturation at 94°C for thirty seconds, annealing at 55°C for thirty seconds, and extension at 72°C for one minute. A final extension was performed at 72°C for five minutes. The PCR products were separated by electrophoresis on a 1.5% agarose gel.

2.9. Luciferase reporter assay

The 293T cells were transfected with 100ng of each reporter plasmid and 5ng of a renilla-luciferase plasmid (used as an internal control) in 48-well plates at a density of 1×10^5 cells per well using Lipofectamine according to the manufacturer's instructions to test the activity of VAE-96 on the FLT3 promoter activity. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan), and the promoter activity was characterized using the ratio of Firefly to Renilla luciferase activities.

2.10. Data Analysis

The tests were performed three times for validity. To compute and analyze the data, we used GraphPad Prism 9.0.0. P values less than 0.001 (****), 0.01 (***), 0.10 (**), and 0.5 (*) were considered statistically significant. The data was displayed using a mean and standard deviation format.

3. Results and Discussion

3.1. VAE-96 induced apoptosis

The results showed that MOLM-13 cell death and apoptosis increased after 16 h of exposure to VAE-96 at a concentration of 50 µg/mL from 0.3% to 93.6% (Figure 1, left panel). The western blot analysis was carried out on apoptosis maker proteins. The bands exhibited the dimness of procaspase-9, procaspase-3, and PARP, while the bands of cleaved proteins were bold (Figure 1, right panel). Cleaved PARP, cleaved caspase-9, and cleaved

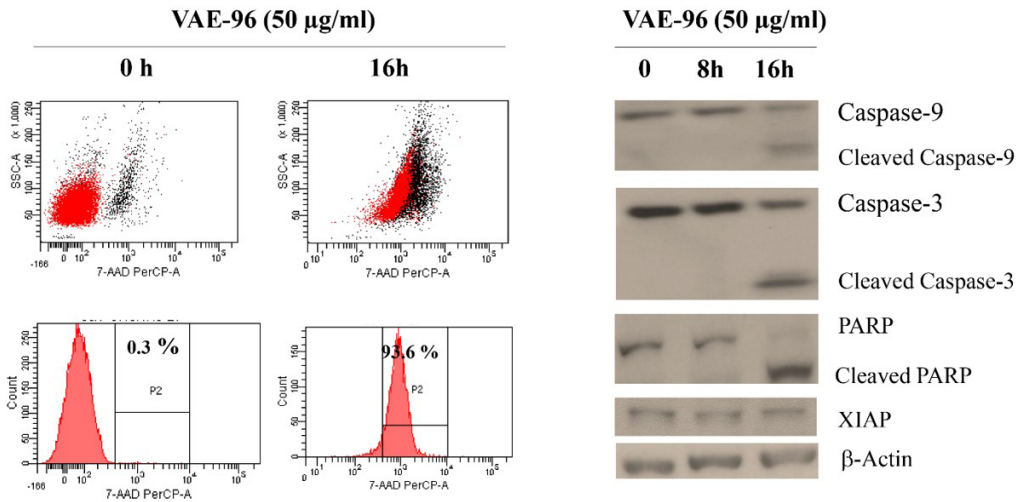


Figure 1. VAE-96 induces apoptosis in MOLM-13 cells. 1×10^5 cells/ml were co-cultured with VAE-96 (50 µg/ml) for 0, 8, and 16 hours. After that, the total cell lysates were prepared, and western blot analysis with the antibodies, including caspase-9, caspase-3, XIAP, and PARP, was performed. 7-AAD stain flow cytometry exhibited an increase of death cells and apoptosis cells on MOLM-13 cells after 16h of VAE-96 (50 µg/ml) exposure.

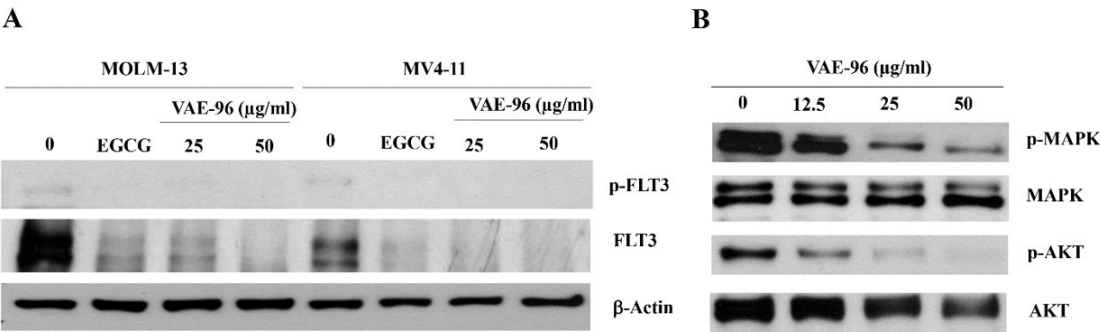


Figure 2. VAE-96 inhibited FLT3 protein expression and caused a decrease in the PI3K/AKT and MAPK signalling pathways. (A) MOLM-13 and MV4-11 cells (1×10^5 cells/ml) were incubated with various concentrations of VAE-96 (25 and 50 µg/ml), EGCG (60 µM), or DMSO (control) for 16 hours (B) MOLM-13 cells (1×10^5 cells/ml) were cultured with varying concentrations of VAE-96 (12.5, 25, and 50 µg/ml) or DMSO (control) for 16 hours. Western blot analysis was then performed on total cell lysates using the indicated antibodies.

caspase-3 were observed after 16 hours of incubation with 50 µg/ml extract, illustrating the cell death by apoptosis induced by VAE-96.

3.2. VAE-96 reduces FLT3 protein expression and suppresses the activity of AKT and MAPK

A western blot assay was conducted on MV4-11 and MOLM-13 cells, both with and without VAE-96 treatment, to investigate the potential impact of VAE-96 on FLT3 expression. EGCG was employed as a positive control (Ly et al., 2013). The results indicated a dose-dependent reduction in FLT3 expression in MV4-11 and MOLM-13 cells after treatment with VAE-96, as depicted in Figure 2A.

FLT3 signalling impacts downstream factors such as MAPK and AKT (Takahashi, 2011). Western blotting with antibodies against MAPK and AKT proteins was used to examine the systemic effects of VAE-96 on

the FLT3-related signalling pathway. As a result of the suppression of FLT3, the expression of downstream proteins was interrupted. The data revealed a time and dose-dependent downregulation of both total protein and phosphorylation, as shown in Figure 2B. The cellular and molecular findings provide support for the assertion that VAE-96's cytotoxic effect on MOLM-13 cells is mediated by inhibiting FLT3 synthesis.

3.3. Suppression of FLT3 gene expression by VAE-96

To determine if the decreased expression of FLT3 protein by VAE-96 was partially due to a decline in its transcriptional level, we examined the effect of VAE-96 on FLT3 transcriptional regulation in MOLM-13 and MV4-11 cells treated with or without VAE-96 (50 µg/ml) for 8 hours. EGCG served as a positive control (Ly et al., 2013). As depicted in Figure 3A, treatment of MV4-11 and

MOLM-13 cells with VAE-96 significantly decreased *FLT3* mRNA levels. We conducted a transient transfection experiment using the *FLT3* promoter-reporter construct, which involved cloning a portion of the *FLT3* gene's 5' flanking region. As demonstrated in Figure 3B, VAE-96 significantly inhibited *FLT3* promoter activity.

3.4. Plant phytochemical

A standard method was utilized to assess the phytochemical composition of VA (Ciulei et al., 1993). The results revealed that the dried bitter leaf powder samples had a relatively limited chemical composition, focusing on three major groups of compounds: polyphenols (primarily flavonoids), terpenoids, and alkaloids. The flavonoid reactions predicted high concentrations of this group of compounds (Table 1). According to Food and Drug Administration data, approximately 40% of molecules derived from or inspired by natural compounds are employed in cancer therapy, with 74% used (Seca and Pinto., 2018). The presence of secondary metabolites implies the potential for cancer regression. Moreover, The polyphenol content determined in VAE-96 is 37.77 mg GAE/g d.w.

3.5. The potential of alkaloid fractions effect on MOLM-13

The extract analysis revealed that the two prominent compounds in VA extract were polyphenols (flavonoids) and alkaloids. Figure 4A showed that the inhibitory effect on cell proliferation did not show a specific correlation with the quantitative polyphenol content in crude extract. A high correlation negative coefficient was observed between the total polyphenol content of dry herbal powder and the extract's cytotoxicity on both MOLM-13 and MV4-11. Still, this correlation was deemed non-statistically significant (Figure 4B). Therefore, the group of alkaloid compounds was further analyzed. Using column chromatography with a solvent system of increasing polarity consisting of n-hexane, ethyl acetate, and methanol, 16 chromatographic

lines were observed and grouped into nine fractions named VA-1 to VA-9 (Supplementary Material Fig. S2). The alkaloid fractions showed a robust cytotoxic effect with over 50% cell death at 30 µg/ml. The difference was statistically significant compared to the control group (Figure 5). Only about 10% of the cells survived under the influence of VA-2, VA-3, and VA-7 on MOLM-13, showing 0.78 ± 1.05 ; 6.44 ± 3.20 ; 5.38 ± 1.17 , respectively. It was suggested that the solvent of 60:40 or 55:45 n-hexane: ethyl acetate be used to obtain the alkaloid fraction that affected MOLM-13 the most.

4. Discussion

Our previous paper demonstrated that VAE-96 inhibited the cell growth of MV4-11 and MOLM-13 (Supplementary Material Fig. S3) (Nguyen et al., 2022) but had no significant effect on the healthy human peripheral blood mononuclear cells (Supplementary Material Fig. S3B). Flow cytometry was performed in this test to clarify the cell death types of MOLM-13 under the effect of VAE-96 based on 7-AAD stain. The fluorescent 7-AAD is selectively bound to the G/G region of DNA, causing fluorescence (Schmid et al., 1992). Therefore, cell luminescence upon staining with 7-AAD indicates membrane damage that leaks dye into the nucleus. The level of 7-AAD expression in cells means whether the cells are alive (7-AADNegative), in apoptosis (7-AADDim), or dead (7-AADbright) cells (Zembruski et al., 2012). Figure 1 shows that VAE-96 caused AML cell death and apoptosis, as evidenced by the presence of cleaved PARP, cleaved caspase-9, and cleaved caspase-3 after 16 hours of incubation with 50 µg/ml extract.

VA accentuated the study lethality of cancer cells at a low dosage (Fachrunisa et al., 2019; Hasibuan et al., 2020; Iweala et al., 2015; Mohd et al., 2016). Apoptosis-inducing capacity was recorded as an activity of VA extract (Fachrunisa et al., 2019). Two aspects of apoptosis

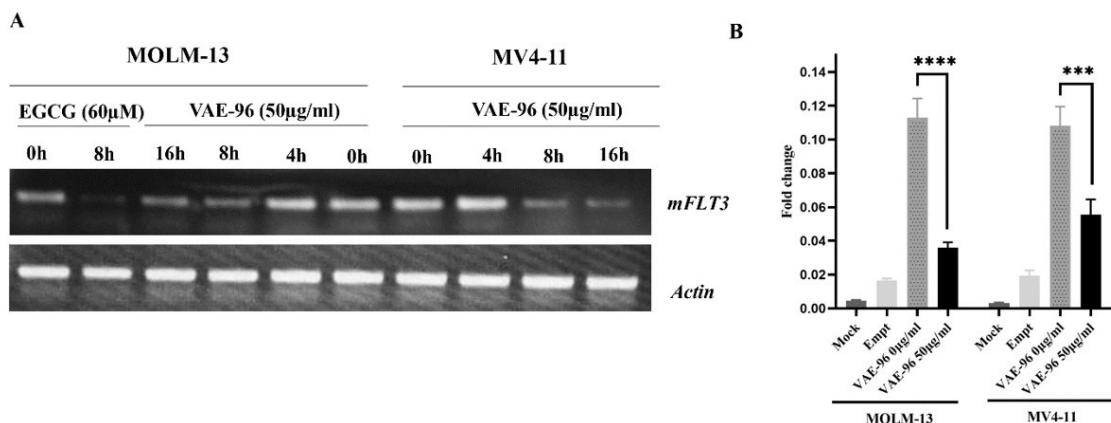


Figure 3. VAE-96 inhibited *FLT3* transcription and its promoter activity in MV4-11 and MOLM-13 cells. (A) Semi-quantitative reverse transcription PCR was performed on MOLM-13 and MOLM-14 cells cultured at a density of 1×10^5 cells/ml and treated with VAE-96 (50 µg/ml), EGCG (60 µM), or DMSO (control) for a specified time. The total RNA was obtained, and cDNA was synthesized using the designated primers for PCR analysis. (B) The inhibition of *FLT3* promoter activity by VAE-96 (50 µg/ml) was assessed using a reporter assay ($n=6$, mean $\pm$ SD). The luciferase activities of the reporter series were measured in the presence or absence of VAE-96 (50 µg/ml).

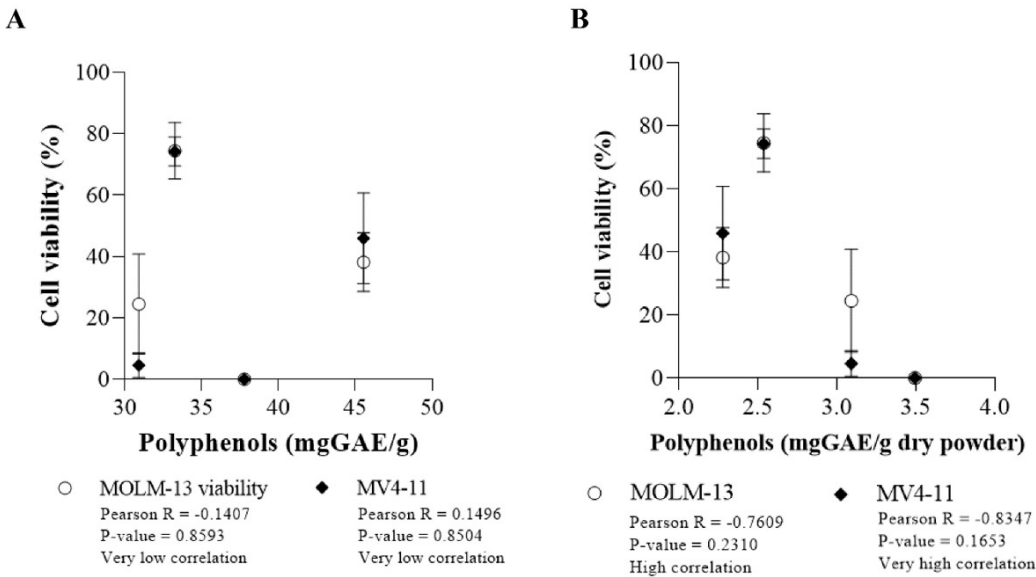


Figure 4. Polyphenols (mgGAE/g (A) and mgGAE/g dry powder (B)) were not found to positively affect the cytotoxic activity towards MOLM-13 and MV4-11 cells. A correlation coefficient analysis was conducted to assess the association between polyphenols and cytotoxic capacity. Both cells exhibited negative correlation coefficients, indicating that the anti-leukaemia effect was less effective with higher polyphenol content. Therefore, this secondary metabolic compound did not significantly contribute to the cytotoxic ability of VA extract.

Table 1. Phytochemical screening results.

Compound	Phytochemical screening results			Conclusion
	Diethyl ether fraction	Ethanol fraction	Water fraction	
Fat	-			-
Carotenoid	-			-
Essential oil	-			-
Triterpenoid	+			+
Alkaloid	+	-		+
Coumarin	-	-		-
Anthraglycosid	-			-
Flavonoid	++	+++	+	+++
Cardiac glycosid		-	-	-
Anthocyanosid		-	-	-
Proanthocyanidin		-		-
Tannin		-		-
Saponin		-		-
Organic acid		++		++
Reducing compound		+++		+++

+: weak intensity reaction; ++: Medium intensity reaction; +++: Strong intensity reaction; -: No reaction.

induction were examined, including cell membrane damage and protein expression in the cells. Membrane bleeding indicates ongoing apoptosis, which harbours changes in the permeability of cell membranes or hollow structures that allow extracellular components to enter the cells (Kurokawa

and Kornbluth, 2009). The fluorescence appearance of 7-AAD in the intracellular space indicated the non-integrity of the cell membrane under the impact of VAE-96.

Moreover, the increased activation of procaspase-3 and procaspase-9 had occurred and manifested through

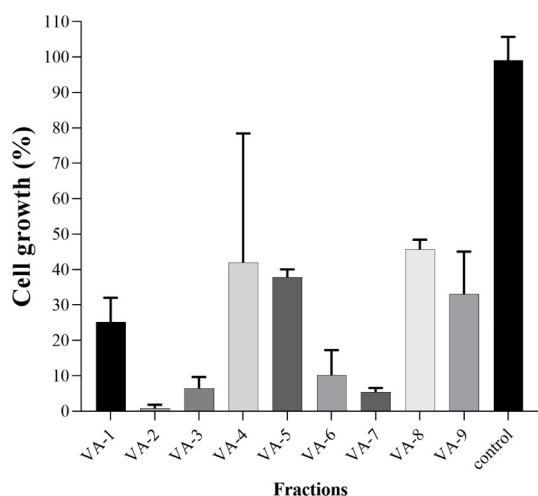


Figure 5. The cytotoxic effect of nine alkaloid fractions obtained from VAE-96 on MOLM-13 cells. The cells were treated with these alkaloid fractions for 72 hours at 1×10^5 cells/ml density.

cleavage, leading to the cleavage and inactivation of a DNA repair factor, PARP. The cleavage of caspase and PARP and the maintenance of expression of the anti-apoptosis factor, XIAP, are considered to be the hallmarks of cell apoptosis (Ly et al., 2013; Takahashi, 2011). Manifestation of apoptosis was also previously reported in other leukaemia cell lines, including HL-60 and cells from adult leukaemia patients after exposure to VA extract (Iweala et al., 2015). The apoptosis-inducing effect expressed by the increased activation of caspase-9 by VAE-96 was predicted to be mediating through signal reception from the cell membrane receptor, especially the tyrosine kinase receptor, because the regulation of caspase-9 action had been found to depend on kinase signalling pathways, which have been described before Cytochrome c - dependent activation (Francisco et al., 2017).

Kinetic signalling plays a crucial role in the origination and progression of AML (Riccioni et al., 2011; Takahashi, 2011). Kinetic gene mutations are common in AML cases, accounting for 30% of cases, detected as FLT3 mutant accumulation (Nakao et al., 1996). FLT3 is crucial in developing, surviving, and increasing normal stem/progenitor cells in myeloid and lymphoid lineages (Beaudin et al., 2014). Overexpression of FLT3 was detected at a rate of 10-15% and negatively affected the overall and event-free survival in cytogenetically regular AML patients (Riccioni et al., 2011). On the other hand, FLT3-ITD has been suggested to play an irreplaceable role in AML pathogenesis and survival (Nakao et al., 1996). Targeting FLT3 in treating AML is of research interest, and many chemotherapy drugs targeting this molecule are being used (Geburu and Wang, 2020). In 2022, an inhibiting interaction of FLT3 phosphorylation induced by VA extract was described for the first time, leading to predictions of the anti-leukemic activity of VA mediated through FLT3 activity inhibition (Hoang and Bui, 2021). FLT3 glycosylation determines protein activity and the location of anchorage (Reiter et al., 2018; Schmidt-

Arras et al., 2005). The glycosylated FLT3 (150-kDa) is expressed predominantly on the plasma membrane, while the immature FLT3 (130-kDa) is prominently expressed in the endoplasmic reticulum (Schmidt-Arras et al., 2005). Interestingly, in this test, the down-expression of both FLT3 and glycosyl-FLT3 was depicted in the presence of VAE-96. The same parallel results were observed on MOLM-13 and MV4-11 (Figure 2). The membrane-persistent FLT3-ITD strongly activated the PI3K and AKT signalling pathways, whereas it started the STAT5 pathway if distributed in the endoplasmic reticulum (Köthe et al., 2013). The total MAPK and AKT content seemed to be maintained under the impact of VAE-96. In contrast, the decrease in the entire FLT3 content resulted in poor activation of downstream signalling pathways that manifested as the collapse of phosphorylated MAPK and phosphorylated AKT. The inhibitory effects of kinase signalling pathways, including PI3K and MAPK, on VA extract and VA-derived compounds have been reported previously (Liu et al., 2022; Takahashi, 2011; Wu et al., 2022). The sharp decline in FLT3 content indicated reduced protein synthesis in transcription or translation (Elmore, 2007).

The results in Figure 3 reflected the decrease in *FLT3* mRNA level after 8 hours of exposure to VAE-96 in both MOLM-13 and MV4-11 cell lines. The interaction of the natural compound from herbs on the promoter structure has been described before (Seca and Pinto, 2018). Notwithstanding the comprehensive research, the evidence for promoter inhibition of VA extracts has been minimal. However, the interaction of VA extract with DNA synthesis has been described (Izevbogie et al., 2004). In this study, the reduced response of fireflies to renilla luciferase reflected the decrease in the activity of the FLT3 promoter in the presence of VAE-96, which led to the mRNA *FLT3* synthesis interruption.

The limitation of this study is that we only focused on the impact of VAE-96 on the FLT3 signal transduction pathway, from inhibiting *FLT3* mRNA biosynthesis through its promoter regulation mechanism to inhibit phosphorylation and activate AKT and MAPK pathways leading to induction of programmed cell death. Meanwhile, many other pathways must be examined, such as the Mitochondria Pathway and Death Receptor Pathway of Cellular Apoptosis.

The anti-leukaemic ability of the VAE-96 was considered to be dependent on the active phytochemical presence. As shown in Table 1, 3 main groups of bioactive compounds were discovered in VA, of which the polyphenol component gave the most vehemently positive reaction, followed by alkaloids. However, the relationship between polyphenol content and cell proliferation inhibitory activity was disputed. Therefore, the alkaloid from VAE-96, the bioactive phytochemical proven to inhibit leukaemia, was selected to be analyzed and exhibited a strong ability to inhibit AML cells. The results in Figure 5 showed that the alkaloid fractions obtained from VAE-96 derived total alkaloids using solvents of 60:40 or 55:45 n-hexane: ethyl acetate showed the most substantial inhibitory effect on the proliferation of AML cells, which needs to be further in-depth studied to clarify the mechanism. Long story short, it suggests that the VAE-96 inhibits FLT3 biosynthesis, which causes a

corresponding decrease in protein levels, interrupting the proliferative signalling pathway and inducing apoptosis in experimental leukemic cells. This effect is predicted to be contributed by the alkaloids present in the extract.

5. Conclusion

VAE-96 showed the most inhibitory effect on AML cell lines consisting of MOLM-13 and MV-4-11 in a dose-dependent manner that allowed apoptosis to occur and down-regulated FLT3 expression that led to interrupted PI3K/AKT and MAPK signalling pathways (Supplementary Material Fig. S4).

Acknowledgements

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.02-2019.50

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. VA plant morphology description. (A) Whole tree overview. The height of the tree ranges from 3 to 10 meters. (B) Flower branch shape. Alternate leaves and flowers grow at the ends of branches. (C) The shape of a leaf. The lamina is elliptical, up to 8 - 12 cm in length and 4-5 cm in width. (D) Flower clusters. Flowers grow in clusters of 8 - 12 flowers. (E) Flower shape. Flowers hermaphroditic, 5 stamens, 1 ovule, 2 carpels, 1 filamentous style, and 2 stigmas. (F) Fruit shape. Cylindrical brown fruit, hairy on the surface.

Figure S2. The procedure for obtaining nine fractions of alkaloids from VAE-96. Six hundred grams of VAE-96 crude extract were dissolved into 6 liters of 5% tartaric acid. The filtrate from the mixture was alkalized with NH_4OH to pH 9-10 and then shaken with CHCl_3 until the solution no longer reacted with the Dragendorff reagent on the thin-layer chromatography. The crude alkaloid extract was collected by rotating the solution until thoroughly dried. The alkaloid fraction was separated using a chromatographic column and a solvent system of increasing polarity composed of n-hexane, ethyl acetate, and methanol in appropriate proportions. The results were obtained into 16 coloured bands and grouped into nine fractions after using the TLC method for classification.

Figure S3. VAE-96 inhibited MOLM-13 and MV4-11 cell growth reported in the previous paper. (A) The effect of VAE-96 was recorded in a dose-dependent manner on both MV4-11 ($\text{IC}_{50} = 16,08 \pm 1,21 \mu\text{g/mL}$) and MOLM-13 ($\text{IC}_{50} = 11,45 \pm 2,12 \mu\text{g/mL}$). Midostaurin (nM) was used as control. (B) The effect of VAE-96 on healthy human peripheral blood mononuclear cells (PBMC). (C) The cell's morphology changes under 72-hour of VAE-96 treatment.

Figure S4. It suggested that VAE-96 down-regulated FLT3 expression, leading to the related signal pathways, including MAPK and PI3K/AKT interruption.

This material is available as part of the online article from <https://doi.org/10.1590/1519-6984.287203>