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Original Research

The cytotoxic effect of *Vernonia amygdalina* Del. extract on myeloid leukemia cells

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ABSTRACT

Introduction: This study aimed to demonstrate the cytotoxic effect of a bitter leaf (*Vernonia amygdalina* Del.) ethanol extract on myeloid leukemia cells. **Methods**: The plant extract was prepared using the maceration method. The toxicity assays used the trypan blue exclusion method. Flow cytometry and reverse transcription PCR methods were used to deduce the mechanism of action. **Results**: The *V. amygdalina* Del. extract strongly affected K562 cells, with a half-maximal inhibitory concentration of 8.78 ± 2.224 µg/mL. The extract could induce apoptosis and arrest the cell cycle in K562 cells. The extract increased the mRNA levels of caspase 3 (CASP3), baculoviral IAP repeat containing 5 (BIRC5/survivin), and phosphatidylinositol 3-kinase (PI3K) and decreased the mRNA levels of retinoblastoma transcriptional corepressor 1 (RB1/pRB), B cell lymphoma/leukemic 2 (BCL2), BCL2-like 1 (BCL2L1/BCL-XL), caspase 9 (CASP9), and the breakpoint cluster region (BCR)-Abelson (ABL) fusion gene. **Conclusion**: The *V. amygdalina* Del. extract strongly inhibited the acute myeloid leukemia cell line K562. It was found to arrest the cell cycle and induce apoptosis by regulating the expression of related genes that predicted targeting BCR-ABL downregulation.

Key words: apoptosis, BCR-ABL, leukaemia, transcriptional expression, V. amygdalina Del.

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INTRODUCTION

Cancer is a leading cause of mortality worldwide, with >19 million new cases and nearly 10 million deaths[1,2]. Unfortunately, cancer cases are expected to increase significantly over the next decade[3]. The economic burden on patients and their families is enormous, significantly affecting public health, the national economy, and social security[4]. Therefore, medical research is racing to develop effective cancer treatments to prolong patient lives. However, current treatments remain largely ineffective[5]. One of the least treatable cancers is leukemia, which causes > 250,000 deaths and nearly 500,000 new diagnoses[1,2]. Despite advances in knowledge and medical techniques, leukemia-related mortality remains high[6].

Phytochemical compounds are being explored as potential treatments for blood cancer[7,8]. Various plant compounds have shown inhibitory effects on leukemia cell proliferation. For example, maytansinoids and their derivatives extracted from *Maytenus serrata* inhibited tubulin, alvocidib extracted from *Dysoxylum binectariferum* inhibited cyclin-dependent kinase 9 (CDK9) activity, and omacetaxine mepesuccinate extracted from *Cephalotaxus harringtonia* has been approved by the US Food and Drug Administration[9–11].

Bitter leaf (*Vernonia amygdalina* Del.) is among the major sources of compounds with scientifically demonstrated anticancer activity. Bitter leaf extract disrupts the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) signaling pathway, the mitogen-activated protein kinase (MAPK) pathway, and fms-related receptor tyrosine kinase 3 (FLT3) phosphorylation, inhibiting cancer cell proliferation[12–15]. Studies have found the bitter leaf to be cytotoxic in breast cancer (half-maximal inhibitory concentration [IC_{50}]: MCF-7 = 50.36 µg/mL, 4T1 = 25.04 ± 0.36 µg/mL, and T47D = 59.19 ± 0.55 µg/mL), neuroblastoma (IC_{50}: U-87 = 18.80 ± 1.11 µg/mL), prostate cancer (IC_{50}: PC-3 = 196.60 µg/mL and DU145 = 40.10 ± 4.30 µg/mL), and acute myeloblastic leukemia (IC_{50}: HL-60 = 5.58 µg/mL, THP-1 = 24.17 ± 3.33 µg/mL, MOLM-13 = 11.45 ± 2.12 µg/mL, and MV4-11 = 16.08 ± 1.21 µg/mL) cells[16–21]. However, few studies have examined bitter leaf's effect on leukemia cells, especially chronic myeloid leukemia, one of the four main leukemia groups. Therefore, this study aimed to investigate the cytotoxicity of a bitter leaf ethanol extract on chronic myeloid leukemia cell line K562 and determine its mechanism of action.

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METHODS

Plant extraction

The bitter leaves were harvested from Xuyen Moc district, Ba Ria–Vung Tau province, Vietnam. Dang Le Anh Tuan, Ph.D., of the Botany Laboratory in the Department of Ecology and Evolutionary Biology, Faculty of Biology and Biotechnology, University of Science, Vietnam National University, Ho Chi Minh City, performed the botanical identification (voucher: PHH0004908; **Supplementary Figure 1**). After washing and thoroughly drying at 40^{o}C, the leaves were ground to a powder, which was then suspended in 96% ethanol (1:10 w/v). The plant extract was collected and rotary evaporated to obtain a crude extract. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) was used to dissolve the crude *V. amygdalina* extract (VAE) into a solution for use, which was stored at −20^{o}C until needed.

Cell culture

The human leukemia cell line K562 was obtained from Prof. Yuko Sato (Tokyo, Japan)[22,23]. The K562 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Sigma-Aldrich, USA) supplemented with 10% inactivated fetal bovine serum (Thermo Fisher Scientific, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich, USA) at 37^{o}C with 5% CO_2. Fibroblast cells were cultured in Dulbecco's modified Eagle's medium (StemCell, Singapore) prepared similarly to RPMI 1640.

Cytotoxicity effect of VAE extract

The toxicity of the VAE on K562 cells was evaluated using the trypan blue exclusion method in a six-well plate[24]. Briefly, 1500 μL of K562 cells at a density of 2×10^5 cells/mL was added to each experimental well before the same volume of VAE at 0 to 100 μg/mL was added. The plates were incubated for 72 hours at 37^{o}C with 5% CO_2. Then, cell viability was calculated as the percentage difference between the treated and negative control groups.

The toxicity of the VAE on fibroblasts was determined using the 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, USA). Briefly, 100 μL of fibroblasts at a density of 2×10^5 cells/mL was added to each experimental well and incubated in a cell culture incubator. After 24 hours, 100 μL of the VAE at 0 to 200 μg/mL was added. After 72 hours, the viability of the fibroblasts was measured using the MTT assay.

Untreated cells were used as negative controls. Moreover, the effect of the DMSO (Protide, USA) was evaluated at 0.1%, corresponding to the solvent content in the highest VAE treatment.

Annexin V/PI analysis

K562 cells at a density of 10^5 cells/mL were exposed to the VAE at 50 and 100 μg/mL. After 24 hours, the K562 cells were collected and washed twice with phosphate-buffered saline (PBS; TBR company, Vietnam). Then, the cells were stained according to the ANNEX100B protocol (BioRad, USA). Briefly, cell pellets were resuspended in 195 μL of 1× binding buffer before adding 5 μL of Annexin V. After incubation for 15 minutes in the dark, the cells were washed with 200 μL of binding buffer and resuspended in 190 μL of binding buffer before adding 10 μL of propidium iodide (PI). The stained cells were analyzed using a BD Accuri C6 Plus Flow Cytometer (BD Biosciences, USA).

mRNA expression analysis

K562 cells at a density of 10^5 cells/mL were exposed to the VAE at 50 and 100 μg/mL. After 16 hours, the K562 cells were collected and washed with PBS. Next, their RNA was extracted according to the TRIzol reagent guidelines (Thermo Fisher Scientific, USA). Then, mRNA expression was detected using the SensiFAST SYBR No-ROX One-Step Kit (Meridian Bioscience, USA) with the primers listed in **Table 1**. Gene expression was determined using reverse transcription quantitative PCR and the $2^{-\Delta\Delta CT}$ method[25].

Data Analysis

Experiments were repeated at least three times, and data are presented as mean ± standard deviation. Statistical analyses were conducted using GraphPad Prism software (version 9.0.0) with $\alpha = 0.05$.

RESULTS

VAE strongly inhibited myelocytic leukemia cells

K562 cell viability was greater with (116.90% ± 16.92%) than without 0.1% DMSO ($P = 0.0002$). In contrast, 0.1% DMSO did not significantly affect fibroblast viability ($P = 0.0786$). Therefore, 0.1% DMSO was considered benign for evaluating cell growth (**Figure 1**). The VAE significantly decreased leukemia cell proliferation but did not significantly affect fibroblast proliferation (**Figure 2 and Supplemental Figure 2**). The IC_{50} values for the VAE were 8.78 ± 2.22 μg/mL for K562 cells and > 200 μg/mL

Table 1: Primers used for analysis

Gene	Sequences (5' – 3')	Reference
TP53	TGTGGAGTATTTGGATGACA	Kang Pa Lee, *et al.*[26]
	GAACATGAGTTTTTTATGGC	
pRB	ACTCCGTTTTCATGCAGAGACTAA	Deborah L. Burkhart, *et al.*[27]
	GAGGAATGTGAGGTATTGGTGACA	
Bcl-XL	TTGGACAATGGACTGGTTGA	Suresh Kumar, *et al.*[28]
	GTAGAGTGGATGGTCAGTG	
Bcl-2	AAGATTGATGGGATCGTTGC	M. Jaberipour, *et al.*[29]
	GCGGAACACTTGATTCTGGT	
Bax	TGGCAGCTGACATGTTTTCTGAC	Kostas V Floros, *et al.*[30]
	TCACCCAACCACCCTGGTCTT	
Survivin	GTTGCGCTTTCCTTTCTGTC	Sang Il Kim, *et al.*[31]
	TCTCCGCAGTTTCCTCAAAT	
Caspase-3	GAACTGGACTGTGGCATTGA	Sadia Perveen, *et al.*[32]
	CCTTTGAATTTCGCCAAGAA	
Caspase-9	GGTGATGTCGGTGCTCTTGA	IDT, Inc.
	CGACTCACGGCAGAAGTTCA	
BCR-ABL	CGGGAGCAGCAGAAGAAGTTGTTC	Nga Nguyen, *et al.*[33]
	CAGGCACGTCAGTGGTGTCTCTGTG	
MAPK	TGAAATGACAGGCTACGTGG	Liping Jiang, *et al.*[34]
	GACTTCATCATAGGTCAGGC	
Pi3K	GGTTGTCTGTCAATCGGTGACTGT	Ismael Riquelme, *et al.*[35]
	GAACTGCAGTGCACCTTTCAAGC	
GADPH	GAAGGTGAAGGTCGGAGTC	Qiuying Chen, *et al.*[36]
	GAAGATGGTGATGGGATTTC	

for fibroblasts. The effects of VAE on K562 cells were classified as selective based on an estimated selective index (SI) of 22.78.

VAE induced apoptosis in K562 cells

K562 cells were further examined by staining with PI and Annexin V after 24 hours of VAE exposure. Cell death increased with VAE concentration. The percentages of Annexin V-positive and PI-positive cells were higher among cells treated with 50 or 100 μg/mL VAE than among untreated control cells ($P < 0.0001$). Most cells died due to apoptosis (6.10% $\pm$ 0.10% for 50 μg/mL and 6.50% $\pm$ 0.44% for 100 μg/mL VAE) or necrosis (5.52% $\pm$ 0.50% for 50 μg/mL and 6.59% $\pm$ 0.52% for 100 μg/mL VAE; **Figure 3**). In addition, the VAE tended to arrest cells at G2/M (9.95% $\pm$ 0.84% for 50 μg/mL and 10.67% $\pm$ 0.40% for 100 μg/mL VAE) or S (2.08% $\pm$ 0.07% for 50 μg/mL and 3.26% $\pm$ 0.03% for 100 μg/mL VAE) checkpoints since the percentage of cells in these phases increased after exposure in a dose-dependent manner (**Figure 4**).

VAE regulates mRNA expression in K562 cells

We examined the expression of genes involved in apoptosis, the cell cycle, and breakpoint cluster region (BCR)-Abelson (ABL) pathway signaling (**Figure 5**). The control group comprised healthy cells at the same density as the experimental groups. When $2^{-\Delta\Delta CT}$ values were compared between the 50 and 100 μg/mL VAE experimental groups, the mRNA levels of the following target genes decreased with increasing VAE

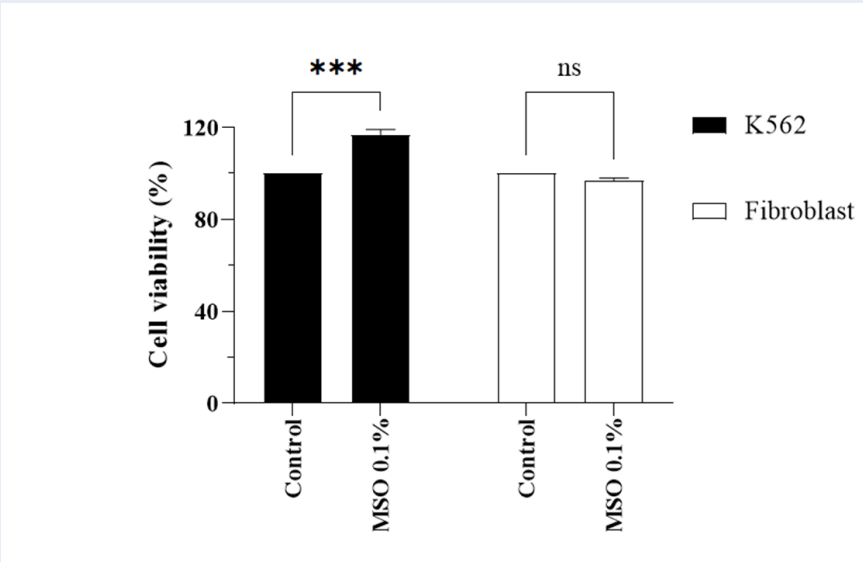

Figure 1: **Cytotoxicity of the DMSO 0.1% on evaluated cell lines.** There was no statistically significant difference recorded in the survival rates of fibroblast cells. DMSO 0.1% induced mild proliferative stimulation on the K562 cell line, p-value < 0.0002. **Abbreviation**: **DMSO**: Dimethyl sulfoxide

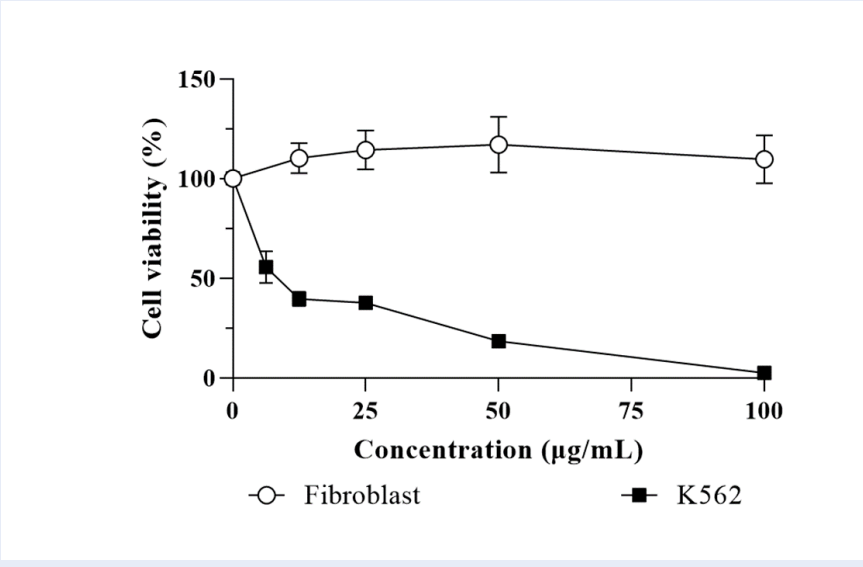

Figure 2: **Cytotoxic effect of VAE on evaluated cells.** The VAE affected K562 proliferation in a dose-dependent manner. No toxicity of VAE was observed at concentrations below 100 μg/mL in fibroblast cells (p-value > 0.9999). **Abbreviations**: **DMSO**: Dimethyl sulfoxide, **VAE**: *Vernonia amygdalina* Del. ethanol extract

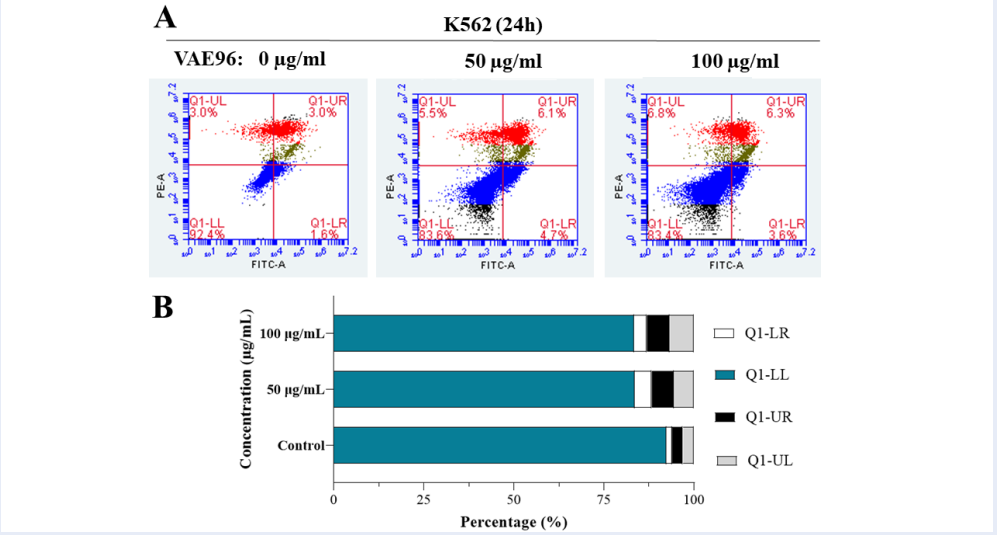

Figure 3: **The VAE induced apoptosis and necrosis on K562**. (**A**) Cell populations positive for PI (PE-A channel) and Annexin V (FITC-A channel) increased under the influence of VAE. (**B**) K562 cells were induced into early apoptosis (quartile Q1-LR), apoptosis (quartile Q1-UR), and necrosis or late apoptosis (quartile Q1-UL), which increased sharply after the effect of VAE. The effect of VAE at a concentration of 50 µg/mL was more effective than this at 100 µg/mL. **Abbreviations**: **DMSO**: Dimethyl sulfoxide, **PI**: Propidium iodide, **VAE**: *Vernonia amygdalina* Del. ethanol extract

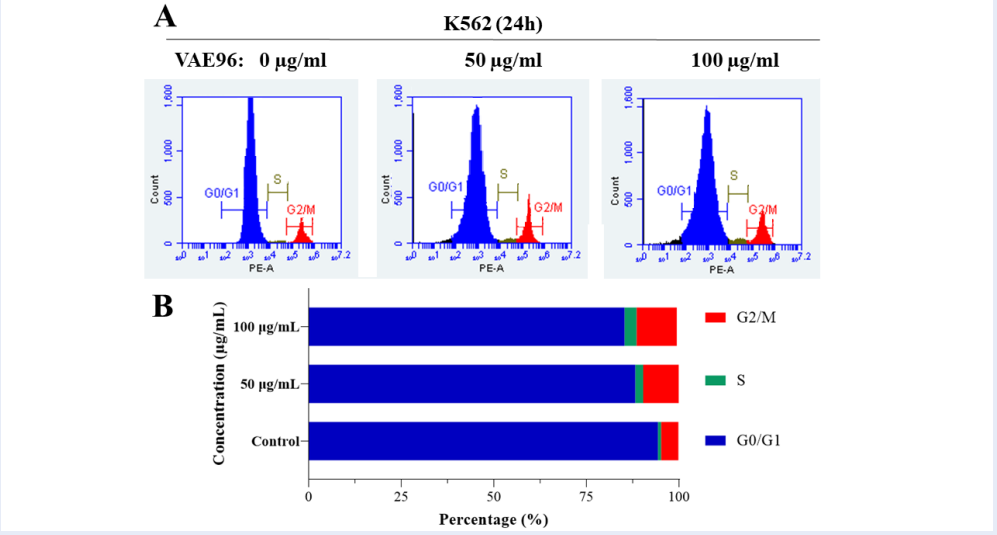

Figure 4: **The VAE-induced K562 cell cycle arrest**. (**A**) The number of cells in the G0/G1 phase decreased gradually under the influence of VAE, while the number of cells increased in the S and G2/M phases. The effect was recorded in a dose-dependent manner. (**B**) K562 cells tended to be trapped in the S and G2/M phases. **Abbreviations**: **VAE**: *Vernonia amygdalina* Del. ethanol extract

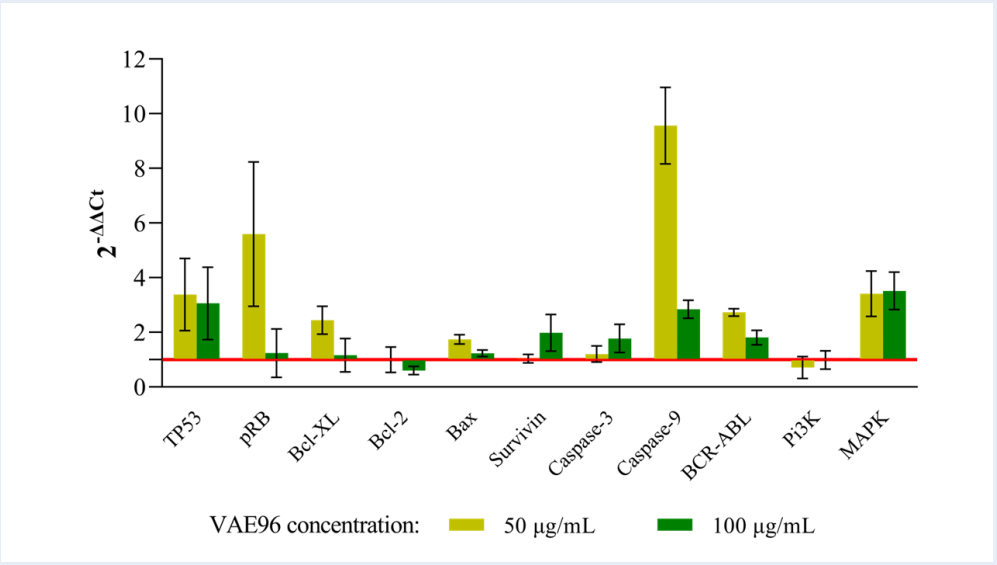

Figure 5: **Examination of the mRNA expression of several genes in K562 cells**. Alterations in mRNA expression of genes involved in apoptosis, cell cycle arrest, and the BCR-ABL signaling pathway under the effect of VAE. The extract down-regulated the mRNA expression of *pRB, BCl-XL, BCl-2, Bax, Caspase-9, and BCR-ABL*, and up-regulated the mRNA expression of *Survivin, Caspase-3, and Pi3K*. **Abbreviations**: **VAE**: *Vernonia amygdalina* Del. ethanol extract

concentration: retinoblastoma transcriptional corepressor 1 (*RB1*/*pRB*; from 5.59 ± 2.63 to 1.24 ± 0.88), B cell lymphoma-leukemia 2 (*BCL2*; from 1.00 ± 0.46 to 0.60 ± 0.15), BCL2-like 1 (*BCL2L1*/*BCL-XL*; from 2.44 ± 0.51 to 1.16 ± 0.61), BCL2-associated X apoptosis regulator (*BAX*; from 1.75 ± 0.17 to 1.23 ± 0.12), caspase 9 (*CASP9*; from 9.57 ± 1.40 to 2.84 ± 0.32), and the *BCR-ABL* fusion gene (from 2.73 ± 0.13 to 1.84 ± 0.33). In contrast, the mRNA levels of the following target genes increased with increasing VAE concentration: baculoviral IAP repeat containing 5 (*BIRC5*/*survivin*; from 1.04 ± 0.15 to 2.00 ± 0.67), caspase 3 (*CASP3*; from 1.21 ± 0.29 to 1.78 ± 0.51), and *PI3K* from 0.71 ± 0.40 to 1.00 ± 0.33). However, VAE did not affect tumor protein p53 (*TP53*) and *MAPK* mRNA levels.

DISCUSSION

DMSO is widely used in herbal pharmacology[37]. However, DMSO easily permeates cells at high concentrations, causing hemolytic toxicity[38,39]. In this study, 0.1% DMSO showed no cytotoxicity, leading to no data distortion. The VAE, which had an IC_{50} of 8.78 ± 2.22 µg/mL for the K526 cell line, is a potential cytotoxic crude extract according to the US National Cancer Institute criteria[40]. Its toxicity has also been reported in several other cancer cell lines[16–20].

A *V. amygdalina* Del. extract was previously reported to have a prominent inhibitory effect on the acute myeloid leukemia cell line HL-60, with an IC_{50} of 5.58 µg/mL[41]. Another *V. amygdalina* Del. extract was reported to have a strong cytotoxic effect on acute myeloid leukemia cell lines THP-1 (IC_{50} = 24.17 ± 3.33 µg/mL), MOLM-13 (IC_{50} = 11.45 ± 2.12 µg/mL), and MV4-11 (IC_{50} = 16.08 ± 1.21 µg/mL)[21]. Moreover, a *V. amygdalina* Del. root extract had a remarkably toxic effect in a clinical trial, killing 50%–75% of acute myeloid and lymphocytic leukemia patient-derived tumor cells[42]. However, leukemic inhibitory activity has also been reported for extracts from other plants of the same *Vernonia* genus, such as *V. condensate*, which had an IC_{50} of 24.20 µg/mL for HL-60 cells[43,44].

Its diversity of phytochemical compounds may contribute to the anti-leukemia effects of *V. amygdalina* Del., including vernodalin, which requires further in-depth research[45]. Furthermore, the effects of the VAE appear highly selective based on its SI of 22.78, facilitating its further evaluation[46]. A previous study reported that a VAE could prevent the phosphorylation of FLT3. Inhibiting the FLT3 pathway could reduce cell proliferation and enhance cell death through apoptosis[15]. Interestingly, *V. amygdalina* Del. induced apoptosis in breast cancer cells by regulating

the expression procaspases and the BCL2 family [18,19]. In our study, the concomitant increases in PI-positive and Annexin V-positive cells after VAE treatment suggests that VAE induces apoptosis in K562 cells. Moreover, PI stain analysis suggests VAE induces cell cycle arrest in K562 cells, consistet with its reported affects on MCF-7 and MDA-MB-231 cells [18,19].

Decreased *pRB* expression has been closely associated with cell cycle arrest [47]. In addition, the decreased expression of genes such as *BCL-XL* and *BCL2* and the increased expression of *CASP3* were found to promote apoptosis in K562 cells [48]. Moreover, *V. amygdalina* Del. extract has been shown to prevent tyrosine kinase receptor phosphorylation activity [15]. We found that the VAE decreased the expression of the *BCR-ABL* fusion gene, suggesting that it injured K562 cells through the BCR-ABL pathway. However, since changes in mRNA levels indirectly reflect changes in the protein levels and activities [49–51], further in-depth research is required to confirm our results at the protein level.

CONCLUSIONS

The *V. amygdalina* Del. ethanol extract showed a potent, selective inhibitory effect on the chronic myeloid leukemia cell line K562. Our results show that its mechanism of action was via apoptosis induction, which evidence suggests is through the BCR-ABL pathway.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide, **IC50**: The half maximal inhibitory concentration, **SI**: Selective index, **VAE**: *Vernonia amygdalina* Del. ethanol extract

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AUTHOR'S CONTRIBUTIONS

NTQ, BTKL, and HTC designed the study, NTQ performed the experiments and data acquisition, and all authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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