RESEARCH ARTICLE

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Cancer Chemotherapeutic Effect of Vernonia Amygdalina Delile on Glioblastoma Brain Cancer Cell

Fathul Huda[1]*, Muhammad Hasan Bashari[1], Denny Satria[2], Adam Hermawan[3], Poppy Anjelisa Zaitun Hasibuan[4], Resti Gradia Dwiwina[1], Rohim Hermawan[5]

Abstract

Objective: This study is targeted at assessing the chemotherapy factor of the ethanol extract of Vernonia amygdalina Delile (VAD). **Methods:** U87 glioblastoma cells were treated with extract and a fraction of Vernonia amygdalina Delile (VAD) was harvested from the herbarium. Cytotoxicity was evaluated to determine the IC50 through microscopic observation followed by an MTT assay. Subsequently, flow cytometry with a FACS type was employed to conduct cell cycle and apoptosis analyses. Annexin V/PI and PI markers were used to assess apoptosis and cell cycle progression. **Result:** The ethanol extract and ethyl acetate fraction of VAD showed promising effects as cancer chemotherapy in glioblastoma cells. The IC50 values for the extract and fraction were notably low, at 37.65 µg/ml and 10.12 µg/ml, respectively, for U87 cells. Analysis of apoptosis using FACS revealed a more pronounced apoptotic effect of the 15 µg/ml fraction of VAD on both early and late apoptosis compared to the 75 µg/ml extract of VAD. Although some differences in cell cycle properties were observed, there were no significant differences in cell cycle analysis between the extract and fraction. **Conclusion:** These findings underscore the efficacy of VAD's ethanol extract and ethyl acetate fraction as chemotherapeutic agents against U87 cancer cells. The low IC_{50} values and significant induction of early apoptosis highlight the cytotoxic effects of these treatments on U87 cells.

Keywords: Antineoplastic agents- Bitter leaf- Glioblastoma- Phytomedicine

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Introduction

Glioblastoma multiforme (GBM), a grade IV glioma with a less than 5% 5-year survival rate, is an aggressive brain cancer. The incidence of this cancer varies worldwide, ranging from 0.59 to 3.69 per 100,000 persons [1]. Despite treatment modalities such as surgical resection, chemotherapy, and chemoradiotherapy, the median overall survival for GBM patients remains less than 15 months [2], largely due to the major challenge of treatment resistance [3]. Resistance mechanisms include the presence of Methyl guanine methyl transferase (MGMT) [4], which enhances DNA repair, as well as the presence of M2 macrophages and non-coding RNA in the GBM microenvironment [5].

While treatment options are advancing, long-term treatment for glioblastoma remains largely ineffective, necessitating the development of new and effective treatments [6, 7]. One potential alternative treatment is the use of Vernonia amygdalina Delile (*V. amygdalina Delile*) ethanol extract, derived from the bitter leaf plant. *V. amygdalina Delile*, which grows year-round in tropical Africa and is also cultivated in Indonesia, has demonstrated pharmacological actions against malaria, diabetes, worms, cancer, and bacterial infections [8–13]. One of its major compounds, amygdalin, is believed to be a potent anticancer agent, inducing apoptosis by modulating mitochondrial signaling pathways [14, 15].

Research has shown that *V. amygdalina Delile* exhibits cytotoxic activity with a low IC50 value and induces apoptosis in the HepG2 cell line [16], as well as cell cycle arrest in PC3 cells [17]. Additionally, the chloroform extract of *V. amygdalina Delile* has been shown to enhance the sensitivity of paclitaxel and doxorubicin, suppressing growth in HEP 3B cells [18]. Furthermore, *V. amygdalina Delile* extract has demonstrated antioxidant effects in several assays [19]. Given these promising findings, we are interested in investigating the effects of *V. amygdalina Delile* ethanol extract and its fractions from African leaves on the development of GBM cell lines.

*[1]Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia. [2]Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Sumatera Utara, Indonesia. [3]Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Sumatera Utara, Indonesia. [4]Laboratory of Macromolecular Engineering, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. [5]Medical Doctor Program, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia. *For Correspondence: fathul@unpad.ac.id*

This research aims to develop a model that can be used to overcome anticancer resistance in GBM treatment, with a focus on examining the extract and fraction's anticancer activity using in vitro models.

Materials and Methods

Sample preparation

V. amygdalina Delile were harvested from the herbarium of Faculty of Farmacy, Universitas Sumatera Utara, in Medan, Indonesian early 2022. The plant subsequently chopped and macerated with 100% ethanol. To gain a concentration of 40.000 µg/mL, ethanol extract of *V. amygdalina Delile* was dissolved using 100% dimethyl sulfoxide (DMSO) (Sigma, cat.no. D8418, USA). The fraction of *V. amygdalina Delile* was achieved by fractionated using ethyl acetate solvents. This fractionation was conducted using the liquid-liquid extraction method as mentioned previous study [20, 21]. This solution would dissolve the compounds from the *V. amygdalina Delile* extract to their solubility and affinities.

Cell line

The research was conducted at the Cell Culture and Cytogenetic Laboratory, Faculty of Medicine, Universitas Padjadjaran. The U87 glioblastoma cell line was cultured in RPMI1640 medium (Gibco, cat.no. 11875-093, USA) supplemented with 1% Penicillin/Streptomycin (Sigma, cat.no. 15140122), 10% Fetal Bovine Serum (FBS) (Gibco, cat.no. 10270-106, USA), and Phosphate Buffered Saline (PBS) (Gibco, cat.no. 70011044, USA) at 37 °C and 5% CO2 in a cell culture incubator (Thermo Scientific model 3429, USA, 2014). Cell detachment was achieved using Trypsin-EDTA (Gibco, cat.no. 25200056, USA). Tissue Culture flasks (TPP, cat.no. 90025, USA) were used for cultivation, 96-Well Tissue Culture Plates (TPP, cat. no. 92096, USA) for the MTT assay, and 6-Well Tissue Culture Plates (TPP, cat.no. 92006, USA) for the cell cycle and apoptosis assays.

Cytotoxic activity with MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, also known as MTT (cat. no. M6494, Sigma Aldrich, USA), is an assay used to evaluate cell viability and proliferation [22]. This assay serves as a gold standard for assessing drug activity in cell lines under controlled conditions and procedures [23]. MTT is converted to formazan crystals by NAD(P)H-dependent oxidoreductase, resulting in a violet color. The intensity of the violet color is then measured by a photometric microplate reader [24, 25], indicating the number of viable cells. To adjust for medium intensity (DMSO), each concentration was subtracted from the blank well containing DMSO.

In this study, the MTT assay was performed to analyze the cytotoxic activity of *V. amygdalina Delile* extract and fraction, following a previously described protocol. The assay was conducted with triplicate samples and repeated three times. U87 cells were cultured for 24 hours, followed by a 72-hour incubation with the extract and fraction. Cellular observations were made using an inverted microscope (Olympus CK40, Japan) equipped with a microscope camera (C-mount camera, China). The MTT assay solution was terminated with DMSO and read at 550 nm with a photometric microplate reader (Thermo Scientific® Multiscan EX, Singapore).

Apoptosis and cell cycle assay

We utilized the Apoptosis Kit with Annexin V FITC and PI (Invitrogen, cat. no. V13242) for FACS analysis after 72 hours of incubation with the ethanol extract and ethyl acetate fraction of *V. amygdalina Delile*. The accuracy of this assessment depends on the integrity and permeability of the plasma membrane. Additionally, cell cycle analysis was performed using PI (Invitrogen, P3566). This analysis characterized the different amounts of DNA in each phase of the cell cycle, with PI serving as a marker of the DNA amount. For both analyses, we utilized an extract concentration of 15 µg/ml and a fraction concentration of 75 µg/ml. Cells were harvested and stained with the marker according to the protocol. The cell suspension was then analyzed using BD FACSFlow (Geneaid, EH22202-03901) for apoptosis and cell cycle. Based on the DNA content, the proportion of cells in each phase of the cell cycle (G1/S and G2/M) will be calculated using the Watson model, and the proportion of cells in the apoptosis phase will be determined using FlowJo (BD, v10.9.0).

Evaluation

Data processing, dose-response curve fitting, bar graph creation, and statistical analysis were conducted using GraphPad Prism v9.0 (GraphPad Software, San Diego, CA). A sigmoidal 4PL model was employed for the dose-response curve and determination of the 50% inhibitory concentration (IC50). One-way ANOVA followed by post hoc analysis was performed using GraphPad Prism. A p-value of less than 0.05 was considered statistically significant (*= <0.05, ** = <0.01, ***=<0.001, ns=not significant). Flow cytometry analysis for apoptosis and cell cycle, pseudo-color density plot generation, and cell cycle analysis were carried out using FlowJo™ v10.8 Software (BD Life Sciences).

Results

Pre-treatment Morphology of U87 Cells

We utilized an inverted microscope to observe U87 cells, focusing on their attachment to the plate and the quality of their morphology. As depicted on Figure 1, microscopic examination revealed that the majority of the cells had appropriately adhered to the plate surface and developed pedicles for intercellular communication. Following these observations, we proceeded to assess the cytotoxic activity of the extract and ethyl acetate fraction to confirm their effects.

Effect of ethanol extract and ethyl acetate fraction on cytotoxic activity of U87 cells

Through the MTT assay, a toxicity test was conducted to determine the dose-response curve and IC50 of

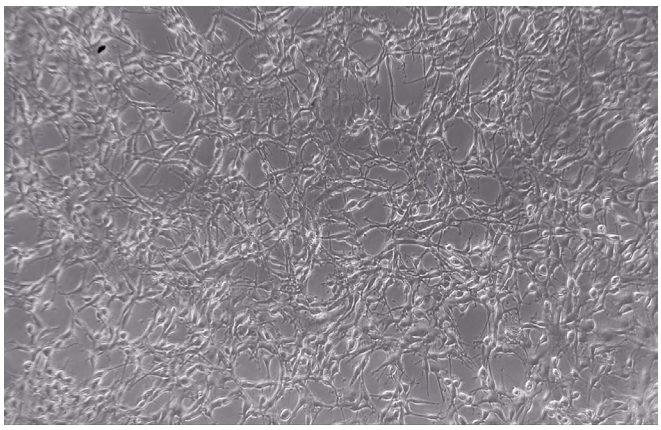

Figure 1. Morphological Appearance before Treatment

preparations against U87 cells. The preparations used were ethanol extracts and ethyl acetate fractions from Vernonia amygdalina Delile. Figure 1 shows morphological appearance of U87 before treatment. Figure 2 illustrates the dose-response curve of the extract and ethyl acetate fraction of Vernonia amygdalina Delile on U87 cells, showing that both the ethanol extract and ethyl acetate fraction potentially exhibited cytotoxic activity against U87 cells, with IC50 values of 37.65 µg/ml and 10.12 µg/ml, respectively. Additionally, microscopic observation of U87 cells revealed that an increasing dose of treatment resulted in a decrease in the number of cells, with cells exhibiting improper growth characterized by slightly elongated plasma cells.

Effect of ethanol extract and ethyl acetate fraction on apoptosis activity of U87 cell

Furthermore, the results of the MTT assay were evaluated, and apoptotic analysis was performed using flow cytometry. Figure 3 illustrates the analysis of apoptosis using FACS compared to the control. The treatment of U87 cells showed a greater apoptotic effect in both early and late apoptosis. Early and late apoptosis can be observed by the shift of dots in Figure 2B to quadrant 3 and quadrant 2, respectively. We found that ethyl acetate had a greater effect in inducing both early and late apoptosis than the ethanol extract. To evaluate statistically, the percentage of apoptotic stages on U87 cells was analyzed. The statistical tests showed that both ethanol extracts and ethyl acetate fractions significantly induced late and early apoptosis on U87 cells ($p < 0.001$).

Effect of ethanol extract and ethyl acetate fraction on cell cycle activity of U87 cells

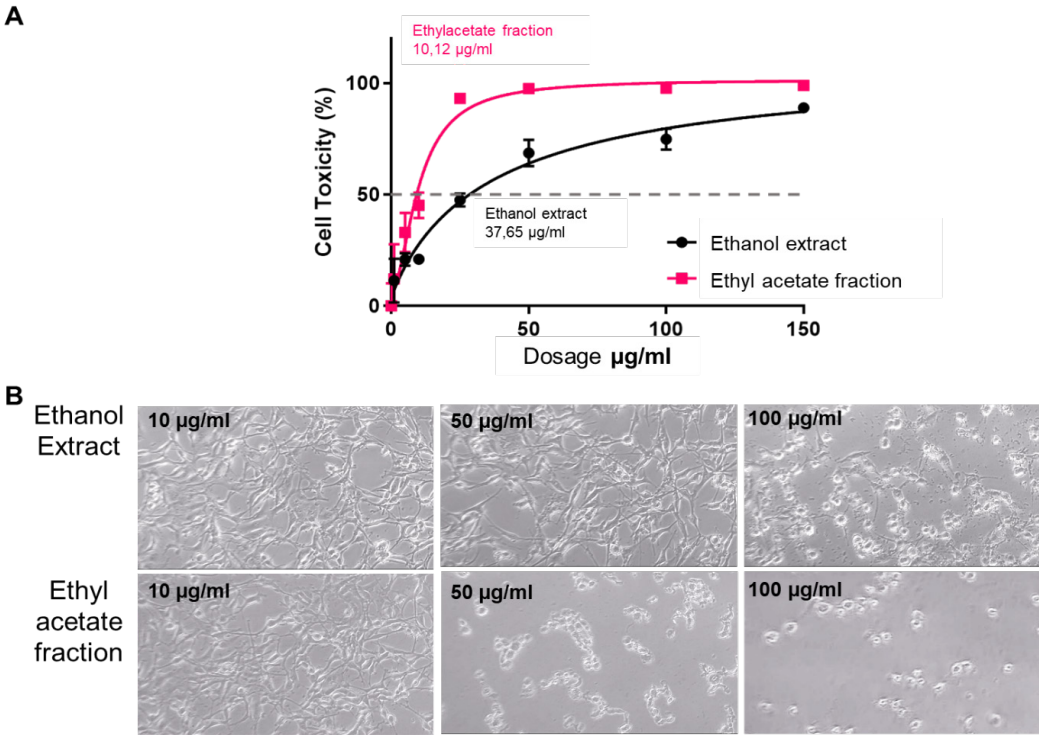

Figure 2. MTT Assay/Toxicity assay Results for *V. amygdalina* Delile Extract and Ethyl Acetate Fraction on U87 Cells. (A) Dose-response curve using 4-parametric regression. (B) Morphological appearance after 72-h treatment

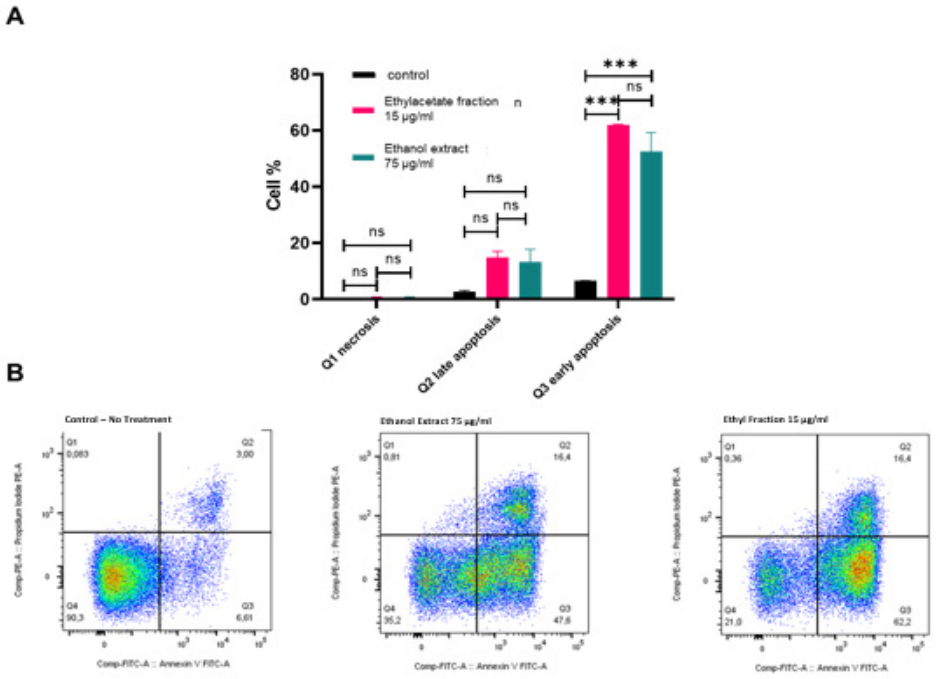

Figure 3. Apoptotic Analysis Using Flowcytometry. A. Apoptosis phase depicted in early and late apoptosis. B. Pseudo color density plot for U87 control, treated with ethanol extract and ethyl acetate fraction. ns: not significant, *: p<0.05, **: p<0.01, ***: p<0.001

For our final analysis, we conducted an examination of cell cycle progression using a flow cytometer, an instrument for investigating cellular dynamics. The data obtained were processed and analyzed with FlowJo software, employing the Matson method to ensure precision and reliability. While we observed

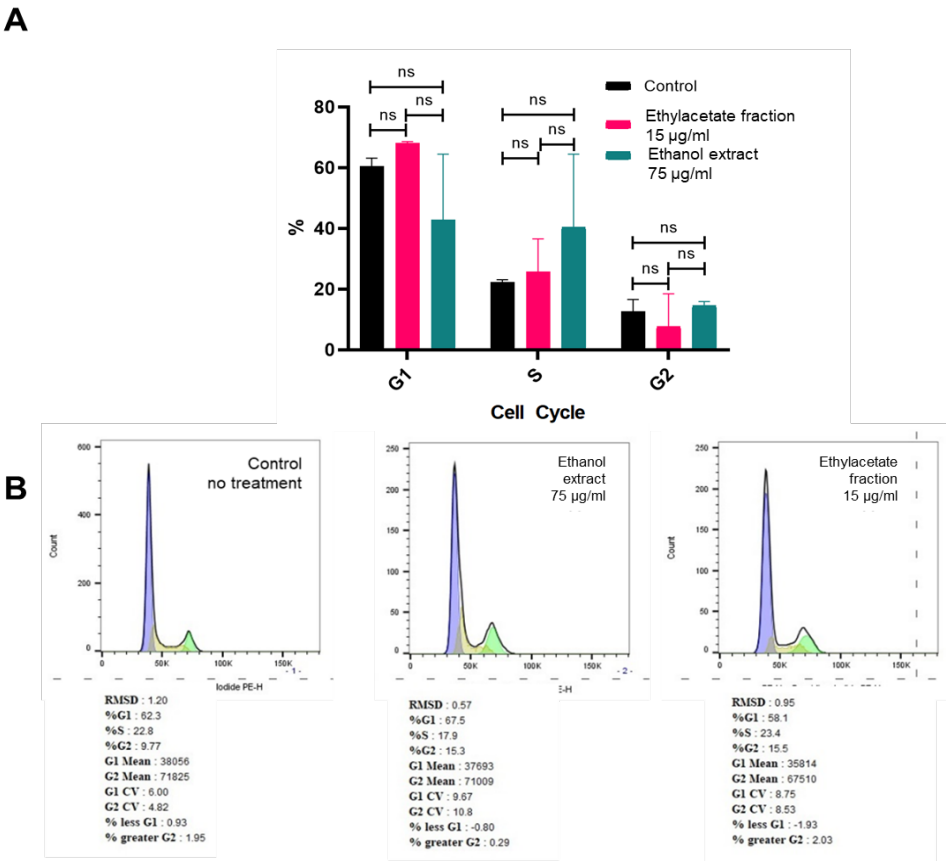

Figure 4. Cell Cycle Analysis Using Flowcytometry. A. Bar graph of cell cycle of U87 cell line and B. cell cycle curve that was treated by ethanol extract and ethyl acetate fraction. ns: not significant

slight variations in the percentage distribution of cell cycle stages, our analysis did not reveal any significant differences in the cell cycle profiles on cells treated with ethanol extracts and those treated with ethyl acetate fractions in the U87 cell line. This finding indicated that both treatments exerted similar effects on the cell cycle dynamics of U87 cells, as depicted in Figure 4.

Discussion

Cancer cells exhibit distinct physiological characteristics compared to normal cells, often evading cell death mechanism such as autophagy, apoptosis, and necrosis. Among tehese, apoptosis is the most common mechanism of cell death in normal cells [26, 27]. Cancer cells, however, can evade cell death and sustain proliferation and noursihment by inducing vasculature, enabling them to become more aggressive and metastasize [28]. GBM, a type IV glioma, is one of the most aggressive brain cancers, with several subtypes including Isocitrate dehydrogenase (IDH)-wild type (most common type), IDH-mutant, Glioblastoma not otherwise specified, and not-elsewhere-classified [1].

Amygdaline, also known as vitamin B17 or laetrile, is a cyanogenic glycoside known for its potential health benefits, especially in cancer treatment [8–13, 15]. It has some degradation enzymes, such as β-glycosidases. This enzyme hydrolized α-glucosidic bond to form benzaldehyde and hydrogen cyanide [29]. Despite the toxic nature of hydrogen cyanide, amygdaline has shown anticancer effects in vitro against various cancers [27, 30–34].

In our study, we used MTT assay to assess the cytotoxic activity of Vernonia amygdaline delile. This assay measure cell viability by detecting the reduction of MTT to formazan, a violet-blue water-insoluble molecule [24]. Treatment with different doses showed a reduction formazan formation with increasing dose. Comparing ethanol extract and ethyl acetate fraction on U87 cells, the ethyl acetate fraction exhibited higher cytotoxic activity, with a lower IC50 of 10.12 µg/ml compared to 37.65 µg/ml for ethanol extract. Other studies have reported IC50 values of *V. amygdalina Delile* ranging from 0 to 60 mg/ml for various cancers [30–33].

Amygdaline has been shown to induce apoptosis in cancer cells through various mechanisms, including the stimulation of pro-apoptotic proteins (Bax, pro-caspase 3, caspase-12, and p38 MAPK) and inhibition of anti-apoptotic protein (Bcl-2) [27, 30–33]. It is also inbibits other anti-apoptotic genes such as survivin and XIAP [30]. *V. amygdalina Delile* inhibits phosphatidylinositol 3'- kinase/protein kinase B (PI3K/Akt) pathway [18], a crucial signaling network involved in various cellular processes like proliferation, growth, and metabolism [35]. However, Vernonia amygdaline delile also has potentially antioxidant activity. This activity was conducted by various assays including 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), Cupric Reducing Antioxidant Capacity (CUPRAC), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydroxyl radical scavenging, and phenanthroline techniques. The study exhibited positive activity across a range of concentration [19]. In our study, we observed ethanol extract and ethyl acetat fraction of *V. amygdalina Delile* induced apoptosis on U87 cells, as evidenced by Annexin V/PI assay, with significant early apoptotic effects. However, the induction of late apoptosis was not as significant as early apoptosis.

U87 cells possess unique characteristics that allow them to resist treatment, underscoring the complexity of cancer cell responses to therapeutic interventions. Previous studies have indicated that U87 cells exhibit overexpression of MGMT [36], which enhances their DNA repair capabilities [4]. Conversely, Vernonia amygdalina Delile has been reported to cause DNA damage in leukemia cells [37]. Given the high levels of MGMT in U87 cells and the DNA-damaging potential of Vernonia amygdalina Delile, it was anticipated that the ethanol extract and ethyl acetate fraction of Vernonia amygdalina Delile might affect the cell cycle of U87 cells. However, our analyses revealed no significant impact on the cell cycle stages of U87 cells, suggesting that the observed resistance may be due to underlying mechanisms yet to be fully understood.

In conclusion, the findings of this study suggest that Vernonia amygdaline delile has potential as a chemotherapeutic agent against U87 cancer cells. It is evidenced by its low IC50 and significant induction of early apoptosis indicating cytotoxic effects of the ethanol extract and ethyl acetate fraction on U87 cells. However, this study did not investigate the specific mechanism by which Vernonia amygdaline Delile induces cell death in U87 cells. Further research is necessary to analyze the active compounds responsible for its anticancer effects and elucidate the underlying mechanisms. Overall, these findings contribute to the growing body of evidence supporting the potential of natural products, such as Vernonia amygdalina Delile, as novel and effective treatments for cancer.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: FH, MHB, DS; data collection: FH, MHB, RH; analysis and interpretation of results: FH, MHB, RGD; funding: FH, RGD, DS, AH, PAZ; draft manuscript preparation: FH, MHB, RH; final draft and revision: FH, RH. All authors reviewed the results and approved the final version of the manuscript.

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Data availability

Not applicable as we used information from previously published articles.

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Approved by any scientific body

Not applicable as the manuscript is not a part of any student thesis or study.

Ethical issues and approval

The ethical aspects of this research were reviewed and approved by Universitas Padjadjaran Research Ethics Committee.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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