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Investigation of the Anticancer Potential of *Vernonia amygdalina* Methanol Extract: A New Hope for MDA-MB-231 Breast Cancer Therapy

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Abstract

MDA-MB-231 is a breast cancer cell line derived from metastatic adenocarcinoma and classified as a triple-negative breast cancer (TNBC) subtype**.** This breast cancer subtype is highly aggressive, has a high recurrence rate, and offers limited treatment options due to the absence of estrogen, progesterone, and HER-2 receptor expression. Therefore, the development of advanced therapeutic strategies is urgently needed to inhibit the metastasis of TNBC cancer cells. This study aimed to investigate the potential of the methanol extract of *Vernonia amygdalina* leaves as a natural chemotherapeutic agent for metastatic breast cancer therapy, with a focus on inducing cell death through apoptosis mechanisms. This study was an experimental research that began with the collection and identification of raw materials, followed by the preparation of simplicia, extraction processes, and phytochemical screening. Subsequently, cytotoxicity testing was conducted using the MTT assay, cell cycle analysis was performed using the PI-staining assay, and apoptosis was assessed using the Annexin V/PI-staining assay, all of which were analyzed through flow cytometry. The methanol extract of *V. amygdalina* demonstrated a moderate cytotoxic effect with an IC_{50} value of 109.36 µg/mL. The extract induced S-phase cell cycle arrest in a dose-dependent manner, indicating its ability to reduce cell viability by inhibiting DNA replication. In the apoptosis assay, the highest percentage of cell death (3.12%) was observed at the concentration of 54.5 µg/mL, suggesting that this dose produced the strongest apoptotic response among the treatments tested. The methanol extract of *V. amygdalina* leaves shows potential as a natural chemotherapeutic agent for metastatic breast cancer. The extract can induce cancer cell death through apoptosis, indicating its promise for further development as a supportive anticancer therapy.

Keywords: Apoptotic; Breast Cancer Therapy; MDA-MB-231; *Vernonia amygdalina* leaves; Methanol extract.

Abbreviations: Annexin V–Propidium Iodide (Annexin V–PI); Half Maximal Inhibitory Concentration (IC_{50}); Human Epidermal Growth Factor Receptor-2 (HER-2); Malondialdehyde (MDA); Microculture Tetrazolium Technique (MTT); Nuclear Factor Kappa-B (NF-κB); Phosphate Buffered Saline (PBS); Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/Akt); Propidium Iodide (PI); Reactive Oxygen Species (ROS); Stem Cell and Cancer Research (SCCR); Thin Layer Chromatography (TLC); Triple-Negative Breast Cancer (TNBC); Ultraviolet (UV); and World Health Organization (WHO)

INTRODUCTION

According to GLOBOCAN 2020 data, a total of 2,261,419 women were diagnosed with breast cancer, with an estimated 684,996 deaths reported globally (Farmasita et al., 2021). MDA-MB-231 is a breast cancer cell line derived from metastatic adenocarcinoma and classified as a triple-negative breast cancer (TNBC) subtype. This cell line lacks estrogen, progesterone, and HER-2 receptors, making it unresponsive to hormonal therapy. As a result, MDA-MB-231 cells exhibit aggressive behavior and possess a high capacity for metastasis to distant organs (Huang et al., 2020). Chemotherapy remains the primary treatment option for various types of cancer, including triple-negative breast cancer (TNBC). However, this therapeutic approach has limitations such as severe side effects, the potential to promote cancer cell dissemination, and the risk of drug resistance. Consequently, natural compounds are increasingly being explored as alternative strategies for cancer therapy.

V. amygdalina, particularly its leaves, holds significant potential for further investigation as a breast cancer therapeutic agent (Hasibuan et al., 2020). The leaves of this plant are known to contain various bioactive compounds—such as flavonoids, terpenoids, and sesquiterpene lactones—that have been reported to exhibit cytotoxic activity against cancer cells (Ndayambaje et al., 2025). With its diverse phytochemical constituents and strong biological activities, *V. amygdalina* leaves represent a promising

candidate for development as an alternative or complementary therapy for triple-negative breast cancer (TNBC). Investigating the potential of these compounds is essential for identifying safer and more effective treatment options capable of inhibiting the growth and metastasis of cancer cells.

The flavonoids present in *V. amygdalina* leaves function as antioxidants by neutralizing free radicals and reactive oxygen species (ROS) in the body. Free radicals and ROS can induce oxidative stress, which contributes to cellular damage and various degenerative diseases. By inhibiting pro-oxidant enzyme activity and enhancing antioxidant enzyme activity, flavonoids help protect cells from oxidative injury (Tumilaar et al., 2024). In addition, these compounds exhibit anticancer potential due to their ability to induce apoptosis, a programmed cell death process that plays a key role in eliminating cancer cells. Flavonoids act by modulating various signaling pathways that regulate the cell cycle and trigger the death of abnormal cells. They also contribute to inhibiting cancer cell proliferation by suppressing the expression of proteins that drive uncontrolled cell growth, thereby slowing tumor progression. Another important mechanism is the modulation of the NF-κB signaling pathway, which plays a crucial role in regulating inflammation and cancer development (Pandey et al., 2025).

The bioactive compounds contained in the methanol extract of *V. amygdalina* leaves—particularly flavonoids—demonstrate promising potential as antimetastatic agents, especially against triple-negative breast cancer (TNBC) such as the MDA-MB-231 cell line. This cancer subtype is known for its aggressive behavior and resistance to conventional hormonal therapies, making alternative natural-based approaches highly relevant. To date, no studies have reported an investigation into the potential of *V. amygdalina* leaf extract as a natural anticancer agent capable of inhibiting metastasis by inducing apoptosis in metastatic breast cancer cells such as MDA-MB-231. Therefore, exploring the anticancer potential of the methanol extract of *V. amygdalina* leaves may open new opportunities for developing more effective and less toxic supportive therapies for this breast cancer subtype.

MATERIALS AND METHODS

The study was conducted at the Pharmacy Laboratory of the Bachelor of Pharmacy Study Program, Universitas Kadiri, Kediri, East Java, Indonesia, and at the Stem Cell and Cancer Research (SCCR) Laboratory in Semarang, Central Java, Indonesia. The research began with plant identification to ensure the authenticity of the plant material used. The plant sample was identified at Materia Medica Batu (MMB), Batu, Malang, East Java, Indonesia, where it was confirmed as *V.amygdalina*. After identification, fresh leaves of *V. amygdalina* were collected and prepared for further processing. The leaves were first washed with clean water to remove impurities and then air-dried at room temperature without direct exposure to sunlight in order to preserve the stability of the bioactive compounds. Once completely dried, the leaves were ground into fine powder to obtain plant simplicia suitable for extraction.

Extraction of the powdered leaves was carried out using methanol through the maceration method for 72 hours with periodic stirring to maximize the dissolution of phytochemical constituents. Methanol was selected as the extraction solvent because of its high efficiency in extracting polar secondary metabolites. After the maceration process, the mixture was filtered to separate the filtrate from the plant residue. The filtrate was then concentrated using a rotary evaporator at a low temperature of approximately 40–45°C to remove the solvent and obtain a thick methanol extract. The resulting concentrated extract was subsequently subjected to phytochemical screening using Thin Layer Chromatography (TLC) to identify the presence of bioactive compounds. The TLC analysis employed Silica Gel 60 F_{254} as the stationary phase, while the mobile phase consisted of n-butanol, acetic acid, and distilled water in a ratio of 4:1:5. The appearance of yellowish-green fluorescence under ultraviolet (UV) light at 366 nm indicated the presence of flavonoid compounds in the extract.

To evaluate the biological activity of the extract, a cytotoxicity assay was performed using the Microculture Tetrazolium Technique (MTT) method against metastatic breast cancer MDA-MB-231 cells. Confluent cells were harvested and seeded into 96-well plates, followed by treatment with serial concentrations of the methanol extract in triplicate. The treated cells were incubated for 24 hours to allow interaction between the extract and the cancer cells. After incubation, the treatment medium was removed and MTT reagent was added to each well, followed by a 4-hour incubation period to allow the formation of formazan crystals. A stopper reagent was then added, and the plate was incubated overnight to ensure complete solubilization of the formazan. Absorbance values were subsequently measured using an ELISA reader at a wavelength of 595 nm to determine cell viability and calculate the inhibitory concentration.

Further analysis was conducted to investigate the mechanism of action of the extract through cell cycle analysis using propidium iodide (PI) staining and flow cytometry. In this procedure, treated cells were washed with phosphate-buffered saline (PBS), and both the culture medium and PBS wash were collected in the same conical tube. Cells were harvested using trypsin, followed by centrifugation to obtain cell pellets. The supernatant was discarded, and the cell pellets were fixed before the addition of 400 µL of PI staining solution containing 1 mg/mL PI, 10 mg/mL RNase, and 0.1% Triton X-100. The cells were then resuspended, incubated to allow staining of

cellular DNA, transferred into flow cytometry tubes, and analyzed to determine the distribution of cells across different phases of the cell cycle. In addition, apoptosis analysis was performed using Annexin V–PI staining followed by flow cytometry. Treated cells were harvested using trypsin and centrifuged to obtain cell pellets. After discarding the PBS from the foil-covered conical tube, 120 µL of binding buffer was added to resuspend the cells. Annexin V and PI reagents were then added at a volume of 2 µL each, followed by the addition of 300 µL of binding buffer. The samples were gently mixed and subsequently analyzed using a flow cytometer to determine the proportion of cells undergoing early apoptosis, late apoptosis, or necrosis.

RESULTS AND DISCUSSION

Result

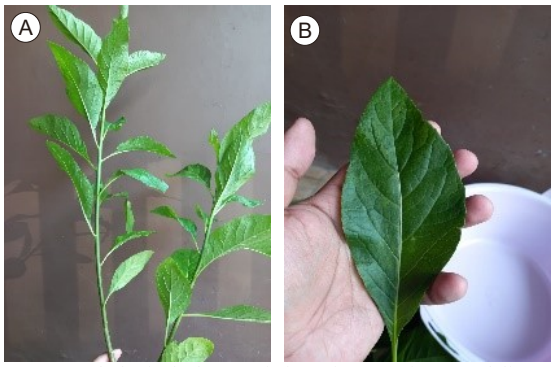

Figure 1. Morphological characteristics of Vernonia amygdalina Delile: (A) plant habitus; (B) leaf morphology.

The results of plant identification confirmed that the collected sample was *V. amygdalina*, commonly known as African leaf. Processing of the plant material produced dried leaf powder with a yield of 42.85% and a moisture content of 7.33%, indicating that the drying process was effective and within the acceptable moisture limit for dried plant materials. Extraction of the powdered simplicia using methanol produced approximately 5 grams of concentrated extract with an extraction yield of 23.07%. Phytochemical screening using TLC revealed that the methanol extract contained several classes of secondary metabolites, including flavonoids, saponins, and alkaloids, which are compounds commonly associated with various biological activities such as antioxidant and anticancer effects (Table 1).

Table 1.Phytochemical Screening Results of Vernonia amygdalina Methanol Extract.

Constituent	Mobile Phase	Phytochemical Screening Results				
		UV 254 nm	UV 366 nm	Reference Standard	Rf Value	Result
Flavonoid	Chloroform: Ethyl acetate (6:4)	Yellow–green	Yellow–green	Quercetin	0,65	Positive
Saponin	Chloroform: Methanol: Water (64:50:1)	Blue–brick red	Blue–brick red	-	0,86	Positive
Alkaloid	Toluene:Ethyl acetate:Diethylamine (7:2:1)	Brown–orange	Brown–orange	-	0,70	Positive

Note: (-) Negative

The cytotoxicity of the methanol extract of *V. amygdalina* leaves was evaluated using the Microculture Tetrazolium Technique (MTT) assay on MDA-MB-231 metastatic breast cancer cells. This method is widely used to determine the inhibitory effect of compounds on cell viability based on the metabolic activity of living cells. In this assay, viable cells with active mitochondrial enzymes reduce MTT into insoluble purple formazan crystals, whereas dead or damaged cells lose this metabolic capability. After treatment with various concentrations of the extract, the cells were incubated to allow interaction between the bioactive compounds and the cancer cells. Subsequently, MTT reagent was added and further incubated to enable the enzymatic reduction process. The resulting formazan crystals were then dissolved using a stopper solution, and the absorbance was measured using an ELISA reader at a wavelength of 595 nm. The absorbance values obtained are directly proportional to the number of viable cells present in each well.

Based on the analysis of the absorbance data, the methanol extract of *V. amygdalina* leaves demonstrated

inhibitory activity against MDA-MB-231 cells, with an IC_{50} value of 109.36 µg/mL (Figure 2). The IC_{50} (half maximal inhibitory concentration) represents the concentration of a compound required to inhibit 50% of cell viability compared to untreated control cells. This value indicates that at a concentration of 109.36 µg/mL, the extract is capable of reducing the viability of MDA-MB-231 breast cancer cells by half. The decrease in cell viability observed in this assay suggests that the extract contains bioactive compounds capable of interfering with cellular metabolic processes and survival mechanisms in cancer cells. Such activity may be associated with the presence of phytochemical constituents identified in the extract, particularly flavonoids, which are known to exhibit antiproliferative and cytotoxic effects through mechanisms such as oxidative stress modulation, disruption of mitochondrial function, and induction of programmed cell death. Therefore, although the IC_{50} value indicates moderate cytotoxic activity, the results provide preliminary evidence that the methanol extract of *V. amygdalina* leaves possesses potential anticancer properties against metastatic breast cancer cells.

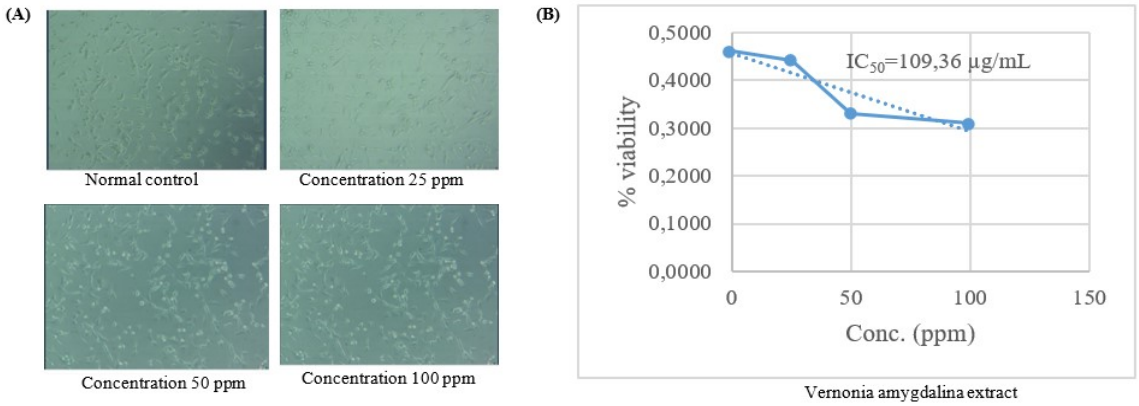

Figure 2. Cytotoxic activity of *Vernonia amygdalina* extract on MDA-MB-231 cells. (A) Cell morphology. (B) MTT dose–response curve (IC_{50} = 109.36 µg/mL).

Cell cycle analysis was performed to further evaluate the effect of the methanol extract of *V. amygdalina* leaves on the progression of the cell cycle in MDA-MB-231 breast cancer cells. This analysis aimed to determine whether the observed cytotoxic activity was associated with alterations in the distribution of cells across different phases of the cell cycle. The assay was conducted using propidium iodide (PI) staining followed by flow cytometry analysis. Propidium iodide is a fluorescent DNA-binding dye that intercalates into double-stranded DNA, allowing quantification of cellular DNA content. By measuring the intensity of fluorescence emitted from stained cells, flow cytometry can distinguish cells in different stages of the cell cycle, including the G0/G1 phase (cell growth), S phase (DNA synthesis), and G2/M phase (preparation for mitosis and cell division).

In this experiment, MDA-MB-231 cells were treated with the methanol extract and then harvested for analysis. The cells were washed with phosphate-buffered saline (PBS) to remove residual medium and treatment compounds, followed by fixation to preserve cellular DNA content and structure. After fixation, the cells were stained with PI solution containing RNase to eliminate RNA interference during fluorescence detection. The stained cells were then analyzed using a flow cytometer, which measured DNA content and generated histograms representing the distribution of cells in each phase of the cell cycle.

The results of the PI-staining flow cytometry analysis indicated that treatment with the methanol extract influenced cell cycle progression in MDA-MB-231 cells. Changes in the proportion of cells in specific cell cycle phases were observed when compared with the untreated control group. Such alterations suggest that the bioactive compounds present in the extract may interfere with regulatory mechanisms controlling cell cycle progression. In many anticancer studies, plant-derived compounds are known to inhibit cancer cell proliferation by inducing cell cycle arrest at particular phases, thereby preventing cells from completing the replication and division processes. The distribution pattern shown in the flow cytometry histogram, presented in the Figure 3, illustrates the proportion of cells in the G0/G1, S, and G2/M phases after treatment, providing insight into how the methanol extract may suppress cancer cell growth by disrupting normal cell cycle regulation.

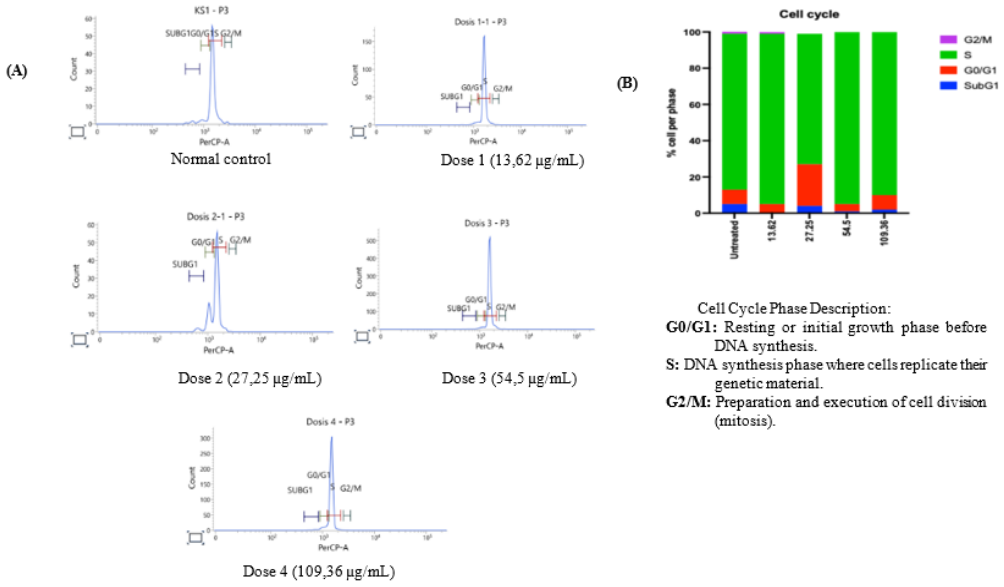

Figure 3. Cell cycle distribution of MDA-MB-231 cells after treatment with *Vernonia amygdalina* extract analyzed by PI flow cytometry showing S-phase arrest.

Apoptosis analysis was conducted to determine whether the cytotoxic effect of the methanol extract of *V. amygdalina* leaves on MDA-MB-231 cells was associated with the induction of programmed cell death. The assay was performed using Annexin V–Propidium Iodide (Annexin V–PI) staining followed by flow cytometry analysis. This method is widely used to differentiate viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells based on changes in cell membrane integrity and phosphatidylserine externalization. During the early stage of apoptosis, phosphatidylserine residues that are normally located on the inner leaflet of the plasma membrane become exposed on the outer surface of the cell membrane. Annexin V has a high affinity for phosphatidylserine and binds to these exposed molecules, thereby serving as a marker for early apoptotic cells. Meanwhile, Propidium Iodide (PI) is a DNA-binding fluorescent dye that can only penetrate cells with compromised membrane integrity, allowing the identification of late apoptotic or necrotic cells.

In this analysis, MDA-MB-231 cells were treated with the methanol extract and then harvested for staining. The cells were first collected by trypsinization and centrifugation to obtain cell pellets. After removing the supernatant, the cells were resuspended in binding buffer to maintain appropriate ionic conditions for Annexin V interaction. Subsequently, Annexin V and PI reagents were added to the cell suspension and incubated in the dark to allow proper staining. The samples were then analyzed using a flow cytometer, which detects fluorescence signals from both Annexin V and PI and categorizes the cells into four populations: viable cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), late apoptotic cells (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+).

The results of the apoptosis assay indicated that treatment with the methanol extract of *V. amygdalina* leaves increased the proportion of apoptotic cells compared with the untreated control group. An elevation in the populations of early apoptotic and late apoptotic cells was observed, suggesting that the extract is capable of triggering programmed cell death in MDA-MB-231 breast cancer cells. This finding indicates that the anticancer activity of the extract may involve apoptosis induction as one of its primary mechanisms. The presence of bioactive compounds such as flavonoids in the extract is likely to contribute to this effect, as flavonoids are known to regulate multiple molecular pathways involved in apoptosis, including mitochondrial dysfunction, activation of caspase enzymes, and modulation of signaling pathways that control cell survival and death. The flow cytometry dot plot illustrating the distribution of viable, apoptotic, and necrotic cells after treatment is presented in the Figure 4.

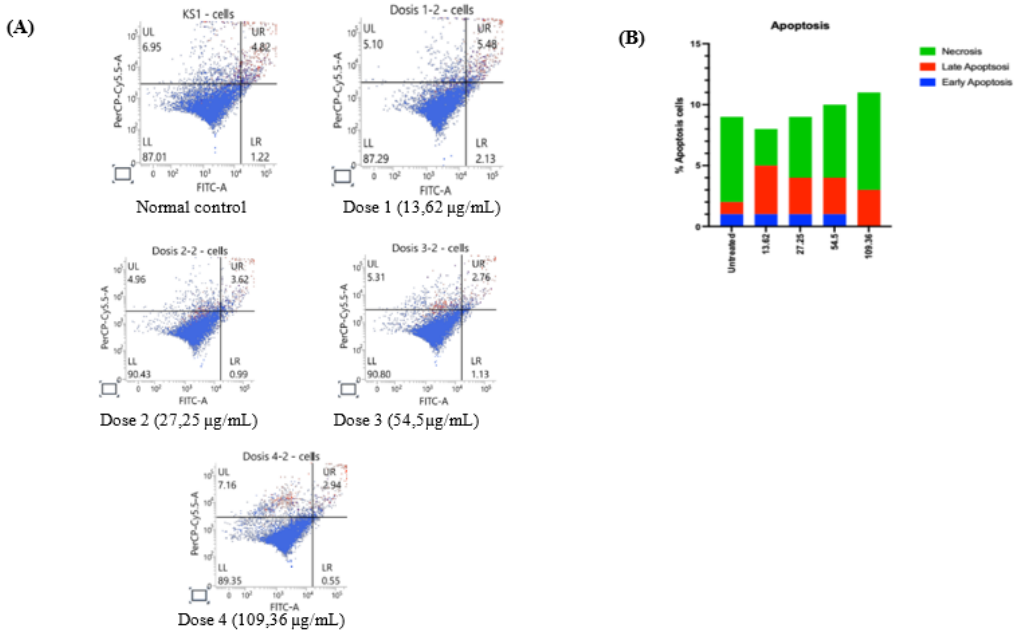

Figure 4. Apoptosis of MDA-MB-231 cells treated with *Vernonia amygdalina* extract analyzed by Annexin V–PI flow cytometry. (A) Dot plots. (B) Cell population distribution.

Discussion

The discussion of this study begins with plant identification conducted at Materia Medica Batu (MMB), which confirmed that the sample used was *V. amygdalina*. Botanical identification is an essential step in medicinal plant research to ensure the authenticity and purity of the raw material, since misidentification may affect phytochemical composition and biological activity of plant extracts (WHO, 2018). The processing of simplicia showed that the dried leaves produced powder with a yield of 42.85% and a moisture content of 7.33%. This moisture level is within the acceptable limit for dried plant materials, which generally recommends moisture content below 10% to prevent microbial growth and degradation of active compounds (Kemenkes RI, 2017). The powder yield also indicates that the drying and grinding processes were effective in producing a suitable material for extraction (Valentine, 2017).

Extraction using methanol produced a concentrated extract with a yield of 23.07%. Methanol is widely recognized as an effective solvent for extracting polar secondary metabolites such as flavonoids, alkaloids, and saponins, which are commonly reported in *V. amygdalina* leaves (Alara et al., 2019; Kadiri & Olawoye, 2016). The relatively high extraction yield suggests that the leaves contain abundant polar phytochemicals that can be efficiently extracted using methanol.

Phytochemical screening using thin layer chromatography (TLC) revealed the presence of flavonoids, saponins, and alkaloids. These compounds have been widely reported as major constituents of *V. amygdalina* and are associated with various biological activities including antioxidant, antimicrobial, and anticancer effects (Ndayambaje et al., 2025). In particular, flavonoids are known to exhibit antiproliferative activity against cancer cells through mechanisms such as oxidative stress modulation and apoptosis induction (Degu et al., 2024; Ugbogu et al., 2021).

The cytotoxicity assay using the MTT method demonstrated that the methanol extract of *V. amygdalina* exhibited an IC_{50} value of 109.36 µg/mL against MDA-MB-231 cells. Based on the classification of cytotoxic activity for plant extracts, an IC_{50} value within the range of 100–500 µg/mL is categorized as weak cytotoxic activity (Adascalului et al., 2020; Amarante-Mendes et al., 2018). Although this value indicates relatively low cytotoxic potency, interpretation should consider the characteristics of the cancer cell line used in this study.

The cytotoxicity test using the MTT assay on the methanol extract of *V. amygdalina* showed an IC_{50} value of 109.36 µg/mL. Based on the cytotoxicity classification for plant extracts, an IC_{50} value within the range of 100–500 µg/mL is categorized as weak cytotoxic activity (Adascalului et al., 2020; Amarante-Mendes et al., 2018). This indicates that the crude methanol extract does not exhibit strong cytotoxic potential; however, it still provides an initial indication of relevant biological activity against cancer cells.

The MDA-MB-231 cell line represents triple-negative breast cancer (TNBC), one of the most aggressive and therapy-resistant breast cancer subtypes. TNBC lacks estrogen, progesterone, and HER-2 receptors, making it unresponsive to hormonal therapy and targeted HER-2 therapy (Bianchini et al., 2016; Garrido-Castro et al., 2019). Due to its highly proliferative and resistant nature,

many plant-derived extracts that exhibit strong activity in other cancer models often show reduced cytotoxicity against TNBC cells. Therefore, even a moderate cytotoxic response may still indicate biologically relevant anticancer potential.

Another factor influencing the IC_{50} value is the use of crude extract. Methanol extract contains a complex mixture of secondary metabolites that may interact synergistically or antagonistically, which can affect the overall biological response (Chen et al., 2022; Mohammed et al., 2020). Consequently, the cytotoxic activity observed in crude extracts may underestimate the potency of individual bioactive compounds.

Several studies have reported that *V. amygdalina* contains bioactive flavonoids such as luteolin, which demonstrate significant antiproliferative activity against various cancer cells including TNBC. Luteolin is known to modulate multiple signaling pathways involved in cancer progression, including PI3K/Akt, NF-κB, and MAPK pathways, while also inducing apoptosis through oxidative stress and caspase activation (Imran et al., 2019; Wu et al., 2023). These findings suggest that the anticancer activity observed in this study may be associated with flavonoid constituents present in the extract. To further investigate the mechanism underlying the observed cytotoxic activity, cell cycle analysis was performed using PI-staining flow cytometry. The results demonstrated alterations in the distribution of cells across different cell cycle phases, including G0/G1, S, G2/M, and sub-G1. The increase in the sub-G1 population observed at certain treatment concentrations indicates DNA fragmentation, which is a characteristic feature of apoptotic cell death (Peng et al., 2023). In addition, the accumulation of cells in the S phase suggests that the extract may interfere with DNA replication, thereby inhibiting cancer cell proliferation.

Apoptosis analysis using Annexin V–PI staining further confirmed that treatment with the methanol extract induced programmed cell death in MDA-MB-231 cells. The flow cytometry results showed an increased proportion of cells in the early apoptotic (Annexin+/PI–) and late apoptotic (Annexin+/PI+) populations compared with the control group. These findings indicate that the extract is capable of triggering apoptosis in a dose-dependent manner.

The induction of apoptosis observed in this study is consistent with previous reports describing the apoptotic activity of flavonoids found in *V. amygdalina*. Compounds such as luteolin have been shown to activate intrinsic apoptotic pathways through mechanisms involving reactive oxygen species (ROS) generation, inhibition of PI3K/Akt signaling, and activation of caspase cascades (Nugraha et al., 2020; Wu et al., 2023). Therefore, the apoptosis induction detected in this study may be attributed to the presence of these bioactive flavonoid compounds.

Overall, the methanol extract of *Vernonia amygdalina* demonstrates preliminary anticancer potential against TNBC cells. Although the IC_{50} value indicates weak cytotoxic activity, further analyses revealed that the extract can modulate cell cycle progression and induce apoptosis in MDA-MB-231 cells. These findings suggest that the extract contains bioactive constituents, particularly flavonoids, that contribute to its antiproliferative effects. Further fractionation and purification studies are necessary to isolate and characterize the active compounds responsible for these anticancer activities.

CONCLUSIONS

This study demonstrates that the methanol extract of *V. amygdalina* leaves exhibits anticancer potential against metastatic breast cancer cells. The extract reduced cell viability and induced apoptosis, accompanied by S-phase cell cycle arrest and a dose-dependent apoptotic response. Among the tested concentrations, 54.5 µg/mL showed the highest apoptotic activity with minimal necrosis. These findings suggest that *V. amygdalina* may serve as a promising source of bioactive compounds for the development of natural-based therapies targeting metastatic breast cancer.

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Authors' Contributions: Ivan Junius Mesak designed the study, conducted the experiments, analyzed the data, and drafted the manuscript. Ardhi Broto Sumanto supervised the research, contributed to data interpretation, and revised the manuscript. All authors read and approved the final manuscript.

Competing Interests: The authors declare no conflicts of interest.

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