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Dichloromethane Fraction of *Vernonia Amygdalina* Delile Enhances the Cytotoxicity of Doxorubicin Against Triple-Negative Breast Cancer (TNBC) Cells Through Apoptosis Induction and Cell Cycle Modulation

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ABSTRACT

Doxorubicin (Dox) is a chemotherapeutic agent commonly used to treat nonspecific cancer types, including triple-negative breast cancer (TNBC). Owing to its toxicity to normal cells, Dox is usually combined with another agent that increases anticancer potency or reduces adverse effects. This study investigated the potential of African bitter leaf (*Vernonia amygdalina* Delile) extracts in combination with Dox to increase cytotoxicity against TNBC cells by modulating cell cycle and inducing apoptosis. The MTT cell viability assay on 4T1 and MDA-MB-231 cells confirmed that only the dichloromethane (DCM) fraction showed cytotoxic activity on both cells at a low grade. The DCM fraction increased the cytotoxic activity of Dox in 4T1 cells. The synergistic effect of DCM and Dox correlated with their activities in cell cycle modulation to accumulate cells in the sub-G1 phase and considerably induced apoptosis. In conclusion, the DCM fraction is a potential co-chemotherapeutic agent for Dox to suppress TNBC cell growth through apoptosis induction.

Keywords: *Vernonia amygdalina* Delille, TNBC, cotreatment, cell cycle, apoptosis

INTRODUCTION

Triple-negative breast cancer (TNBC) has a high proliferation rate and tends to undergo aggressive progression, with an increased risk of metastasis (Kumar & Aggarwal, 2016). Specific therapies are limited because of the absence of hormone receptors and HER2/neu protein expression in TNBC, and this limitation has led to the use of primarily conventional chemotherapy (Al-Mahmood et al., 2018), which has potentially severe side effects (O'Reilly et al., 2021). In this context, exploratory research into new chemotherapeutic agents for TNBC has explored their unique biological characteristics. The aggressiveness of TNBC and its resistance to conventional therapeutic modalities emphasize the urgent need to develop effective and safe therapeutic strategies, such as co-chemotherapy based on doxorubicin (Dox). Intrinsic resistance associated with TNBC is essential to the identification and development of novel chemotherapeutic agents that can induce apoptosis and cell cycle arrest (Obidiro et al., 2023). Therefore, continued research for identifying and testing these agents is crucial for the development of specific and effective therapies for TNBC.

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The African leaf (*Vernonia amygdalina* Delile; family Asteraceae) is a traditional African medicinal plant. It is originally from savanna woodland in tropical West Africa (Ohigashi et al., 1991) and can be found in Asian countries, including Indonesia. *V. amygdalina* has been referred to as bitter leaf because of its bitter taste and distinct odor (Wong et al., 2013). It contains flavonoids (Igile et al., 1994), sesquiterpene lactones (Sinisi et al., 2015), and steroidal saponins (Quasie et al., 2016). The bioactive compounds in African leaf play critical roles in its pharmacological activities, such as antioxidant, antimalaria, antimicrobial, antidiabetic, and anticancer activities (Ijeh & Ejike, 2011; Yeap et al., 2010). Vernodalinol, which is an isolated active compound of *V*. *amygdalina* (Luo et al., 2011), exerts a cytotoxic effect on MCF-7, demonstrating its potential as a chemotherapeutic agent. In addition, *V. amygdalina* contains some cardiac glycosides that play a considerable role in the protection of cardiac cells from oxidant materials, including chemotherapeutic agents.

Dox is one of the chemotherapeutic agents that are highly cytotoxic to cardiomyocytes because it generates ROS (Asensio-López et al., 2016). However, Dox is still preferred over other chemotherapeutic agents because of its effectiveness and low cost (Obidiro et al., 2023). Therefore, the development of strategies for reducing the side effects of Dox is needed. People in indigenous communities utilize natural resources in combination with chemotherapeutic agents, such as Dox, to treat cancer. However, information about the potential of co-chemotherapeutic agents remains limited. Thus, natural sources, such as galangal (Ahlina et al., 2020), soursop leaf (Salsabila et al., 2021), mangostin (Sarmoko et al., 2023), *citrus sinensis* peel (Zufairo' et al., 2023), kirinyuh leaf and bay leaf (Putri et al., 2023; Putranti et al., 2024), should be explored further. The crucial aspects of this endeavor are discovering alternative natural resources, such as common plants, as co-chemotherapeutic agents for Dox with precise dose, attenuating adverse effects, and preventing side effects. *V. amygdalina* is a viable co-chemotherapeutic agent because of its chemical contents.

This study evaluated the potential cytotoxic effects of *V. amygdalina* extracts and their combination with Dox to TNBC cells. Given that compounds in *V*. *amygdalina* leaves have varying polarity, we fractionated and tested them *in vitro*. The 4T1 and MDA-MB-231 cells were employed in this study to represent TNBC cells. In addition, we explored the fractions on cell cycle progression and apoptosis induction in cell lines. The results may provide valuable insights into the synergistic effect of both materials and their impact on the physiological processes closely related to changes in cancer cell viability.

MATERIALS AND METHODS

Dried *V. amygdalina* leaf extracts were obtained through reflux with n-hexane (HEX) and methanol extraction. The resulting methanol extract (MeOH) was fractionalized with a liquid–liquid extraction method using dichloromethane (DCM), ethyl acetate, and n-butanol (BuOH).

4T1 Breast Cancer Cells and Cytotoxic Assay

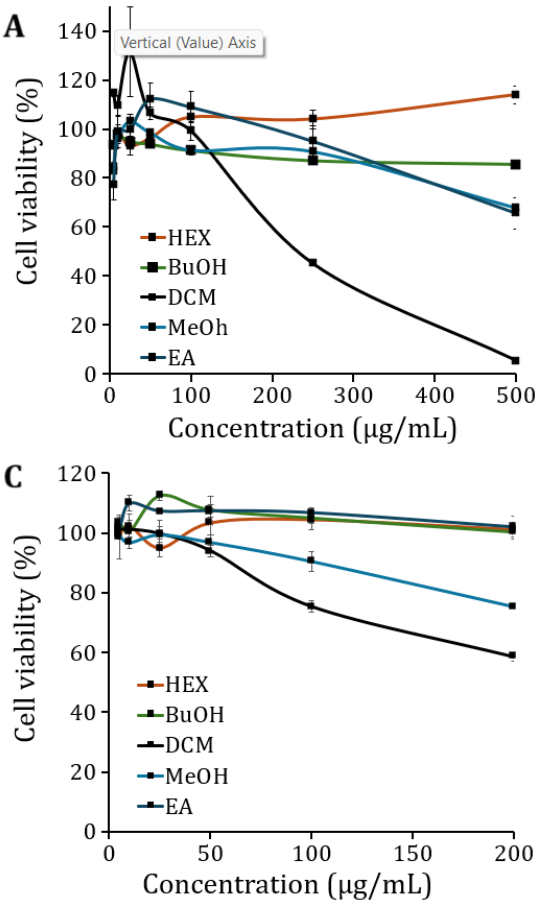
Breast cancer cells (4T1) were obtained from Prof. Masashi Kawaichi at the Nara Institute of Science and Technology (NAIST), Japan, and cultured under approved conditions. The cytotoxicity of all extracts and fractions, including the DCM fraction, Dox, and DCM–Dox combination, were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The 4T1 cells (8×10^3 cells/well) were seeded in 96-well plates to reach 80% confluence within 24 h. Subsequently, the cells were treated with varying concentrations (up to 500 µg/mL for *V. amygdalina* extracts and fractions and 10 nM for Dox) in culture media for 24 h. After treatment, an MTT solution was added, and formazan crystals were dissolved. Absorbance at 595 nm was measured using a microplate reader for triplicate IC_{50} calculation.

$$IC_{50} = \frac{cell\ absorbance\ with\ treatment - media\ control\ absorbance}{cell\ control\ absorbance - media\ control\ absorbance}$$

In the MTT test of combination treatment, compound concentrations (about ⅛, ¼, and ½ IC_{50}) were determined according to the IC_{50} value of a single treatment. The Chou–Talalay method assessed combination index (CI) values for synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1). The CI value was calculated as follows:

$$CI = \frac{D_1}{Dx_1} + \frac{D_2}{Dx_2},$$

where Dx_1 and Dx_2 denote the concentrations of individual agents that produce an x% effect, whereas D_1 and D_2 are the concentrations of these agents in combined treatment.



B

Compounds	IC_{50} value
HEX	n.d.
BuOH	n.d.
DCM	290 µg/mL
MeOH	n.d.
Ethyl Acetate (EA)	n.d.
Doxorubicin	570 nM

D

Compounds	IC_{50} value
HEX	n.d.
BuOH	n.d.
DCM	214 µg/mL
MeOH	n.d.
Ethyl Acetate (EA)	n.d.

Figure 1. Cytotoxic activity of *V. amygdalina* extracts/fractions and Dox on 4T1 (A, B) and MDA-MB-231 cells (C, D). Cytotoxic assays were performed with an MTT colorimetric system. The 4T1 cells (A, B) were treated with *V. amygdalina* extract and fraction and Dox for 24 h for the calculation of IC_{50} values in 4T1 cells. MDA-MB-231 cells (C, D) were treated with different extracts and fractions for 24 h for the calculation of IC_{50} values in MDA-MB-231 cells. HEX = hexane extract, MeoH = methanolic extract, DCM = dichloromethane fraction, BuOH = n-butanol fraction, EA = ethyl acetate fraction. Data presented as mean ± SD (n = 3).

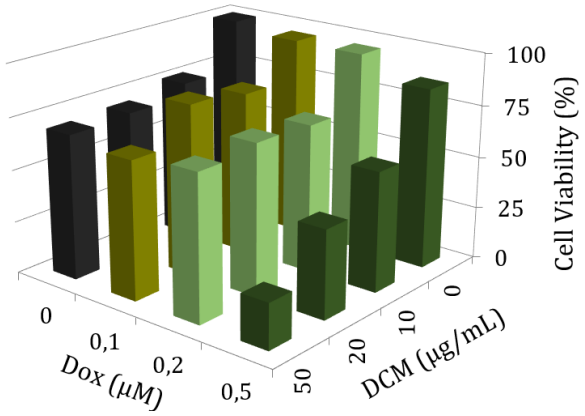
MDA-MB-231 Breast Cancer Cells and Cytotoxic Assay

MDA-MB-231 cells were obtained from ECACC (92020424). The cells were grown in an L-15 medium supplemented with 15% FBS in a humid incubator at 37 °C. A confluent culture of MDA-MB-231 cells was trypsinized, and the cells were seeded onto a 96-well plate at 5000 cells/well and incubated overnight inside a CO_2 incubator. The following day, the cells were treated with a series concentration of Hex, MeOH, DCM, BuOH, or DMSO as the control solvent and incubated for 24 h. At the end of incubation, the cells were treated with an MTT reagent. The resulting formazan was dissolved in DMSO after 2–3 h of incubation. The absorbance was measured at 570 nm with a microplate reader for the calculation of cell viability.

Cell Cycle Modulation

4T1 cells (3×10^5 cells/well) were treated with DCM or Dox or both at ¼ and ½ IC_{50} for 24 h. Then, the cells were harvested through trypsinization, washed with PBS once, and permeated at 2500 rpm for 5 min. The cells were fixed with 70% ethanol, stained with PI/RNase staining buffer solution (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions, and 0.1% Triton X-100. The fluorescence of DNA–propidium iodide (PI) complexes in the cells was analyzed using a BD FACS Aria III instrument (Rifai et al., 2024).

A



B

Dox (μM)	DCM (μg/mL)		
	10	20	50
0.1	0.108	0.205	0.253
0.2	0.110	0.170	0.306
0.5	0.127	0.124	0.169

Figure 2. Cytotoxic evaluation of the combination of DCM and Dox on 4T1 cells. Cytotoxic assays were performed using the MTT colorimetric system. The cells were treated with serial concentrations of ⅛, ¼, and ½ IC_{50} of the fraction and Dox for 24 h. Cell viability profile of 4T1 cells after treatment with DCM and Dox (A). Quantification of CI (B). DCM = dichloromethane fraction, Dox = doxorubicin. Data presented as mean (n = 3).

Apoptosis Profile Detection

Apoptosis in 4T1 cells was comprehensively assessed through flow cytometry employing annexin V and PI staining. The cells were treated with DCM and Dox at concentrations equivalent to half of their respective IC_{50} values. The evaluation of apoptosis was conducted meticulously at two distinct time points after 24 h of incubation. This systematic approach facilitated the comprehensive examination of cell death mechanisms, offering valuable insight into the specific impact of DCM fraction and Dox treatments on apoptosis during designated incubation periods.

Statistical Analysis

Statistical analysis was performed using SPSS version 25, and results were presented as mean ± SD. One-way ANOVA and Tukey post-hoc tests were employed, and significance was denoted by *p*-values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

RESULTS AND DISCUSSION

Cytotoxic Activity of *V. amygdalina* Extracts and Fractions

MTT cytotoxic assays were performed to test the cytotoxicity of the *V. amygdalina* extracts and fractions to MDA-MB-231 after 24 h of incubation. The test concentrations of up to 200 µg/mL were used. The DCM fraction showed the lowest IC_{50} value (214 µg/mL ± 0.76) among the extracts or fractions (Figures 1 C, D). MTT assays of the extracts and fractions were also performed in another TNBC cell line (4T1) after 24 h of incubation. The test concentration was up to 500 µg/mL. Dox was tested as a positive control with a concentration of up to 10 µM. In line with the test result in the MDA-MB-231 cells, the MTT assay in the 4T1 cells indicated that the DCM fraction had the lowest IC_{50} value (290 µg/mL ± 0.73) among the *V. amygdalina* extracts or fractions, indicating it had the highest level of cytotoxicity (Figures 1 A, B). In addition, the chemotherapy agent Dox showed high cytotoxic effect, with an IC_{50} value 570 ± 0.12 nM (Figures A, B).

Given that the DCM fraction had the highest potential among other *V. amygdalina* extracts or fractions, we further tested the combination effect of DCM and Dox on 4T1 cells with combinatorial cytotoxic tests. The serial concentrations of DCM and Dox were ⅛, ¼, and ½ and the combination effects were considered synergistic when a CI value was below 1.

Our study showed that combining DCM and Dox at concentrations lower than the IC_{50} concentration resulted in a 50% reduction in cell viability. This effect could explain the increase in antitumor activity (Figure 2). The DCM fraction might inhibit cellular signaling pathways that support cancer growth, and Dox might act directly on cancer cell DNA, enhancing its cytotoxic effects. The DCM fraction from *V. amygdalina* might contain bioactive compounds with antioxidant and anticancer potential. Therefore, combining Dox with DCM would exert a synergistic effect by optimizing the cytotoxic effect against breast cancer cells.

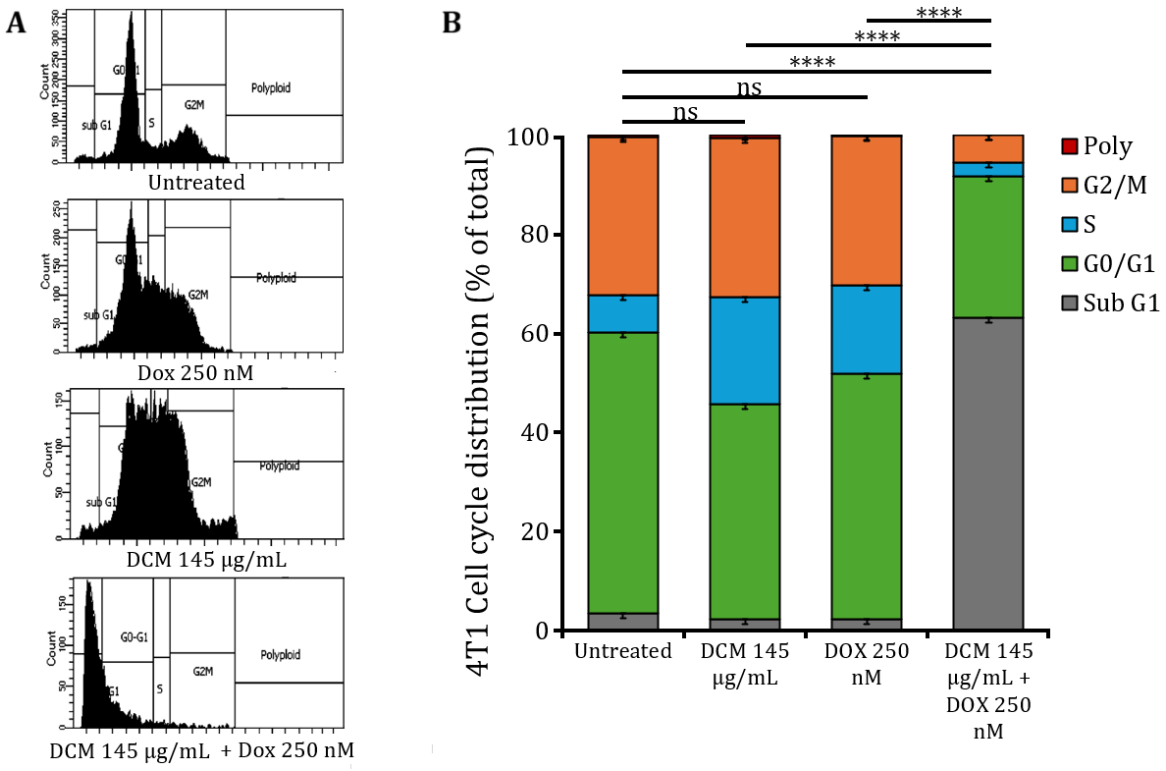


Figure 3. Cycle distribution of 4T1 cells during 24 h of treatment. Cell cycle test using propidium iodide staining assay by flow cytometry. Cells were treated with serial concentrations of ½ IC_{50} DCM, Dox, and a combination of the two samples for 24 h. Poly = polyploid cells; DCM = dichloromethane fraction; Dox = doxorubicin. Data presented as mean ± SD (n = 3).

Synergy analysis showed CI values less than 0.4 for all treatment combinations, indicating that the combined effect of the two agents was more significant than their individual effects. These findings indicated that combining DCM fractions from *V. amygdalina* with Dox is a practical therapeutic approach and potentially reduces the dose of Dox required for chemotherapy, thereby reducing the risk of side effects and drug resistance while treating breast cancer.

Enhancing Dox-mediated cell cycle arrest with the DCM fraction from *V. amygdalina* extract

Cell cycle profile testing is pivotal to the advancement of novel drug compounds for breast cancer. In this study, the cell cycle testing used the ½ IC_{50} values of DCM (145 µg/mL) and Dox (250 nM) in single or combination treatments on 4T1 cells.

Notably, the DCM fractions promoted cell accumulation in the synthesis phase of the 4T1 cell cycle. Similarly, Dox increased the synthesis phase cell accumulation even at a low dose. Intriguingly, the combined treatment yielded distinctive outcomes: a noteworthy surge in the sub-G1 phase ($p < 0.001$), signifying substantial cell death (Figure 3). This unique event occurred in the 4T1 cell cycle distribution during the 24 h treatment and was observed through a cell cycle assay utilizing PI staining via flow cytometry. These findings suggest a synergistic interplay when the DCM fraction and Dox coalesce, shedding light on the molecular intricacies governing their collaborative cytotoxicity.

DCM enhanced 4T1 apoptosis induction by Dox

For the apoptosis assay, 4T1 cells were treated with each DCM (145 µg/mL) and Dox (250 nM) at ½ IC_{50} concentrations. Single and combination tests were performed using flow cytometry. In a single treatment, 145 ug/mL DCM fraction showed an increase in apoptotic cells compared with nontreated cells, and Dox treatment caused a higher apoptosis rate than treatment with the DCM fraction. Interestingly, their combination increased the rate of apoptosis, considerably increasing late apoptosis events (Figure 4).

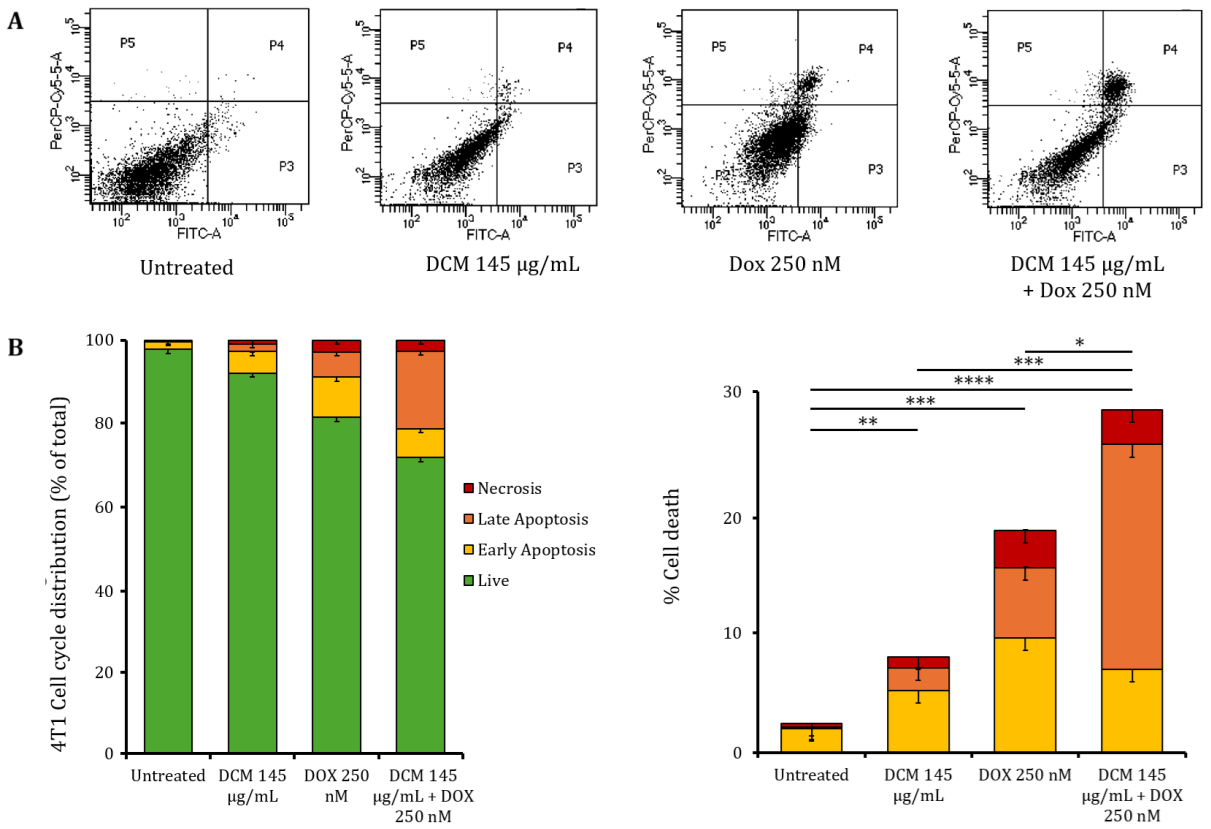


Figure 4. Apoptotic profile of 4T1 after treatment with DCM and Dox. The test was carried out using a flow cytometry assay with annexin V PI staining. Cells were treated with serial concentrations of ½ IC50 DCM, Dox, and a combination of DCM–Dox for 24 h. DCM = dichloromethane fraction; Dox = doxorubicin. Data presented as mean + SD (n = 3).

Natural products are potential resources for drug development and co-chemotherapeutic agents to increase the efficacy of chemotherapeutic agents and reduce side effects (Handayani et al., 2012). Several compounds or extracts exert a synergistic effect with Dox on several cancer cell lines. This research presents a novel challenge: using *V. amygdalina* to improve Dox's ability to suppress the growth of 4T1 cells. Interestingly, the synergistic effect that suppresses cell growth cells is correlated to an effect promoting cell cycle arrest prior to the initiation of apoptosis. The DCM fraction had the highest potential to suppress cell growth, but it had low cytotoxicity against 4T1 and MDA-MB-231 cells, i.e., this extract alone is not a potent anticancer agent. Nevertheless, the synergistic effect of the DCM extract and Dox on 4T1 cells indicated the potential of DCM as a co-chemotherapeutic agent.

The synergism of two agents is due to different target mechanisms (Caesar & Cech, 2019; Jenie et al., 2020; Rahmawati et al., 2023). Some flavonoids, such as genistein and hesperidin, show synergism with Dox because of different molecular targets. The flavonoids inhibit cell proliferation by targeting protein kinases or inhibit drug efflux by suppressing the activity of efflux pump Pg-P, whereas Dox inhibits DNA synthesis by intercalating the double-stranded DNA. Therefore, they show different effects on cell cycle progression. Flavonoids suppress cell cycle at the G-1 phase, and Dox suppresses the cell cycle in the S-phase (Ponte et al., 2021; Sutejo et al., 2019). Thus, DCM and Dox suppress the cell cycle at the S-phase. This phenomenon is typical for Dox, which has specific targets on DNA, but DCM might target S-phase regulatory proteins rather than directly interacting with DNA because DCM alone only elicits a weak cytotoxic activity. This possibility is an interesting issue that could be a concern of further exploration.

The DCM fraction of *V. amygdalina* contains flavonoids, luteolin, and their derivatives (Igile et al., 1994), and some cardiac glycosides may prevent cardiac tissue damage by inhibiting the Na+/K+ pump (Harahap et al., 2021). Apart from lowering the risk of cardiac cellular damage (Harahap et al., 2021), these compounds exert cytotoxic effects, especially in the S-phase. Luteolin and glycoside compounds scavenge some free radicals, such as ROS, in cells, preventing the side effects of chemotherapeutic agents. These findings offer prospects for research on the benefits of *V. amygdalina*.

CONCLUSION

This study highlights the potential of the DCM fraction from *V. amygdalina* to induce cytotoxicity and enhance apoptotic effects when combined with Dox. Further research is needed to elucidate physiological changes after cotreatment, such as changes in cell cycle progression and levels of apoptosis, the specific mechanisms, and potential therapeutic applications of this combination to TNBC treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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