



## Research article

The anticancer activities of *Vernonia amygdalina* Delile. Leaves on 4T1 breast cancer cells through phosphoinositide 3-kinase (PI3K) pathwayPoppy Anjelisa Zaitun Hasibuan<sup>a,\*</sup>, Urip Harahap<sup>a</sup>, Panal Sitorus<sup>b</sup>, Denny Satria<sup>b</sup><sup>a</sup> Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Sumatera Utara, 20155, Indonesia<sup>b</sup> Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Sumatera Utara, 20155, Indonesia

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## ABSTRACT

*Vernonia amygdalina* Delile (Asteraceae) is used in traditional medicine to treat diabetes mellitus, and some research provides its activity to treat breast cancer. The aim of this study is to assess the anticancer activity of *Vernonia amygdalina* Delile leaves fractions on 4T1 breast cancer cells. Analysis of phytochemical compounds were carried out with LC-MS/MS. Cytotoxic activity was determined using the MTT method in the 4T1 cell line. Apoptosis, the cell cycle, and PI3K and mTOR profiles were analyzed with flow cytometry. The phytochemicals found were diterpene (ingenol-3-angelate) and some phenolics (chlorogenic acid and 4-methoxycinnamic acid), flavonoids (apigenin, apigenin, luteolin, diosmetin, baicalin, rhoifolin, and scutellarin), and coumarins (7-hydroxycoumarin, 4-methylumbelliferone, and 4-methylumbelliferyl glucuronide). The results of the MTT assay showed that the IC<sub>50</sub> values *n*-hexane fraction, ethylacetate fraction (EAF), and ethanol fractions were 1,860.54 ± 93.11, 25.04 ± 0.36, and 1,940.84 ± 96.37 µg/mL, respectively. EAF induced early and late apoptosis, inhibited cell cycle progression on the G<sub>2</sub>/M phase, and inhibited PI3K and mTOR expression. The EAF of *Vernonia amygdalina* Delile leaves showed anticancer activity on 4T1 breast cancer cells through induction of apoptosis, enhanced cell accumulation on G<sub>2</sub>/M phases in the cell cycle, and inhibited expression of PI3K and mTOR.

## 1. Introduction

Breast cancer is a result of breast cells growing uncontrollably. Cells could invade nearby tissues and spread throughout the body. Any kind of tissue in the breast can form a cancer, but the most common type of breast cancer starts in the milk ducts or glands. The factors that influence the risk of breast cancer are reproductive history (e.g., no children and first pregnancy at an advanced age), the length of time of exposure to hormones or hormone replacement therapy, dietary factors, lack of physical activity, radiation during breast development, and breast cancer-related congenital genetic factors (e.g., the presence of gene mutations) [1]. In 2012, the WHO reported that breast cancer is one of the main causes of death and the most common incidence of cancer type among women worldwide [2].

The phosphoinositide 3-kinase (PI3K) pathway regulates cell growth and proliferation and is often dysregulated in cancer because of mutation, amplification, deletion, methylation, and post-translational modifications. This pathway is an intracellular signaling pathway that is of vital importance for apoptosis, malignant transformation, tumor

progression, metastasis, and radioresistance. Phosphatase and tensin homolog (PTEN) is a negative regulator of the PI3K/Akt/mTOR pathway. Because of the important role of the PI3K pathway in cancer research, many valuable inhibitors targeting one signal node (single inhibitor) or two nodes at the same time (dual inhibitor) in the pathway have been developed in recent years. In the last decade, significant progress has been made in developing combination therapy using the PI3K inhibitor together with other therapies for a more effective treatment [3, 4, 5, 6, 7].

*Vernonia amygdalina* Delile, from the family of Asteraceae, comes from West Africa. Several studies found some of its chemical constituents, such as flavonoids, sesquiterpene lactones, fatty acids, and steroidal saponins [8, 9, 10, 11, 12, 13, 14], and indicated some of its pharmacological activities, such as anti-malaria, anti-inflammation, anti-tumor, and anti-obesity [15, 16, 17, 18, 19, 20, 21, 22]. The aim of this study was to evaluate the anticancer activities of *Vernonia amygdalina* Delile leaves extract on 4T1 breast cancer cells through the PI3K pathway.

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## 2. Materials and methods

### 2.1. Plant and chemicals material

Fresh leaves of *Vernonia amygdalina* Delile were collected from the Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia. *Vernonia amygdalina* Delile was identified in Herbarium Medanense, Department of Biology, Faculty Mathematic and Natural Sciences, Universitas Sumatera Utara, and the voucher specimen was deposited in a herbarium (No. 1712/MEDA/2017). The chemicals used were acetonitrile (Merck), Annexin-V (BioLegend), anti-PI3K FITC (Bioss) and anti-mTOR PE (Biolegend), distilled water (BrataChem), dimethyl sulfide (Sigma), ethanol (Merck), ethylacetate (Merck), formic acid (Merck), *n*-hexane (Merck), propidium iodide (PI) (BioLegend), and [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma).

### 2.2. Preparation of fractions

The air-dried and powdered leaves of *Vernonia amygdalina* Delile (500 g) were repeatedly fractionated by maceration with *n*-hexane (3 × 3 day, 7.5 L). The powder was dried in the air and fractionated with ethylacetate (3 × 3 day, 7.5 L) at room temperature with occasional stirring. Then, the powder was dried in the air and fractionated with ethanol (3 × 3 day, 7.5 L). The filtrate was collected, evaporated under reduced pressure to give a viscous fraction, and then freeze-dried [23, 24, 25].

### 2.3. Phytochemical constituent analysis with LC-MS/MS

Analysis of phytochemicals from ethylacetate fraction (EAF) was carried out with TSQ Exactive (Thermo) (LSIH, Brawijaya University) with mobile phase A (0.1% formic acid in water) and phase B (0.1% formic acid in acetonitrile) following the gradient method. A flow rate of 40 µL/min was used for the Hypersil GOLD aQ column, 50 × 1 mm × 1.9 µm, and the time for analysis was 70 min. The results were analyzed using the Compound Discoverer software with mzCloud [26].

### 2.4. Cytotoxicity activity

#### 2.4.1. Cell culture

The 4T1 cell line was obtained from the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

#### 2.4.2. Cytotoxicity assay

The cells were treated with *n*-hexane fraction, EAF, and ethanol fraction. In this test, the 4T1 cell line was grown in a Dulbecco's Modified Eagle Medium, containing 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco), and 0.5% fungizone (Gibco) in a flask in a humidified atmosphere (5% CO<sub>2</sub>) at 37 °C. The inoculums were seeded at 1 × 10<sup>4</sup> cells/mL at an optimal volume of 0.1 mL per well. After 24 h incubation, the medium was discharged and treated by fractions and doxorubicin. After 24 h incubation, the cells were incubated with 0.5 mg/mL MTT for 4 h at 37 °C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, 10% SDS as the stopper (Sigma) in 0.01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for 24 h at room temperature and protected from light. After incubation, the cells were shaken, and absorbance was measured using a microplate reader at λ 595 nm. The data that were absorbed from each well were converted to percentage of viable cells [27, 28].

### 2.5. Preparation of cells for flow cytometry analysis

4T1 cells (5 × 10<sup>5</sup> cells/well) were seeded into a six-well plate and incubated for 24 h. After that, the cells were treated with EAF and then incubated for another 24 h. Both floating and adherent cells were collected in a conical tube using 0.025% trypsin. The cells were washed thrice with cold phosphate-buffered saline (PBS) and centrifuged at 2500

rpm for 5 min. The supernatant was separated, whereas the sediment was collected [29].

#### 2.5.1. Cell cycle analysis

Cells were fixed in cold 70% ethanol in PBS at –20 °C for 2 h. They were washed thrice with cold PBS, resuspended and incubated in 70% ethanol for 60 min, and then centrifuged at 3000 rpm for 3 min, and the PI reagent (containing 40 µg/mL PI and 100 µg/mL RNase) was added to the sediment and resuspended and incubated at 37 °C for 30 min. The samples were analyzed using a FACScan flow cytometer. On the basis of DNA content, the percentage of cells in each stage in the cell cycle (G<sub>1</sub>, S, and G<sub>2</sub>/M) was calculated using ModFit Lt. 3.0 s [30, 31].

#### 2.5.2. Apoptosis analysis

The Annexin-V reagent was added to the sediment and suspended and incubated at 37 °C for 30 min. The samples were analyzed using the FACScan flow cytometer [27].

### 2.6. PI3K and mTOR expression analysis

4T1 cells (5 × 10<sup>5</sup> cells/well) were seeded into a six-well plate and incubated for 24 h. After that, the cells were treated with EAF and then incubated for 24 h. Both floating and adherent cells were collected in a conical tube using 0.025% trypsin. The cells were washed thrice with cold PBS and centrifuged at 2500 rpm for 5 min. The supernatant was separated, whereas the sediment was collected. The sediment cells were fixed with 70% ethanol and allowed to stand for 2 h at –20 °C, and PI3K FITC and mTOR PE antibodies were added and incubated at 37 °C for 10 min. The samples were analyzed using the FACScan flow cytometer [32].

### 2.7. Statistical analysis

Data were expressed as mean ± SD. All statistics were analyzed using the SPSS 21 software.

## 3. Results

### 3.1. Phytochemical constituent analysis of EAF

Phytochemical constituent analysis of EAF was carried out with LC-MS/MS to obtain information about its compounds. The results are given in Table 1.

The results showed that EAF contains diterpene (ingenol-3-angelate) and some phenolics (chlorogenic acid and 4-methoxycinnamic acid), flavonoids (apigenin, apigenin, luteolin, diosmetin, baicalin, rhoifolin, and scutellarin), and coumarines (7-hydroxycoumarine, 4-methylumbelliferone, and 4-methylumbelliferyl glucuronide).

### 3.2. Inhibitory concentration 50% (IC<sub>50</sub>)

The MTT method was used to determine cell viability after incubation for 24 h. Table 2 shows the IC<sub>50</sub> values of *Vernonia amygdalina* Delile fractions for every treatment.

### 3.3. Effect on cell cycle and apoptosis

To evaluate the effect of EAF (see Figure 1) on the increase of cell death by modulating the cell cycle, we used the flow cytometry method. In contrast, treatment with EAF at 12.5 µg/mL caused cell accumulation at the G<sub>2</sub>/M phase (34.22%) and for control cells (17.93%).

Evaluation of apoptosis induction was performed using the flow cytometry method with Annexin-V as shown in Figure 2. The cells in the upper and lower right quadrants represent late apoptotic/necrotic and early apoptotic cells, respectively. The percentage of control cells and cells treated with EAF at 12.5 µg/mL was 0.55% and 3.13% in early

**Table 1.** Phytochemical constituent analysis of EAF with LC-MS/MS.

| No | Name                             | Formula   | Molecular Weight | Retention Time (min) |
|----|----------------------------------|---|------------------|----------------------|
| 1  | Diosmetin                        | C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>  | 300.0633         | 0.785                |
| 2  | 4-Methylumbelliferyl glucuronide | C <sub>16</sub> H <sub>16</sub> O <sub>9</sub>  | 352.0769         | 0.955                |
| 3  | Chlorogenic acid                 | C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>  | 354.0949         | 2.703                |
| 4  | 4-Methylumbelliferone            | C <sub>10</sub> H <sub>8</sub> O <sub>3</sub>   | 176.0475         | 4.354                |
| 5  | Scutellarin                      | C <sub>21</sub> H <sub>18</sub> O <sub>12</sub> | 462.0797         | 5.164                |
| 6  | Rhoifolin                        | C <sub>27</sub> H <sub>30</sub> O <sub>14</sub> | 578.1634         | 5.346                |
| 7  | 7-Hydroxycoumarine               | C <sub>9</sub> H <sub>6</sub> O <sub>3</sub>    | 162.0316         | 5.424                |
| 8  | Apigetrin                        | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub> | 432.1059         | 5.564                |
| 9  | Apigenin                         | C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>  | 270.0521         | 5.566                |
| 10 | Baicalin                         | C <sub>21</sub> H <sub>18</sub> O <sub>11</sub> | 446.0849         | 5.628                |
| 11 | Luteolin                         | C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>  | 286.0467         | 6.888                |
| 12 | Ingenol-3-angelate               | C <sub>25</sub> H <sub>34</sub> O <sub>6</sub>  | 430.2353         | 8.126                |
| 13 | 4-Methoxycinnamic acid           | C <sub>10</sub> H <sub>10</sub> O <sub>3</sub>  | 178.0629         | 15.929               |

**Table 2.** The IC<sub>50</sub> values of *Vernonia amygdalina* fractions with the MTT assay of 4T1 cells (mean ± SD, three times of replication).

| Treatment                 | IC <sub>50</sub> (µg/mL) |
|---------------------------|--------------------------|
| <i>n</i> -Hexane fraction | 1,860.54 ± 93.11         |
| Ethylacetate fraction     | 25.04 ± 0.36             |
| Ethanol fraction          | 1,940.84 ± 96.37         |

apoptosis, 0.52% and 5.12% in late apoptosis/early necroptosis, and 2.99% and 22.75% in late necroptosis, respectively.

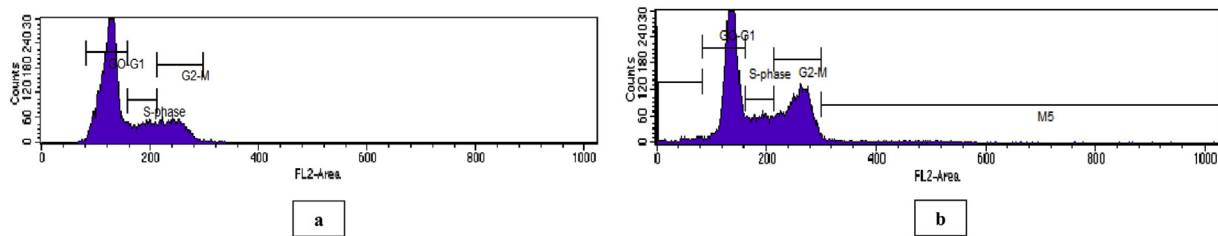
**3.4. Analysis of PI3K and mTOR expression**

To evaluate the effect of EAF on the decrease of PI3K and mTOR expression, we used the flow cytometry method. In contrast, treatment of EAF at 12.5 µg/mL caused cell accumulation in the M2 area (7.59%) and for control cells (4.27%). Evaluation of PI3K expression was performed using the flow cytometry method with PI3K antibody that conjugated with FITC as shown in Figure 3.

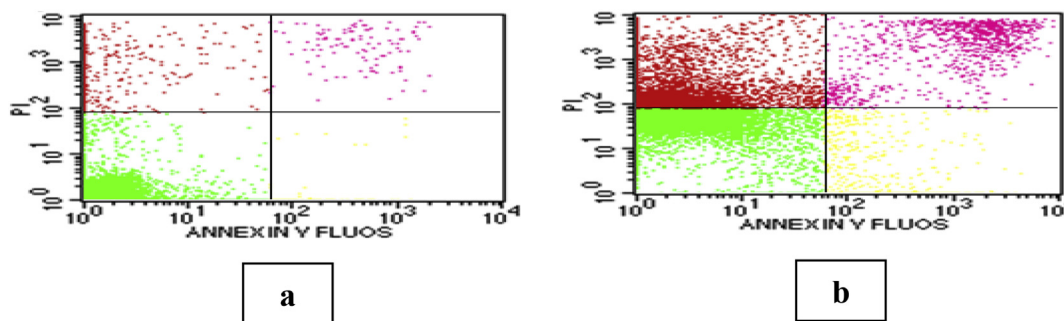
Moreover, treatment with EAF at 12.5 µg/mL caused cell accumulation in the M2 area (9.22%) and for control cells (1.04%). Evaluation of mTOR expression was performed using the flow cytometry method with mTOR antibody that conjugated with PE as shown in Figure 4.

**4. Discussion**

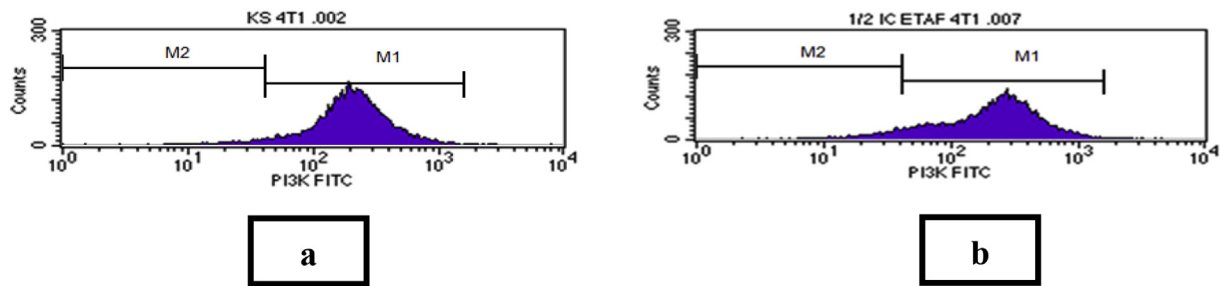
The profile of *Vernonia amygdalina* Delile. was predicted on the basis of the molecule weight and fragmentation pattern. Predictions were analyzed on the basis of the percentage of similarity between the target compound and a compound library using computer software [27]. Cell viability was determined by the MTT method after incubation for 24 h. In every treatment, fractions are shown to inhibit cell growth. The IC<sub>50</sub> from *n*-hexane fraction, EAF, and ethanol fraction were 1,860.54 ± 93.11, 25.04 ± 0.36, and 1,940.84 ± 96.37 µg/mL, respectively. The cytotoxicity of the natural product is related to the content of active compounds in some plants. *Vernonia amygdalina* Delile. contains diterpene (ingenol-3-angelate), phenolics (chlorogenic acid and 4-methoxycinnamic acid), flavonoids (apigetrin, apigenin, luteolin, diosmetin, baicalin,



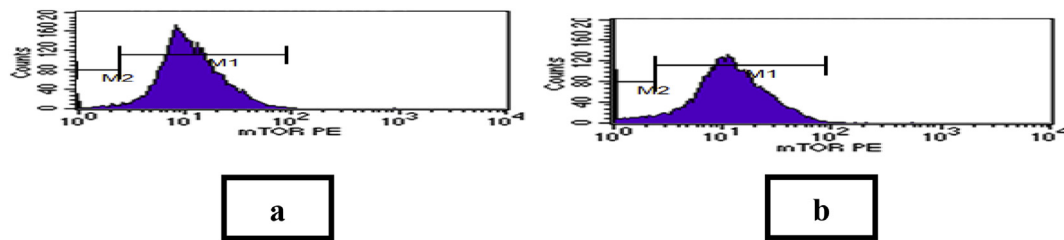
**Figure 1.** Representative images showing cell cycle analysis after treatment with EAF, which inhibits cell cycle progression on 4T1 cells. Percentage of the cell cycle phase of 4T1 cells treated with EAF for 24 h. (a) Control cells. (b) Cells treated with EAF at 12.5 µg/mL.



**Figure 2.** Representative images showing apoptosis analysis using flow cytometry after treatment with EAF, which stimulates the apoptosis process on 4T1 cells. 4T1 cells were treated with EAF for 24 h and stained using Annexin-V. (a) Control cells. (b) Cells treated with EAF at 12.5 µg/mL. EAF was increased at early apoptosis and late apoptosis.



**Figure 3.** Representative images showing the analysis of PI3K expression after treatment with EAF, which decreased the expression of PI3K protein on 4T1 cells. PI3K analysis was carried out using the flow cytometry method. 4T1 cells were treated with EAF for 24 h and stained using PI3K antibody. (a) Control cells. (b) Cells treated with EAF at 12.5 µg/mL.



**Figure 4.** Representative images showing the analysis of mTOR expression after treatment with EAF decreased the expression of mTOR protein on 4T1 cells. The mTOR analysis was carried out using the flow cytometry method. 4T1 cells were treated with EAF for 24 h and stained using mTOR antibody. (a) Control cells. (b) Cells treated with EAF at 12.5 µg/mL.

rhoifolin, and scutellarin), and coumarines (7-hydroxycoumarin, 4-methylumbelliferone, and 4-methylumbelliferyl glucuronide) as active compounds [33, 34]. Apigenin (5,7,4'-trihydroxyflavone) has anticancer activity in various cancer cells. Combining apigenin with 5-fluorouracil and cisplatin inhibits the growth of SCC25 and A431 cells and induces cell cycle arrest in the G2/M phase. The  $IC_{50}$  value of apigenin in ER(-) breast cancer cells is  $60.4 \pm 15.8 \mu\text{M}$  [35]. Luteolin (5,7,3',4'-tetrahydroxyflavone) is a well-known chemopreventive and cytotoxic agent that increases expression of p53 and p21 in A549 cells. Combining luteolin with gemcitabine or 5-fluorouracil synergistically brings about anti-proliferative activity in U251MG and U87MG cells [36]. Coumarines such as imperatorin and esculetin inhibit the proliferation of cancer cells through cell cycle arrest [37, 38]. EAF has increased the cells' likelihood of undergoing apoptosis, both early apoptosis and late apoptosis, compared with the control cells. Cells that underwent apoptosis, a programmed cell death process, have altered morphology, membrane blebbing, and chromatin changes [39, 40]. Some of the isolated polyphenols from plants such as kaempferol, quercetin, coumarin acid, anthocyanins, and ellagic acid inhibit the growth (inhibit cell cycle progression and induce apoptosis) of human breast cancer cells [41, 42, 43]. Apigenin releases cytochrome c; activates the caspase cascade; downregulates antiapoptosis protein such as Bcl-xL, Bcl-2m, Mcl-1; and upregulates Bax, Bad, and Bak in T24 cells [44].

## 5. Conclusion

In conclusion, this study has revealed that EAF is endowed with the many bioactive compounds that possess anticancer activity in 4T1 breast cancer cells.

## Declarations

### Author contribution statement

D. Satria: Performed the experiments; Analyzed and interpreted the data.

P.A.Z. Hasibuan: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

U. Harahap: Conceived and designed the experiments; Wrote the paper.

P. Sitorus: Analyzed and interpreted the data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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