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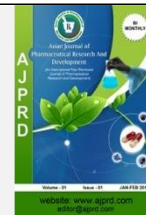

Open Access Research Article

PHYTOCEMICALS SCREENING AND CELL CYCLE ARREST ACTIVITY OF *n*-HEXANE EXTRACT OF *Vernonia amygdalina* Delile LEAVES AGAINST PANCREATIC CANCER CELL LINE

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ABSTRACT

Objective: This study aims to determined phytochemical compounds of simplex and *n*-hexane extract (nHE) of *Vernonia amygdalina* Delile Leaves and cell cycle arrest activity against PANC-1 cells.
Methods: The leaves of *Vernonia amygdalina* Delile were dried and extracted with *n*-hexane, followed by evaporation and freeze-drying. Phytochemicals screening were analyzed with standard procedures. Cytotoxic activity was carried out using MTT assay method. PANC-1 cells were treated with different concentrations of nHE (500 ug/mL, 250 ug/mL, 125 ug/mL, 61.5 ug/mL and 31.25 ug/mL) for 24 hours to obtained IC_{50} values. The cell cycle inhibition activity of nHE was carried out used flowcytometry method and apoptosis activity used double staining method.
Results: Then HE was identified contains steroids/triterpenoids. The IC_{50} was114.80 ± 1.21 ug/mL. The nHE inhibited cell cycle PANC-1 on M1 phase (67.39%) and it was induced apoptosis process on PANC-1 cells.
Conclusions: Our results suggest that extracts*n*-hexane of *Vernonia amygdalina* Delile Leaves had as cancer chemoprevention activities with inhibits cell cycle and spur apoptosis process on PANC-1 cells.

Keywords: *Vernonia amygdalina* Delile, Phytochemcial, Cytotoxic, Cell Cycle, apoptosis

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INTRODUCTION

Cancer was become a very scary disease because of high amount new and death cases occurred in the world[1]. Pancreatic cancer was a type of disease that continued to develop in the world. It was a type of cancer that attack human's organ called pancreas [2]. Around 400 thousand new cases occurred in 2018 [3], with 331 thousand case leaded a death every year [4]. Lack of data about pancreatic cancer, the absence of specific symptoms, fast metastasis and resistance incidence of chemotherapeutics were the causes of high death cases by pancreatic cancer [5-6].

Efforts to treated pancreatic cancer can be done with some method such as surgery, radiotherapy and chemotherapeutic. Traditional medicine that was rich a Phytochemical compounds such as alkaloid, glycoside, polyphenol, saponin and steroid/ triterpenoid can used as alternative drug to pancreatic cancer[7]. *Vernonia amygdalina* Delile (VAD) was an *Asteraceae* plant, used as vegetable by west and center Africa people. Some research has done against VAD to find out the benefits in a health field between antimicrobial activity, antidiabetic activity and anticancer activity [8-9]. As a anticancer, VAD has been tested in vitro and in vivo, among others against nacofaring cancer cell, skin cancer cell [10], prostate cancer cell [11] and breast cancer cell [12].

VAD activity for inhibit development some kind of cancer cells was a reason to do screening Phytochemical compounds

in VAD. Phytochemical compounds in VAD can use as a drug cancer, especially for pancreatic cancer. As a potential pancreatic cancer drug, VAD must through some tests. Cytotoxic test, cell cycle inhibition and spur apoptosis into a test that can be done to determined the anticancer activity against pancreatic cancer cell.

MATERIAL AND METHODS

Material

All chemicals and reagents were procured from certified suppliers and were of the highest analytical standard. The pancreatic cancer cell (PANC-1) was obtained from Laboratory of Parasitology, Universities Gadjah Mada, Yogyakarta, Indonesia.

Sampel Preparation and Extraction

The leaves of *Vernonia amygdalina* Delile collected from garden of medical plants Faculty of Pharmacy, Universities Sumatera Utara, Medan, Indonesia. 1000 g of powdered were extracted by cold maceration with *n*-hexane (3 x 3 d, 7,5 L). The filtrate was collected and the evaporated under reduced pressure to give a viscous extract and then freeze-dried to give a dried extract [13-14].

Phytochemical Screening

Phytochemical screening to determine alkaloids, glycosides, flavonoids, saponins, steroid/ triterpenoids and tannins were carried out according to standard procedures [14].

Cytotoxicity Test

PANC-1 cell line was grown in DMEM medium containing 10% Fetal Bovine Serum (Gibco), 1% penicillin-streptomycin (Gibco) and fungizone 0,5% (Gibco) in a flask with condition a humidified atmosphere (5% CO_2) at 37^0C. The inoculums seeded at 1 x 10^4 cells/mL at an optimal volume of 0,1 mL per well. After 24 h incubated, the medium was discharged and treated by nHE. After 24 h incubated, the cells were incubated with 0,5 mg/mL MTT for 4 h at 37^0C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, SDS 10% as a stopper (Sigma) in 0,01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for 24 h in room temperature and protected from light. After incubated, the cells were shaken and absorbance was measured using microplate reader at λ595 nm. The data which were absorbed from each well were converted to the percentage of viable cells [13-17]. The equation to determination viability of cells:

$$\frac{Abs\ of\ treatment - Abs\ of\ medium}{Abs\ of\ control\ cells - Abs\ of\ medium}\ x\ 100\%$$

Cell Cycle Inhibition Test

PANC-1 cells (1 x 10^6 cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated nHE with IC_{50} concentration and incubated for 24 h. The cells were collected in a conical tube using trypsin 0,025% and were washed with PBS, after that centrifugated at 2500 rpm for 5 min. The sediment was collected and fixed in cold 70% ethanol in PBS at 4°C for 1 h. After that, the cells were washed with cold PBS and resuspended the centrifuged at 3000 rpm for 3 min and PI kit. The sediment was re suspended and incubated at 37°C for 30 min. The samples were analyzed using FAC Scan flowctometry. The percentage of cells were calculated using Mod Fit Lt. 3.0.s [13, 18, 20].

Observation of Apoptosis

PANC-1 cells (1 x 10^5 cells/well, 24 - well plate) were plated on cover slips and incubated for 24 h. After that, the cells were treated nHE with IC_{50} concentration and incubated for 24 h. The medium was removed and washed using PBS. Cover slips were taken and put on glass slide before added with 10µL acridine orange-ethidium bromide and incubated for 15 minutes. Then were observed under the confocal microscope [20].

Statistical analysis

All data were anelyzed with regression analysis using SPSS 22.

RESULT AND DISCUSSION

VAD activity as anticancer agent was reported. In this study was showed nHE can inhibit development of pancreatic cancer. nHE was obtained steroids/triterpenoids metabolite secondary. Phytochemicals content of simplex VAD and nHE can be seen on Table 1. Steroids/ triterpenoids were indentified active as inhibited the development cell cancers [21-23]. This was supported by cytotoxic nHE activity, the ability to inhibit the cell cycle and trigger apoptosis of PANC-1 cells.

Table 1 Phytochemicals content of simplex VAD and nHE

No	Metabolite secondary	Simplex	nHE
1	Alkaloids	-	-
2	Flavonoid	+	-
3	Glycoside	+	-
4	Steroid/triterpenoid	+	+
5	Saponin	+	-
6	Tannin	+	-

Description: (+) shows that the simplicia VA and nHE contains secondary metabolite, (-) shows that the simplex VA and nHE not contain secondary metabolite. VA: *Vernonia amygdalina*, nHE: Extract *n*-hexane *Vernonia amygdalina*. Phytochemical compounds in simplex VA and nHE were different.

Cytotoxic effect of nHE against PANC-1 cells was carried out by MTT method [13,15].PANC-1 cells were treated with some nHE concentrations (500 ug/mL, 250 ug/mL, 125 ug/mL, 61.5 ug/mL and 31.25 ug/mL). After that, IC_{50} were measured using a microplate reader at 595 nm.The result of IC_{50} can be seen in the Table 2.nHE was showed have cytotoxic activity. nHE has an IC_{50} 114, 80 ± 1, 21 ug/mL against PANC-1 cell. nHE was declared less active as anticancer because the IC_{50} < 100 ug/mL. However, nHE can still be developed as an anticancer because it has an IC_{50} value < 500 ug/mL (22).

Table 2 IC_{50} (ug/mL) nHE against PANC-1 cell

Sample	IC_{50} (ug/mL)
nHE	114.80 ± 1.21

Description: IC_{50} was measured using MTT method, nHE: Extract *n*-hexane *Vernonia amygdalina*.

nHE cytotoxic activity was also showed by changes in PANC-1 cells morphology and viability data after treatment. PANC-1 cells morphology and viability data can be seen in figure 1 and figure 2.When viewed from figure 1, nHE leaded death in PANC-1 cell. Cell morphology was changed and having damaged [25]. If concentration of nHE was increased, then it will cause percentage of viability will decreased. Figure 2 showed, an increased in nHE concentration caused decreased percentage of viability at 85.66%, 83.33%, 48.33%, 17.66% and 3.57%.

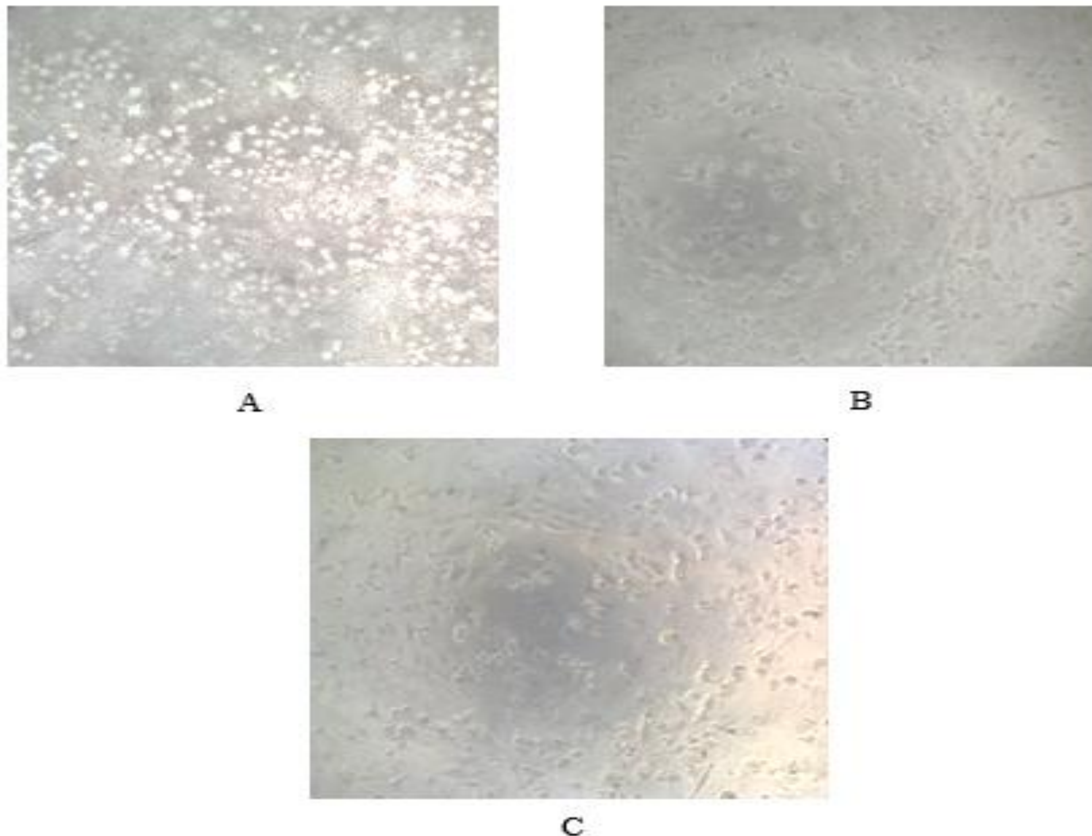

Figure 1: The cytotoxic effect of the sample on PANC-1 cell. The observation was performed under inverted microscope with 100x magnification.

The (blue arrows) showed normal cells and (black arrows) signs of morphological changes. A: nHE 500 ug/mL, B: nHE 31.25 ug/mL, C: Control Cell

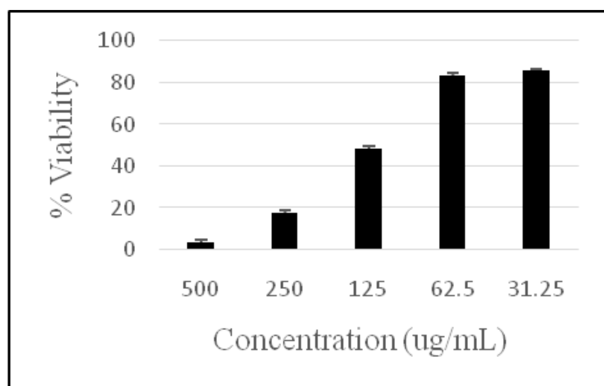

Figure 2: Percentage of viability Extract *n*-hexane *Vernonia amgydalina* (500 ug/mL, 250 ug/mL, 125 ug/mL, 62.5 ug/mL and 31.25 ug/mL) on PANC-1 cell.

As an anticancer, nHE must be able to inhibit the cell cycle. PANC-1 cell cycle inhibition activity of nHE was do flowcytometry method. PANC-1 cell was treated with nHE concentration 115 ug/mL. Cell cycle inhibition effect can be seen in Figure 3. PANC-1 cell was treated with nHE concentration 115 ug/mL. nHE leaded inhibition of PANC-1 cell cycle in M1 phase with cell accumulation at 67.39% and cell control at 17.32%. This report was followed with decreasing cell cycle accumulation in G0-G1 phase, nHE at 19.36% and cell control at 50.51%. This percentage was showed that the nHE can lead cell cycle inhibition in M1 phase. In the cell cycle analysis, nHE can decrease cell cycle accumulation in G0-G1 phase if compared with cell control. nHE can increased cell cycle accumulation in M1 phase if compared with cell control. M1-M5 phase was a phase who showed apoptosis accumulation of PANC-1 cell [26-28]. Based on the cell cycle inhibition tested, then the nHE can leaded apoptosis in PANC-1 cell.

Apoptosis can be defined as a process of programmed cell death and this process depend on biochemical mechanism of cell [29]. In this study, Figure 4 showed nHE was spurred apoptosis. Apoptosis PANC-1 cells was observation used confocale microscope. PANC-1 cell was treated with nHE concentration 115 ug/mL. Before observation, thesample was first staining with 10 µL acridine orange-ethidium bromide and incubated for 15 minutes.

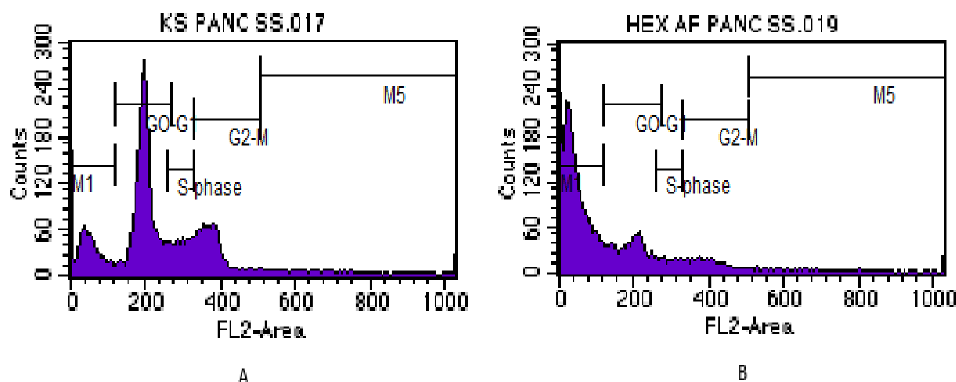

Figure 3: Cell cycle analysis using flowcitometry. PANC-1 cell was treated with nHE 115 ug/mL and stained using propidium iodide. A: Cell controls, B: Extract *n*-hexane *Vernonia amygdalina* 115 ug/mL.

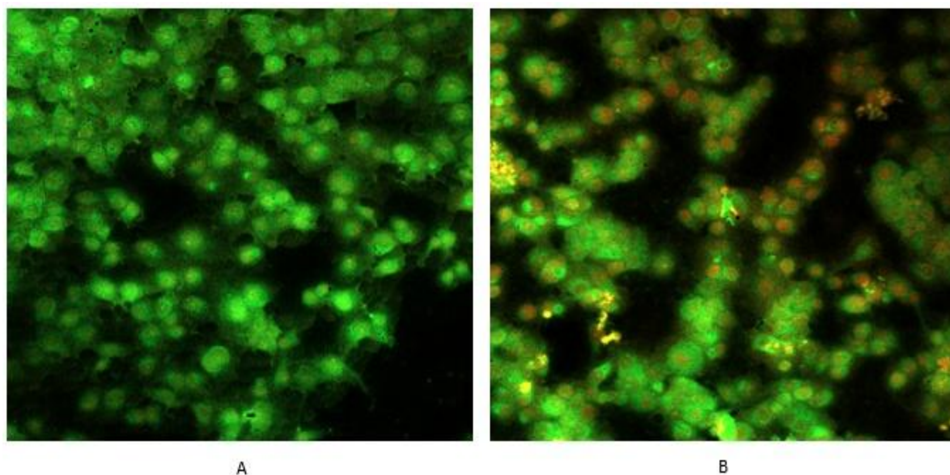

Figure 4: Observation of apoptosis in fluorescence microscope with 40x magnification and 3 x zoom out. A: Control cell, B: Extract *n*-Hexane *Vernonia amygdalina* with concentration 115 ug/mL.

Apoptosis testing was done used double staining method. Green fluorescent cells show living cells, while red fluorescent cells show dead cells (30). On the control cells, green fluorescent can be seen because it only absorbs acridine orange. Ethidium bromide cannot enter to the cell control because cell integrity is still good. In Figure 4B showed, cells with treatment 150ug/mL nHE was spurred apoptosis. Red fluorescent in the cells was showed the cells loss of membrane permeability and leaded ethidium bromide can enter the cells. It was as an indicator that cells death [31].

CONCLUSION

The activity shown by nHE in inhibiting the development of PANC-1 cells was good. There needs to be futher exploration of the nHE mechanism as an anticancer pancreas. Exploring of the effect nHE in inhibiting gene expression associated with the cell cycle and apoptosis like p53, cyclin D, PI3K/Akt/mTOR is an interesting thing to do. By examining gene expression, it will show the nHE mechanism of action.

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