© 2024 Journal of Pharmacy & Pharmacognosy Research, 12 (5), 870-880, 2024
ISSN 0719-4250
https://jppres.com

DOI: https://doi.org/10.56499/jppres23.1748_12.5.870

Original Article

Combination of cisplatin and ethyl acetate extract of *Vernonia amygdalina* Delile induces cell cycle arrest and apoptosis on PANC-1 cells via PI3K/mTOR

[Combinación de cisplatino y extracto de acetato de etilo de *Vernonia amygdalina* Delile induce la detención del ciclo celular y la apoptosis en células PANC-1 vía PI3K/mTOR]

Poppy Anjelisa Zaitun Hasibuan[1*], Jane Melita Keliat[2], Muhammad Fauzan Lubis[3]

[1]Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia.
[2]Department of Pharmaceutical and Food Analysis, Faculty of Vocational, Universitas Sumatera Utara, Medan, 20155, Indonesia.
[3]Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia.
*E-mail: poppyanjelisa@usu.ac.id

Abstract

Context: *Vernonia amygdalina* Delile (VAD) is known as a potential plant with a wide variety of medicinal properties, including anticancer.

Aims: To evaluate the combination effect of VAD extract with cisplatin (CIS) against PANC-1 cells, focusing on cell cycle arrest and apoptosis activities.

Methods: This study is an experimental study using PANC-1 cells as objects. The phytochemical compounds were analyzed with LC-MS/MS. The cytotoxic activity of extracts and their combinations was determined using the MTT assay method in the PANC-1 cell line. Apoptosis, cell cycle arrest, and PI3K/mTOR profiles were analyzed with flow cytometry. Immunocytochemistry was used to determine Bcl-2, Cyclin D1, and p53 expression.

Results: The phytochemicals found were five compounds with retention times of 8.96, 9.85, 10.52, 12.59, and 10.36. The results of the MTT assay showed that the IC_{50} values of extract and CIS were 21.83 ± 0.46 µg/mL and 3.02 ± 0.44 µg/mL, respectively. The combination index (CI) value of extract and CIS had a synergistic effect. Combination extract and CIS induced early and late apoptosis, inhibited cell cycle progression on the G1 phase, inhibited Bcl-2 and cyclin D1 expression, induced p53 expression, and inhibited PI3K and mTOR expression.

Conclusions: The combination of extract and CIS showed anticancer activity against PANC-1 cells through induction of apoptosis and cell cycle arrest via inhibition of PI3K and mTOR expressions.

Keywords: apoptosis; cell cycle arrest; cisplatin; herbal medicine; pancreatic cancer.

Resumen

Contexto: *Vernonia amygdalina* Delile (VAD) es conocida como una planta potencial con una amplia variedad de propiedades medicinales, incluyendo anticancerígenas.

Objetivos: Evaluar el efecto combinado del extracto de VAD con cisplatino (CIS) contra las células PANC-1, centrándose en las actividades de detención del ciclo celular y apoptosis.

Métodos: Este estudio es un estudio experimental utilizando células PANC-1 como objetos. El análisis de los compuestos fitoquímicos se llevó a cabo con LC-MS/MS. La actividad citotóxica del extracto y sus combinaciones se determinó mediante el método de ensayo MTT en la línea celular PANC-1. La apoptosis, la detención del ciclo celular y los perfiles PI3K/mTOR se analizaron con citometría de flujo. Se utilizó inmunocitoquímica para determinar la expresión de Bcl-2, ciclina D1 y p53.

Resultados: Los fitoquímicos hallados fueron cinco compuestos con tiempos de retención de 8,96, 9,85, 10,52, 12,59 y 10,36. Los resultados del ensayo MTT mostraron que los valores de IC_{50} del extracto y del CIS fueron 21,83 ± 0,46 µg/mL y 3,02 ± 0,44 µg/mL, respectivamente. El valor del índice de combinación (IC) del extracto y el CIS tuvo un efecto sinérgico. La combinación de extracto y CIS indujo la apoptosis temprana y tardía, inhibió la progresión del ciclo celular en la fase G1, inhibió la expresión de Bcl-2 y ciclina D1, indujo la expresión de p53 e inhibió la expresión de PI3K y mTOR.

Conclusiones: La combinación de extracto y CIS mostró actividad anticancerígena contra células PANC-1 a través de la inducción de apoptosis y detención del ciclo celular vía inhibición de las expresiones PI3K y mTOR.

Palabras Clave: apoptosis; detención del ciclo celular; cisplatino; fitoterapia; cáncer de páncreas.

ARTICLE INFO
Received: July 27, 2023.
Accepted: January 30, 2024.
Available Online: April 16, 2024.

AUTHOR INFO
ORCID:
0000-0001-9122-711X (PAZH)
0000-0002-8366-3795 (JMK)
0000-0001-9651-904X (MFL)

INTRODUCTION

Pancreatic adenocarcinoma is a lethal condition with poor outcomes and an increasing incidence (Werner et al., 2013). This is reinforced by the state of resistance of cancer cells to chemotherapy regimens in the treatment of pancreatic cancer and late cancer detection (Karanikas et al., 2016). Drug resistance is a common and well-understood occurrence among anticancer treatments, and platinum drugs are no exception (Housman et al., 2014). Extrinsic and intrinsic cell resistance makes it difficult to utilize these medicines in chemotherapy (Nikolaou et al., 2018). This causes the use of chemotherapy to be combined, such as a combination of gemcitabine (GEM) and cisplatin (CIS) (Housman et al., 2014). The use of this combination chemotherapy can increase survival and reduce symptoms of the disease, but it can also increase its toxicity (Ergun et al., 2018). Several studies reported the side effects of the application of GEM and CIS, such as thrombocytopenia, which caused the quality of life to decrease (Chao et al., 2013). On the other hand, the hematological toxicity of this combination was often reported (Heinemann et al., 2000). Furthermore, other ways need to be considered to treat pancreatic cancer.

Co-chemotherapy is a solution to the multi-drug resistance (MDR) phenomena (Lubis et al., 2022). Co-chemotherapy combines phytochemical compounds from natural materials with chemotherapeutic agents to increase efficacy and reduce chemotherapy toxicity against normal tissues (Febriansah et al., 2014). *Vernonia amygdalina* Delile leaves (VAD) is a plant (*Asteraceae*) that has been widely reported to have anticancer properties (Hasibuan et al., 2021). Several studies found that some of its chemical constituents, such as flavonoids, sesquiterpene lactones, fatty acids, and steroidal saponins, have an anticancer effect (Hasibuan et al., 2020b). The combination test of VAD leaf extract with CIS will show the results of the interaction between these ingredients.

Considering that VAD leaves have prospects as a chemopreventive agent, its strong antitumor ability to interfere with cancer cell growth and action tracking as an initiative towards developing new strategies for cancer treatment (Fachrunisa et al., 2019; Hasibuan et al., 2020a; 2000b). Also, the combination of VAD leaves with CIS as a chemotherapeutic agent provides for potentiating the efficacy of the latter at a lower dose and thus reduces the side effects in normal cells and increases the effectiveness of CIS (Kim et al., 2014; Tseng et al., 2016). In this study, secondary metabolite compounds in VAD leaves were investigated, determining the combined index value of CIS and VAD leaves to determine the nature of their interactions, tracing the mechanism of action of medicinal substances as anti-pancreatic cancer through cell cycle inhibition tests, promoting apoptosis, and influencing PI3K/mTOR proteins expression in PANC-1 cell lines.

MATERIAL AND METHODS

Materials

Cisplatin (Platinol), DMEM medium (Sigma Aldrich), 0.25 % trypsin EDTA (Gibco, USA), fetal bovine serum (Gibco, USA), Fungizone® (Gibco, USA), 0.4% trypan blue (Gibco, USA), dimethyl sulfoxide (DMSO) (Sigma, USA), penicillin-streptomycin (Sigma, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent) (Sigma, USA), 96-well plate (Corning, USA), 24-well plate (Corning, USA), ethanol (Merck, USA), Ethyl acetate (Merck, USA), n-hexane (Merck, USA), propidium iodide kit (Biolegend), PI3K and Akt genes (Biossusa), and ELISA reader (Merck), FACS flow cytometer (Biorad).

Plant material

Vernonia amygdalina leaves were obtained from Deli Serdang Regency, North Sumatera, Indonesia. The leaf was found in the Tumpatan Nibung Subdistrict of Deli Serdang Regency, at elevations ranging from 4-30 meters above sea level and located at 98°49'3.339'' East longitude and 3°35'34.112'' North latitude. The leaf was identified as *V. amygdalina* by Medanese Herbarium, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara Indonesia (voucher number: 228/UN5.2.1.1.2.4/HMed/2023).

Extract preparation

The maceration technique was used to make the ethyl acetate extract of *Vernonia amygdalina* (EAV). The dry powder was weighed and placed in a container weighing 1000 g. For 24 h, the dry powder was submerged in 10 L of ethyl acetate, agitated, and left to stand. This can be done up to three times in a row. The filtrate was separated from the residue and collected in a container. To get a thick extract, the entire filtrate was evaporated with a rotary evaporator (Hasibuan and Sumaiyah, 2019).

Secondary metabolite identification

The gradient technique was used to analyze phytochemicals from EAV using TSQ Exactive (Thermo) (LIPI, Indonesia) with mobile phase A (0.1 percent formic acid in water) and phase B (0.1% acid in ace-

tonitrile). The Hypersil GOLD aQ column had a flow rate of 40 L/min and a diameter of 50 µm × 1 mm × 1.9 m, and an analysis duration of 70 min. The data were evaluated using mzCloud and the Compound Discoverer program (Tine et al., 2017).

Cell culture

The PANC-1 cell line was obtained from the parasitology laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. The cells were maintained in DMEM, 10% fetal bovine serum, and 1% Abam. The conditions of this process were secured under 37°C, 5% CO_2 atmospheric, and 95% humidity. After incubating for 24 h, the viable number of cells was calculated with the addition of Trypan blue staining in the hemocytometer. Meanwhile, the Vero cells were conducted to provide the effect of EAV and CIS against normal cells. The cultured cell process was similar to PANC-1 cells (Hasibuan et al., 2023).

Cytotoxic MTT assay

In 96-well plates, 1 × 10^4 PANC-1 cells were planted per well and incubated for 48 h. For 24 h, cells were exposed to increasing concentrations of the extract or cisplatin, either alone or in combination. The cells were washed in PBS after the culture media was removed. Each well received 0.5 mg/mL MTT in the media and was incubated for 3–4 h. The MTT reaction was halted by adding 10% SDS to 0.01 N HCl and incubating in the darkroom overnight. The absorbance was measured at 595 nm with a microplate reader. Each treatment was done in triplicate, and the absorbance data is expressed as a percentage of the control cells' vitality (untreated) (Satria et al., 2019).

Cell cycle and apoptosis induction by flow cytometry assay

In a 6-well plate, about 5 × 10^5 PANC-1 cells were grown and incubated for 48 h. The extract and cisplatin were then given to the cells for 24 h, either alone or in combination. The cells were collected using trypsin EDTA, rinsed in phosphate-buffered saline (PBS), and centrifuged for 5 min at 500 rpm (Dalimunthe et al., 2017). For apoptosis induction, cells were treated in the dark for 15 min with annexin V FITC and propidium iodide before being examined using a flow cytometer. Cells were fixed in 70% cold ethanol for 30 min, washed in PBS, and centrifuged at 500 rpm for 5 min to evaluate cell cycle distribution. Cells were then resuspended in PBS with 40 g/mL propidium iodide, 20 g/mL RNAse, and 0.1% Triton X114 for 15 min in the dark before flow cytometry detection (Hasibuan et al., 2015).

Immunocytochemistry

In a 24-well plate, PANC-1 cells (5 × 10^4 cells/well) were planted on coverslips and incubated for 24 h. The cells were subsequently treated with EAV and CIS, either alone or in combination, and incubated for 24 h. After incubation, the cells were rinsed in PBS and then fixed in cold methanol for 10 min. After that, the cells were washed with PBS and blocked in hydrogen peroxide blocking solution for 10 min at room temperature before being incubated for 1 h with primary antibodies against Bcl-2, cyclin D1, and p53, then washed three times with PBS before being incubated with secondary antibody for 10 min. The cells were rinsed in PBS and then incubated for 10 min in a 3,3-diaminobenzidine (DAB) solution before being washed in distilled water. After that, the cells were counterstained for 5 min with Mayer hematoxylin, and the coverslips were removed and rinsed with distilled water before being submerged in xylol and 70% ethanol. A light microscope was used to observe protein expression (Nikon YS100). The brown color was provided by cells expressing a specific protein, while the blue color was provided by cells not expressing a specific protein (Illian et al., 2019; Istiqomah et al., 2020).

PI3K and mTOR expression analysis

In a six-well plate, PANC-1 cells (5 × 10^5 cells/well) were planted and incubated for 24 h. The cells were then cultured for 24 h after being treated with EAV and CIS, either alone or in combination. 0.025% trypsin was used to collect both floating and adhering cells in a conical tube. The cells were rinsed three times with cold PBS and centrifuged for five minutes at 2500 rpm. The sediment was collected while the supernatant was separated. The sediment cells were fixed in 70% ethanol for 2 h at 20°C, then PI3K FITC and mTOR PE antibodies were added and treated for 10 min at 37°C. The FACScan flow cytometer was used to examine the samples (Hasibuan et al., 2020b).

Data analysis

To calculate the IC_{50} value from a single cytotoxicity assay, we plotted linear regression of concentration and % cell viability using Excel MS Office 2020. The entire data obtained from the flow cytometer was analyzed using BD Accuri C6 software and the SPSS 22 ANOVA test. Meanwhile, the combination treatment on PANC-1 cells was evaluated by calculating the Combination Index (CI) value using the following formula [1].

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \quad [1]$$

Where D_1 and D_2 are the doses of the two drugs in combination, that inhibit the proliferation of the cells by x%, and Dx_1 and Dx_2 are the doses of the drugs alone, that are expected to be necessary to achieve the experimentally measured response by x%.

The CI values were determined for each combination of LBE and doxorubicin using CalcuSyn® (Biosoft, Cambridge, UK) (Sutejo et al., 2019).

RESULTS

Identification of secondary metabolites

LCMS/MS analysis was used to conduct a phytochemical ingredient study. Compounds with retention times of 8.96, 9.85, 10.52, 12.59, and 10.36 min are among the five compounds of concern to be discussed. These are compounds that have a significant peak area. Based on the examination results, the active compounds' molecular weights were 361.12 m/z, 469.29 m/z, 621.30 m/z, 871.57 m/z, and 593.27 m/z. Furthermore, the identification results of compounds identified as vernomenin-6-(2-hydroxy-methyl)-acrylate, 18-hydroxy-27-norolean-12,14-dien-30-al-28-oic acid, dibritanilactone B, candidate $C_{54}H_{78}O_9$, and candidate $C_{25}H_{46}O_{14}$. The chromatogram can be seen in Fig. 1.

Cytotoxic activity by single treatment of EAV and cisplatin

EAV and CIS were employed as materials in this work, with concentrations of 31.25, 62.5, 125, 250, and 500 µg/mL, respectively, to examine their cytotoxic capability against PANC-1 cells. Table 1 shows the IC_{50} value of EAV and CIS.

Based on these findings, EAV has cytotoxic properties against PANC-1 cells**,** as indicated by the IC_{50} value of 21.83 ± 0.46 µg/mL. Based on the IC_{50} value of EAV, it was stated that EAV had a strong potential to inhibit PANC-1 cells (Syari et al., 2019). We investigated the effect of the extract and CIS in combination on PANC-1 cells at various concentrations. Furthermore, the EAV is categorized as having high selectivity**,** while CIS as a positive control is categorized as not selective.

Combinatorial effect of EAV and cisplatin on PANC-1 cells

In this study, the treatment with the series concentrations of each drug under IC_{50} values was used to examine the combinatorial impact of these agents. The results revealed that increasing the concentration of EAV 3.75, 7.5, 15, and 30 µg/mL in conjunction with CIS 0.375, 0.75, 1.5, and 3 µg/mL did not result in decreased cell viability, yielding a combination index (CI) value of 0.017–0.238. The CI value can be seen in Table 2.

From all the test formations carried out between the combination of EAV and CIS, the CI value <1 was obtained. This provides information that the combined effect that occurs showed a synergistic effect (Febriansah et al., 2014).

Cell cycle progression and apoptosis induction

The combined impact of CIS and extract in inhibiting cell growth might be due to a change in the cells' physiological processes (Kotawong et al., 2018). Flow cytometry was used to study the effect of combinatorial therapy on PANC-1 cell cycle progression and cell death in order to better understand the physiological processes of the cells (Lubis et al., 2019). We selected one combination, EAV-CIS, with a concentration of 30:1.5 µg/mL. Fig. 2 depicts the treatment's cell cycle histogram. G1 arrest was marginally caused by a single dose of CIS 1.5 µg/mL (68.1%), compared to cell control (65.6%). Whereas EAV caused inhibition of cells in the S phase (29.4%), compared to cell control (20.8%). The combined effect of EAV and CIS provided inhibition of the G1 phase (67.9%).

We stained treated cells with propidium iodide–annexin V and submitted them to flow cytometry to investigate the cell death process in the cell population and if the synergistic combination was mediated by apoptosis (Szliszka et al., 2009). The result of apoptosis can be seen in Fig. 3. In comparison to untreated cells (3.9%), the apoptotic population was dramatically enhanced in PANC-1 cells treated with a combination of EAV-CIS (13.8%). When compared to EAV (52.5%) or CIS (14.9%) alone, the combination of EAV-CIS had fewer apoptotic cells but showed the greatest percentage of necrotic cells (32.4%). To confirm the cell cycle arrest activity and cell death mechanism in PANC-1 cells of the single and combination treatment, the expression level of Bcl-2, cyclin D1, and p53 were analyzed by immunocytochemistry, and PI3K/mTOR by flow cytometer with PE and FITC-A reagents were shown in cells treated with single CIS and its combination with EAV. The results are shown in Fig. 4A-B.

The results of the protein expression assessment showed a significant difference between the control group and the group test ($p<0.05$). Bcl-2 and cyclin D1 expression on PANC-1 cells showed a significant decrease in CIS, EAV, and combination groups compared with the control group ($p<0.05$). In contrast, the expression of p53 on PANC-1 cells showed a significant increase in CIS, EAV, and combination groups compared with the control group ($p<0.05$). This explains that the test group affects cell cycle activity and apoptosis in PANC-1 cells through the expression of Bcl-2, cyclin D, and p53 proteins.

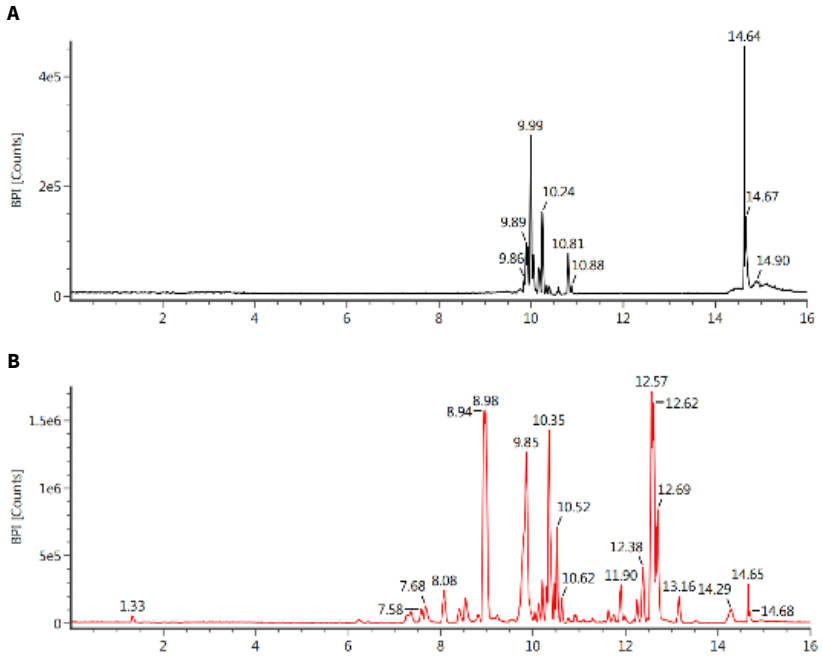

Figure 1. The phytochemical content of acetate extract of *Vernonia amygdalina* (EAV) using LCMS/MS.
(A) Blank; **(B)** EAV.

Table 1. The IC_{50} value of acetate extract of *Vernonia amygdalina* (EAV) and cisplatin (CIS) against PANC-1 and Vero cells.

Compounds	IC_{50} (µg/mL)		Index selectivity
	PANC-1 cells	Vero cells	
EAV	21.83 ± 0.46	153.78 ± 1.52	7.04 ± 0.76
CIS	3.02 ± 4.44	6.32 ± 3.42	2.09 ± 0.65

IC_{50} Values from three independent experiments were given as means ± SD, and the standard deviations were less than 10%. The index selectivity values described a selectivity effect of EAV and CIS. The sample showed a selective effect if the index selectivity value ≥3.

Table 2. The value of cisplatin's combination index (CI) in combination.

Treatment	Concentration (µg/mL)		Viability (%)	CI
	EAV	CIS		
Combination 1	30	3	63.91 ± 1.34	0.238
Combination 2	15	3	84.94 ± 1.56	0.104
Combination 3	7.5	3	79.38 ± 1.47	0.071
Combination 4	3.75	3	79.04 ± 2.68	0.052
Combination 5	30	1.5	83.79 ± 2.41	0.166
Combination 6	15	1.5	94.37 ± 3.21	0.080
Combination 7	7.5	1.5	94.16 ± 2.51	0.047
Combination 8	3.75	1.5	55.17 ± 0.92	0.051
Combination 9	30	0.75	83.92 ± 1.72	0.158
Combination 10	15	0.75	92.53 ± 3.64	0.075
Combination 11	7.5	0.75	95.93 ± 3.01	0.039
Combination 12	3.75	0.75	91.72 ± 2.23	0.024
Combination 13	30	0.375	99.18 ± 3.56	0.130
Combination 14	15	0.375	103.93 ± 4.32	0.063
Combination 15	7.5	0.375	105.96 ± 4.87	0.032
Combination 16	3.75	0.375	108.61 ± 4.92	0.017

Synergistic effects are indicated by a C<1, additive effects by a C=1, and antagonistic effects by a CI>1.

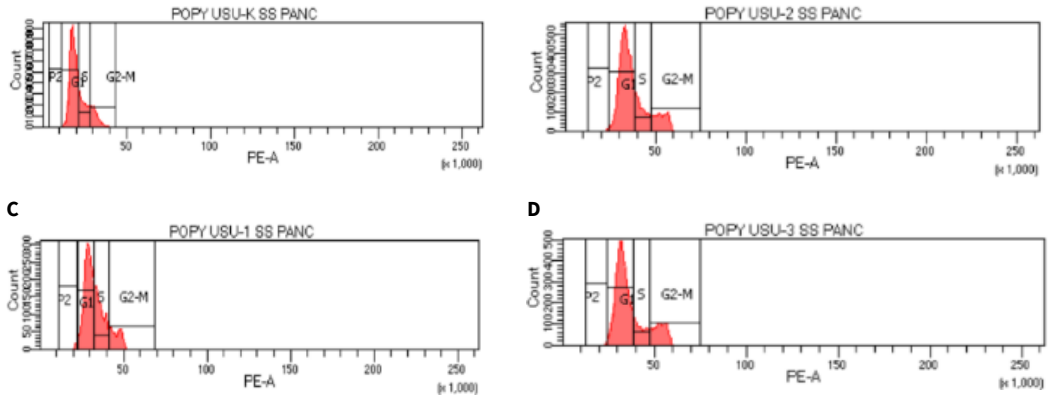

Figure 2. The effect of acetate extract of *Vernonia amygdalina* (EAV), cisplatin (CIS), and their combination on cell cycle distribution.

(A) Cell control, **(B)** CIS 1.5 µg/mL, **(C)** EAV 30 µg/mL, **(D)** EAV and CIS combination. Cells were treated for 24 h and stained with the PI reagent. Each sample was subjected to a flow cytometer.

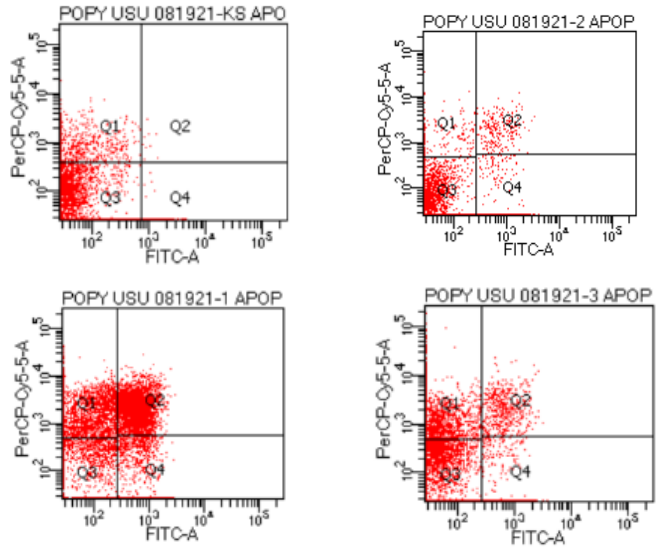

Figure 3. The effect of acetate extract of *Vernonia amygdalina* (EAV), cisplatin (CIS), and their combination on cell apoptosis.

(A) Cell control, **(B)** CIS 1.5 µg/mL, **(C)** EAV 30 µg/mL, **(D)** EAV and CIS combination. Cells were treated for 24 h and stained with PI-Annexin V reagent, and each sample was subjected to a flow cytometer.

The activity of CIS, EAV, and their combination was also observed in PI3K and mTOR proteins. Based on the results, a combination of CIS and EAV groups showed a significant decrease in the PI3K and mTOR expression. The expression of these PI3K and mTOR proteins decreased from 26.5% to 11.7% and 93.4% to 9.0%, respectively ($p<0.05$). The same thing happened to the administration of CIS on PANC-1 cells. However, decreased expression of PI3K did not occur with EAV 30 µg/ml ($p<0.05$). This explains why the combination of CIS and EAV is mutually beneficial. Reduce the risk of side effects and increase effectiveness in inhibiting PANC-1 cancer cells.

DISCUSSION

The use of additional chemotherapy in cancer treatment is one effort that can be made to increase the effectiveness and reduce the risk of side effects from conventional chemotherapy. Additional chemotherapy derived from natural ingredients is currently very popular because it has been proven to be efficacious and can interact synergistically with conventional chemotherapy. One of them is the use of EAV as additional chemotherapy. This research succeeded in proving the efficacy of EAV as an anti-pancreatic cancer in single therapy against PANC-1 cells with an IC_{50} of 21.83 ± 0.46 µg/mL and was proven to be selective with a selectivity index value of 7.04 ± 0.76. This activity was described in the previous study by Hasibuan et al. (2024), that the single treatment of EAV against PANC-1 cells has the strongest activity with IC_{50} of 21.19 ± 0.76 µg/mL. The cytotoxic effect of EAV cannot be separated from the active compound content. This research shows that EAV contains at least three terpene compounds, namely vernomenin-6-(2-hydroxymethyl)-acrylate, 18-hydroxy-27-norolean-12, 14-dien-30-al-28-oic acid, and dibritanilactone B. In previous studies, these compounds have been tested for cytotoxic activity against several cancer cell lines (Topçu et al., 2021; Tuasha et al., 2022; Zhang et al., 2015).

Cisplatin is a potent chemotherapeutic drug that is commonly used to treat pancreatic cancer (Kong et al., 2020). Due to the danger of cardiotoxicity, nephrotoxicity, and resistance progression, its use in chemotherapy is frequently limited (Aldossary, 2019).

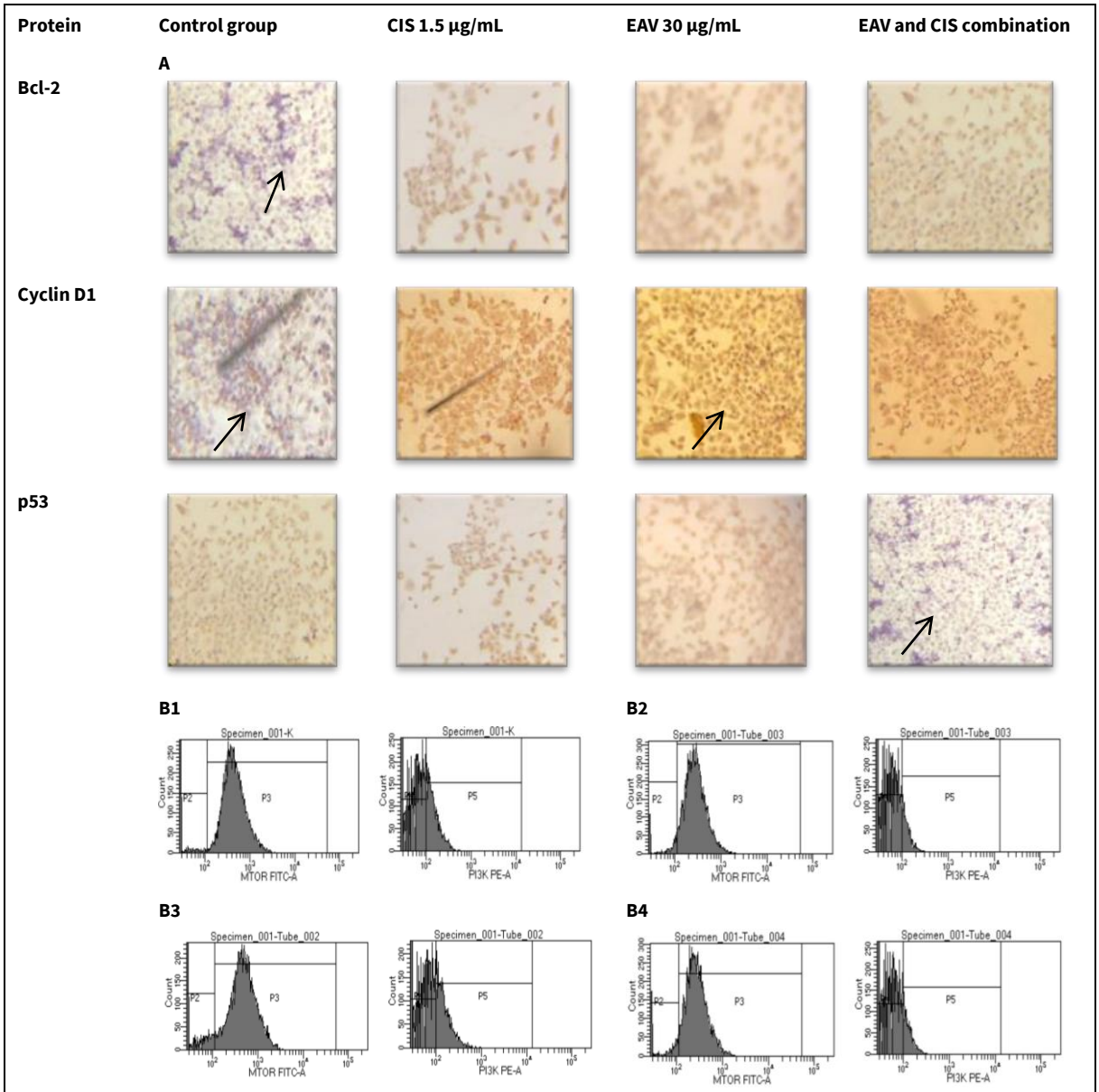

Figure 4. The effect of acetate extract of *Vernonia amygdalina* (EAV), cisplatin (CIS), and their combination on Bcl-2, Cyclin D1, p53, PI3K, and mTOR expressions.

(B1) control group; **(B2)** CIS 1.5 µg/mL; **(B3)** EAV 30 µg/mL; **(B4)** CIS and EAV combination (1.5: 30). Cells were treated for 24 h, and Bcl-2, cyclin D1, and p53 expressions were analyzed using the immunocytochemical method. PI3K and mTOR expressions were obtained using a flow cytometer with FITC-A and PE as reagent. The black arrow shows the protein expression of the PANC-1 cells.

Concerns about the serious side effects of cisplatin have limited the use of cisplatin as the main chemotherapy in the treatment of pancreatic cancer (Barabas et al., 2008). One possible technique for improving cisplatin anticancer activity while reducing toxicity is to combine it with natural chemopreventive medicines (Xiao et al., 2020). Therefore, we investigated the modulatory effect of EAV combined with cisplatin on cytotoxicity, cell cycle progression, and apoptosis induction in the PANC-1 cancer cell line. The MTT assay demonstrated that single EAV, as well as cisplatin, significantly reduced the viability of PANC-1 cells in this investigation. We also used an MTT experiment with the extract and cisplatin to determine the CI value. The CI is commonly regarded as the simplest method for assessing synergism or antagonism in pharmacologic drug interactions (Kapadia et al., 2013). Synergism interaction would be extremely beneficial in the treatment of horrible diseases like cancer (Dong et al., 2014). The key benefits include achieving a synergistic therapeutic impact, lowering doses and toxicity, and preventing or delaying drug resistance (Ruzzolini et al., 2018). The combination of cisplatin 0.375, 0.75, 1.5, and 3 µg/mL with EAV 3.75, 7.5, 15, and 30 µg/mL (equivalent to 1, 1/2, 1/3, and 1/4 of IC_{50}, respectively) exhibited a synergistic inhibitory

effect. As a result, the combination extract may minimize cisplatin dosage treatment while also lowering the risk of cardiotoxicity and nephrotoxicity.

Cytotoxic action is dependent on cell cycle regulation and apoptosis induction. The cell cycle is a well-organized and strictly regulated system for cell division that includes four phases: G1, S (synthesis), G2, and M (mitosis) (Teodoro et al., 2012). Abrogation of cell cycle checkpoints at crucial times is predicted to target cell cycle regulatory faults in order to achieve cancer cell-specific cytotoxicity and render tumor cells vulnerable to apoptosis (Kabała-Dzik et al., 2018; Uroz et al., 2018). To overcome cell cycle-mediated drug resistance and improve cytotoxic effectiveness, these medicines are now coupled with traditional chemotherapeutic drugs (Haryanti et al., 2016). Our results confirmed that a single treatment of CIS 1.5 µg/mL and EAV 30 µg/mL induced cell accumulation at different phases, G1 and S phase, respectively. The combination of CIS with EAV enhanced cell accumulation at G1, compared to the untreated cells and each single treatment. These findings revealed that EAV enhanced the cytotoxic action of CIS, resulting in cell death.

The cell cycle arrest represents a cancer cell's survival mechanism for repairing its damaged DNA (Williams and Stoeber, 2012). When a particular chemical disrupts cell cycle checkpoints before DNA repair is completed, the apoptotic pathway is activated, leading to cell death (Eldhose et al., 2014). In comparison to untreated cells and each treatment, the combination of CIS and the extract boosted apoptotic and necrotic cell induction in our study. ICC and flow cytometer analysis showed expression of Bcl-2, p53, PI3K, and mTOR in the treatment of single CIS and its combination with EAV, confirming apoptosis induction. Multiple signaling pathways are implicated in apoptosis-related cell death, with the Bcl-2 family playing a vital role in regulating caspase activation (Aamazadeh et al., 2020). Pro-apoptotic initiators, pro-survival guardians, and pro-apoptotic effectors are all members of the Bcl-2 family (Chen et al., 2010). The most commonly characterized protein expression pattern implicated in apoptotic cancer cell death is increased expression of Bax (a pro-apoptotic protein) with a simultaneous drop in Bcl-2 (a pro-survival protein) (Ohno et al., 2010).

Because of mutations, amplification, deletion, methylation, and post-translational alterations, the phosphoinositide 3-kinase (PI3K) pathway governs cell growth and proliferation and is frequently dysregulated in cancer (Akinleye et al., 2013). Apoptosis, malignant transformation, tumor development, metastasis, and radioresistance all rely on this intracellular signaling system (Mao et al., 2016). Phosphatase and tensin homolog (PTEN) is a PI3K/Akt/mTOR pathway negative regulator. Many useful inhibitors targeting one signal node (single inhibitor) or two signal nodes at the same time (dual inhibitor) in the PI3K pathway have been created in recent years due to the pathway's vital involvement in cancer research (Li et al., 2019; Wang et al., 2020). In the recent decade, tremendous progress has been made in the development of combination therapy that combines a PI3K inhibitor with other medicines to provide a more effective treatment (Istiqomah et al., 2020). In this study showed the combination activity of CIS and EAV to decrease the expression of PI3K and mTOR compared with the untreated cell group and single group.

CONCLUSION

The combination of EAV and CIS showed anticancer activity on PANC-1 cells through induction of apoptosis and cell cycle arrest and inhibited expression of PI3K/mTOR pathways. These results are essential to be shared with another researcher as information for other tests. More tests, such as *in vivo* and toxicity effects of this combination, are still needed to prove the combination's ability to fight pancreatic cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This article was supported by a research grant from the Indonesia Ministry of Education, Culture, Riset, and Technology, PDUPT scheme grant 2021.

REFERENCES

Aamazadeh F, Ostadrahimi A, Rahbar Saadat Y, Barar J (2020) Bitter apricot ethanolic extract induces apoptosis through increasing expression of Bax/Bcl-2 ratio and caspase-3 in PANC-1 pancreatic cancer cells. Mol Biol Rep 47: 1895–1904. https://doi.org/10.1007/s11033-020-05286-w

Akinleye A, Avvaru P, Furqan M, Song Y, Liu D (2013) Phosphatidylinositol 3-kinase (PI3K) inhibitors as cancer therapeutics. J Hermatol Oncol 6: 88. https://doi.org/10.1186/1756-8722-6-88

Aldossary SA (2019) Review on pharmacology of cisplatin: Clinical use, toxicity and mechanism of resistance of cisplatin. Biomed Pharmacol J 12: 7–15. https://doi.org/10.13005/bpj/1608

Barabas K, Milner R, Lurie D, Adin C (2008) Cisplatin: a review of toxicities and therapeutic applications Vet Comp Oncol 6: 1–18. https://doi.org/10.1111/j.1476-5829.2007.00142.x

Chao Y, Wu CY, Wang JP, Lee RC, Lee WP, Li CP (2013) A randomized controlled trial of gemcitabine plus cisplatin versus gemcitabine alone in the treatment of metastatic pancreatic cancer. Cancer Chemother Pharmacol 72: 637–642. https://doi.org/10.1007/s00280-013-2239-1

Chen XL, Cheng QY, She MR, Wang Q, Huang XH, Cao LQ, Fu XH, Chen JS (2010) Expression of sonic hedgehog signaling components in hepatocellular carcinoma and cyclopamine-induced apoptosis through Bcl-2 downregulation *in vitro*. Arch Med Res 41: 315–323. https://doi.org/10.1016/j.arcmed.2010.06.003

Dalimunthe A, Hasibuan PAZ, Satria D (2017) Cell cycle arrest activity of *Litsea cubeba* Lour: Heartwood and fruit extracts against T47D breast cancer cells. Asian J Pharm Clin Res 10: 404–406. https://doi.org/10.22159/ajpcr.2017.v10i11.20204

Dong Q, Ling B, Gao B, Maley J, Sammynaiken R, Yang J (2014) *Hedyotis diffusa* water extract diminished the cytotoxic effects of chemotherapy drugs against human breast cancer MCF7 cells. Nat Prod Commun 9: 699–700. https://doi.org/10.1177/1934578x1400900529

Eldhose B, Gunawan M, Rahman M, Latha MS, Notario V (2014) Plumbagin reduces human colon cancer cell survival by inducing cell cycle arrest and mitochondria-mediated apoptosis. Int J Oncol 45: 1913–1920. https://doi.org/10.3892/ijo.2014.2592

Ergun Y, Ozdemir NY, Guner EK, Esin E, Sendur MA, Koksoy EB, Demirci NS, Eren T, Dede I, Sezer A, Engin H, Oksuzoglu B, Yalcin B, Utkan G, Zengin N, Urun Y (2018) Comparison of gemcitabine monotherapy with gemcitabine and cisplatin combination in metastatic pancreatic cancer: a retrospective analysis. J BUON 23: 116–121. https://pubmed.ncbi.nlm.nih.gov/30722120/

Fachrunisa D, Hasibuan PAZ, Harahap U (2019) Cell cycle inhibition and apoptotic induction of *Vernonia amygdalina* Del. leaves extract on MCF-7 cell line. Open Access Maced J Med Sci 7: 3807–3810. https://doi.org/10.3889/oamjms.2019.509

Febriansah R, Putri DD, Sarmoko, Nurulita NA, Meiyanto E, Nugroho AE (2014) Hesperidin as a preventive resistance agent in MCF-7 breast cancer cells line resistance to doxorubicin. Asian Pac J Trop Biomed 4: 228–233. https://doi.org/10.1016/S2221-1691(14)60236-7

Haryanti S, Pramono S, Murwanti R, Meiyanto E (2016) The synergistic effect of doxorubicin and ethanolic extracts of *Caesalpinia sappan* L. wood and *Ficus septica* Burm. f. leaves on viability, cell cycle progression, and apoptosis induction of MCF-7 cells. Indones J Biotech 21: 29–37. https://doi.org/10.22146/ijbiotech.26105

Hasibuan PAZ, Chrestella J, Satria D (2015) Combination effect of ethylacetate extracts of *Plectranthus amboinicus* (Lour.) Spreng. with doxorubicin againts T47D breast cancer cells. Int J Pharm Pharm Sci 7: 156–159.

Hasibuan PAZ, Harahap U, Sitorus P, Lubis MF, Satria D (2021) *In-silico* analysis of vernonioside D and vernonioside E from *Vernonia amygdalina* Delile. leaves as inhibitor of epidermal growth factor receptor (EGFR) and mammalian target of rapamycin (mTOR). Rasayan J Chem 14: 1539–1543. https://doi.org/10.31788/RJC.2021.1436092

Hasibuan PAZ, Harahap U, Sitorus P, Satria D (2020b) The anticancer activities of *Vernonia amygdalina* Delile. leaves on 4T1 breast cancer cells through phosphoinositide 3-kinase (PI3K) pathway. Heliyon 6: e04449. https://doi.org/10.1016/j.heliyon.2020.e04449

Hasibuan PAZ, Keliat JM, Lubis MF, Nasution A (2024) The ethyl acetate extract of *Vernonia amygdalina* leaf ameliorates gemcitabine effect against migration and invasion of PANC-1 cells via down-regulation the VEGF, COX_2, and RAS/MEK pathways. Saudi Pharm J 32: 101872. https://doi.org/10.1016/j.jsps.2023.101872

Hasibuan PAZ, Lubis MF, Keliat JM, Azizah N (2023) Cytotoxic test combination of ethyl acetate extract africant leaves (*Vernonia amygdalina* Delile) and gemcitabine on PANC-1 cells. AIP Conference Proceedings 2626: 030004. https://doi.org/10.1063/5.0149754

Hasibuan PAZ, Munir D, Pertiwi D, Satria D, Lubis MF (2020a) Flavonoids constituent analysis and cell cycle inhibition activity of ethylacetate extract of *Vernonia amygdalina* Delile. leaves on lung cancer cell line. Rasayan J Chem 13: 2577–2581. https://doi.org/10.31788/RJC.2020.1345625

Hasibuan PAZ, Sumaiyah S (2019) The anti-proliferative and pro-apoptotic properties of ethanol *Plectranthus amboinicus* (Lour.) Spreng. leaves ethanolic extract nanoparticles on T47D cell lines. Asian Pac J Cancer Prev 20: 897–901. https://doi.org/10.31557/APJCP.2019.20.3.897

Heinemann V, Wilke H, Mergenthaler HG, Clemens M, König H, Illiger HJ, Arning M, Schalhorn A, Possinger K, Fink U (2000) Gemcitabine and cisplatin in the treatment of advanced or metastatic pancreatic cancer. Ann Oncol 11: 1399–1403. https://doi.org/10.1023/a:1026595525977

Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, Sarkar S (2014) Drug resistance in cancer: An overview. Cancers (Basel) 6: 1769–1792. https://doi.org/10.3390/cancers6031769

Illian DN, Hasibuan PAZ, Sumardi S, Nuryawan A, Wati R, Basyuni M (2019) Anticancer activity of polyisoprenoids from *Avicennia alba* Blume. in WiDr cells. Iran J Pharm Res 18: 1477–1487. https://doi.org/10.22037/ijpr.2019.1100719

Istiqomah, Meighina A, Hasibuan PAZ, Sumaiyah S, Yusraini E, Oku H, Basyuni M (2020) Anticancer effects of polyisoprenoid from *Nypa fruticans* leaves by controlling expression of P53, EGFR, PI3K, AKT1, and MTOR genes in colon cancer (WiDr) cells. Nat Prod Commun 15: 1–8. https://doi.org/10.1177/1934578X20918412

Kabała-Dzik A, Rzepecka-Stojko A, Kubina R, Iriti M, Wojtyczka RD, Buszman E, Stojko J (2018) Flavonoids, bioactive components of propolis, exhibit cytotoxic activity and induce cell cycle arrest and apoptosis in human breast cancer cells MDA-MB-231 and MCF-7 - a comparative study. Cell Mol Biol (Noisy-le-grand) 64: 1–10. https://pubmed.ncbi.nlm.nih.gov/29981677/

Kapadia GJ, Rao GS, Ramachandran C, Iida A, Suzuki N, Tokuda H (2013) Synergistic cytotoxicity of red beetroot (*Beta vulgaris* L.) extract with doxorubicin in human pancreatic, breast and prostate cancer cell lines. J Complement Integr Med 10: 113–122. https://doi.org/10.1515/jcim-2013-0007

Karanikas M, Esempidis A, Chasan ZT, Deftereou T, Antonopoulou M, Bozali F, Amarantidis K, Man YG (2016) Pancreatic cancer from molecular pathways to treatment opinion. J Cancer 7: 1328–1339. https://doi.org/10.7150/jca.15419

Kim M, Kim YS, Kim KM, Ko HC, Kim SJ, Kim JH, Kim Y (2014) Combination of *Sasa quelpaertensis* Nakai leaf extract and cisplatin suppresses the cancer stemness and invasion of human lung cancer cells. Integr Cancer Ther 13: 529–540. https://doi.org/10.1177/1534735414534462

Kong F, Liu X, Zhou Y, Hou X, He J, Li Q, Miao X, Yang L (2020) Downregulation of METTL14 increases apoptosis and autophagy induced by cisplatin in pancreatic cancer cells. Int J Biochem Cell Biol 122: 105731. https://doi.org/10.1016/j.biocel.2020.105731

Kotawong K, Chaijaroenkul W, Muhamad P, Na-Bangchang K (2018) Cytotoxic activities and effects of atractylodin and β-eudesmol on the cell cycle arrest and apoptosis on cholangiocarcinoma cell line. J Pharmacol Sci 136: 51–56. https://doi.org/10.1016/j.jphs.2017.09.033

Li Y, Wang T, Sun Y, Huang T, Li C, Fu Y, Li Y, Li C (2019) p53-Mediated PI3K/AKT/mTOR Pathway Played a Role in $Ptox^{Dpt}$-Induced EMT Inhibition in Liver Cancer Cell Lines. Oxid Med Cell Longev 2019: 2531493. https://doi.org/10.1155/2019/2531493

Lubis MF, Hasibuan PAZ, Harahap U (2019) Phytochemicals screening and cell cycle arrest activity of n-Hexane extract of *Vernonia amygdalina* Delile leaves against pancreatic cancer cell line. Asian J Pharm Res Dev 7: 12–16. https://doi.org/10.22270/ajprd.v7i4.533

Lubis MF, Hasibuan PAZ, Harahap U, Satria D, Syahputra H, Muhammad M, Astyka R (2022) The molecular approach of natural products as pancreatic cancer treatment: a review. Rasayan J Chem 15: 1362–1377. https://doi.org/10.31788/RJC.2022.1526765

Mao Y, Xi L, Li Q, Cai Z, Lai Y, Zhang X, Yu C (2016) Regulation of cell apoptosis and proliferation in pancreatic cancer through PI3K/Akt pathway via Polo-like kinase 1. Oncol Rep 36: 49–56. https://doi.org/10.3892/or.2016.4820

Nikolaou M, Pavlopoulou A, Georgakilas AG, Kyrodimos E (2018) The challenge of drug resistance in cancer treatment: A current overview. Clin Exp Metastasis 35: 309–318. https://doi.org/10.1007/s10585-018-9903-0

Ohno I, Eibl G, Odinokova I, Edderkaoui M, Damoiseaux RD, Yazbec M, Abrol R, Goddard WA 3rd, Yokosuka O, Pandol SJ, Gukovskaya AS (2010) Rottlerin stimulates apoptosis in pancreatic cancer cells through interactions with proteins of the Bcl-2 family. Am J Physiol Gastrointest Liver Physiol 298: G63–G73. https://doi.org/10.1152/ajpgi.00257.2009

Ruzzolini J, Peppicelli S, Andreucci E, Bianchini F, Scardigli A, Romani A, la Marca G, Nediani C, Calorini L (2018) Oleuropein, the main polyphenol of *Olea europaea* leaf extract, has an anti-cancer effect on human braf melanoma cells and potentiates the cytotoxicity of current chemotherapies. Nutrients 10: 1950. https://doi.org/10.3390/nu10121950

Satria D, Silalahi J, Haro G, Ilyas S, Hasibuan PAZ (2019) Chemical analysis and cytotoxic activity of n-hexane fraction of *Zanthoxylum acanthopodium* DC. fruits. Rasayan J Chem 12: 803–808. https://doi.org/10.31788/RJC.2019.1225180

Sutejo IK, Putri H, Handayani S, Jenie RI, Meiyanto E (2019) *In vitro* study of the combination of doxorubicin, *Curcuma xanthorrhiza*, *Brucea javanica*, and *Ficus septica* as a potential novel therapy for metastatic breast cancer. Indones J Pharm 30: 15–24. https://doi.org/10.14499/indonesianjpharm30iss1pp15

Syari DM, Rosidah R, Hasibuan PAZ, Haro G, Satria D (2019) Evaluation of cytotoxic activity alkaloid fractions of *Zanthoxylum acanthopodium* DC. fruits. Open Access Maced J Med Sci 7: 3745–3747. https://doi.org/10.3889/oamjms.2019.495

Szliszka E, Czuba ZP, Domino M, Mazur B, Zydowicz G, Krol W (2009) Ethanolic extract of propolis (EEP) enhances the apoptosis- inducing potential of TRAIL in cancer cells. Molecules 14: 738–754. https://doi.org/10.3390/molecules14020738

Teodoro AJ, Oliveira FL, Martins NB, Maia Gde A, Martucci RB, Borojevic R (2012) Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. Cancer Cell Int 12: 36. https://doi.org/10.1186/1475-2867-12-36

Tine Y, Yang Y, Renucci F, Costa J, Wélé A, Paolini J (2017) LC-MS/MS analysis of flavonoid compounds from *Zanthoxylum zanthoxyloides* extracts and their antioxidant activities. Nat Prod Commun 12: 1865–1868. https://doi.org/10.1177/1934578x1701201213

Topçu G, Ayral MN, Aydin A, Gören AC, Chai HB, Pezzuto JM (2001) Triterpenoids of the roots of *Lavandula stoechas* ssp. stoechas. Pharmazie 56: 892–895. https://pubmed.ncbi.nlm.nih.gov/11817178/

Tseng CY, Lin CH, Wu LY, Wang JS, Chung MC, Chang JF, Chao MW (2016) Potential combinational anti-cancer therapy in non-small cell lung cancer with traditional Chinese medicine Sun-Bai-Pi extract and cisplatin. PLoS One 11: e0155469. https://doi.org/10.1371/journal.pone.0155469

Tuasha N, Escobar Z, Seifu D, Gadisa E, Petros B, Sterner O, Oredsson S (2022) Cytotoxic and other bioactivities of a novel and known sesquiterpene lactones isolated from *Vernonia leopoldi* (Sch. Bip. ex Walp.) Vatke in breast cancer cell lines. Toxicol Rep 9: 382–392. https://doi.org/10.1016/j.toxrep.2022.02.011

Uroz M, Wistorf S, Serra-Picamal X, Conte V, Sales-Pardo M, Roca-Cusachs P, Guimerà R, Trepat X (2018) Regulation of cell cycle progression by cell-cell and cell-matrix forces. Nat Cell Biol 20: 646–654. https://doi.org/10.1038/s41556-018-0107-2

Wang X, Wang X, Xu Y, Yan M, Li W, Chen J, Chen T (2020) Effect of nicastrin on hepatocellular carcinoma proliferation and apoptosis through PI3K/AKT signalling pathway modulation. Cancer Cell Int 20: 91. https://doi.org/10.1186/s12935-020-01172-4

Werner J, Combs SE, Springfeld C, Hartwig W, Hackert T, Büchler MW (2013) Advanced-stage pancreatic cancer: therapy options. Nat Rev Clin Oncol 10: 323–333. https://doi.org/10.1038/nrclinonc.2013.66

Williams GH, Stoeber K (2012) The cell cycle and cancer. J Pathol 226: 352–364. https://doi.org/10.1002/path.3022

Xiao Z, Jiang Y, Wang CQ, Hu SS, Huang XR, Chen XF, Huang J, Shan LJ, Tang YH, Wang YH, Gong QH, Feng JH, Xiao X, Li XF (2020) Clinical efficacy and safety of aidi injection combination with vinorelbine and cisplatin for advanced non-small-cell lung carcinoma: A systematic review and meta-analysis of 54 randomized controlled trials. Pharmacol Res 153: 104637. https://doi.org/10.1016/j.phrs.2020.104637

Zhang X, Ren J, Cheng X, Jin H, Zhang W (2015) One new unusual sesterterpenoid and four new sesquiterpene dimers from *Inula britannica*. RSC Advances 5: 1979–1982. https://doi.org/10.1039/C4RA11171K

AUTHOR CONTRIBUTION:

Contribution	Hasibuan PAZ	Keliat JM	Lubis MF
Concepts or ideas	x		
Design	x		
Definition of intellectual content	x	x	x
Literature search	x	x	x
Experimental studies		x	x
Data acquisition	x	x	x
Data analysis	x	x	x
Statistical analysis		x	x
Manuscript preparation		x	x
Manuscript editing		x	x
Manuscript review	x	x	x

Citation Format: Hasibuan PAZ, Keliat JM, Lubis MF (2024) Combination of cisplatin and ethyl acetate extract of *Vernonia amygdalina* Delile induces cell cycle arrest and apoptosis on PANC-1 cells via PI3K/mTOR. J Pharm Pharmacogn Res 12(5): 870–880. https://doi.org/10.56499/jppres23.1748_12.5.870