



# 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<sub>2</sub>, and RAS/MEK pathways

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## ABSTRACT

Individuals diagnosed with cancer often turn to the use of herbal remedies with the intention of treating and ameliorating the condition, impeding the progression of metastasis, enhancing immune function, mitigating stress, and inducing relaxation. Recently, medicinal plants were combined with conventional chemotherapy to decrease the side effects and increase the effectiveness of chemotherapy. This study showed the effectiveness of gemcitabine (Gem) was significantly increased after being used together with ethyl acetate extract obtained from *Vernonia amygdalina* (Eav) leaves. The combination doses of Eav and Gem were determined based on cytotoxic activity using the MTT assay method. The anticancer effect of this combination was identified by several parameters including the apoptosis effect, anti-migration, and anti-invasion activities of PANC-1 cells. Furthermore, this effect was explained via protein expression evaluation using immunohistochemical and flow cytometry. The Eav has a better Inhibitory Concentration 50 (IC<sub>50</sub>) than Gem of 21.19 ± 0.64 µg/mL and 164.78 ± 1.40 µg/mL. The combination of Eav and Gem at IC<sub>50</sub> (1:1) has the strongest activity than Eav and Gem alone at 500.00 µg/mL. The anti-cancer effect of this combination showed significantly increased levels of apoptosis, particularly in the early phase of 17.46 ± 0.35 % (p < 0.0001) than Eav and Gem alone of 7.76 ± 0.25 % and 7.06 ± 0.20 %. A similar impact was evaluated in the migration and invasion of PANC-1 cells after the combination treatment. The % relative migration and cell invasion were significantly decreased compared to the control group and Eav or Gem alone by 21.49 ± 0.96 % and 125.25 ± 5.25 cells, respectively (p < 0.0001). This study found that signature molecules of VEGF, COX<sub>2</sub>, RAS, and MEK were down-regulated after treatment. Our study suggested that the Eav ameliorates the Gem effect against PANC-1 cells through apoptosis, migration, and invasion influence via RAS/MEK pathways.

## 1. Introduction

Pancreatic cancer is a significant unresolved healthcare issue worldwide, with the highest mortality rate among all primary cancers. In the United States alone, it is projected to cause the deaths of over 49,830 individuals in 2022 (Park et al., 2021). Pancreatic cancer is a challenging malignancy to identify. Upon diagnosis, over 50 % of pancreatic cancer cases have already metastasized, resulting in a more 3 % five-year survival rate for affected individuals. The imprecise

administration of chemotherapy contributes to the challenging nature of treating pancreatic cancer in this phase (McGuigan et al., 2018; Hu et al., 2021).

The present chemotherapy regimen, predominantly employing gemcitabine as the benchmark (with or without nab-paclitaxel or cisplatin), has exhibited marginal enhancements. However, they are considerably inadequate in attaining satisfactory outcomes (Saung et al., 2017; Springfield et al., 2019). The FOLFIRINOX regimen, consisting of folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin, not only has

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demonstrated increased efficacy but also has a concomitant increase in toxicity (Suker et al., 2016; Conroy et al., 2018). Tyrosine-kinase inhibitors, exemplified by erlotinib, have demonstrated efficacy in pre-clinical studies, but have not yielded similar outcomes in the clinical context (Lakkakula et al., 2019). Clinical testing has demonstrated the ineffectiveness of antiangiogenic treatments (Tamburrino et al., 2013). The aforementioned reasoning is based on the fact that antiangiogenic therapy exacerbates hypoxia and reduces vascularization in pancreatic cancer, thereby limiting the efficacy of additional chemotherapeutic agents in accessing the tumor (Annese et al., 2019). An urgent requirement exists for a novel combination chemotherapy regimen to address the treatment of pancreatic cancer (Miller et al., 2020).

The utilization of natural products as a source of bioactive anticancer agents has been extensively demonstrated. Natural products possess the advantage of exhibiting lower toxicity in comparison to conventional chemotherapy drugs (Kashyap et al., 2021; Huang et al., 2021; Lubis et al., 2022). The co-administration of natural compounds and conventional chemotherapeutic agents has the potential to produce additive or synergistic effects in the eradication of cancerous cells. This approach may facilitate the use of reduced and less hazardous dosages (Tan and Norhaizan., 2019; Ashrafizadeh et al., 2020). A study reported a synergistic effect in inhibiting pancreatic cancer cell lines when gemcitabine was combined with extracts of *Clinacanthus nutans* (Hii et al., 2019). Another study demonstrated the similar impact of the combination of gemcitabine and *Pao pereira* extract on PANC-1 cells (Yu et al., 2013). Furthermore, the concomitant administration of a Chinese herbal blend and gemcitabine exhibited a synergistic impact on PANC-1 cells (Pak et al., 2021). Further investigation is required to examine the potential impact of incorporating *Vernonia amygdalina* leaf extract in conjunction with gemcitabine, as an additional outcome of utilizing natural products.

The leaf of *Vernonia amygdalina* has been documented as a co-chemotherapeutic agent (Alara et al., 2017). According to various studies, the extract derived from *Vernonia amygdalina* leaf has demonstrated the ability to inhibit the growth of cancer cell lines such as breast cancer cells (Yedjou et al., 2008; Oyugi et al., 2009; Wong et al., 2013; Yedjou et al., 2013), colon cancer cells (Bestari et al., 2017; Burhan et al., 2022), prostate cancer cells (Johnson et al., 2017), liver cancer cells (Thongnest et al., 2019), and pancreatic cancer cells (Lubis et al., 2022). According to the report, the *Vernonia amygdalina* leaf's ethyl acetate extract was found to induce cell death in 4 T1 cells through the activation of the phosphoinositide 3-kinase (PI3K) pathway (Hasibuan et al., 2020). The observation of the extract activity has the potential to induce cell death in T47D cells through the activation of cell cycle arrest and apoptotic pathways (Lifiani et al., 2018). The ethanolic extract of *Vernonia amygdalina* leaf has been observed to induce apoptosis in MDA-MB-231 and MCF-7 cancer cell lines through the mediation of caspase and p53. This effect has been noted in other cancer cell lines as well (Wong et al., 2013). The n-hexane extract of *Vernonia amygdalina* leaf was utilized to induce cell cycle arrest and apoptosis in PANC-1 cells (Fauzan et al., 2019). The leaves of *Vernonia amygdalina* have demonstrated anticancer properties. Nevertheless, there is currently no report on the combined effect of *Vernonia amygdalina* and chemotherapy.

The present study aims to investigate the synergistic impact of *Vernonia amygdalina* leaf in combination with gemcitabine on PANC-1 cells. The leaves of *Vernonia amygdalina* contain phytochemical compounds such as polyphenols, flavonoids, glycosides, vernoniosides, vernodaline, and vernomygdine. These compounds have been found to exhibit anticancer activity against cancer cell lines. Additionally, *Vernonia amygdalina* leaf has been shown to enhance the effectiveness of gemcitabine through various mechanisms (Evbuomwan et al., 2018; Yusoff et al., 2020; Oladele et al., 2021). The identification of the anticancer mechanism can be accomplished through the observation of cancer cell migration and invasion (Wang et al., 2018). The RAS/MEK pathways exhibit a noteworthy association with the migratory and invasive behavior of PANC-1 cells at the molecular level (Buscaill et al., 2020).

The study reports that the combination of ginsenoside and cisplatin effectively inhibited cell migration in HeLa cells by suppressing the RAS/MEK/ERK pathways (Li et al., 2023). Anthocyanins derived from black rice were found to inhibit cell migration and invasion by suppressing MEK in various other cell lines (Chen et al., 2015). Therefore, in order to address the treatment challenges associated with pancreatic cancer, it is imperative to evaluate the efficacy of gemcitabine and *Vernonia amygdalina* combination therapy against PANC-1 cells, with a focus on the proteins that regulate their migration and invasion.

## 2. Materials and methods

### 2.1. Cell lines and reagents

The PANC-1 human pancreatic cancer cell lines were procured from the culture collection of the parasitology laboratory at Universitas Gadjah Mada, Indonesia. The PANC-1 cell lines was maintained in vitro at a temperature of 37 °C under a gaseous atmosphere consisting of 5 % CO<sub>2</sub>, and 95 % air. The recommended culture medium supplemented with 10 % fetal bovine serum was utilized to support cell growth. The leaf of *Vernonia amygdalina* was collected from the botanical garden located at the Faculty of Pharmacy, Universitas Sumatera Utara. The chemical reagents utilized in this investigation possess a certification from Sigma Aldrich. The study employed a detection kit with DAB, FITC goat anti-rabbit IgG, annexin V, and propidium iodide (PI) that was procured from Abclonal for the primary and secondary antibodies in the immunohistochemical analysis.

### 2.2. Extract preparation

The maceration method was employed to prepare the ethyl acetate extract of *Vernonia amygdalina* leaf, denoted as Eav. In summary, a quantity of 500 g of dry *Vernonia amygdalina* leaf powder was subjected to a soaking process with 5 L of ethyl acetate for a duration of 3 cycles of 24 h each, while being stored in a light-free environment. The specimen was obtained from a macerated sample after the evaporation of ethyl acetate via employment of a rotary evaporator (Type RE100-S, Dlab). The specimen is stored at a temperature of 2 °C until it is utilized for the examination (Rosidah et al., 2021; Lubis et al., 2022).

### 2.3. Phytochemical analysis

The present study involved the examination of phytochemicals obtained from the ethyl acetate extract of *Vernonia amygdalina*. The analysis was conducted using TSQ Exactive (Thermo) (LSIH, Brawijaya University) and involved the use of mobile phase A (0.1 % formic acid in water) and phase B (0.1 % formic acid in acetonitrile) in accordance with the gradient method. The Hypersil GOLD aQ column, measuring 50 × 1 mm × 1.9 μm, was subjected to a flow rate of 40 μL/min during the analysis, which lasted for a duration of 70 min. The outcomes were scrutinized through the utilization of Compound Discoverer software, employing mzCloud (Hasibuan et al., 2020).

### 2.4. Viability test

The viability of cells was evaluated through the utilization of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after 24 h of treatment. The study involved subjecting cells in the exponential growth phase to serial dilutions of Eav, Gemcitabine (Gem), or a combination of the two for a period of 24 h. The cells did not attain a state of plateau phase within the designated period of incubation. The concentration of a drug that resulted in a 50 % inhibition of cell growth relative to the untreated control was determined as the Fifty Percent Inhibitory Concentration (IC<sub>50</sub>) (Hasibuan et al., 2013; Lubis et al., 2022).

## 2.5. Apoptosis detection by flow cytometry

The cells were subjected to a 48-hour exposure to IC<sub>50</sub> concentrations of Eav, Gem, or a combination of both. The cells underwent a washing process utilizing PBS, followed by resuspension in binding buffer. Subsequently, they were subjected to staining with FITC conjugated annexin V and propidium iodide (PI) in accordance with the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). Flow cytometry was employed to analyze the cells. The present study identified Annexin V positive cells as early-stage apoptotic cells, while Annexin V-PI double positive cells were identified as late-stage apoptotic cells. Necrotic cells were identified as PI single-positive cells (Hasibuan et al., 2019; Lubis et al., 2023).

## 2.6. Cell migration assay

The PANC-1 cells were cultured in 35 mm dishes until they reached full confluence, at which point a wound was created by scratching the surface with a sterile pipette tip. Subsequently, the cells were subjected to treatment with IC<sub>50</sub> Eav, either alone or in combination with Gem at IC<sub>50</sub>, for a duration of 24 h. The photomicroscope was utilized to capture images of cells for the purpose of evaluating cell migration. Statistical analysis was conducted based on a minimum of three biological replicates (Harahap et al., 2018).

## 2.7. Cell invasion assay

The experiment involved conducting a cell invasion assay through the utilization of a polyethylene terephthalate membrane (with a pore size of 8 μm) in conjunction with Millicell hanging cell culture inserts and BD Matrigel (Millipore). The PANC-1 cells that were introduced into the upper chamber underwent treatment with IC<sub>50</sub> Eav, either alone or in combination with Gem of IC<sub>50</sub>. In this experiment, PANC-1 cells were subjected to invasion assay by incubating them with a conditioned medium containing 10 % (v/v) FBS in each well. The cells were allowed to penetrate through the membrane during a 24-hour incubation period at 37 °C. The cells that traversed the filter pores were immobilized with methanol, and subjected to crystal violet staining, and the invasiveness of PANC-1 cells was assessed through microscopic observation by quantifying the count of cells that had invaded. Cell invasion evaluation was conducted using three biological replicates (Luo et al., 2020).

## 2.8. Immunohistochemical analysis

The PANC-1 cells were seeded onto a 24-well plate, with a coverslip included in each well. The experiment involved subjecting the cells to IC<sub>50</sub> Eav treatment, both in the presence and absence of combined treatment with Gem of IC<sub>50</sub>, for a duration of 24 h. In addition, the cells were rinsed with phosphate-buffered saline (PBS) and transferred to a glass receptacle where they were subjected to incubation at ambient temperature for a duration of 10–15 min. Following incubation, the cells were subjected to two washes with phosphate-buffered saline (PBS) and subsequently exposed to monoclonal antibodies targeting vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX2). The cells were then incubated for a duration of 1 h at ambient temperature. The cells underwent a PBS rinse and were subsequently treated with a secondary antibody, followed by incubation at ambient temperature for a duration of 10 min. The expression of proteins was observed and documented using a light microscope. The data was quantified as a proportion of cells that exhibited protein expression in 10 distinct fields of view for every group (Sari et al., 2018).

## 2.9. RAS and MEK expression analysis

A total of  $5 \times 10^5$  PANC cells were seeded per well in a six-well plate and incubated for 24 h. Subsequently, the cells were subjected to IC<sub>50</sub>

Eav treatment, either alone or in conjunction with Gem at IC<sub>50</sub>, for a duration of 24 h. Both floating and adherent cells were harvested into a conical tube utilizing 0.025 % trypsin. The cellular samples underwent three rounds of washing with cold phosphate-buffered saline (PBS) and were subsequently subjected to centrifugation at a rate of 2500 rpm for a duration of 5 min. The liquid portion was isolated, while the solid residue was gathered. The sediment cells were treated with 70 % ethanol and left to incubate for a duration of 2 h at a temperature of –20 °C. Following this, RAS FITC and MEK PE antibodies were introduced and left to incubate at a temperature of 37 °C for a duration of 10 min. The specimens underwent analysis through employment of the FACScan flow cytometer (Hasibuan et al., 2020).

## 2.10. Statistical analysis

The statistical analysis for this study was conducted utilizing the GraphPad Prism software. The data were presented as the mean value plus or minus the standard deviation and were analyzed using the student's *t*-test to determine the statistical significance of the differences observed between the two groups. Each assay was replicated biologically a minimum of three times. Statistical significance was determined based on the P-values of < 0.05, <0.001, or < 0.0001.

## 3. Results

### 3.1. Phytochemical compounds of extract

The 49.50 g Eav was obtained after the maceration process using ethyl acetate for 3 × 24 h. It means this extraction was given % a yield extract of 9.90 %. Phytochemical constituent analysis of EAF was carried out with LC-MS/MS to obtain information about its compounds. The results are given in Fig. 1.

The results showed that Eav contains various phytochemicals. Five compounds were successfully identified based on the structure fragmentation of compounds. That is C<sub>19</sub>H<sub>20</sub>O<sub>7</sub> candidate absence, C<sub>29</sub>H<sub>40</sub>O<sub>5</sub> candidate, C<sub>34</sub>H<sub>46</sub>O<sub>9</sub> candidate, C<sub>25</sub>H<sub>46</sub>O<sub>14</sub> candidate, and C<sub>54</sub>H<sub>78</sub>O<sub>9</sub> candidate with retention time sequentially 8.96 min, 9.85 min, 10.52 min, 10.36 min, and 12.59 min. Other tests need to be carried out to determine these compounds.

### 3.2. Percentage viability of PANC-1 cells

The viability of PANC 1 cells was evaluated using the MTT assay 24 h after Eav and Gem treatment. The results revealed that Eav and Gem treatment inhibited cell viability in a dose-dependent manner (Fig. 2A). The PANC-1 cells were treated using various concentrations of Eav and Gem of 7.81 μg/mL, 15.62 μg/mL, 31.25 μg/mL, 62.50 μg/mL, 125.00 μg/mL, 250.00 μg/mL, and 500.00 μg/mL, respectively. The Eav concentrations led to significant differences in % cell viability (*p* < 0.0001). The 500.00 μg/mL Eav was the strongest concentration causing PANC-1 cell death with a % viability of 21.43 ± 0.40 %. Meanwhile, 500.00 μg/mL Gem treatment was identified lead PANC-1 cell death but the % viability (56.70 ± 0.19 %) is significantly different compared to the Eav group (*p* < 0.0001), indicating the cytotoxic activity of Gem was resistant although the concentrations have increasing.

The IC<sub>50</sub> was utilized to characterize the cytotoxicity of the samples. This concentration elucidates that the potency of the samples results in a 50 % fatality rate of cells. A lower IC<sub>50</sub> value in samples would indicate a higher potential as an anticancer agent (Paudel et al., 2019; Hasibuan et al., 2020). In this study, the IC<sub>50</sub> of Eav and Gem can be shown in Fig. 2B. The cytotoxic activity of Eav is better than Gem described from IC<sub>50</sub> of 21.19 ± 0.64 μg/mL and 164.78 ± 1.40 μg/mL. This study determined the % viability of PANC-1 cells after treatment with the combination of Eav and Gem of IC<sub>50</sub> and ½ IC<sub>50</sub>, respectively (Fig. 2C). % Viability of these combinations shows slightly down compared with Gem of 500.00 μg/mL. Furthermore, the combination of Eav and Gem at

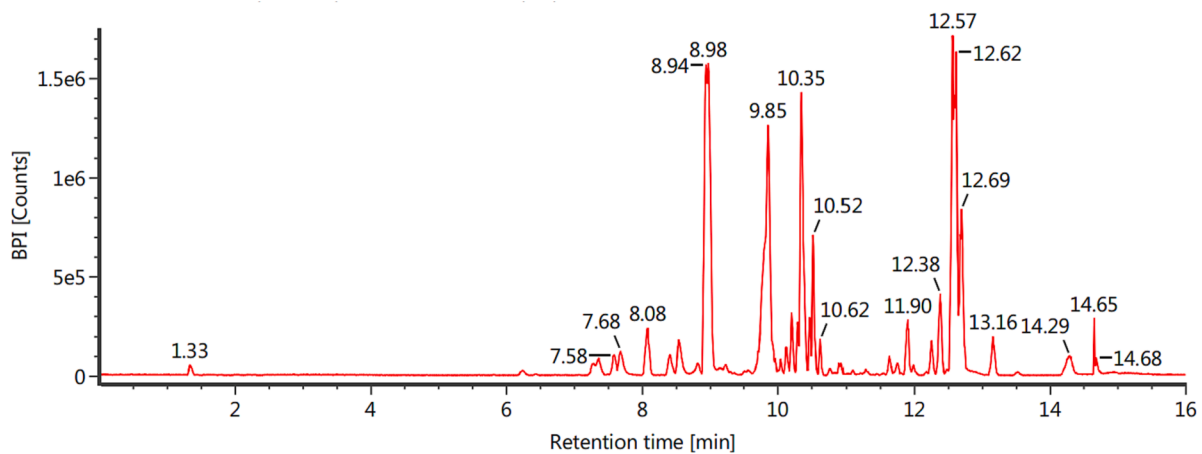


Fig. 1. LC-MS/MS analysis of Eav compounds identification. The analysis was carried out using 0.1 % formic acid in water (Solvent A), and 0.1 % formic acid in acetonitrile (Solvent B) in accordance with the gradient method. Meanwhile, the Hypersil GOLD aQ column (50 × 1 mm × 1.9 μm) as a coloumn system.

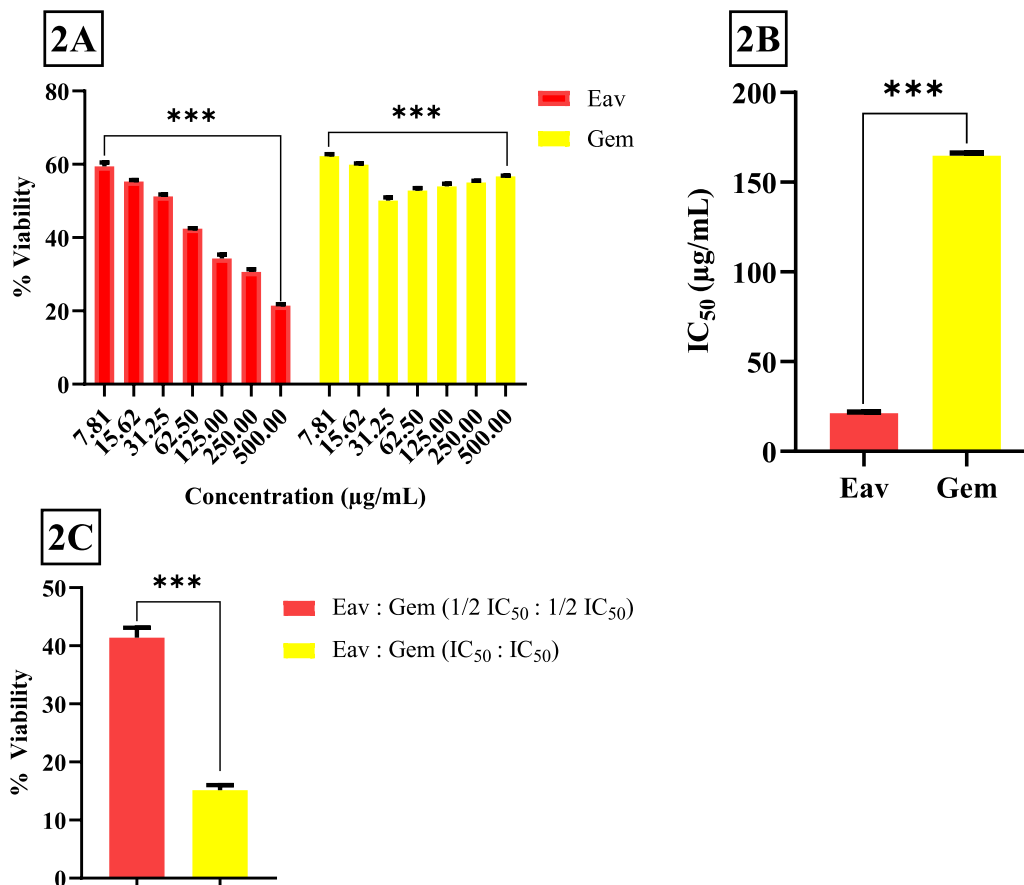


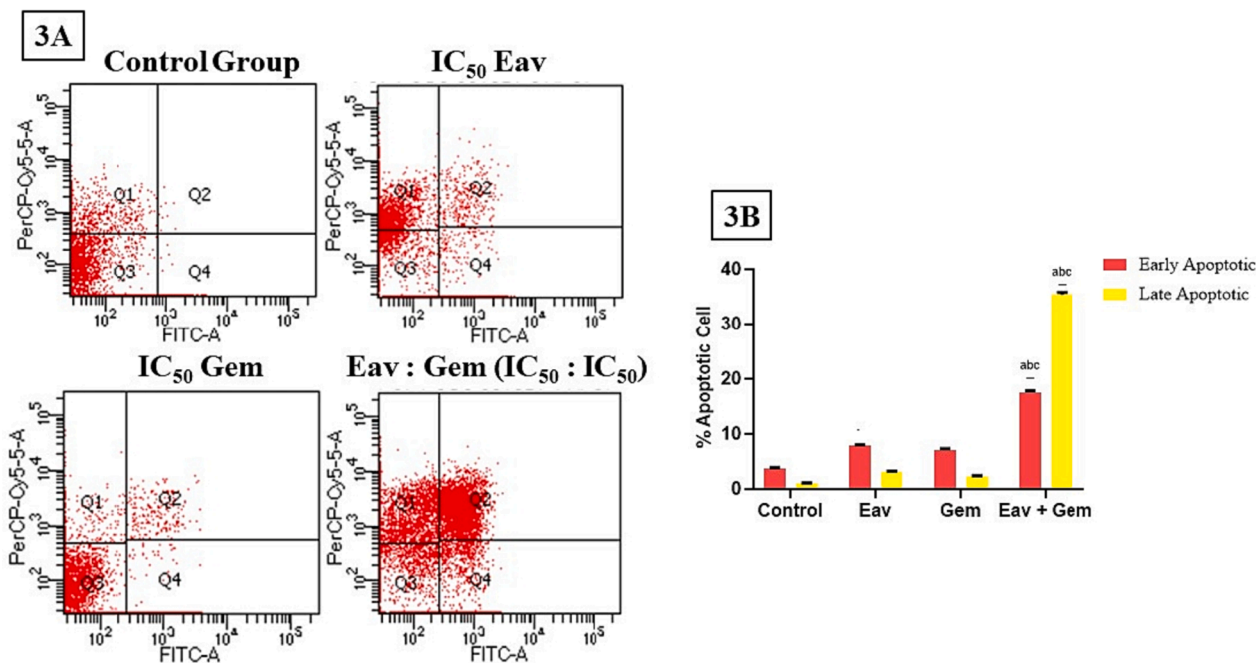
Fig. 2. The percentage cell viability of PANC-1 cells after being treated with various concentrations of Eav, Gem, and/or co-treatment of Eav-Gem. \*\*\* The results showed a significant difference between group tests with  $p < 0.0001$ . (2A) % viability of PANC-1 cells, (2B) Inhibitory concentration 50 ( $IC_{50}$ ) of Eav and Gem, (2C) % viability of PANC-1 cells after treatment with combination of Eav and Gem.

$IC_{50}$  (1:1) has activity strongest than Eav alone at 500.00 μg/mL. This result indicated potential anti-pancreatic activity after Eav combined with Gem at  $IC_{50}$  (1:1).

### 3.3. Analysis of apoptotic cell death

In order to examine the impact of Eav on apoptotic processes, PANC-1 cells were subjected to a 72-hour treatment with Eav and/or Gem. The

resulting number of apoptotic cells was subsequently analyzed through Annexin V/PI double staining (Fig. 3A). The present study observed a significant increase ( $p < 0.0001$ ) in the levels of early apoptotic in PANC-1 cells after Eav treatment ( $7.76 \pm 0.25$  %) in comparison to the control group ( $3.70 \pm 0.17$  %) (Fig. 3B). In addition, the levels of early apoptotic following Eav treatment ( $7.76 \pm 0.25$  %) are significantly different ( $p < 0.05$ ) than the gemcitabine-induced levels of early apoptotic ( $7.06 \pm 0.20$  %). Moreover, the levels of early apoptotic



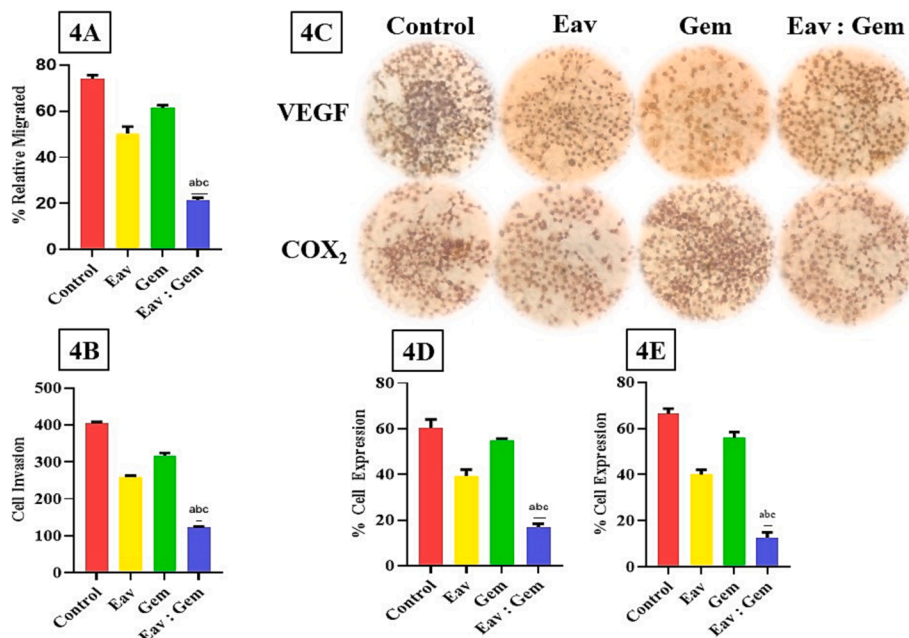
**Fig. 3.** The percentage of apoptotic cells after treatment with Eav and Gem in combination or alone is significantly different ( $p < 0.0001$ ). (3A) The distribution of PANC-1 cells using Annexin V/PI double staining, (3B) % apoptotic cell, <sup>a</sup> the result is significantly different with the control group ( $p < 0.0001$ ), <sup>b</sup> the result is significantly different with Eav group ( $p < 0.0001$ ), <sup>c</sup> the result is significantly different with Gem group ( $p < 0.0001$ ).

following the co-treatment were significantly different increased ( $17.46 \pm 0.35$  %) than Eav or Gem alone ( $p < 0.0001$ ). The evaluation of late apoptotic events was conducted after the administration of Eav and/or Gem (Fig. 3A). The co-treatment resulted in a significant increase ( $p < 0.0001$ ) in the levels of late apoptotic, which were measured at  $35.46 \pm 0.45$  %. In comparison, the levels of late apoptotic following Eav and Gem treatment were  $3.11 \pm 0.10$  % and  $2.26 \pm 0.15$  %, respectively (Fig. 3B). The general pattern exhibited a strong resemblance to that which was noted in the context of initial apoptosis. The findings indicate

that the combined treatment may exhibit a synergistic impact on apoptotic and cytotoxic functions.

**3.4. Co-treatment inhibited PANC-1 cell migration and invasion**

To evaluate the potential antimetastatic effect of Eav and/or Gem, we analyzed its ability to inhibit the migration and invasion of PANC-1 cells. It was demonstrated that co-treatment inhibits migration of PANC-1 cells significantly different ( $p < 0.0001$ ) with the control group, Eav,



**Fig. 4.** The effect of Eav and Gem Combination Against migration and invasion of PANC-1 cells. The results showed significantly different ( $p < 0.0001$ ) with each group treatment. (4A) %relative migrated of PANC-1 cells, (4B) Cell invasion value after treatment with each group, (4C) Immunohistochemical analysis of VEGF and COX2 proteins in PANC-1 cells, (4D) % expression of VEGF in PANC-1 cells, (4E) % expression of COX2 in PANC-1 cells.

and Gem with % relative migrated index subsequently  $21.49 \pm 0.96 \%$ ,  $74.00 \pm 1.70 \%$ ,  $50.32 \pm 2.99 \%$ , and  $61.35 \pm 1.30 \%$  (Fig. 4A). The following observation was to determine the co-treatment effect of PANC-1 cell invasion. These results are not different from migration results, that co-treatment has strongest activity inhibited PANC-1 cells invasion ( $p < 0.0001$ ) than the control group, Eav, and Gem with invaded cells result of  $125.25 \pm 5.25$  cells,  $405.73 \pm 5.15$  cells,  $260.15 \pm 5.70$  cells, and  $315.35 \pm 5.40$  cells (Fig. 4B). Hence, these results suggested that the co-treatment is stronger than Eav and Gem alone in inhibiting migration and invasion of PANC-1 cells.

### 3.5. Effect of co-treatment on the VEGF, COX<sub>2</sub>, RAS, and MEK expressions

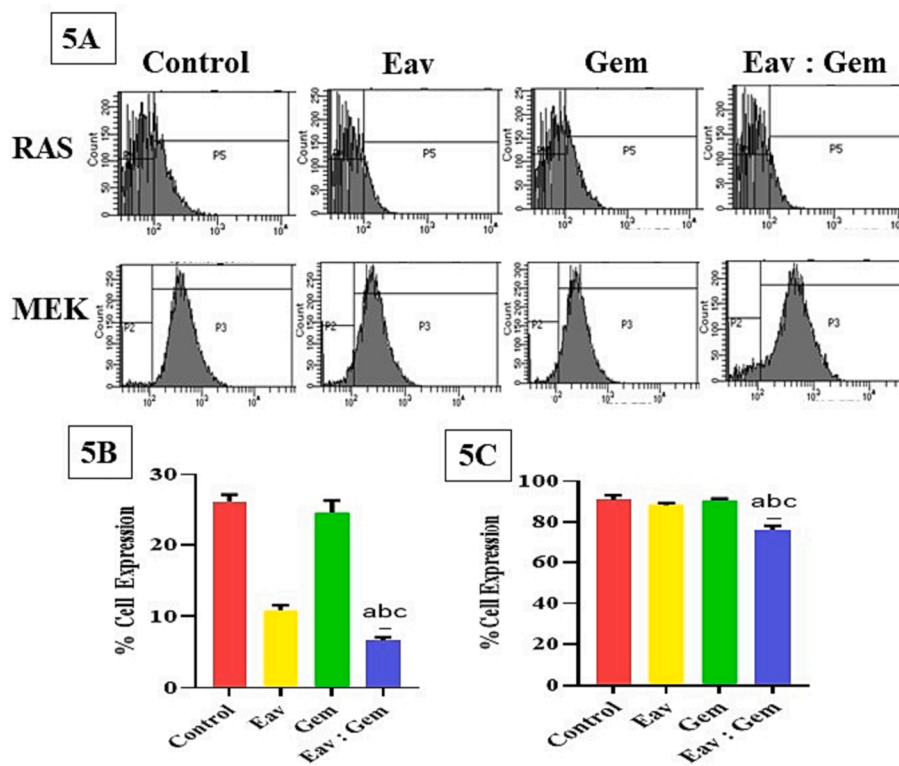
Migration and invasion of PANC-1 cells are dependent on protein expressions like VEGF and COX2 (Shi et al., 2015). In this study, we investigated VEGF and COX2 levels on PANC-1 cells after treatment with Eav and/or Gem. The expression of proteins was carried out using immunohistochemical analysis (Fig. 4C). The expressions of VEGF were significantly decreased with co-treatment compared to the control group of  $16.88 \pm 1.45 \%$  and  $60.51 \pm 3.87 \%$ , respectively. The co-treatment ( $16.88 \pm 1.45 \%$ ) of Eav with Gem is superior to each treatment of Eav and Gem. The percentage expression showed significant differences compared with single treatments of  $39.14 \pm 1.91 \%$  and  $55.70 \pm 0.63 \%$  (Fig. 4D). The following test described the effect of co-treatment on COX2 expression (Fig. 4C). The percentage of COX2 expression measured with the co-treatment significantly decreased COX2 expression compared with the control group, Eav, and Gem of  $10.70 \pm 1.51 \%$ ,  $68.70 \pm 2.31 \%$ ,  $39.70 \pm 1.81 \%$ , and  $56.70 \pm 1.96 \%$  were obtained (Fig. 4E). These results showed possible mechanisms of co-treatment of Eav and Gem against inhibiting migration and invasion of PANC-1 cells.

Furthermore, the flow cytometer was utilized to determine the

expressions of RAS and MEK. The combined treatment of Eav and Gem resulted in a significant reduction of RAS expression in PANC-1 cells, as compared to both the control group and individual treatments of Eav and Gem (Fig. 5A). In contrast, the co-treatment of Eav and Gem caused cell accumulation in the P5 area ( $6.66 \pm 0.49 \%$ ). While the control group and each treatment with Eav/Gem have a cell accumulation in the P5 region of  $26.23 \pm 0.96 \%$ ,  $10.88 \pm 0.69 \%$ , and  $24.69 \pm 1.65 \%$  were obtained (Fig. 5B). On the other hand, MEK expression was obtained after PANC-1 cells were treated using the combination of Eav and Gem. The PANC-1 cells have accumulated in the P3 region of  $76.06 \pm 2.34 \%$ . This value is significantly decreased if compared with the control group and each treatment of Eav and Gem of  $90.95 \pm 2.18 \%$ ,  $88.28 \pm 1.18 \%$ , and  $90.90 \pm 0.57 \%$  (Fig. 5C), respectively. Furthermore, the PANC-1 cells have described overexpression of RAS and MEK, and the combination of Eav and Gem significantly reduced RAS and MEK expression compared to other groups.

## 4. Discussion

The limited availability of efficacious chemotherapeutic agents has been a significant challenge in the management of pancreatic cancer. Currently, Gem is utilized as the primary treatment for pancreatic cancer. However, it yields minimal effects on the median survival rate of individuals diagnosed with locally advanced or metastatic pancreatic cancer (Schultheis et al., 2017). According to another study, the tumor inhibitory effects of Gem were observed only during the initial stages of the treatment regimen when the tumors were small. The drug was found to be ineffective in inhibiting tumor metastasis (Jamshed et al., 2020). This study reports a reduction in Gem activity as observed in the migration and invasion assays conducted on PANC-1 cells. The investigation of combination chemotherapeutic strategies was undertaken to aid in the advancement of a more efficacious treatment for pancreatic



**Fig. 5.** The expression of RAS and MEK proteins using a flow cytometer. (5A) Distribution of cells is significantly different after the treatment of samples with  $n = 3 \pm$  SD. The P5 and P3 area was described as cell distributions after treatment with each sample. The increasing cell accumulation in P5 and P3 illustrated the expression of each protein after being treated with each sample, (5B) % expression of RAS in PANC-1 cells is significantly different with  $p < 0.001$ , (5C) % expression of MEK in PANC-1 cells is significantly different with  $p < 0.001$ .

cancer. Several studies have indicated that natural products may inhibit the growth of pancreatic cancer by triggering apoptosis, suppressing migration and invasion, and blocking critical cellular signaling pathways (Wang et al., 2013). Nevertheless, the studies were limited to the utilization of monotherapy exclusively in cancer cell lines, and there was a dearth of information regarding the impact of combining Eav with conventional chemotherapeutic agents. The current investigation aimed to assess the potential of Eav alone or in conjunction with Gem to elicit enhanced anticancer outcomes.

The study at hand has documented that Eav, a form of traditional medicine, could potentially serve as a valuable adjunct to chemotherapeutic agents in enhancing the effectiveness of chemotherapy for pancreatic cancer cells. This is achieved through a reduction in the required dosage of Gem, thereby minimizing the treatment volume. The study conducted by Hasibuan et al. (2020) provided evidence that the expression of PI3K/Akt/mTOR pathways mediated the eradication of breast cancer cells by Eav. These pathways have been linked to the migration and invasion of cancer cells (Xiao et al., 2021). In the present study, secondary metabolites of Eav were identified using LC-MS/MS (Fig. 1). Five compounds success in determining, which C<sub>19</sub>H<sub>20</sub>O<sub>7</sub> candidate, C<sub>29</sub>H<sub>40</sub>O<sub>5</sub> candidate, C<sub>34</sub>H<sub>46</sub>O<sub>9</sub> candidate, C<sub>25</sub>H<sub>46</sub>O<sub>14</sub> candidate, and C<sub>54</sub>H<sub>78</sub>O<sub>9</sub> candidate with retention time sequentially 8.96 min, 9.85 min, 10.52 min, 10.36 min, and 12.59 min. In a previous study, LC-MS/MS analysis of Eav identified that some of components, including diosmetin, 4-methylumbelliferyl glucuronide, chlorogenic acid, 4-methylumbelliferone, scutellarin, rhoifolin, 7-hydroxycoumarine, apigenin, baicalin, luteolin, ingenol-3-angelate, and 4-methoxycinnamic acid (Hasibuan et al., 2020). Meanwhile, our study reported Eav contains vernonioside D and vernonioside E and these compounds were shown activity to bond with epidermal growth factor and mammalian target of rapamycin proteins (Hasibuan et al., 2021). Another study reported apigenin affects vascular epidermal growth factor (VEGF), inhibiting migration and invasion of pancreatic cancer cells (Ashrafizadeh et al., 2020). King et al. (2012) reported that apigenin-induced apoptosis in human pancreatic cells through p53 modulation. Similarly, luteolin was found to induce apoptosis through BCL-2-mediated pathways (Li et al., 2018).

Initially, the optimization of the treatment conditions for PANC-1 cells with Eav was conducted in the presence or absence of Gem. The viability of PANC-1 cells was found to be inhibited by Eav treatment via the induction of apoptosis. Subsequently, it was found that Eav triggers cellular apoptosis, a process that differs significantly from the early apoptotic effects of Gem. The preceding research demonstrated the apoptotic-inducing efficacy of Eav against HTB-182 cells (Hasibuan et al., 2020). The findings indicate that the combined administration of Eav and Gem exhibited a significantly higher efficacy in triggering early apoptotic events, as opposed to the singular administration of either treatment. This study has demonstrated the potential efficacy of combining Eav and Gem therapies in inhibiting the migration and invasion of PANC-1 cells. The efficacy of this combination in reducing the percentage of relative migration and inhibiting cell invasion is superior to that of individual treatments with Eav and Gem. The combined treatment exhibited increased inhibitory activity on proteins associated with migration and invasion, such as VEGF, COX2, RAS, and MEK, compared to the individual treatments of Eav or Gem.

The RAS/MEK pathway is a crucial factor in the metastasis of pancreatic cancer (Bylsma et al., 2020). According to the report, a mutation in over 90 % of the RAS gene was observed in pancreatic cancer, leading to the excessive activation of the downstream signaling pathway (Bannoura et al., 2021). Several genetic studies have demonstrated that the activation and mutations of RAS are essential for the initiation of pancreatic cancer (Di Magliano and Logsdon., 2013). Previously, it has been reported that the *Vernonia amygdalina* leaf compounds (Vernonioside D and Vernonioside E) inhibit EGFR and mTOR (Hasibuan et al., 2021). EGFR is essential for RAS oncogene-driven pancreatic cancer (Shankar et al., 2020). The EGFR protein is a member of the erbB family

of tyrosine kinase receptors and plays a crucial role in the processes of tumor growth, metastasis, and disease recurrence (Zakaria et al., 2019). The research findings suggest that Eav has the potential to hinder migration and invasion through the inhibition of the RAS/MEK signaling pathway. The concurrent administration of Gem and Eav demonstrates a notable decrease in the expressions of RAS and MEK, in comparison to the individual treatments. The results of this study suggest that Eav has potential as a monotherapy or adjunctive therapy in combination with the standard chemotherapy drug Gem for managing pancreatic cancer. A forthcoming investigation will verify the safety and effectiveness of combined therapy in vivo, with the aim of establishing its theoretical foundation for clinical implementation. Furthermore, the potential impact of Eav in conjunction with other chemotherapeutic agents will be investigated.

## 5. Conclusion

The present investigation has furnished initial proof that Eav has the capability to trigger apoptosis and hinder the migration and invasion of PANC-1 cells in an in vitro setting. The results were obtained after the combination of Eav and Gem in each IC<sub>50</sub> (1:1). This study succeeded in providing a significantly different effect of the combination effect than the control group and Eav or Gem alone in entire parameters. The potential of Eav to enhance Gem-induced cytotoxicity against PANC-1 cells warrants further investigation to elucidate the underlying synergistic mechanisms. The potential toxic and anticancer properties of this combination require in vivo testing as a preliminary step in the development of clinical trials.

## CRedit authorship contribution statement

**Poppy Anjelisa Zaitun Hasibuan:** Conceptualization, Supervision, Methodology, Data curation, Validation, Formal analysis, Investigation, Funding acquisition, Writing – original draft. **Jane Melita Keliat:** Data curation, Validation, Formal analysis. **Muhammad Fauzan Lubis:** Resources, Data curation, Conceptualization, Methodology, Formal analysis. **Annisa Nasution:** Project administration, Software, Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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