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**A COMPARATIVE STUDY INCLUDING ACEFYLLINE AND  
AMYGDALIN AS CHALLENGING ANTIPROLIFERATIVE AGENTS  
IN MOUSE EHRLICH ASCITES CARCINOMA**

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**ABSTRACT**

Many malignancies originate from prolonged inflammation where the tumor microenvironment, which is predominantly regulated by inflammatory cells, is a crucial player in the neoplastic process, supporting proliferation and migration. Phosphodiesterase-4 inhibitors and amygdalin analogs showed great potential to treat inflammatory diseases such as psoriasis and become challenging for hindering malignant ascites. Ascites was induced by intraperitoneal inoculation of Ehrlich ascites carcinoma (EAC) in mice then after 14 days mice were distributed into four groups; the positive control, group treated with cisplatin, and two groups received cisplatin in combination with acefylline or amygdalin for seven days. Flow cytometry was used to evaluate cell cycle phases and expression of cyclin B1 (CDK-B1), p53, and p21 as cell cycle regulators. Proliferation was assessed by Ki-67 expression and proliferation index was estimated. Apoptosis was determined by analysis of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 expression. Both acefylline and amygdalin combination with cisplatin showed a reduction in Ki-67 expression and arrested the cell cycle. Amygdalin treatment showed a significant reduction in S-phase and proliferation index. This combination also restored the cell's apoptotic ability proved by the apoptosis index and increased p53, p21, and Bcl-2 expression while reducing Bax expression in ascites. Results showed a distinct effect

of amygdaline associated with a significant reduction in the ascites burden with markedly decreased proliferation and induced apoptosis. These findings provide a basis for the theory that anti-inflammatory agents could be used efficiently to delay tumor growth and hinder cell cycle by their effective antiproliferative activity.

**Keywords: Acefylline, p21, amygdalin, flow cytometry, Ki-67, proliferation**

## INTRODUCTION

Since the pathophysiology of malignant ascites is complicated, it is typically protein-rich, with increased capillary permeability caused by cytokines in addition to impaired lymphatic drainage and hormonal processes. Cytokines are important mediators of inflammation, various immune processes, even cancer, and chronic tissue remodeling where uncontrolled proliferation alters the expression of proteins involved in cell cycle checkpoint regulators [1]. In fact, p53 mutations are observed in chronic inflammatory disorders such as rheumatoid arthritis and inflammatory bowel disease at a frequency similar to those in malignancies. The presence of Ki-67 in the G1, S, and G2 phases of the cell cycle, but not in quiescent or resting cells in the G0 phase, has been shown in several publications from the cell cycle study in the nucleus, suggesting its role as a cell proliferation marker in many malignancies [2]. In clinical settings, a high Ki-67 index often indicates a bad prognosis. In addition, Ki-67 has consensus sites for cyclin-dependent kinase 1. (CDK1). Ki-67 is remarkable for localizing granular nucleolar components to mitotic chromosomes, which makes it crucial for

nucleolar segregation between daughter cells [3]. Research has revealed that Ki-67 has an involvement in rRNA transcription that explains the association with cell growth during ribosome biogenesis.

CDK-1 is regarded as the primary regulatory kinase that directs the cell entry into mitosis. Cell cycle progression is directly regulated by Cyclin B1/CDK1. Ki-67 is phosphorylated by CDK-1 during mitosis and the cell continues to divide until the sister chromatid separation then nucleus reassembly is initiated during the metaphase to anaphase transition [4].

Overexpression of p21, a universal CDK inhibitor, may block the cyclin B1-CDK1 and cyclin A-CDK1/2 complexes and cause cell cycle arrest during the G2/M transition. P21's impact on CDK is stoichiometry-controlled and does not always render it inactive. P21 forms complexes with the kinases CDK1, CDK2, CDK3, CDK4, and CDK6 in conjunction with certain cyclins. Furthermore, In p21 checkpoint-capable cells, where Ki-67 controls cell cycle progression, reduction of Ki-67 delayed S phase entrance [5].

p21 mediates anti-tumor activity by G1 cell cycle arrest through activation by p53 and CDK regulation. High p53 concentrations leads to cell cycle arrest and apoptosis due to increased p53 transcriptional activity [6]. Significantly, several genes that are transcriptionally regulated by p53 are affected, such as cyclin B1 and B2.

Besides, p53 builds up in the cytosol and interacts with B-cell lymphoma-2 (Bcl-2) family members and engage directly in the intrinsic apoptotic process. Cell death was induced through activation of the Bcl2-associated X (Bax) protein by p53. Contrarily, Bcl-2 reduces the pro-apoptotic Bax activity to stop apoptosis [7].

In clinical trials, combination therapy regimens are frequently used to combat chemotherapy resistance. A recent report showed that sequential treatment might be more effective than combined treatment to block drug resistance [8]. Our objective is to study the effect of Acefylline piperazine, a methylated xanthine drug and amygdaline, a naturally aromatic cyanogenic compound, with cisplatin and their potential effects on proliferation and apoptosis of malignant ascites.

## MATERIAL AND METHODS

### 2.1. Experimental animals

Adult female Wistar albino mice weighing 20-25 gm were purchased from the Egyptian Organization for Biological Products and Vaccines (Cairo, Egypt).

Animals were kept under conventional laboratory conditions (room temperature 24–27°C and 55 ± 10% humidity) with alternating 12h light and dark cycles. Animals were fed normal chow and were permitted water *ad libitum*. They were left in the animal house at faculty of pharmacy, MSA university for acclimatization for one week before the start of the study.

### 2.2 Transplantation of tumor cells

Ehrlich ascites carcinoma cells were collected from donor mice gotten from Egyptian National Cancer Institute, Cairo University, and suspended in sterile isotonic saline. A fixed number of viable cells were transferred to healthy mice through intraperitoneal (i.p.) transplantation of  $10^6$  cells (in 0.2 ml of PBS/animal). Then, the growth of EAC was distinguished by captivating photographs at 1st, 5th, 10th, and 14th days.

### 2.3. Drugs and chemicals

Amygdaline was purchased from Sigma Chemicals (MO, USA). Cisplatin and Acefylline were obtained from pharmacy. (Cairo, Egypt).

### 2.4. Experimental design

After two weeks, mice were divided into four groups (n = 8) as follows:

EAC group: mice inoculated with Ehrlich cell line as previously described.

EAC+Cis group: EAC bearing mice treated with single dose of cisplatin (5 mg/kg; i.p.)

EAC+Cis+Ace group: EAC bearing mice treated with single dose of cisplatin (5 mg/kg; i.p) and received acefylline daily (75 mg/kg; i.p.) for 7 days.

EAC+Cis+Amy group: EAC bearing mice treated with single dose of cisplatin (5 mg/kg; i.p) and received amygdaline daily (100 mg/kg; i.p.) for seven days.

### 2.5. Sample collection

Mice were euthanized and the ascites fluid was collected from the intraperitoneal cavity and sent for assessment by flow cytometry technique.

### 2.6. Cell cycle analysis

The ascites fluid from all groups were collected from mice and washed thrice with the cold PBS. After fixation with 70% ethanol for 24 h at 4 °C the cells were then washed thrice with cold PBS. Finally, the cells in 1 ml PBS were treated with 50 l of RNase A (1 mg/ml) for 30 min at 37 °C followed by staining with 5 µl of Propidium Iodide (1 mg/ml) in dark at 4 °C for 5 min before analyzing using flow cytometry. The fractions of cells in G0/G1, S and G2/M phase were analyzed by a FACS Flow cytometer (Partec CyFlow SL, Germany).

### 2.7. Flow cytometric analysis of ki-67, CDK-B1, p53, p21, Bax and Bcl2.

For detection of parameters cell surface expression, ascites fluid was used then single cell suspensions were washed with staining buffer (PBS containing 1% FBS). Cells were then incubated with biotin-

conjugated rat anti-human ki-67, CDK-B1, p53, p21, Bax and Bcl2 antibodies at a concentration of 20 mL/1 x 10<sup>6</sup> cells for 30 minutes on ice. After washing with staining buffer, the cells were mixed with Streptavidin-phycoerythrin and immediately analyzed with a flow cytometer FACScan and CellQuest Software. Background staining was determined by staining cells with biotin-conjugated isotype control rat IgG2a followed by Streptavidin-phycoerythrin incubation.

### 2.8. Statistical analysis

Data were expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey-Kramer post-Hoc test. GraphPad Prism 8 for Windows (GraphPad Software, Inc, La Jolla, United States) was used in all analyses. P value < 0.05 was considered significant.

## RESULTS

### Cell cycle analysis of ascites fluid cells in EAC bearing mice.

DNA flow cytometry was used to study the effect of cisplatin and its combination with acefylline or amygdaline on the cell cycle distribution of malignant ascites (EAC) induced by inoculation of Ehrlich cell line in mice. Cisplatin produced nonsignificant changes in the nonproliferating cell fraction (G0 /G1 phase), whereas its combination with acefylline or amygdaline significantly

increased G0 /G1 phase by 3.59% and 26.87% respectively compared with the untreated EAC bearing mice (Figure 1a-e).

Both combination groups showed nonsignificant change compared to cisplatin only treated group.

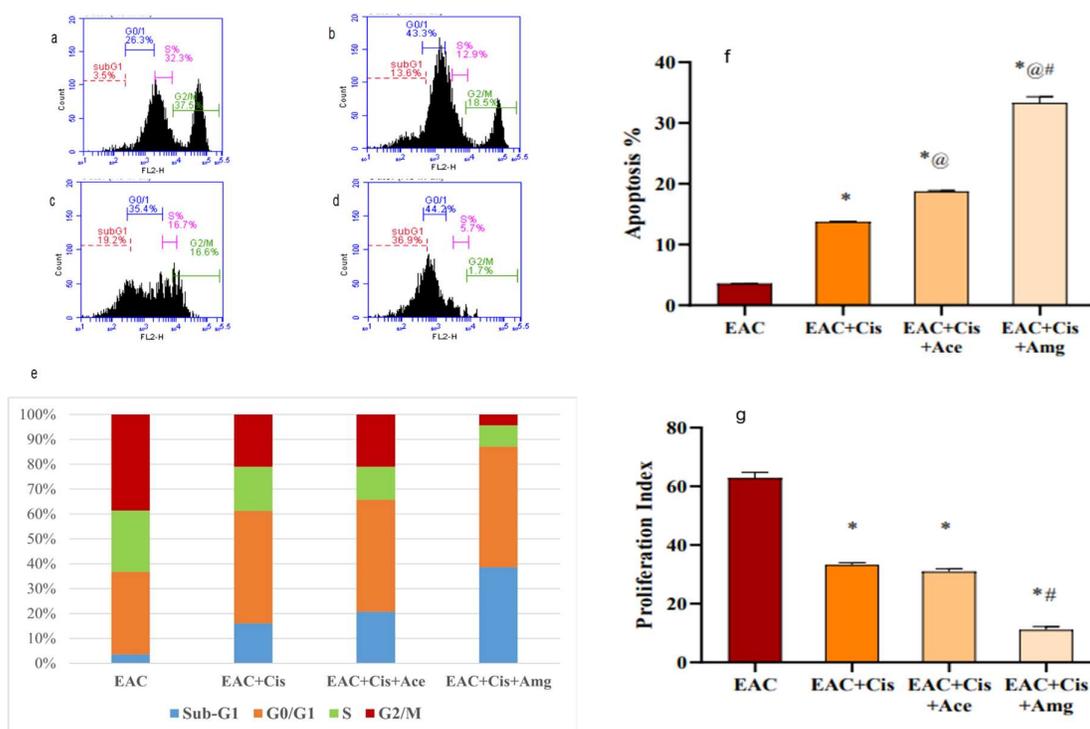


Figure 1: Flow cytometric analysis of the effect of cisplatin alone or in combination with acefylline or amygdalin on cell cycle in the cells of malignant ascites fluid

Cell cycle distribution was determined using DNA flow cytometry analysis, the histograms (a), (b), (c), and (d) represent the DNA content of the cells in each of the cell cycle phases (Sub-G1, G0 /G1, S, and G2 /M) in malignant ascites group (EAC), cisplatin single treatment group (EAC+Cis), cisplatin in combination with acefylline (EAC+Cis+Ace), and cisplatin in combination with amygdaline group (EAC+Cis+Amy), respectively. Different cell phases were plotted (e) as percentage of total events. The apoptotic cells were plotted separately (F) as percentage of the total

events. Proliferation index (S% phase + G2 /M% phase) of all groups was plotted (g). Data are expressed as mean ± SEM (n=6). (\*), (@) and (#) indicate significant difference from EAC, EAC+Cis and EAC+Cis+Ace, respectively at  $P < 0.05$  using one-way ANOVA followed by Tukey-Kramer post-Hoc test. **EAC**: Ehrlich ascites carcinoma; **Cis**: Cisplatin; **Ace**: Acefylline; **Amy**: Amygdaline. Cisplatin, and the combination therapy markedly decreased the S-phase cell population by 37.63%, 51.13%, and 70.17%, respectively, compared with the

EAC mice (**Figure 1a-e**). The group treated with combination of acefylline showed nonsignificant change in S phase, but amygdaline treated group showed significant decrease by 52.17% compared to the cisplatin only treated mice.

No apparent change in the G2 /M-phase cell population was observed with combination of acefylline with cisplatin (**Figure 1a-e**), whereas cisplatin combination with amygdaline group showed significant decrease by 78.83% compared to the cisplatin single treatment. Cisplatin and its combination therapy with acefylline or amygdaline markedly decreased the G2 /M-phase cell population by 53.08%, 50.52% and 90.07%, respectively, compared with the EAC untreated mice (**Figure 1a-e**).

Cisplatin monotherapy resulted in significant accumulation of dead cells in sub-G1 phase by 283.72%, whereas the combination groups of cisplatin with acefylline or amygdaline showed more accumulation of the dead cells in sub-G1 phase by 423.26% and 829.58% respectively compared with untreated EAC mice (**Figure 1a-f**). The groups of the combination therapy showed potentiated effect on sub-G1 phase by 36.36% and 142.25% respectively compared with cisplatin monotherapy mice. On top of that, amygdaline treatment showed significant increase in apoptosis by 77.65% compared to the acefylline treated mice.

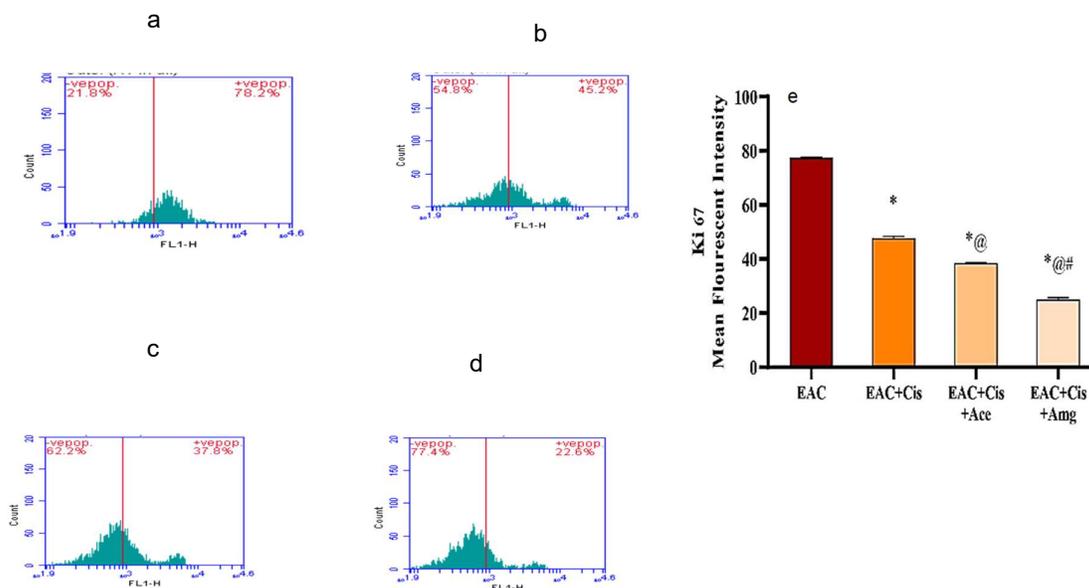
On the other hand, there was a significant inhibition of the proliferation index (S% phase and G2 /M% phase) by 47.07%, 50.75%, and 82.32% in cisplatin single treatment group, and groups of combination therapy with acefylline or amygdaline, respectively, compared with EAC bearing group (**Figure 1g**). Combination therapy with amygdaline showed marked inhibition by 66.61% when compared to cisplatin treated group, however, combination with acefylline did not significantly affect the proliferation index compared with cisplatin treated group. Treatment with amygdalin significantly showed inhibition by 64.11% compared to the acefylline treated mice.

### **1.Flow cytometric analysis of proliferation marker Ki-67 in ascites fluid in EAC bearing mice treated with cisplatin alone or in combination with acefylline or amygdaline.**

Flow cytometric analysis of Ki-67 expression (**Figure 2e**) on ascites fluid cells showed the highest level of expression in EAC bearing mice (**Figure 2a**). EAC bearing mice treated with cisplatin monotherapy showed significant reduction in the expression of Ki-67 on ascites cells by 38.34%, compared to the untreated EAC bearing mice (**Figure 2b**). Ki-67 expression showed significantly more decrease in cisplatin together with acefylline treated group and cisplatin together with amygdaline treated mice by 19.42% and

47.6% respectively compared to the group treated with cisplatin alone (**Figure 2c and 2d**). Moreover, the group treated with

amygdaline showed significant reduction in Ki-67 expression by 34.97% compared to acefylline-treated mice (**Figure 2e**).



**Figure 2: Flow cytometric analysis of the effect of cisplatin monotherapy or in combination with acefylline or amygdalin on Ki-67 expression in the cells of malignant ascites fluid**

Histogram plots of EAC group (a), EAC+Cis group (b), EAC+Cis+Ace group (c) and EAC+Cis+Amg group (D). Mean fluorescence intensity of Ki-67 is presented (e). Data are expressed as mean  $\pm$  SEM (n = 6). (\*), (@) and (#) indicate significant difference from EAC, EAC+Cis and EAC+Cis+Ace, respectively at  $P < 0.05$  using one-way ANOVA followed by Tukey-Kramer post-Hoc test. **EAC**: Ehrlich ascites carcinoma; **Cis**: Cisplatin; **Ace**: Acefylline; **Amg**: Amygdaline.

## 2. Cell cycle regulators expression analysis in ascites fluid by flow cytometry.

### 2.1. CDK-B1 expression in ascites fluid cells treated with cisplatin alone or in combination with acefylline or amygdaline.

EAC bearing mice showed the lowest expression of CDK-B1 on ascites fluid cells (**Figure 3a**). Cisplatin monotherapy (EAC+Cis) showed a significant increase in the expression of CDK-B1 on ascites cells by 156.95% compared to the untreated EAC bearing group (**Figure 3b**). CDK-B1 expression showed significantly increase in the group treated with cisplatin and acefylline (EAC+Cis+Ace) and mice treated with and cisplatin and amygdaline

(EAC+Cis+Amy) by 24.33% and 27.95% respectively compared to the group treated with cisplatin alone (Figure 3c and 3d).

Treatment with amygdalin showed no significant difference compared to acefylline-treated mice (Figure 3e).

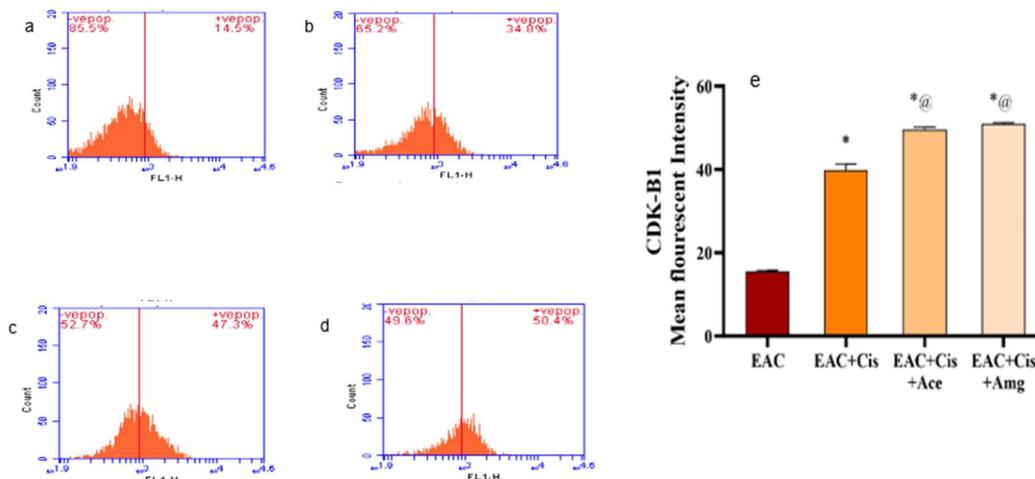
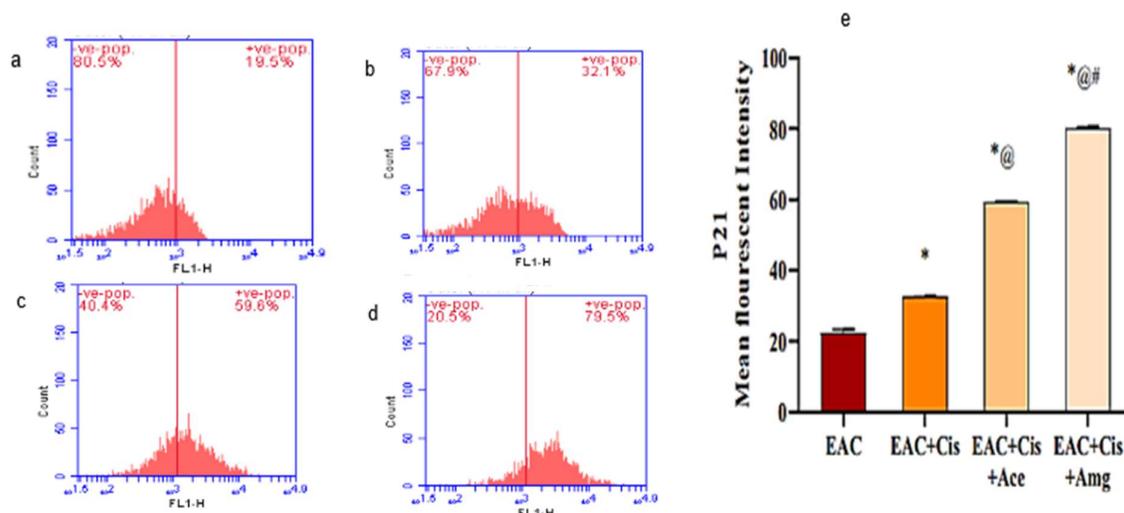


Figure 3: Flow cytometric analysis of the effect of cisplatin monotherapy or in combination with acefylline or amygdalin on CDK-B1 expression in the cells of malignant ascites fluid

Histogram plots of EAC group (a), EAC+Cis group (b), EAC+Cis+Ace group (c) and EAC+Cis+Amg group (D). Mean fluorescence intensity of CDK-B1 is presented (e). Data are expressed as mean  $\pm$  SEM (n = 6). (\*), (@) and (#) indicate significant difference from EAC, EAC+Cis and EAC+Cis+Ace, respectively at  $P < 0.05$  using one-way ANOVA followed by Tukey-Kramer post-Hoc test. **EAC**: Ehrlich ascites carcinoma; **Cis**: Cisplatin; **Ace**: Acefylline; **Amg**: Amygdaline.

## 2.2. Expression of p21 in the ascites fluid cells treated with cisplatin alone or in combination with acefylline or amygdaline

Ascites fluid cells showed low expression of p21 in EAC bearing mice (Figure 4a). Treatment with cisplatin as monotherapy (EAC+Cis) showed a significant increase in p21 expression on ascites cells by 45.75% compared to the untreated EAC bearing group (Figure 4b). The groups treated with cisplatin and acefylline (EAC+Cis+Ace) and cisplatin and amygdaline treated group (EAC+Cis+Amy) showed significant increase in p21 expression by 165.36% and 146.17% respectively compared to the mice administered cisplatin alone (EAC+Cis) (Figure 4c and 4d). Furthermore, treatment with amygdalin showed significant increase by 35.21% compared to acefylline treated mice (Figure 4e).



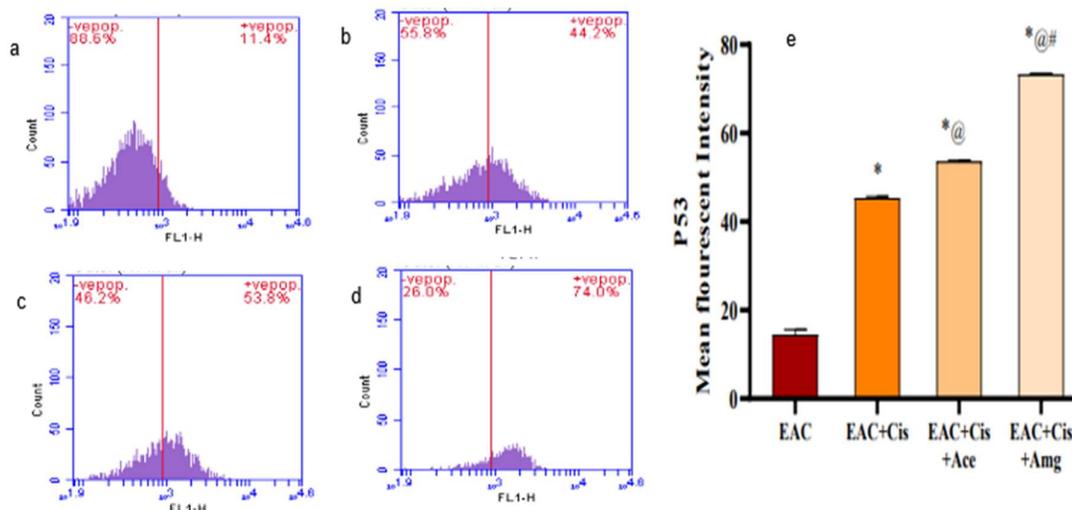
**Figure 4: Flow cytometric analysis of the effect of cisplatin monotherapy or in combination with acefylline or amygdalin on p21 expression in the cells of malignant ascites fluid**

Histogram plots of EAC group (a), EAC+Cis group (b), EAC+Cis+Ace group (c) and EAC+Cis+Amg group (d). Mean fluorescence intensity of p53 and p21 is presented (e). Data are expressed as mean  $\pm$  SEM (n = 6). (\*), (@) and (#) indicate significant difference from EAC, EAC+Cis and EAC+Cis+Ace, respectively at  $P < 0.05$  using one-way ANOVA followed by Tukey-Kramer post-Hoc test. **EAC:** Ehrlich ascites carcinoma; **Cis:** Cisplatin; **Ace:** Acefylline; **Amg:** Amygdaline

### 2.3. Ascites fluid cells analysis for p53 in EAC mice treated with cisplatin alone or in combination with acefylline or amygdaline.

Expression of p53 in EAC bearing mice was low in ascites fluid cells (Fig.5a). EAC

bearing mice administered cisplatin monotherapy (EAC+Cis) showed significant increase in p53 expression on ascites cells by 212.61% compared to the untreated EAC bearing mice (Fig. 5b). Treatment with cisplatin and acefylline (EAC+Cis+Ace) and cisplatin and amygdaline treated group (EAC+Cis+Amy) showed significant increase in p53 expression by 18.29% and 61.58% respectively compared to treatment with cisplatin alone (EAC+Cis) (Fig. 5c and 5d). In addition, mice treated with amygdaline showed a significant increase by 36.59% compared to the group treated with acefylline (Fig. 5e).



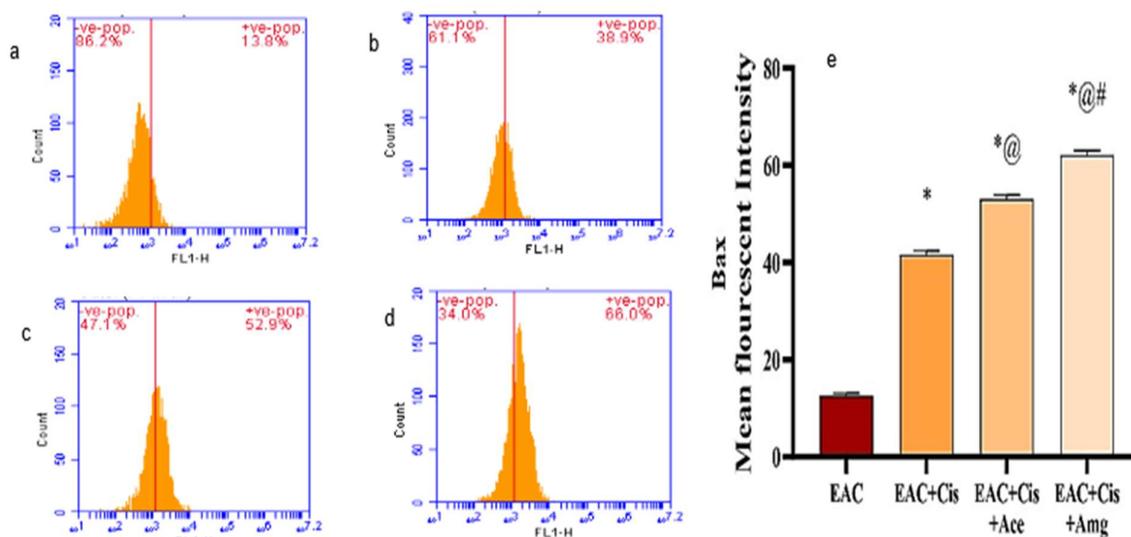
**Figure 5:** Flow cytometric analysis of the effect of cisplatin monotherapy or in combination with acefylline or amygdalin on p53 expression in the cells of malignant ascites fluid

Histogram plots of EAC group (a), EAC+Cis group (b), EAC+Cis+Ace group (c) and EAC+Cis+Amg group (d). Mean fluorescence intensity of p53 and p21 is presented (e). Data are expressed as mean  $\pm$  SEM (n = 6). (\*), (@) and (#) indicate significant difference from EAC, EAC+Cis and EAC+Cis+Ace, respectively at  $P < 0.05$  using one-way ANOVA followed by Tukey-Kramer post-Hoc test. **EAC:** Ehrlich ascites carcinoma; **Cis:** Cisplatin; **Ace:** Acefylline; **Amg:** Amygdaline

### 3. Apoptosis analysis in mice ascites fluid by flow cytometry.

#### 3.1. Analysis of Bax in ascites fluid cells upon treatment with cisplatin alone or in combination with acefylline or amygdaline.

Analysis of Bax expression in EAC bearing mice showed low levels in ascites fluid cells (**Figure 6a**). Cisplatin monotherapy (EAC+Cis) showed a significant increase in Bax expression on ascites cells by 228.41% compared to the group of EAC bearing mice (**Fig. 6b**). Cisplatin and acefylline treatment (EAC+Cis+Ace) and cisplatin and amygdaline treated group (EAC+Cis+Amy) showed significant increase in Bax expression by 31.25% and 51.69% respectively compared to treatment with cisplatin alone (EAC+Cis) (**Figure 6c and 6d**). Amygdaline treated mice showed significant increase by 15.57% compared to the group treated with acefylline (**Figure 6e**).

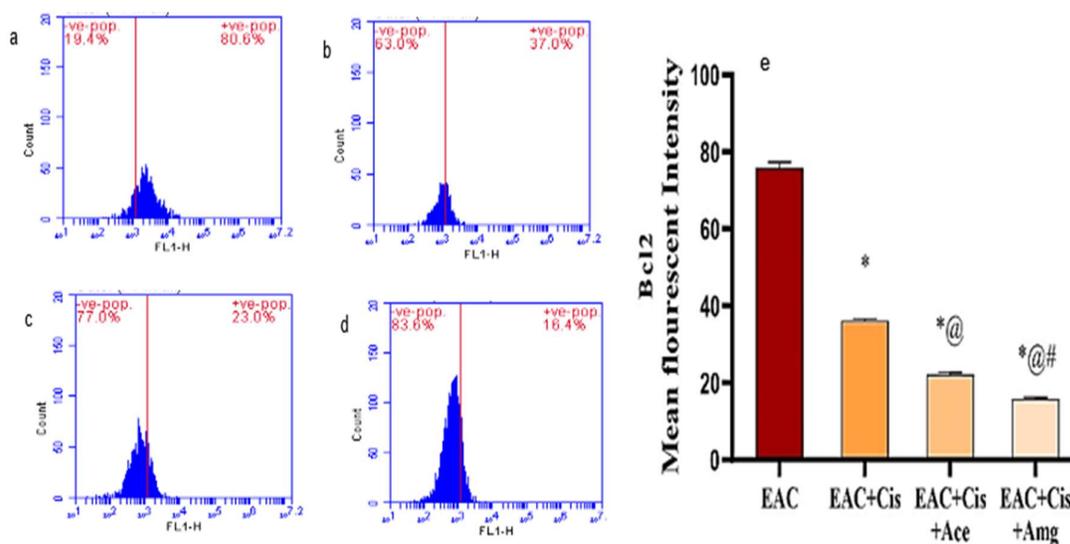


**Figure 6:** Flow cytometric analysis of the effect of cisplatin monotherapy or in combination with acefylline or amygdalin on Bax expression in the cells of malignant ascites fluid

Histogram plots of EAC group (a), EAC+Cis group (b), EAC+Cis+Ace group (c) and EAC+Cis+Amg group (d). Mean fluorescence intensity of Bax and Bcl2 is presented (e, j). Data are expressed as mean  $\pm$  SEM (n = 6). (\*), (@) and (#) indicate significant difference from EAC, EAC+Cis and EAC+Cis+Ace, respectively at  $P < 0.05$  using one-way ANOVA followed by Tukey-Kramer post-Hoc test. **EAC:** Ehrlich ascites carcinoma; **Cis:** Cisplatin; **Ace:** Acefylline; **Amg:** Amygdaline.

### 3.2. Bcl2 analysis for expression in ascites fluid cells when treated with cisplatin alone or in combination with acefylline or amygdaline.

Expression of Bcl2 showed high levels in ascites fluid cells of EAC bearing group (**Figure 7a**). The group treated with cisplatin monotherapy (EAC+Cis) showed a significant decrease in Bcl2 expression on ascites cells by 53.09% compared to the group of EAC bearing mice (**Figure 7b**). The groups treated with cisplatin and acefylline (EAC+Cis+Ace) and cisplatin and amygdaline (EAC+Cis+Amg) showed a significant decrease in Bcl2 expression by 38.58% and 55.85% respectively compared to treatment with cisplatin alone (EAC+Cis) (**Figure 7c and 7d**). Mice treatment with amygdalin showed a significant decrease by 28.13% compared to mice treated with acefylline (**Figure 7e**).

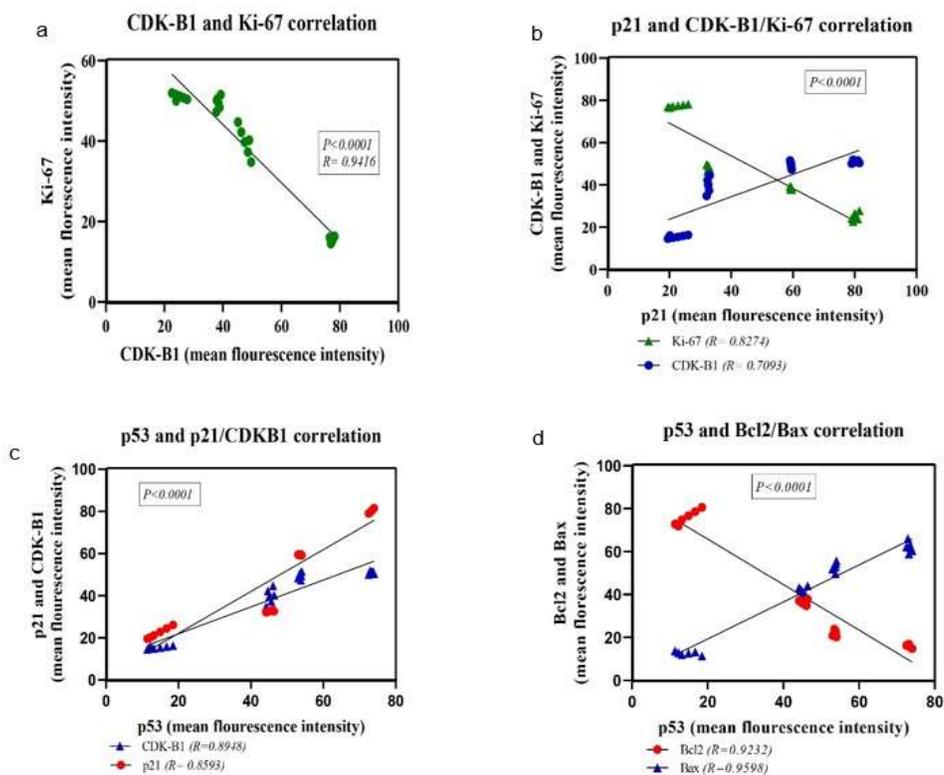


**Figure 7: Flow cytometric analysis of the effect of cisplatin monotherapy or in combination with acefylline or amygdalin on Bax expression in the cells of malignant ascites fluid**

Histogram plots of EAC group (a), EAC+Cis group (b), EAC+Cis+Ace group (c) and EAC+Cis+Amg group (d). Mean fluorescence intensity of Bax and Bcl2 is presented (e, j). Data are expressed as mean  $\pm$  SEM (n = 6). (\*), (@) and (#) indicate significant difference from EAC, EAC+Cis and EAC+Cis+Ace, respectively at  $P < 0.05$  using one-way ANOVA followed by Tukey-Kramer post-Hoc test. **EAC:** Ehrlich ascites carcinoma; **Cis:** Cisplatin; **Ace:** Acefylline; **Amg:** Amygdaline

#### **4. Correlation between the assessed cell regulators with proliferation and apoptotic parameters as well as between CDK-B1 and Ki-67.**

The scatter plots show correlations in all study groups where negative correlation between CDK-B1 expression and Ki-67 expression is exhibited on malignant ascites (**Figure 8a**). In addition, p21 expression correlated with the ascites proliferation markers is displayed, a positive correlation with CDK-B1 expression (**Figure 8b**), as well as a negative correlation with Ki-67 expression (**Figure 5B**). Also, both p21 and CDK-B1 expression are positively correlated to p53 ascites expression (**Figure 8c**). Furthermore, p53 is correlated with apoptotic markers where it is positively correlated with Bax (**Figure 8d**) while a negative correlation has been shown with Bcl2 (**Figure 5d**) in all study groups.



**Figure 8:** Correlation between the assessed cell regulators with proliferation and apoptotic parameters (a) Strong negative correlation between CDK-B1 and Ki-67 expression on ascites cells. In addition, (b) p21 expression shows a strong positive correlation with CDK-B1 as an ascites proliferation marker while Ki-67 expression is negatively correlated. Furthermore, (c) p21 has a strongly positive correlation with p53 ascites expression. Also, p53 is strongly correlated with apoptotic markers where positively correlated with Bax while negative correlation has been shown with Bcl2 (d). The solid lines represent the linear regression and correlation coefficient (r), P is the correlation significance level

## 5. Mortality rate differences between cisplatin single treatment and the combination treatment groups.

Mortality is presented in relation to exposure to different treatments (**Figure 9**). Blue line represented the EAC bearing mice where mortality rate increased over time. Cisplatin single treatment (EAC+Cis) is represented by orange line where mortality rate increased in relation to the progress in days of experiment. The group treated with

cisplatin in combination with amygdalin (EAC+Cis+Amg) is represented by grey line where it showed higher rates of mortality among all groups. Acefylline treatment in combination with cisplatin (EAC+Cis+Ace) is represented by yellow line showing lower levels of mortality when compared to cisplatin and amygdalin combined with cisplatin treated groups.

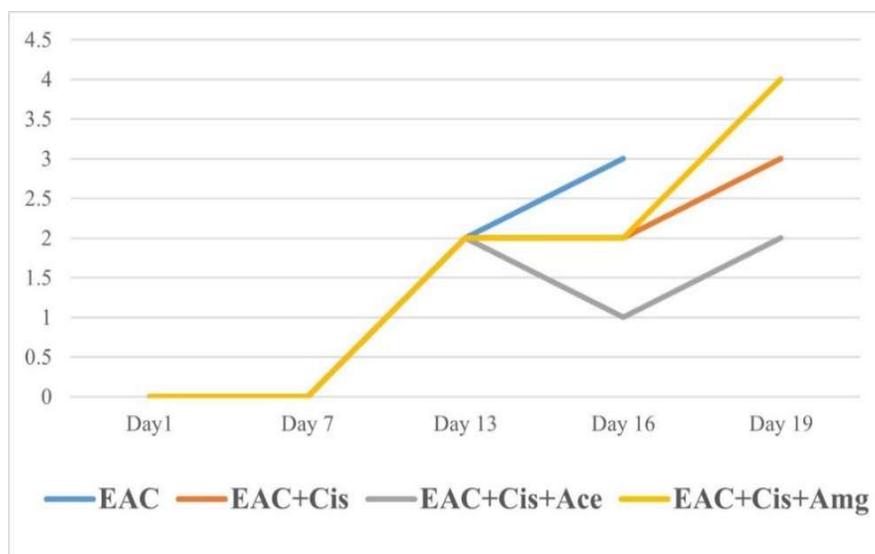


Figure 9: Mortality rate differences between cisplatin and combination groups

Lines represent the change in mortality in relation to exposure to cisplatin, acefylline and amygdalin. Blue line represented EAC group increasing mortality rate. (EAC+Cis) group represented by orange line where mortality rate increased over time. (EAC+Cis+ACE) group represented by grey line where it showed lower rates of mortality. (EAC+Cis+Amg) group represented by yellow line showing increased mortality rate. **EAC:** Ehrlich ascites carcinoma; **Cis:** Cisplatin; **Ace:** Acefylline; **Amg:** Amygdaline

## DISCUSSION

The long-term survival of patients with advanced cancers requires new approaches due to drug resistance. Resistance to platinum-based medications affects treatment effectiveness and prognosis. The mechanisms of drug resistance are complex due to many etiologies, including abnormal

membrane protein expression, enhanced DNA repair functions, and abnormal apoptosis regulation mechanisms [9]. Several studies have found that combining antiproliferative drugs with traditional chemotherapeutics improved the ability to inhibit tumor growth and metastasis.

The present study investigated the chemoprotective and therapeutic action of acefylline and amygdalin when combined with cisplatin on proliferation and apoptosis markers expression by flow cytometry. To determine the differential cytotoxicity towards Ehrlich ascites carcinoma, the effect of cisplatin separately was evaluated as well as in combinations. This evidence may assume particular importance considering the sensible reduction to the resistance of chemotherapeutical agents. The cell cycle is arrested in the G1, S, or G2-M phases because of the cytotoxic effects of

cisplatin that are caused by the formation of mono-, inter-, and intra-strand cisplatin-DNA adducts [5, 10]. Both acefylline and amygdalin have been proven previously as effective treatments for inflammation involved in psoriasis [11]. In the current study, we showed their extending effect as antiproliferative agents through analysis of cell cycle regulators.

Cisplatin and its analogs have been widely used in combination with natural compounds, such as vindesine and curcumin in different cancer types [12]. Amygdalin is a natural compound whose anticancer, anti-inflammatory activity and other medicinal benefits have been known for many years [13]. Its anti-inflammatory activity is related to inhibition of arachidonic acid metabolites and hindering cytokines transcription<sup>9</sup> and Amygdalin's anticancer activity is primarily influenced by the amount of cyanide and benzaldehyde in the cytoplasm that is released during amygdalin decomposition because of the glucosidase enzyme's analytical action.

Acefylline piperazine (AP) is a methylated xanthine drug and serves as an anti-asthmatic, cardiac stimulant, bronchodilator, and diuretic [14]. Literature reveals that introduction of bulky alkyl group in acefylline may lead to compounds with potential anti-cancer activity [15]. Another study on acefylline-oxadiazole hybrids showed the antiproliferative and

anticancer activity of the acefylline itself. Acefylline is a phosphodiesterase inhibitor that may efficiently kill cancer cells in vitro by increasing intracellular cAMP and controlling the enzymes responsible for DNA repair.

DNA flow cytometry was used to assess proliferative activity. This technique allows for the analysis of cellular DNA content and the quantification of cells as percentages within the cell cycle phases G0/G1 (pre-synthesis), S (DNA synthesis), and G2/M (pre-mitosis and mitosis) [16].

Our data revealed that combination therapy with acefylline reduced the ascites burden and showed increased antiproliferative effect when compared to cisplatin monotherapy. Acefylline treatment and analysis of cell cycle by flow cytometry showed increased G0 /G1 phase with decreased S-phase cell population as well as more accumulation of the dead cells in sub-G1 phase. Besides acefylline and cisplatin combination results indicated profound inhibition of the proliferation index (S% phase and G2 /M% phase) when compared to EAC-bearing mice but not to the group treated with cisplatin alone.

A Study agreed that induction of apoptosis in human leukemic cells was through PDE inhibition which does not allow cells to progress to S phase, suggesting that phosphodiesterases play a key role in the G1/S transition [17]. In addition, a study

showed that combination of platinum-based compound with PDE inhibitor on lung cancer cell-line resulted in more cytotoxicity than carboplatin alone and was apparent in induction of apoptosis and interference with cell cycle indicating the antiproliferative effect [18].

In addition, the current study showed that amygdalin in combination with cisplatin exhibited intense results regarding cell cycle arrest compared to cisplatin monotherapy where it significantly increased G0 /G1 phase and markedly decreased the S-phase and G2 /M-phase cell population. Amygdalin distinctly accumulated the dead cells in sub-G1 phase and suppressed the proliferation index. In agreement, it was stated that amygdalin-induced cell cycle arrest in the G0/G1 phases. and inhibits cell transfer from the G1 to S phase, resulting in the inhibition of cell proliferation and growth [19]. Besides, a study on Caco-2 and HT-29 cells, a digested beverage of fruit pulp contains amygdalin inhibited proliferation through an increase in the proportion of cells in the S-phase and a decrease in the number of cells in G0/G1 [20].

Cisplatin is one of the agents with the capacity to increase the intracellular availability of glucosidase enzyme paradoxically increasing the cytotoxicity of amygdalin. This is accomplished by cisplatin's capacity to induce lysosome

membrane permeabilization, which results in the availability of more glucosidase enzymes in the cytoplasm [21]. In a study using Hela cancer cells, the cytotoxicity of cisplatin and amygdalin was shown by growth inhibition and potentiation abilities between them, including amygdalin's capacity to reduce resistance to the cytotoxic effect of cisplatin. The energy deprivation caused by cyanide's action reduces the growth of cancer cells' resistance to cisplatin's ability to cause lysosome membrane permeabilization [22].

In a study on acefylline derivatives, it was shown that they have more reactive anti-proliferative activity against the cancer cell line Hep G2 [23]. Xanthine derivatives decreased the population of tumor proliferating cells by decreasing ki-67 in a dose-dependent manner and restored the p53 pathway signaling that leads to apoptotic cell death as well as disturbed S phase. Malignant ascites is characterized by a high Ki-67 proliferation index, which is associated with tumor aggressiveness and a poor prognosis, while current treatment with acefylline enhanced the survival of mice by reducing the expression of Ki-67 together with an increase in the expression of the pro-apoptotic p53 and cell cycle regulator p21 in ascites cells. Consistently, it has been shown that phosphodiesterase 1A inhibition increases phosphorylation of the tumor suppressor p53 [24]. Additionally,

Phosphodiesterase role and its catalytic activity on cGMP has been shown to influence cell cycle regulatory proteins such as p21 that under phosphodiesterase inhibition delayed cell cycle progression and induced apoptosis [25].

Relocating to amygdalin treatment, the current work demonstrated that amygdalin treatment with cisplatin augmented the anti-tumor activity by reduction of the expression of Ki-67 in malignant ascites and induced cell death by elevating p53 and p21 and was shown as a negative correlation between p21 and Ki-67. These are in accordance with a study [26] that proved amygdalin's inhibitory effect on proliferation activity via ki-67 reduced levels, which were measured in rats with colon cancer. Additionally, by downregulating p53, amygdalin caused apoptosis in squamous cell carcinoma and decreased the mitotic index (ki-67) [27]. Also, it was demonstrated that amygdalin increased the expression of p21 while decreasing the expression of Ki-67 in rat hepatocellular carcinoma [28]. A polysaccharide extract from an Asian traditional medicine that contains amygdalin demonstrated anti-tumor activity by raising levels of p53 and p21 to stop the proliferation of MCF-7 cells and trigger cell apoptosis [29].

p53 activation causes apoptosis by triggering an intrinsic mitochondrial pathway. The balance of expression

between the anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) members of the Bcl-2 family proteins, which regulate this pathway, determines the fate of the cell [30]. Cancer is thought to be characterized by dysregulation of apoptosis, particularly the intrinsic apoptotic pathway. It was previously indicated that Bcl-2 inhibition may improve the chemotherapy effect on ovarian cancer peritoneal metastasis and may help overcome the issue of platinum resistance of MCS [31]. In consensus, we demonstrated that acefylline treatment resulted in a significant decrease in the expression of Bcl-2 and the increase in the expression of Bax when compared to cisplatin alone. In accordance, a study on mutated human granuloma cells showed that combination of cisplatin with PDE inhibitor produced a synergistic effect in induction of apoptosis that was mediated by suppression of Bcl2 where both drugs alone result in a dose-dependent reduction of Bcl2 levels, but the reduction was more significant when used together [32]. A positive correlation was presented between p53 and Bax while Bcl2 was negatively correlated.

Moreover, treatment with amygdalin in combination with cisplatin reduced the expression of Bcl2 together with increased expression of Bax. In mouse neuroblastoma cells, treatment with the amygdalin-containing apricot seed extract increased the expression of the pro-apoptotic protein Bax

and the activity of the caspase-3 enzyme while decreasing the expression of the anti-apoptotic protein Bcl-2 [33]. In addition, amygdalin was found to increase expression of Bax, a pro-apoptotic protein, and decrease expression of Bcl-2, an anti-apoptotic protein in prostate cancer cells [34].

Based on our findings, both acefylline and amygdalin in combination with cisplatin decreased the expression of CDK-B1 compared to cisplatin monotherapy and this decrease was displayed in the correlation with Ki-67, p21 and p53. Cyclin B1 is a key regulator of the G2/M to G0/1 transition, and this is supported by its distinguished role in the cell cycle network. In early mitosis, the cyclin B-CDK1 complex stimulates the anaphase-promoting complex (APC), which then kills it in late mitosis. A non-degradable cyclin B would be anticipated to cause an arrest in M phase because the cyclin B-CDK1 complex must be destroyed to exit mitosis.

It was observed that cyclin B overexpression did not show a detectable arrest in G2/M suggesting that the level of overexpression obtained under the experimental conditions using theophylline small molecule-responsive ribozyme switches is insufficient to undermine the ability of APC [35]. In addition, cyclin B1 level was shown to be rapidly restored to control levels upon addition of pentoxifylline as a methylxanthine drug [36]. This could be

another explanation that underlies the understanding of CDK-B1 in the regulation of cell cycle. Furthermore, binding of amygdalin to CDK1/Cyclin B was illustrated by docking which can give an insight into inhibitory activity of amygdalin [37]. These observations support the assumption that the level of expression of CDK-B1 may be affected by the dose and duration of exposure and the type of cancer cell line.

An ideal G2/M transition marker in the absence of DNA damage is CYCB1;1, one of the four cyclins belonging to the B1-subclass. Under DNA damage, CYCB1;1 expression may be mistakenly interpreted as promoting cell division or as a sign that cells are arrested in G2/M, when it indicates that these cells have been arrested in an S- or early G2-phase. Distinguishing between a true G2/M arrest or stimulation of cell division from a DNA damage response, conclusions about cell cycle and proliferation activity under stress conditions should be better supported by flow cytometry [37].

The present study showed that mortality rates are different among treatment groups. The Cisplatin group showed a comparable high mortality rate to the group treated with combination of cisplatin and amygdalin. The lethal consequences of enzymatically released HCN and non-hydrolyzed cyanogenic glycosides are assumed to be

connected to amygdalin's anticancer activities. However, several studies have demonstrated the amygdalin's anticancer effect as well as its toxicity when administered in high doses due to inactivation of ferric ion-containing enzymes such as respiratory chain-cytochrome oxidase by cyanide compounds [38]. On the other hand, the group treated with a combination of cisplatin and acefylline showed a lower level of mortality among the treatment groups. Acefylline is one of the methylxanthines promising purine-based lead compounds, which is characterized by a beneficial safety profile as compared to theophylline but suffers from poor absorption [39].

## CONCLUSION

The data of the present study supported the hypothesis that anti-inflammatory agents could be used efficiently to hinder tumor growth and arrest the cell cycle. Overall, acefylline and amygdaline combination with cisplatin decreased tumor cell proliferation, induced apoptosis, and dramatically reduced malignant ascites burden. Amygdalin was observed to be significantly effective over acefylline which may strongly underline the employment of natural agents with cisplatin although the mortality rate which has an impact on the agent used as anticancer raises more advantages for the use of acefylline over amygdalin.

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## CONFLICTS OF INTEREST

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## ETHICS

The experimental protocol was approved by the Research Ethics Committee (REC) of Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA University) (PH6/Ec6/2021F).

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