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Biochemical and molecular mechanisms of *Annona sp.* fruits extract in Ehrlich ascites carcinoma-bearing mice

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Abstract

Limitations of chemotherapeutic drugs for cancer treatment could be due to the lack of specificity, rapid drug metabolism, and harmful side effects. Therefore, finding new approaches in neoplastic cancer or targeting drugs depends on the pathways and characteristics of different tumor entities. Natural elements as anticancer agents are an effective strategy in the fight against cancer. This study investigated the biochemical and molecular mechanisms of *Annona sp.* fruits extract (ASFE) in Ehrlich ascites carcinoma (EAC)-bearing mice. Seventy mice were divided into seven groups (n = 10) as follows: Gp1 was used as a negative control, from Gp2 to Gp7 were inoculated with 1×10^6 EAC-cells/mouse, then Gp2 left as EAC-bearing mice, Gp3 was injected with Cis (2 mg/kg), Gp4 was injected with ASFE (200 mg/kg), Gp5 was co-treated with Cis as Gp3 and ASFE as Gp4. Gp6 was injected with a low dose of Cis (0.5 mg/kg), and Gp7 was co-treated with a low dose of Cis as Gp6 and ASFE as Gp4. The body weight change percentages (b.wt%) were calculated. On day 14, all groups were sacrificed, the ascitic fluids were harvested, and the total tumor volume, count, and live and dead tumor cells were measured. The relative expression of P53, Bcl-2, BAX, and caspase-9 genes was determined in EAC cells by RT-PCR. Sera samples were collected for biochemical parameters assessment. Liver tissues were collected for the determination of oxidants/antioxidants biomarkers. The results showed that co-treatment of the high or low doses of Cis with ASFE led to synergistic effects via targeting apoptosis in EAC-cells that can significantly inhibit tumor growth, decrease liver dysfunctions induced by Cis and enhance the hepatic antioxidant status.

Keywords: *Annona sp.*; Antitumor; Antioxidant; Chemotherapy; Cisplatin; Apoptosis.

Introduction

Cancer is a significant public health problem that is caused by DNA mutations leading to uncontrolled cell growth and it has the second highest mortality rate accounting for about 610.000 deaths in 2022 [1].

Apoptosis, the programmed cell death, occurs following DNA damage or during development, which is finely regulated at the gene level resulting in the orderly and efficient removal of damaged cells [2]. Deregulation of this process is associated with

unchecked cell proliferation, development, and progression of cancer and is considered one of the hallmarks of cancer and cancer resistance to drug therapies [3]. The machinery of apoptosis is complex and involves many signaling pathways, it can be triggered in a cell through either the caspase-mediated extrinsic or intrinsic pathways to activate the effector apoptotic caspases resulting ultimately in morphological and biochemical cellular alterations. The balance between the pro-apoptotic and anti-apoptotic protein regulators is a critical key point to determine if a cell undergoes apoptosis, thereby blocking tumor growth [4].

Conventional treatment approaches, including surgery, chemotherapy, and radiotherapy, have been in use, recently, other approaches for instance, stem cell therapy, targeted therapy, ablation therapy, nanoparticles, natural antioxidants, radionics, sonodynamic therapy, and ferroptosis-based therapy have been used [5,6]. Chemotherapy is considered the most effective and widely used modality in treating cancers alone or in combination with other therapies, however, they cause numerous side effects due to increasing the oxidative stress agents and consequently led to toxicity of vital organs [7]. Therapeutic strategies targeting molecules involved in apoptotic resistance, therefore, represent a valid approach to be pursued to restore cancer cells' sensitivity to apoptosis and overcome the ineffectiveness of the treatments [8].

The use of natural plant elements as an alternative to chemotherapy or chemo-preventive drugs is an effective strategy against cancer, especially with the possibility of targeting cancerous cells without healthy cells [9]. Phytochemicals have gained attention in cancer research due to their non-toxic properties and potential anticancer capacities [10]. *Annona sp.* is a deciduous tropical evergreen fruit tree belonging to the *Annonaceae* family that has been widely used globally as a traditional medicine for several diseases. For

instance, anticancer, antidiabetic, antiviral, antihypertensive, and wound healing [11]. The main active components of *Annona sp.* are acetogenin, alkaloids, and flavonoids [12]. Analysis of the compounds in *A. muricata* leaf extract revealed secondary metabolites such as flavonoids, terpenoids, saponins, coumarins, lactones, anthraquinones, glycosides, tannins, and phytosterols [13]. Several biological activities and the general mechanisms underlying the effects of *Annona sp.* have been tested both *in vitro* and *in vivo* [14].

Fadholly *et al.* (2019) reported that *Annona squamosa* extract showed antitumor efficacy against human colon cancer cell lines via potent cytotoxicity, increasing caspase-3 expression significantly and cell cycle arrest in the G2/M phase [15]. According to Al-Nemari *et al.* (2022), the free *A. squamosa* extract increased the expression of proliferative and apoptotic markers, which in turn enhanced the extract's anticancer effect on breast cancer cell lines. Additionally, it was found that the treatment of rats with this fruit extract had an impact on the tumor size, proliferative, and apoptotic indicators [16]. The *Annona muricata* extract has selective inhibitory activity against cancer cells that was modulated by acerogenin activity in the complex I mitochondrial electron transport chain, hampering the process of ATP formation needed for cancer cell growth [17]. Furthermore, the antitumor activity of *A. muricata* extracts against Ehrlich Ascites Carcinoma and Dalton's Lymphoma Ascites mediated tumors in Swiss albino mice have been reported [18]. Therefore, this study was designed to investigate the biochemical and molecular mechanisms of the hydro-ethanolic extract of *Annona sp.* as an anti-cancer agent alone or in combination with the chemotherapeutic drug Cis in Ehrlich ascites carcinoma (EAC)-bearing mice.

Materials and Methods

Chemicals

Chemicals for phytochemical assessments were purchased from Sigma (St. Louis, Mo., USA). Cisplatin (50 mg/50 mL vial) was purchased from Merk Ltd. (Cairo, Egypt). Biochemical kits were purchased from Bio-diagnostic Company, Egypt.

Collection of plant materials and extract preparation

Annona sp fruits were purchased from the local market in Tanta city, Gharbia, Egypt. The plant materials were identified and authenticated by a taxonomist at the Botany Department, Faculty of Science, Tanta University. Leaves were dried, crushed, and a certain weight of powder was mixed with ethanol. The hydroalcoholic *Annona sp* fruits extract (ASFE) was weighed and stored at 4 °C for further processing.

Mice and experimental design

Ehrlich ascitic carcinoma (EAC) cells were collected from the tumor-bearing mice purchased from the National Cancer Institute (NCI, Cairo, Egypt). The number of tumor cells was adjusted at 2×10^6 cells/mouse for intraperitoneal (i.p) inoculation. Seventy male Swiss albino mice (20 ± 2 g) were given drinking tap water and normal experimental pelleted animal food *ad libitum*. Mice were divided into seven groups as follows: Gp1 was used as a negative control, from Gp2 to Gp7 were inoculated with 1×10^6 EAC-cells/mouse, then Gp2 left as a positive control, Gp3 was injected with Cis (2 mg/kg), Gp4 was injected with ASFE (200 mg/kg) [19], Gp5 was co-treated with Cis (2 mg/kg) and ASFE (200 mg/kg). Gp6 was injected with the low dose of Cis (0.5 mg/kg), and Gp7 was co-treated with the low dose of Cis (0.5 mg/kg) and ASFE (200 mg/kg).

All treatments were intraperitoneally (i.p) injections after 24 hours of EAC-cells inoculation for 6 consecutive days. All groups were weighted at the beginning (initial b.wt) and at the end of the experiment (final b.wt). The percentage of the change in the total body weight was calculated. On day 14, mice from all groups were sacrificed. By using 10 mL syringes, the ascitic fluids were harvested from all

groups under the study. The volume of ascitic tumor fluids was measured. To determine the number of live and dead tumor cells, the trypan blue exclusion method was used. EAC cells were isolated from the ascetic fluid to determine the relative expression of apoptosis regulatory genes (P53, Bcl-2, BAX, and caspase-9). The total RNA was extracted from EAC cells by using RNeasy Mini Kit containing DNase. Blood samples were collected, and the sera were separated and frozen at -20 °C until used for the determination of biochemical parameters. Liver homogenates were used for the determination of oxidants/antioxidants biomarkers.

Biochemical analyses

Sera alanine transaminase (ALT), and aspartate aminotransferase (AST) activities were assayed according to the method of Reitman and Frankel (1957) [20]. Alkaline phosphatase was estimated according to Belfield and Goldberg, (1971) [21]. Total proteins were assessed according to Gornall *et al.* (1949) [22]. Superoxide dismutase (SOD) and catalase (CAT) activities were measured [23, 24]. Reduced glutathione was assayed according to the method of Beutler *et al.* (1963) [25]. The malondialdehyde (MDA) levels were assayed according to the method of Esterbauer and Cheeseman (1990) [26].

Molecular analyses

Tumor cells were harvested from tumor-bearing groups of mice under different treatment conditions. Total RNA was extracted following the manufacturer's protocol using the RNeasy Mini kit, and then by using Quant script reverse transcriptase, complementary DNA (cDNA) was formed from 4 µg of the total RNA per sample. Amplification of the cDNA was done with 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer's protocol (Thermo Scientific, # K0221), and gene-specific primers of P53, Bax, caspase 9, and Bcl-2 were used. The reaction volume and qPCR thermal conditions were applied. At the end of the last cycle, the temperature was increased

up to 95 °C to produce a melt curve. The relative change in gene expression was shown as fold change with critical threshold quantities.

Statistical analysis

All data are presented as mean \pm SD. One-way analysis of variance (ANOVA) was used to assess the significant differences among treatment groups. The SPSS statistics program was used for data analysis. The criterion for statistical significance was set at $p < 0.05$.

Results

Effect of ASFE Administration on the body weight changes in EAC-bearing Mice

The results showed that the % b.wt changes of the EAC-bearing mice were significantly increased ($p < 0.05$) up to 38.39 ± 1.45 when compared to the negative control group (19.35 ± 1.34). Treatment of EAC-

bearing mice with the therapeutic dose of Cis (2 mg/kg b.wt) or ASFE (200 mg/kg) for six consecutive days led to a significant decrease in % b.wt changes to 8.73 ± 1.23 , or 11.31 ± 1.93 , respectively when compared to EAC-bearing mice alone. The co-treatment of Cis (2 mg/kg b.wt)/ASFE (200 mg/kg b.wt) led to a much more decrease in the % b.wt changes up to 3.45 ± 0.87 when compared to a single treatment. EAC-bearing mice that were treated with the low dose of Cis (0.5 mg/kg b.wt) showed a significant decrease in % b.wt changes (12.61 ± 1.55) but not as the treatment with the therapeutic dose, however, the combination of low dose of Cis and ASFE led to synergistic effect on the decrease in the % b.wt changes due to the decrease in the ascitic tumor fluid (**Table 1**).

Table (1): Initial body weights, final body weights, % change of body weight of groups under the study

Groups	I. B. wt. (g)	F. B. wt. (g)	% change of B.wt.
Group 1	21.54 ± 1.98^a	25.71 ± 2.41^a	19.35 ± 1.34^a
Group 2	22.24 ± 2.47^a	30.78 ± 3.59^a	38.39 ± 1.45^c
Group 3	22.33 ± 2.21^a	24.28 ± 3.48^a	8.73 ± 1.23^d
Group 4	23.41 ± 2.34^a	26.06 ± 4.84^a	$11.31 \pm 1.93^{a,d}$
Group 5	22.87 ± 1.94^a	$23.68 \pm 2.31^{a,b}$	3.45 ± 0.87^e
Group 6	22.27 ± 2.76^a	$25.08 \pm 2.45^{a,b}$	$12.61 \pm 1.55^{a,d}$
Group 7	22.06 ± 2.84^a	$23.74 \pm 3.48^{a,b}$	7.53 ± 1.65^d

The values represented as means \pm S.D.; Group 1: Negative control; Group 2: EAC-bearing mice; Group 3: EAC/Cis (2 mg/kg b.wt); Group 4: EAC/ASFE (200 mg/kg b.wt); Group 5: EAC/Cis (2 mg/kg b.wt)/ASFE (200 mg/kg b.wt); Group 6: EAC/Cis (0.5 mg/kg b.wt); Group 7: EAC/Cis (0.5 mg/kg b.wt)/ASFE (200 mg/kg b.wt); ASFE: *Annona sp* fruits extract; Cis: Cisplatin; I.B.wt: Initial body weight; F.B.wt: Final body weight. This means that do not share a letter in each column is significantly different ($p < 0.05$).

Total tumor volume, viable and dead EAC-cells after different treatments in the different groups of EAC-bearing mice

The results demonstrated that there were significant decreases ($p < 0.05$) in the total tumor volume of EAC-bearing mice that were treated with the therapeutic dose of Cis (2 mg/kg), ASFE (200 mg/kg), or their combinatorial treatment for six consecutive days (1.4 mL \pm 0.43, 3.5 mL \pm 0.27, 0.9 mL \pm 0.14) when compared to EAC-bearing mice alone (8.7 mL \pm 0.65). Furthermore, EAC-bearing mice that were treated with the low dose of Cis (0.5 mg/kg) showed a significant reduction ($p < 0.05$) in the total tumor volume (3.1 mL \pm 0.41) when compared to its value in EAC-bearing mice alone. However, the co-treatment of EAC-bearing mice with the low dose of Cis and ASFE for six consecutive days led to a synergistic effect on the total tumor volume that represented 1.9 mL \pm 0.33 when compared to the EAC-bearing mice that was treated with the low dose of Cis (**Table 2**).

The treatment with Cis (2 mg/kg/6 days) led to a significant decrease ($p < 0.01$) in the total tumor cell counts (T.T.C) (41 $\times 10^6$ /mouse \pm 3.2) when compared to the EAC-bearing mice. Also, the treatment of EAC-bearing mice with ASFE led to a significant decrease in the T.T.C (217 $\times 10^6$ /mouse \pm 9.3) when compared to EAC-bearing mice alone (575 $\times 10^6$ /mouse \pm 22.3). Furthermore, the co-treatment with the high dose of Cis and ASFE caused a significant reduction in the T.T.C to 29 $\times 10^6$ /mouse \pm 3.5 when compared to a single treatment. Treatment of EAC-bearing mice with a low dose of Cis or in combination with ASFE caused a marked decrease in the T.T.C (67 $\times 10^6$ /mouse \pm 4.5, or 49 $\times 10^6$ /mouse \pm 3.8, respectively). The number of viable tumor cells decreased, and the number of dead EAC-cells was increased after different treatment protocols when compared to their numbers in the EAC-bearing mice (**Table 2**).

Table (2): Total volume, viable and dead EAC-cells in the different groups of EAC-bearing mice.

Groups	T.T.V (ml)	T.T.C ($\times 10^6$ /mouse)	T.L.C ($\times 10^6$ /mouse)	T.D.C ($\times 10^6$ /mouse)
Group 2	8.7 \pm 0.65 ^a	575 \pm 22.3 ^a	552 \pm 24.7 ^a	23 \pm 2.8 ^a
Group 3	1.4 \pm 0.43 ^b	41 \pm 3.2 ^b	12 \pm 2.5 ^b	29 \pm 3.1 ^a
Group 4	3.5 \pm 0.27 ^c	217 \pm 9.3 ^c	116 \pm 6.5 ^c	101 \pm 7.4 ^c
Group 5	0.9 \pm 0.14 ^b	29 \pm 3.5 ^{b,e}	9 \pm 0.8 ^e	20 \pm 1.3 ^a
Group 6	3.1 \pm 0.41 ^c	67 \pm 4.5 ^d	43 \pm 1.4 ^d	24 \pm 1.5 ^a
Group 7	1.9 \pm 0.33 ^{b,c}	49 \pm 3.8 ^b	12 \pm 0.9 ^b	37 \pm 1.9 ^a

The values represented mean \pm SD. T.T.V: Total tumor volume, T.T.C: Total tumor count, T.L.C: Total live cells, T.D.C: Total dead cells. This means that do not share a letter in each column is significantly different ($p < 0.05$).

Co-treatment of Cis with ASFE mitigated liver dysfunctions in EAC-bearing mice

The results showed that in EAC-bearing mice, there were significant increases ($p < 0.05$) in the sera levels of ALT, AST, and ALP enzymes due to liver injury (187.0 ± 9.4 U/L, 212.2 ± 11.4 U/L, and 232.57 ± 12.5 U/L, respectively) when compared to the normal control group (37.6 ± 3.1 U/L, 77.4 ± 4.6 U/L, and 87.65 ± 5.4 U/L, respectively) (**Table 3**). The data of the present study indicated that the treatment of EAC-bearing mice with high or low doses of Cis resulted in significant maintenance of the liver functions by a significant decrease ($p < 0.05$) in the sera ALT, AST, and ALP levels when compared to the group of EAC-bearing mice alone. However, the co-treatment of EAC-bearing mice with ASFE and high or low doses of Cis led to significant alleviation of liver injury much more than single treatments and a significant decrease in the sera activities of ALT, AST, and ALP (**Table 3**). The results demonstrated that the EAC-bearing mice showed a significant decrease ($p < 0.05$) in their sera total protein levels when compared to the negative control group. However, the treatment of EAC-bearing mice with high or low doses of Cis led to a significant increase in the sera protein levels due to improvement in the liver synthetic functions. Moreover, the combinatorial treatment of EAC-bearing mice with ASFE and Cis (high or low doses) resulted in

significant alleviation of liver injury much more than single treatments by a significant increase in the total protein levels (**Table 3**).

Co-treatment of Cis with ASFE improved hepatic antioxidants/oxidants status in EAC-bearing mice

The result showed that EAC-bearing mice showed significant decreased ($p < 0.05$) in the hepatic SOD, CAT, and GSH levels to 2.76 ± 0.15 U/mg protein, 32.21 ± 2.1 U/mg protein, and 1.93 ± 0.27 $\mu\text{mol/g}$ tissue, respectively when compared to the negative control group that represented 6.94 ± 0.57 U/mg protein, 77.35 ± 3.8 U/mg protein, and 6.96 ± 0.54 $\mu\text{mol/g}$ tissue, respectively (**Figures 1**). In contrast, levels of MDA in liver tissues of EAC-bearing mice were significantly increased ($p < 0.05$) up to 79.33 ± 2.4 nmol/g tissue when compared to the normal control group that represented 38.52 ± 1.9 nmol/g tissue (**Figures 1**). The results obtained from the present study revealed that treatment of EAC-bearing mice with high or low doses of Cis led to significant improvement in the antioxidants/oxidants status evidenced by an increase in SOD, CAT, and GSH levels accompanied by a significant decrease in the levels of MDA in the liver tissues homogenates. Moreover, the treatment of EAC-bearing mice with Cis and ASFE showed much more improvement in their antioxidant capacity (**Figure 1**).

Table (3): Serum ALT, AST, ALP, and the total protein levels in the different groups under the study

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	T. protein (g/dL)
Group 1	37.6 ± 3.1^a	77.4 ± 5.6^a	87.65 ± 5.4^a	7.41 ± 0.32^a
Group 2	187.0 ± 9.4^c	212.2 ± 11.4^b	232.57 ± 12.5^b	4.10 ± 0.25^b
Group 3	113.1 ± 6.4^b	143.4 ± 9.3^c	174.52 ± 9.5^c	5.12 ± 0.28^c
Group 4	$97.5 \pm 5.6^{b,d}$	138.2 ± 7.5^c	169.32 ± 8.4^c	5.72 ± 0.27^c
Group 5	87.5 ± 6.5^e	101.5 ± 8.4^d	131.51 ± 7.5^e	$6.18 \pm 0.21^{a,c}$
Group 6	123.2 ± 7.4^b	$167.1 \pm 9.7^{c,e}$	$200.33 \pm 9.5^{b,d}$	$4.87 \pm 0.15^{b,c}$
Group 7	$100.4 \pm 6.7^{b,d}$	137.5 ± 6.9^c	$190.41 \pm 10.3^{c,d}$	$5.97 \pm 0.17^{a,c}$

The values represented as means \pm S.D.; ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase. This means that do not share a letter in each column is significantly different ($p < 0.05$).

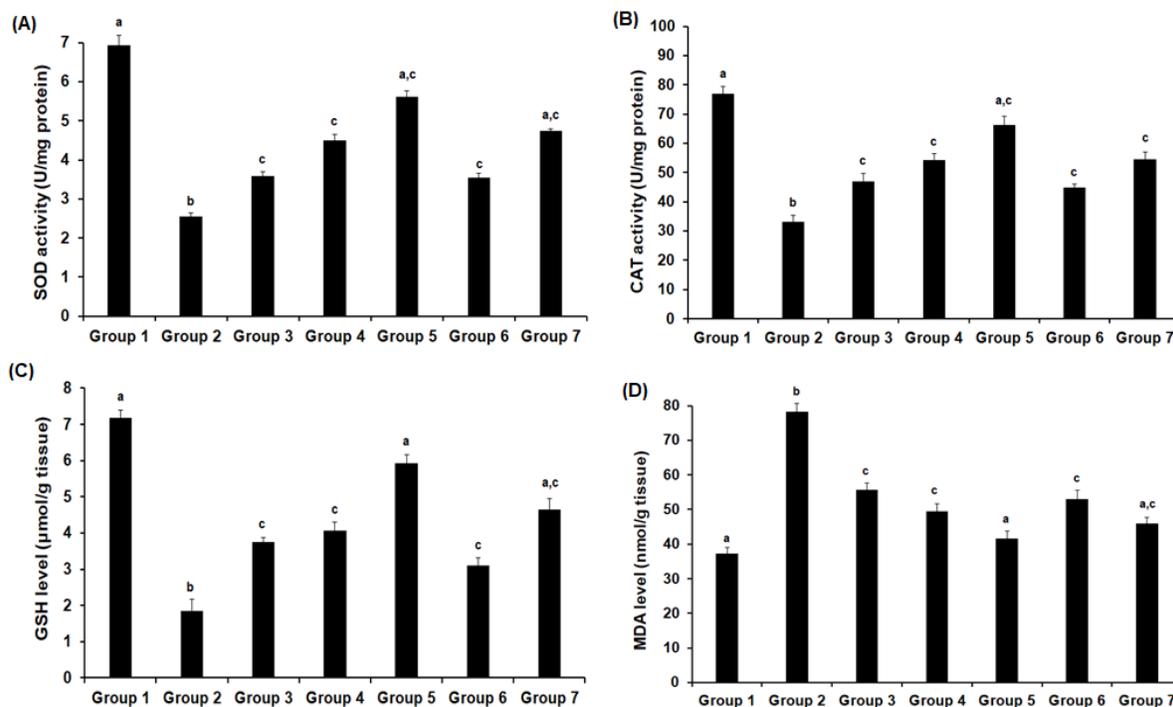


Figure (1): Hepatic superoxide dismutase (SOD) activity (A), catalase (CAT) activity (B), reduced glutathione (GSH) level (C), and malondialdehyde (MDA) (D) level in the different groups under the study. The values represented as means \pm S.D.; Means that do not share a letter are significantly different ($p < 0.05$).

Effect of the treatment with Cis or/and ASFE on gene expression of apoptotic-related genes in EAC-cells

The results of qPCR analysis showed that the treatment with the high dose of Cis induced apoptosis *in vivo* in EAC-cells through significant induction ($p < 0.05$) of the P₅₃ mRNA relative expression level by around 8.79 folds when compared with the EAC-bearing mice alone. Furthermore, EAC-bearing mice that were treated with ASFE also showed a significant increase in the expression level of the P₅₃ gene (6.58 ± 0.16) as determined by RT-PCR when compared to EAC-bearing mice alone (1.00 ± 0.05) (Figure 2A). Co-treatment of EAC-bearing mice with the high dose of Cis/ASFE led to enhancement of apoptosis that was indicated by a significant increase in the P₅₃ gene expression to 13.97 ± 0.28 when compared to the single treatments (Figure 2A). treatment of EAC-bearing mice with high or low doses of Cis resulted in a significant decrease ($p < 0.05$) in the mRNA relative expression level of the Bcl-2 gene to 0.51 ± 0.06 or 0.64

± 0.04 , respectively when compared with an EAC-bearing control group that represented 1.09 ± 0.08 . The results obtained from the gene expression analysis showed that treating EAC-bearing mice with ASFE led to a significant decrease in the mRNA expression level of the antiapoptotic marker Bcl-2 gene (0.59 ± 0.05) when compared with EAC-bearing mice alone. Co-treatment of EAC-bearing mice with Cis/ASFE led to a synergistic effect on the reduction of Bcl-2 gene expression in EAC-cells when compared with single injections (Figure 2B).

The treatment of EAC-bearing mice with high or low doses of Cis for six consecutive days after 24 hours of EAC-cells inoculation led to a significant increase ($p < 0.05$) in the mRNA relative expression level of BAX genes to 10.24 ± 0.11 or 6.85 ± 0.18 , respectively (Figure 2C). EAC-bearing mice that had been administered with ASFE showed a remarkable increase in the relative mRNA expression level of the BAX as an apoptotic marker gene by 6.65 ± 0.07 when

compared with EAC-bearing mice alone. The co-treatment of EAC-bearing mice with Cis/ASFE led to the enhancement of carcinoma cell apoptosis and a synergistic effect on the raising of BAX gene expression in EAC cells when compared with single injections (**Figure 2C**). The results showed that there were significant increases ($p < 0.001$) in the mRNA expression level of the caspase-9 gene in EAC-bearing mice that were treated with the high or low doses of Cis to 8.32 ± 0.45 or 6.77 ± 0.54 , respectively when

compared with EAC-bearing mice alone (**Figure 2D**). The treatment of EAC-bearing mice with ASFE post 24 hours of EAC-cells inoculation resulted in a significant increase ($p < 0.001$) in the mRNA expression of the caspase-9. Co-treatment of EAC-bearing mice with Cis and ASFE showed the highest level of caspase-9 gene expression, which indicated the induction of apoptotic EAC-cells when compared to single treatments (**Figure 2D**).

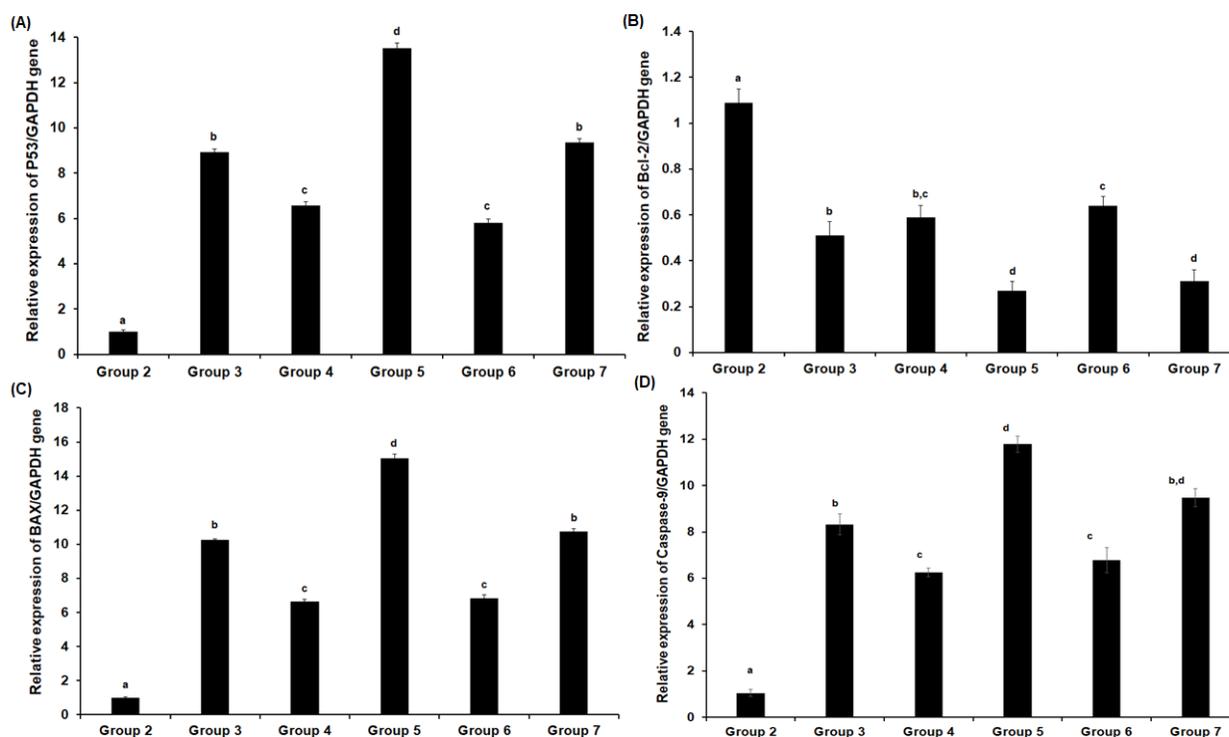


Figure (2): Gene expression analysis by RT-PCR in the different groups under the study shows the relative expression of P53 (A), Bcl-2 (B), BAX (C), and caspase-9 (D) using GAPDH as a housekeeping gene. The values are represented as means \pm S.D. This means that do not share a letter are significantly different ($p < 0.05$).

Discussion

Cancer involves dysregulation of apoptosis, increased proliferation, angiogenesis, immune evasion, inflammatory responses, and metastasis [27]. Conventional chemotherapy including cisplatin (Cis) showed various side effects on vital organs and induces resistance of the tumor cells to the treatment [7].

Several pathways involved in cancer therapy progression and how they can be targeted have improved dramatically, with combinatorial strategies, involving multiple targeted therapies or traditional chemotherapeutics being found to have a synergistic effect [28].

Natural products have been extensively explored for their anticancer potential. The collective efforts of the community have achieved tremendous advancements, bringing natural products to clinical use, and discovering new therapeutic opportunities [29]. The development of new anticancer agents with low toxicity would be a major advance in cancer therapeutics [30].

Phytochemical constituents were reported to contribute to fighting several cancer types. Moreover, phenolic and flavonoid compounds are endowed with several biological activities such as antioxidant and anti-inflammatory properties [31]. *Annona sp.* is a deciduous tropical evergreen fruit tree belonging to the *Annonaceae* family. *Annona muricata* Linn. has recently attracted the attention of scientists for its medicinal value [32]. It has specific bioactive constituents, which can be widely used globally as a traditional medicine for several diseases. For instance, anticancer, antidiabetic, antiviral, antihypertensive, and wound healing [11]. This study investigated the biochemical and apoptotic mechanisms of the ethanolic extract of *Annona sp.* as an anti-cancer agent alone or in combination with the chemotherapeutic drug Cis in Ehrlich ascites carcinoma (EAC)-bearing mice.

Annona sp. contains significant anticancer agents which are called acetogenins and play an important role in several cancer types [33]. This study indicated that EAC-bearing mice showed a significant increase in the percentage of body weight change, and this could be due to the proliferation of EAC-cells inside the peritoneal cavity of mice and the increase in the ascitic fluid. This finding was in agreement with a previous study by El-Naggar et al. (2019), who reported that there was a significant increase in the total body weight change upon the inoculation of EAC-cells in mice compared to naïve mice [34]. Treatment of EAC-bearing mice with Cis or ASFE led to a significant decrease in the percentage of body weight change. This could be due to the inhibition of tumor cell growth in

the peritoneal cavities of mice and hence decrease the ascitic fluid. This finding was in accordance with previous studies [11, 18]. Co-treatment of EAC-bearing mice with high or low doses of Cis and ASFE led to a synergistic effect on the reduction of the % B.wt change this could be due to the inhibition of EAC-cells growth in the peritoneal cavity of mice and a decrease in tumor growth compared to single treatments. Previous studies reported that co-treatment of cisplatin with natural products enhanced the antitumor activity and resulted in a reduction in the percentage of body weight change of EAC-bearing mice [11,31,35, 36].

Our data suggest that ASFE treatment could be a therapeutic tool as an anticancer agent in agreement with Torres et al. (2018) who stated that the *A. muricata* extract significantly reduced Ehrlich tumor cells in Swiss albino mice, and induced apoptosis [37]. In this study, the treatment of EAC-bearing mice with high or low doses of Cis, and ASFE led to a significant reduction in the total tumor volume, total tumor count, and total live tumor cells, however, the low dose of Cis or ASFE did not completely treat the EAC-bearing mice. This could be due to the low doses of Cis not being enough to eliminate or stop the tumor cells completely. Co-treatment with ASFE increased the efficacy of the low doses of Cis as anticancer agents in EAC-bearing mice. This finding was supported by the decrease in the total volume, total tumor count, total live tumor cells, and increase in the total dead tumor cells. These findings were in line with previous studies that reported that the antitumor efficacy of low doses of Cis could be enhanced in EAC-bearing mice by increasing the percentages of dead tumor cells [11,31,35, 36].

The elevation levels of ALT and AST in EAC tumor-bearing mice is an index of deterioration of hepatic functions due to cancer proliferation as observed in the EAC group and it suggested a functional impairment of hepatic cell membranes and a cellular leakage which demonstrated that EAC-induced liver injury [38]. Previous studies reported the ameliorative effect of

Graviola (*A. muricata*) on hepatic injury in experimental animals due to their antioxidant, apoptotic, and anti-inflammatory efficacies [39,40]. In the present study, the protective and antitumor effects of ASFE were investigated in EAC-bearing mice. Cis increased ALT and AST in EAC-bearing mice. The results showed that the treatment with ASFE could protect against liver damage that was induced by Cis. These results agreed with the previous studies that reported the efficacies of the co-treatment with Cis and natural products [35, 41, 42]. Combinatorial treatment with Cis/ASFE led to a significant decrease in the levels of liver transaminases in EAC-bearing mice compared to single injections which indicates the ameliorative effects of ASFE on hepatotoxicity. Decreasing the hepatic toxicity upon treatment with this combination indicates that the ASFE has a protective effect against liver dysfunction and cellular injury of the liver. These findings were in accordance with a previous study that reported a significant impact of ASFE as a hepatoprotective agent by the decrease in sera levels of liver transaminases in EAC-bearing mice [19].

In Cis-induced hepatotoxicity, few mechanisms have been identified, which started as excess generation of reactive oxygen species that leads to oxidative stress, inflammation, DNA damage, and apoptosis in the liver. Various natural products, plant extracts, and oil rich in flavonoids, terpenoids, polyphenols, and phenolic acids were able to minimize oxidative stress by restoring the level of antioxidant enzymes and acting as an anti-inflammatory agent [42]. The present study stated that ASFE induced significant improvement in reversing the alterations in the hepatic antioxidant/oxidant status as it can inhibit lipid peroxidation and prevent oxidative stress. Treatment with ASFE significantly reversed the oxidative stress-associated alterations in EAC-bearing mice, which could be due to the improvement of the antioxidant defense system in agreement with Kataki et al. (2022) who found that *Annona squamosa* L. extract reduced

tumor size and improved liver and kidney functions, and oxidative stress [43]. The results obtained from the present study revealed that treatment of EAC-bearing mice with high or low doses of Cis led to significant improvement in the antioxidants/oxidants status evidenced by an increase in SOD, CAT, and GSH levels accompanied by a significant decrease in the levels of MDA in the liver tissues homogenates. Moreover, the treatment of EAC-bearing mice with Cis and ASFE showed much more improvement in their antioxidant capacity. The same results have been shown that *Annona crassiflora* fruit peel polyphenols preserve antioxidant defense and reduce oxidative damage in mice [44]. Abd-Elghany et al. (2022) assess the potential anticancer activity of the *Annona squamosa* leaves extract in EAC-bearing mice, and reported that liver and kidney functions were improved, and inflammatory markers were decreased. Oxidative stress was improved in tumor, liver, and kidney tissues [45].

The current study revealed that ASFE has a good antitumor potency against Ehrlich ascites carcinoma *in vivo*. The mechanism underlying its antitumor activity may be attributed to its apoptotic effects which increase its potential as a promising anticancer candidate. To investigate the induction of apoptosis in cancer cells, the RT-PCR technique was used to detect the relative expression level of the apoptotic-related genes, P₅₃, Bcl-2, BAX, and caspase-9 genes. The results indicated that there was a significant increase in the mRNA relative expression level of the P₅₃ gene of the EAC-cells in the EAC-bearing mice treated with the high or low dose of Cis alone or in combination with ASFE. These findings indicated the induction of apoptosis *in vivo* in EAC-cells via the mitochondrial-dependent pathways. In the present study, the co-treatment of EAC-bearing mice with Cis and ASFE enhanced the antitumor activity by inducing apoptosis in EAC-cells that was evidenced by a significant decrease in the relative expression level of the anti-apoptotic Bcl-2 gene with a significant increase in the relative

expression level of the apoptotic BAX and caspase-9 genes. These findings agreed with the previous study performed by Hashem et al. (2021) reported the antitumor efficacy of *Arthrospira platensis* and/or cisplatin in a murine model of Ehrlich ascites carcinoma via inducing apoptosis [35]. It has been reported that the *A. squamosa* leaf extract increased the expression of proliferative and apoptotic markers, which in turn enhanced the extract's anticancer effect on breast cancer cell lines. Additionally, they conducted an in vivo investigation, and it was found that the treatment of rats with the extracts had an impact on the tumor size, proliferative, and apoptotic indicators [16]. Furthermore, a previous study could be suggested that *Graviola* exerts its antitumor effect throughout the regulation of the tumor cell cycle as well as inducing apoptotic signals including an increase in antiapoptotic Bcl-2 gene expression [46].

Conclusion

The co-treatment with ASFE and Cis led to ameliorative effects on the hepatotoxicity that were induced by Cis via improvement of liver functions and antioxidant status and exhibits synergistic anticancer activity with Cis through inducing apoptotic signals.

Conflict of interest

All authors declared that there were no conflicts of interest.

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