Journal of Research in Pharmacy

Research Article

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Proanthocyanidin: An interesting option as cisplatin-alternative in solid tumor treatment

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Received: 2 April 2024 / Revised: 23 May 2024 / Accepted: 26 May 2024

ABSTRACT: Cisplatin is an approved chemotherapeutic for treating many solid tumors, but it can cause toxicities e.g. nephrotoxicity, which restrict its application. Therefore, researchers are looking for safer and more effective cisplatin-alternatives. Although proanthocyanidins' protective effects against many cisplatin-induced toxicities have been published and their anticancer activity has been investigated in some research papers, their anticancer efficacy compared to cisplatin has not been investigated yet, making them an interesting candidate. So, we aimed to evaluate the therapeutic efficacy of grape seed proanthocyanidin extract (GSE) in treating solid tumor-bearing mice compared to cisplatin. Sixty adult female Swiss albino mice were grouped (ten mice/group): Normal, EAC ($1x10^6$ Ehrlich ascites carcinoma cells/mouse/once; subcutaneous), EAC+Cisplatin (3.5 mg/kg/once, intraperitoneal), and EAC+50 or 75 or 100 mg/kg/day, oral for 13 days). Blood, livers, and tumors were obtained, and tumor weights, volumes, and hepatic concentrations of malondialdehyde, glutathione, superoxide dismutase, catalase, and nitric oxide were estimated. Serum albumin, alanine aminotransferase, aspartate aminotransferase, cholesterol, triglycerides, lactate dehydrogenase, and creatine phosphokinase were assessed. Survival and lifespan indexes were calculated. GSE treatment boosted antioxidant levels, improved biochemical changes, protected liver and heart tissues from tumor-induced damage, reduced tumor size, and increased median survival time (MST) and percentage increase in lifespan (%ILS). GSE at 100 mg/kg was a more effective antitumor than cisplatin. Finally, our results recommend GSE as a potentially effective cisplatin-therapeutic alternative against solid tumors after more research.

KEYWORDS: Antitumor; Cisplatin; Efficacy; Protective; Heart; Lifespan; Liver; Survival.

1. INTRODUCTION

Cancer, or malignant neoplasm, is a type of disease in which a set of cells grow without control and may even metastasize to affect other sections of the body [1]. Despite significant progress in therapies, cancer remains a top causative factor of death among human diseases [2], with a higher number of deaths recorded in developing nations, including Egypt [3]. In poor communities, infections that lead to cancer, like hepatitis [4], cause up to 30% of cancer cases [5]. Cancer was evaluated as the second most lethal disease globally after cardiovascular diseases, with nearly 18 million cancer-infected people in 2018 [6] and nearly 10 million cancer-related dead persons in 2020 [5, 6]. By 2035, twenty-four million persons are predicted to have cancer, and 14.6 million are expected to die from cancer worldwide [7]. The number of infected people is expected to rise to 35 million by 2050 [8].

Tumor cells' growth and invasion can disturb antioxidant processes by producing high levels of free radicals, which can lead to damage and mutations in healthy tissues. As a result, there is a positive link between changes in antioxidant mechanisms and the proliferation of cancer cells [9]. Although synthetic anticancer agents are frequently used to treat cancer, their use is limited by side effects and the emergence of drug-resistant cancer cells in patients. The constraints of synthetic drugs emphasize the importance of developing anticancer agents from natural products, particularly medicinal plants [10]. Natural products have unique molecular properties that can provide superior efficacy and safety, making them a promising alternative to synthetic drugs [11].

Breast cancer is the most prevalent type of invasive cancer in women globally and also in Egypt [12]. Moreover, it is the second dominant cause of death among females infected with different cancers. However, the most effective treatments for breast cancer are still unclear [13]. Ehrlich ascites carcinoma (EAC) is a

How to cite this article: Abdlsamea FE, Zahran RF, Saad EA. Proanthocyanidin: An interesting option as cisplatin-alternative in solid tumor treatment. J Res Pharm. 2025; 29(2): 751-763.

ISSN: 2630-6344

https://doi.org/10.12991/jrespharm.1664922

mammary adenocarcinoma that occurs spontaneously and is commonly used as a model for experimental cancer research. EAC is similar to human tumors in that it exhibits rapid, undifferentiated proliferation, has a low survival rate, and is 100% malignant [14]. As a result, both the solid and ascitic forms of this tumor are commonly used to study tumor pathogenesis and to develop anti-tumor treatments using different products [15].

Cisplatin is a drug clinically used to treat solid tumors, but it can cause nephrotoxicity, which limits its use [16]. Therefore, scientists are seeking safer and more effective alternatives [8, 17]. Many plant extracts and their products have significant antioxidant properties, which can help alleviate the effects of cancer on the body [18]. Proanthocyanidins are polyphenolic compounds [19] found in plants that are one of the most abundant groups of plant secondary metabolites. They have been described as having antioxidant, antitumor, and anti-inflammatory effects [20]. Grape (*Vitis viniferae*) is one of the biggest fruit crops globally. The grape has medicinal properties in both its fresh and processed forms [13]. Researchers have declared that grape seed extract showed antioxidant, antidiabetic, cardioprotective, hepatoprotective, anticarcinogenic, antimicrobial, and antiviral criteria. Further, in many investigations, as a safe prospective anticancer, grape seed proanthocyanidins hindered the growth of different cancerous cells in vitro and in vivo [21].

This work aimed to assess the antitumor efficacy of grape seed proanthocyanidins extract (GSE) in vitro versus EAC cells and in vivo against mice bearing Ehrlich tumor. The effectiveness of GSE was compared to that of cisplatin.

2. RESULTS

2.1. In vitro cytotoxicity assay

GSE was tested using short-term in vitro cytotoxicity towards EAC cells as a preliminary screening technique with the trypan blue exclusion method (cell viability test) for its cytotoxic potential. The results revealed an increase in the percentage of dead EAC cells as the extract concentration and exposure time increased. The maximum cytotoxic effect of GSE was 96.39 ± 0.56% in 30 minutes of exposure at a concentration of 100 µg/ml, as shown in Figure 1.

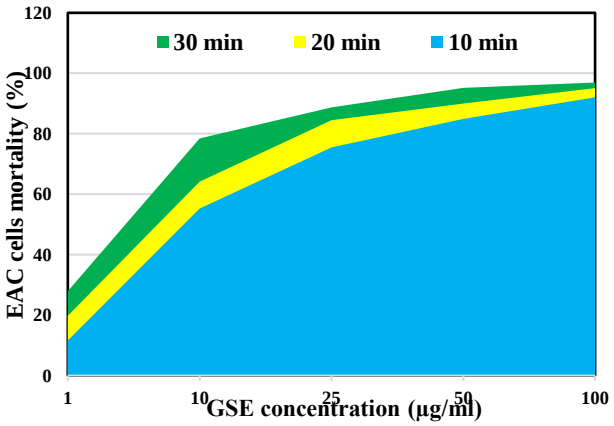

Figure 1. In vitro effect of grape seed extract (GSE) with different concentrations and at different time intervals on Ehrlich ascites carcinoma (EAC) cells mortality.
Results are expressed as mean ±S.D.
n=3 replicates.

2.2. In vivo studies

The potential anticancer effects of GSE were studied in vivo at three different doses. Tumor treatment led to a significant reduction in the tumor burden in a dose-dependent manner as illustrated in Table 1. In particular, treatment with GSE (at a dose of 100 mg/kg) significantly ($P<0.001$) reduced tumor volume by 81.1% and decreased the mean tumor weight to 0.243 ± 0.04 g compared to 1.64 ± 0.62 g in the EAC positive control ($P<0.001$). Regarding treatment with cisplatin, it reduced ($P<0.001$) tumor volume by 70.39% and lowered ($P<0.001$) mean tumor weight to 0.393 ± 0.4 g (Table 2).

Table 1. Solid tumor weights and volumes of tumor-bearing mice with tumor photo for each group

Groups	Tumor	Mice number					
		1	**2**	**3**	**4**	**5**	**6**
EAC	Weight (g)	1.87	0.94	2.1	0.8	2.3	1.85
	Volume (mm^3)	2684	1806	3011	1598.09	3183	2113.11
	Photo						
EAC+50 mg/kg	Weight (g)	1.788	0.45	0.91	0.92	0.48	0.908
	Volume (mm^3)	1190.04	812	900	950	669.045	878
	Photo						
EAC+75 mg/kg	Weight (g)	0.64	0.69	0.59	0.3	0.18	0.61
	Volume (mm^3)	901.5	830.94	620	550	112.32	650
	Photo						
EAC+Cisplatin (3.5 mg/kg)	Weight (g)	0.108	0.22	0.35	0.33	0.15	1.2
	Volume (mm^3)	259.58	346.06	748.16	702.29	152.94	930
	Photo						
EAC+100 mg/kg	Weight (g)	0.3	0.28	0.19	0.18	0.27	0.24
	Volume (mm^3)	615	259.25	146.25	177.97	282.81	209.85
	Photo						

Table 2. Mean solid tumor weight (TW) and volume (TV) of tumor-bearing mice

Groups	TV (mm^3) On day 11	TV (mm^3) On day 24	Decrease in TV (%)	TW (g)
EAC	1029.581 ± 35.47	2399.2 ± 655.02	0.00%	1.64 ± 0.62
EAC+50 mg/kg	1651.04 ± 185.7	899.84 ± 172.15***	45.49%	0.909 ± 0.48*
EAC+75 mg/kg	2023.9 ± 626.43**	610.79 ± 278.12***	69.96%	0.501 ± 0.2***
EAC+Cisplatin (3.5 mg/kg)	1767.45 ± 320.09*	523.17 ± 311.81***	70.39%	0.393 ± 0.4***
EAC+100 mg/kg	1491.46 ± 346.01	281.85 ± 170.79***	81.10%	0.243 ± 0.04***

Results are expressed as mean±S.D.
n=6 mice in each group.
*,**,***: P<0.05, P<0.01, P<0.001, respectively versus EAC group.
W=weight, V=volume, T=tumor.
Day 11 is the first day of treatment, and Day 24 is the day of sacrification.

In addition, the administration of GSE increased the MST of mice with tumors from 21 days in the EAC group to 45 days in the EAC+100 mg/kg group. GSE treatment also prolonged the lifespan of mice by 114.28%. On the other hand, treatment with cisplatin increased the MST from 21 to 43 days and extended the lifespan by 104.76% (Table 3).

Hb content and RBCs count were significantly lower (P<0.001) in mice with EAC than in the normal control group. On the other hand, the WBCs and PLT counts were significantly higher (P<0.001) in EAC-bearing mice than those in the normal control group. The hematological portrait showed dose-dependent significant improvements, towards normal ranges, of Hb, RBCs, WBCs, and PLT counts in the treated mice groups compared to the EAC control group. In fact, the 100 mg/kg GSE dose showed better improvement than the reference drug, cisplatin (Figure 2).

Table 3. Mortality, median survival time (MST), and percentage increase in life span (%ILS) of different mice groups

Days	Mouse 1	Mouse 2	Mouse 3	Mouse 4	MST (Days)	ILS (%)
Normal	√	√	√	√	**>90**	**>328.57**
EAC	x on day 14	x on day 17	x on day 18	x on day 28	**21.0**	**0.00**
EAC+50 mg/kg	x on day 30	x on day 31	x on day 41	x on day 41	**35.5**	**69.04**
EAC+75 mg/kg	x on day 30	x on day 49	x on day 51	x on day 53	**41.5**	**97.61**
EAC+Cisplatin (3.5 mg/kg)	x on day 36	x on day 40	x on day 43	x on day 50	**43,0**	**104.76**
EAC+100 mg/kg	x on day 32	x on day 54	x on day 55	x on day 58	**45.0**	**114.28**

Mice were monitored for 3 months starting from the day of tumor inoculation (day 0) to record mortality.
n=4 mice in each group.
✓: alive.
×: dead.

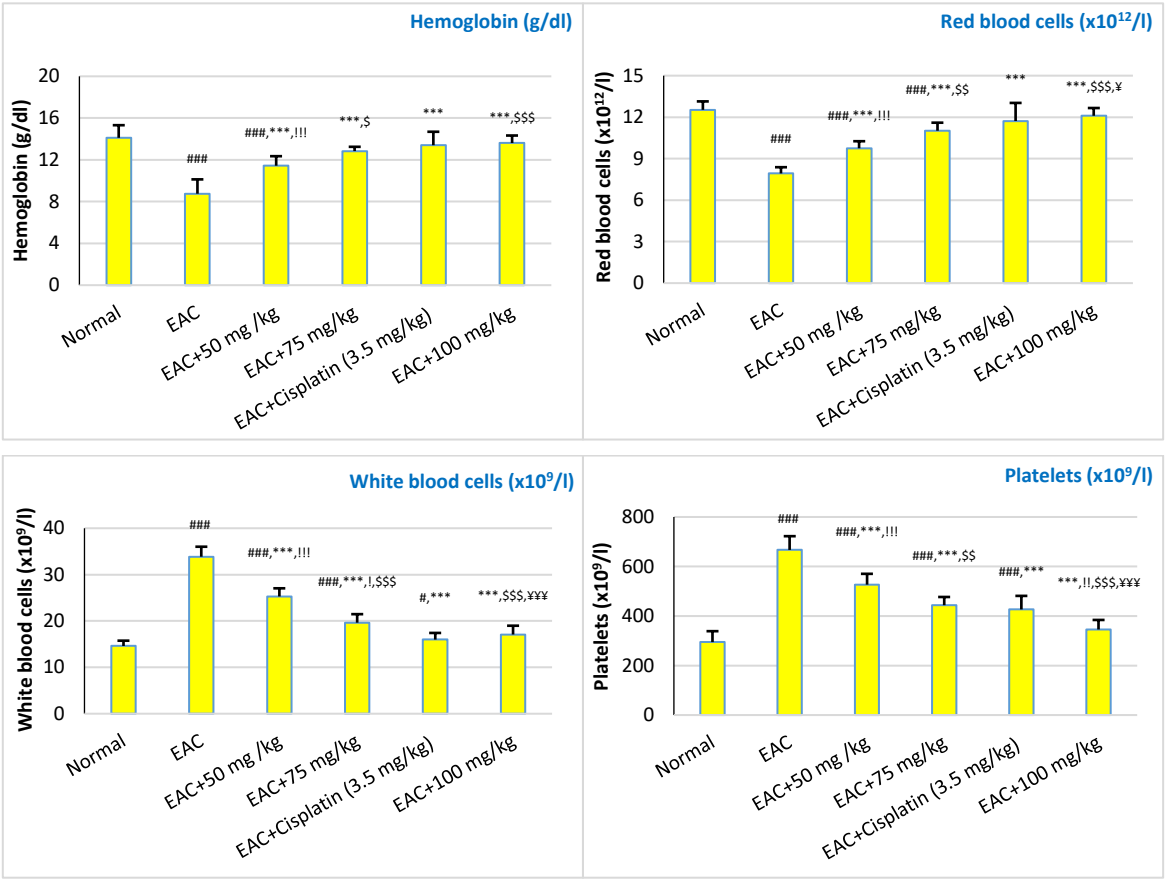

Figure 2. Effect of treatment on hematological parameters of mice in different groups.
Results are expressed as mean±S.D.
n=6 mice in each group.
#, ###: P<0.05, P<0.001, respectively versus normal control group.
***: P<0.001 versus EAC group.
!, !!, !!! : P<0.05, P<0.01, P<0.001, respectively versus EAC+Cisplatin group.
$, $$, $$$: P<0.05, P<0.01, P<0.001, respectively versus EAC+50 mg/kg.
¥, ¥¥¥: P<0.05, P<0.001, respectively versus EAC+75 mg/kg.

Additionally, there was a significant increase (P<0.001) in ALT and AST activities, while there was a significant decrease (P<0.001) in serum albumin levels observed in the EAC control compared to the normal control. After treatment with GSE, serum ALT and AST activities were reduced (P<0.001), while serum albumin levels improved (P<0.001) in a dose-dependent manner compared to EAC positive control. The effect of treatment with cisplatin was inferior to the highest dose of GSE (100 mg/kg) (Figure 3).

After implantation of the tumor, there were significant (P<0.001) increases in cholesterol, triglycerides, LDH, and CPK levels compared to non-tumorized mice. However, after treatment with GSE or cisplatin to kill the tumor cells, all these levels were significantly (P<0.001) reduced. The highest dose of GSE showed the best improvement in reducing the levels of these lipids (Figure 3).

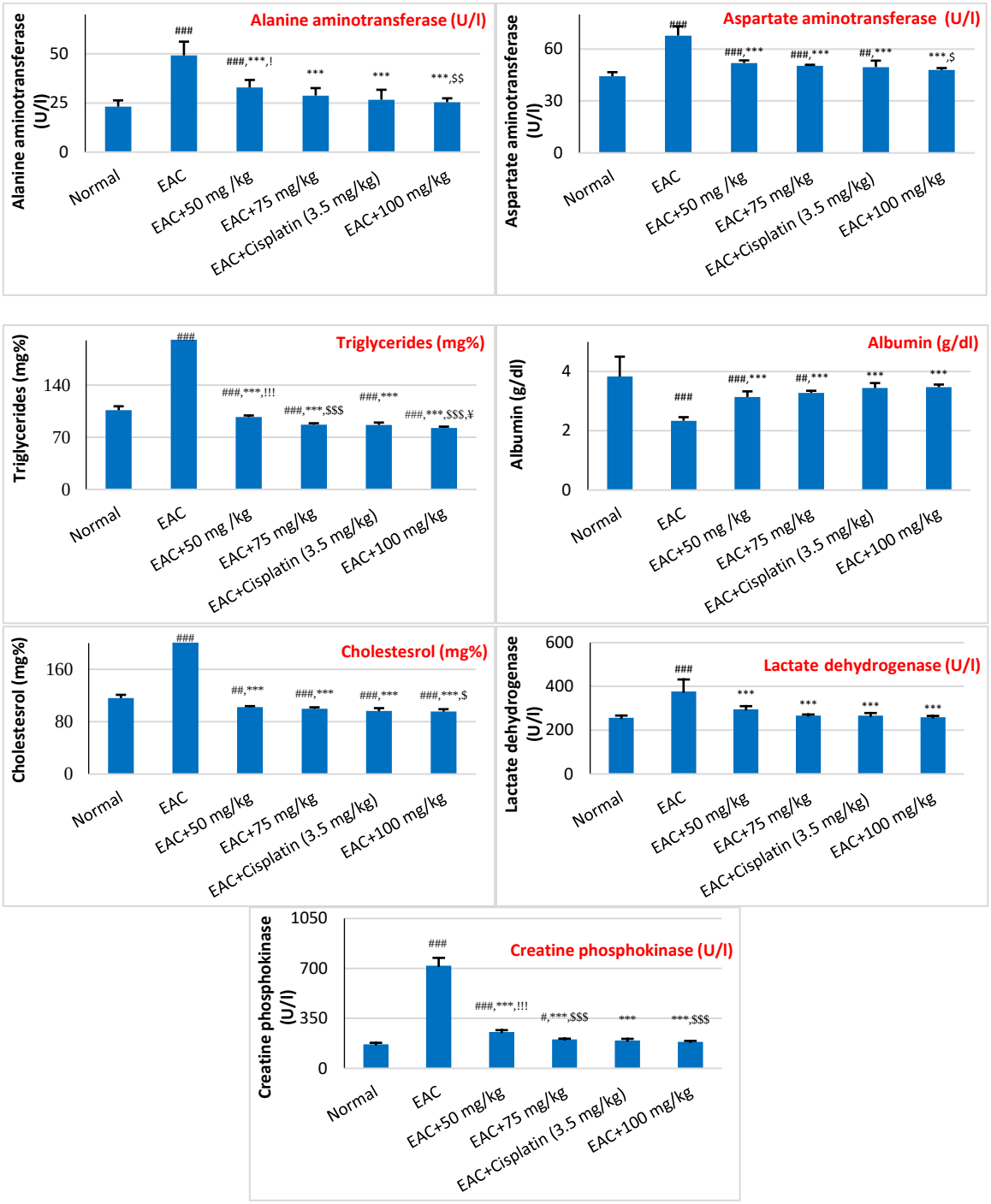

Figure 3. Effect on liver function and heart function tests in addition to cholesterol and triglycerides levels of different mice groups.

Results are expressed as mean±S.D.
n=6 mice in each group.
##, ###: P<0.01, P<0.001, respectively versus normal control group.
***: P<0.001 versus EAC group.
!: P<0.05 versus EAC+Cisplatin group.
$, $$: P<0.05, P<0.01, respectively versus EAC+50 mg/kg.

Tumor progression was associated with increased (P<0.001) levels of MAD and NO and decreased (P<0.001) levels of GSH, SOD, and CAT. Treatment of tumor-bearing mice with GSE resulted in dose-dependent increases in GSH, SOD, and CAT, and decreases in both MDA and NO levels, bringing them closer

to normal ranges compared to untreated mice. The highest dose of GSE showed better results than cisplatin, as shown in Table 4.

Table 4. Effects on malodialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) of different mice groups

Groups	MDA (nM/g tissue)	NO (µM/l homogenate)	SOD (% inhibition)	CAT (U/g tissue)	GSH (mM/g tissue)
Normal	9.65±1.34	10.23±1.06	84.13±3.83	9.05±0.86	7.34±0.66
EAC	31.97±4.93$^{\#\#\#}$	22.35±3.5$^{\#\#\#}$	53.15±2.43$^{\#\#\#}$	3.24±0.45$^{\#\#\#}$	3.14±0.48$^{\#\#\#,***}$
EAC+50 mg/kg	23.59±1.39$^{\#\#\#,***,!!!}$	17.78±0.64$^{\#\#\#,***,!!!}$	68.73±4.35$^{\#\#\#,***,!!!}$	5.44±0.47$^{\#\#\#,***,!!}$	4.44±0.32$^{\#\#\#,***,!!!}$
EAC+75 mg/kg	16.72±0.86$^{\#\#\#,***,\$\$\$}$	14.52±0.63$^{\#\#\#,***,\$\$}$	74.21±1.72$^{\#\#\#,***,\$}$	6.59±0.57$^{\#\#\#,***,\$}$	5.23±0.53$^{\#\#\#,***,\$}$
EAC+Cisplatin (3.5 mg/kg)	14.96±0.99$^{\#\#\#,***}$	12.74±1.95$^{\#,***}$	78.35±7.12$^{\#,***}$	6.91±1.52$^{\#\#\#,***}$	5.52±0.55$^{\#\#\#,***}$
EAC+100 mg/kg	12.52±1.86$^{***,\$\$\$,¥¥}$	12.05±0.99$^{***,\$\$\$,¥}$	79.08±2.14$^{***,\$\$\$}$	8.08±0.49$^{!,\$\$\$,¥¥}$	6.42±0.38$^{\#\#,***,!!,\$\$\$,¥¥¥}$

Results are expressed as mean±S.D.
n=6 mice in each group.
#, ##, ###: P<0.05, P<0.01, P<0.001, respectively versus normal control group.
***: P<0.001 versus EAC group.
!, !!, !!!: P<0.05, P<0.01, P<0.001, respectively versus EAC+cisplatin group.
$, $$, $$$: P<0.05, P<0.01, P<0.001, respectively versus EAC+50 mg/kg,
¥, ¥¥, ¥¥¥: P<0.05, P<0.01, P<0.00, respectively versus EAC+75 mg/kg.

3. DISCUSSION

Cisplatin is commonly utilized to cure different neoplasms [22]. However, it can cause severe adverse effects such as kidney damage, hearing loss, and low blood cell count [23]. Due to these side effects, there is a growing interest in developing efficient anticancer agents with fewer side effects [24]. Natural products or drugs based on natural products are considered to be promising in this regard, as they have been found to have fewer or no adverse effects [25]. Of such likely plants are grapes, which displayed preventive and anticancer effects in several in vitro and animal models [26].

The current research aimed first to test the cytotoxicity of grape seed extract (GSE) against EAC cells. The results showed that GSE has a promising dose- and time-dependent cytotoxic effect against EAC cells. When EAC cells were exposed to a GSE dose of 1 µg/ml, the mortality rate was 11.28%±0.38 in 10 minutes and 27.41%±0.53 in 30 minutes. However, when the concentration was increased to 100 µg/ml, the mortality rate also increased to 91.49%±0.56 in 10 minutes and 96.39%±0.56 in 30 minutes.

We conducted an in vivo experiment to investigate the effects of three different doses of GSE on changes in hematological, biochemical, and antioxidant markers in mice with Ehrlich tumors. The implantation of EAC cells in mice caused disorders in hematological indices, liver and heart functions, lipid profile, and antioxidant levels due to tumor progression. However, administering GSE displayed effective dose-dependent improvements in all these markers toward normal levels. The highest dose of GSE showed even better positive outcomes than the positive effects of cisplatin treatment.

Our results showed that treating Ehrlich solid tumors with GSE caused effective dose-dependent reductions in tumor weight and volume. The average weight of tumors in mice that were not treated was 1.64 g. However, when tumor-bearing mice were treated with GSE (50 mg/kg), the tumor weight decreased to 0.909 g. Whereas, when treated with GSE (100 mg/kg), the tumor weight decreased to 0.243 g. In comparison, the tumor weight decreased to 0.393 g when treated with cisplatin. These results demonstrate the effectiveness of GSE in killing cancer cells. When tumorized mice were treated with GSE (100 mg/kg) or cisplatin, tumor volume decreased by 81.1% and 70.39%, respectively. Compared to cisplatin treatment, the highest dose of GSE proved to be more effective in reducing both tumor weight and volume. These findings are in line with a previous study [27] that reported on the inhibition of Ehrlich tumor development through co- and post-treatment with grape seed proanthocyanidins extract. The increase in tumor size in the Ehrlich tumor-bearing group may be due to morphological and metabolic changes that increase the rates of tumor cell progression [28]. However, treating mice with GSE reduces tumor weight might by inhibiting tumor cell growth and enhancing apoptosis in Ehrlich tumor cells, according to previous studies [29, 30].

Animal lifespan extension is considered a reliable indicator of the effectiveness of an anticancer agent [31]. In the current study, administering GSE to mice with tumors significantly improved their MST and prolonged their lifespan compared to those who were not treated. The tumor-bearing mice had an MST of 21 days, but this was improved by increasing the dose of GSE to 50, 75, and 100 mg/kg, resulting in MSTs of 35.5,

41.5, and 45 days respectively. Surprisingly, the 100 mg/kg dose of GSE was more effective than cisplatin, which only extended the MST to 43 days. The percent increase in lifespan (%ILS) was also higher for the 100 mg/kg dose of GSE, at 114.28%, compared to 97.61% for cisplatin. It can be suggested that the extended lifespan of mice-bearing EAC in response to GSE treatment may be due to a delay in cell division [32]. These results suggest that GSE is a highly effective antitumor agent and even more effective than cisplatin in tumor treatment.

During cancer chemotherapy, the main challenges faced are myelosuppression and anemia, which are primarily caused by iron deficiency resulting from hemolytic or myelopathic conditions. This leads to a decrease in the count of RBCs or Hb content [33]. In the current study, catabolic status was confirmed by a decrease in Hb content, supported by a decline in RBCs count, and an increase in WBCs and PLT counts in the EAC control group. The reduction in Hb and RBCs is due to the destruction of RBCs and/or the inability of the bone marrow to produce them [34]. The development of cancer/tumors can create harmful reactive oxygen species that can cause extensive damage to cells and biomolecules, contributing to the development of cancer. RBC membranes are especially vulnerable to oxidative stress because of their high content of polyunsaturated fatty acids, which are more susceptible to oxidative damage. RBCs are also susceptible to peroxide stress due to their high content of iron, which is a potent catalyst for the production of reactive oxygen species, and due to their continuous exposure to high oxygen tension [35]. Tumor cells can also activate and aggregate PLT [36].

A previous study have shown that one of the most important criteria for evaluating anticancer drugs is their ability to reduce the WBCs count in tumorized animals [23]. Our findings are consistent with Islam et al. (2012) [37], where treatment with GSE restored the Hb content, RBCs, WBCs, and PLT count towards normal levels, indicating that the treatment has a protective effect on the hematopoietic system without causing myelotoxicity. The effects of GSE treatment at a dose of 100 mg/kg were better than the effects of cisplatin on hematological parameters. Most plant-derived extracts/compounds reduce EAC-induced myelotoxicity due to their immune-boosting properties [38], as well as their antioxidant and free radical scavenging activity [25].

Our experimental data revealed that the untreated tumor-bearing group experienced liver function disturbances due to tumor development and progression. This finding is consistent with the studies conducted by El-Emshaty et al. [4], Ozaslan et al. [39], Raju and Arokiasamy [40], and Saad et al. [41]. Mice with Ehrlich solid carcinoma showed significant increases in ALT and AST levels [42], along with a decrease in albumin levels [43], compared to the control group. On the other hand, EAC plus GSE resulted in a significant decrease in ALT and AST levels and an increase in albumin levels, which approached normal ranges. These results suggest that GSE protects the liver from injuries and improves EAC-induced hepatic function disturbances [44].

The present study found that the group with untreated tumors had significantly higher levels of triglycerides and cholesterol compared to the normal group. This could be due to abnormal lipid metabolism caused by excess lipogenesis, which can lead to the development of malignancies. It could also be a result of metabolic disturbance in tumor cells. The treatment groups displayed a decrease in cholesterol and triglyceride levels when compared to the cancer-bearing group. This could be a result of the antioxidant and antiapoptotic effects of proanthocyanidin. These results align with those reported by Abdeen et al. (2018) [45].

Oxidative stress happens when there is too much production of reactive oxygen species (ROS) and the body cannot keep up with its antioxidant defense system. This imbalance can cause harm to the DNA and tissues [46]. Additionally, the process of lipid peroxidation, which is caused by free radicals, has been connected to various cell pathologies [47]. It has been observed in various models of tissue toxicity that oxidative stress induced by toxicity is a crucial factor [16,51]. Free radicals have also been known to cause cancer [52]. Higher ROS levels have been linked to decreased liver antioxidant status [53]. The liver of mice with Ehrlich solid tumors can experience an increase in lipid peroxide due to a chain reaction or indirect mechanisms that surpass the liver's antioxidant capacity [44]. Therefore, antioxidant sources must be increased to protect against liver damage induced by tumors. These antioxidants can be either enzymatic, such as SOD, CAT, glutathione peroxidase (GPX), glutathione-S-transferase (GST), or non-enzymatic, such as vitamin C, GSH, etc. [54]. SOD helps scavenge superoxide radicals by converting them to H_2O_2 and avoiding the formation of hydroxyl radicals. The resulting H_2O_2 is then removed by CAT or GPX [55] by converting it into water. Thus, SOD and CAT play a crucial role in preventing oxidative stress [56]. MDA, the final product of lipid peroxidation, was found to be higher in cancer tissues compared to normal tissues [57]. GSH, a significant non-protein thiol in living organisms, has multiple roles as an antioxidant agent and can protect against cancer

[58]. NO is a diatomic free radical and a short-lived molecule that has high reactivity across a wide range of biomolecules [59]. NO is an important cell signaling molecule whose levels are often elevated in many tumors [60]. This is further supported by the present study, which revealed high levels of NO in untreated mice with tumors. These findings support the idea that NO concentration is closely linked to certain fetal diseases like atherosclerosis, septic shock, endothelial dysfunction, and tumors, as stated by Wang et al. [61].

The current study revealed an oxidative stress state in tumor-bearing mice, as evidenced by significant increases ($P<0.001$) in MDA and NO levels, coupled with significant decreases ($P<0.001$) in GSH, SOD, and CAT levels. Our results align with those obtained by Samudrala et al. [62], who found an increase in MDA and a decrease in the activities of GSH, SOD, and CAT as a result of tumor growth in EAC-bearing mice. Similarly, Elshahawy et al. (2023) [63] found that the levels of MDA and NO significantly increased ($P<0.001$), but the levels of CAT and SOD significantly decreased ($P<0.001$) in the HCC model when compared with the levels in the normal rats.

On the other side, The GSE treated group showed an improvement in antioxidant activity when compared to the EAC group. This suggests that GSE supplementation may protect cells and tissues from oxidative damage, which is caused by reactive oxygen. Similar results obtained by Almajwal and Elsadek [64] and Chis et al. [65] have shown that oral administration of GSE improved CAT levels and reduced the levels of lipid peroxides. This enhances the antioxidant defense against the triggering of reactive oxygen species. Therefore, GSE administration inhibited the lowering of antioxidant levels and rise in MDA and NO levels in EAC-bearing mice, thereby confirming the potent antioxidant and free radical scavenger activity of GSE.

CPK and LDH are considered the best markers of cardiotoxicity as they mainly leak from damaged cardiac tissue due to their tissue specificity and serum catalytic activity [66, 67]. In the present study, LDH and CPK demonstrated highly significant elevations in the EAC group, referring to damage in heart tissue during tumor growth. These findings agree with Saad et al. (2022) [25], who observed CPK and LDH elevations in Ehrlich-induced oxidative tissue damage. Maghamiour and Safaie (2014) [68], reported parallel findings in other tumors, such as prostatic carcinoma and breast cancer. In agreement with Fadillioglu and Erdogan [69], these elevations may be due to the following EAC, like other tumors, requiring ROS for its progression and metastasis. Elevations in these enzymes are attributable to the damaged tissues of the heart due to primarily oxidative stress (increased reactive species over antioxidants) [48, 51], causing leakage of many cytosolic enzymes in serum [70]. These findings are also in harmony with Aboseada et al. (2021) [24], who stated that in the cisplatin-induced cardiotoxicity model, the administration of cisplatin might damage the myocardial cell membrane and increase its permeability with subsequent leakage of these markers into the blood. In addition, the increased production of ROS can lead to an enhancement in the expression of nuclear factor kappa B and the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha, which could intensify the cytotoxic effects of cisplatin. In our study, the highest dose of GSE showed normalization of these levels in the treated tumor-bearing group, suggesting that it is better than cisplatin in protecting heart tissue against heart injury induced by Ehrlich solid tumor.

More studies on varied animal models with different solid tumors are necessary to validate our conclusions.

4. CONCLUSION

In conclusion, based on our study, it was found that treating tumorized mice with GSE resulted in an improvement of hematological and biochemical changes, bringing them closer to normal ranges when compared to EAC non-treated mice. GSE hindered solid tumor growth, enhanced antioxidant levels, protected liver and heart tissues from tumor-induced damage, improved blood picture and lipid profile deterioration, and increased survival. Our results showed that GSE at 100 mg/kg was a more effective antitumor than cisplatin. Therefore, we recommend GSE as a powerful and effective cisplatin-alternative against solid tumors after future studies for more validation.

5. MATERIALS AND METHODS

5.1. Chemicals

Grape seed proanthocyanidin extract (GSE), commercially known as ActiVin®, with 96% purity of proanthocyanidins, purchased from InterHealth Nutraceuticals Inc. (Benicia, CA, USA) was used. Cisplatin

purchased from a local pharmacy was also used. Trypan blue was obtained from Sigma (USA), and other chemicals and reagents of the highest available pure grade were used.

5.2. Animals

Sixty adult female Swiss albino mice weighing between 20 and 25 grams were obtained from the Animal Farm of Vacsera in Helwan, Egypt. The mice were housed according to the guidelines in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science, published by the National Institute of Health, and approved by the Animal House of Biochemistry in our University. The mice were kept under controlled conditions for one week, with a temperature of 23 ± 2 °C, appropriate humidity, and 12-hour light and dark cycles. They were housed in clean polypropylene cages and provided with a standard mice pellet diet and water *ad libitum*. All animal protocols were approved by our Chemistry Department, Faculty of Science, Damietta University, Damietta, Egypt (Board no: 205).

5.3. Parent cell line

The EAC cell line was provided by the Nile Center for Experimental Research in Mansoura, Egypt. The parent cell line was maintained through serial intraperitoneal (i.p.) transplantation of $1x10^6$ viable tumor cells per mouse in 0.2 ml saline.

5.4. Induction of EAC tumor

To induce the tumor, EAC cells were collected 7-10 days after i.p. implantation. For solid tumor inoculation, $1x10^6$ viable cells per mouse were implanted subcutaneously into the right thigh of the hind limb.

5.5. Experimental design

The experimental design involved dividing 60 adult female Swiss albino mice randomly into six groups of ten mice each: *Normal group:* normal healthy mice; *EAC group:* mice injected with $1×10^6$ EAC cells per mouse subcutaneously into the right thigh of the hind limb; *EAC+50 mg/kg group:* mice received GSE (50 milligram (mg) in dist. water/kg/day) by gavage once a day for 13 days, after ten days of tumor inoculation; *EAC+75 mg/kg group:* mice received GSE (75 mg in dist. water/kg/day) by gavage once a day for 13 days, after ten days of tumor inoculation, *EAC+100 mg/kg group:* mice received GSE (100 mg in dist. water/kg/day) by gavage once a day for 13 days, after ten days of tumor inoculation; and *EAC+Cisplatin (3.5 mg/kg) group:* mice were treated i.p. with cisplatin at a dose of 3.5 mg/kg [71] just for one time, after ten days of solid tumor inoculation.

At the end of the treatment, six mice from each group were sacrificed after an eight-hour fast. Their tumor weights and volumes were measured, and blood samples and liver organs were obtained. One portion of each blood sample on EDTA was used for a complete blood count (CBC), while the other portion was centrifuged to obtain serum for estimating the biochemical parameters. The liver was quickly dissected, rinsed with isotonic saline, and dried, and 10% liver tissue homogenate in cold phosphate buffer (w/v) was prepared. After centrifugation, the supernatants were used for estimating hepatic antioxidant parameters. The remaining four mice in each group were kept alive to measure survival parameters.

5.6. Estimation of cytotoxicity against EAC cells

In vitro, EAC cells were incubated with different concentrations of GSE, mixed with trypan blue, and then visually examined to determine whether cells take up (dead) or exclude dye (alive) using a light microscope [72].

5.7. In vivo studies

Each separated tumor was weighed, and its volume was measured using the formula ''tumor volume (mm^3) = 0.52 AB^2, where A represents the minor axis, and B represents the major axis''[6]. The median survival time (MST) was monitored by recording daily mortality for three months, and the percentage increase in lifespan (%ILS) was calculated using the formulas:

MST = (day of first death + day of last death)/2

%ILS = [(MST of treated group/MST of control group) -1] x 100

Hemoglobin (Hb) level, red blood cell (RBC) count, white blood cell (WBC) count, and platelet (PLT) count were estimated using methods of Dacie and Lewis (1984), D'Amour et al. (1965), Wintrobe et al. (1961), and Becton-Dickinson (1996) [73-76]. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine phosphokinase (CPK) activities were determined along with albumin level following the instructions of kits purchased from DIAMOND DIAGNOSTICS in Germany. Albumin level was measured by quantifying its binding to the bromocresol green dye, resulting in blue-green color in an acidic medium. AST and ALT activities were estimated by measuring the amount of oxalate and pyruvate formed over a specific period. CPK activity was measured by following the rate of NADPH formation at 340 nm. Additionally, lactate dehydrogenase (LDH) activity was measured by monitoring the rate of conversion of NADH/NAD^+ at 340 nm utilizing a Chema Diagnostica kit (Italy). Triglycerides and total cholesterol levels were also measured colorimetrically according to the manufacturer's instructions (DIAMOND DIAGNOSTICS, Germany).

Moreover, concentrations of markers for oxidative stress were estimated using kits purchased from the BIODIAGNOSTIC Company located in Giza, Egypt. The markers include malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), nitric oxide (NO), and glutathione (GSH). MDA level was assayed using the thiobarbituric acid method. The activity of SOD was investigated based on its ability to inhibit the phenazine methosulfate-mediated reduction of nitro blue tetrazolium dye. The activity of CAT was measured depending on hydrogen peroxide degradation. NO was assessed by forming a bright reddish-purple azo dye read at 540 nm. The GSH level was assayed by breaking down 5,5¯-dithiobis(2-nitrobenzoic acid), which resulted in a yellow color read at 405 nm.

5.8. Statistical analysis

The data were expressed as mean ± S.D. The statistical analyses were done using the SPSS software package, version 26. Data were analyzed utilizing One-Way ANOVA followed by Tukey post-hoc test to compare between multiple groups. P values >0.05, >0.01, and >0.001 were considered significant, highly significant, and extremely significant, respectively, for all analyses.

Acknowledgements: -
Author contributions: Concept – E.A.S., R.F.Z.; Design – E.A.S., F.E.A.; Supervision – E.A.S., R.F.Z.; Resources – F.E.A.; Materials – F.E.A.; Data Collection and/or Processing – F.E.A..; Analysis and/or Interpretation – E.A.S., F.E.A.; Literature Search – F.E.A., E.A.S.; Writing – F.E.A, E.A.S..; Critical Reviews – E.A.S, R.F.Z, F.E.A.
Conflict of interest statement: The authors declared no conflict of interest.

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