Asian Oncology Research Journal

2(1): 1-12, 2019; Article no.AORJ.51655

Antineoplastic Activities of Grape Seed Proanthocyanidin Extract against Ehrlich Solid Tumor Bearing Mice Induced Alterations in AFP, CEA, TNF-α and DNA Damage

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Authors' contributions

This work was carried out in collaboration among all authors. Author TE designed the study and wrote the protocol. Authors SNEA and MA performed the statistical analysis. Authors HMH and EMM wrote the first draft of the manuscript, managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Nina Tuncel, Department of Physics and Radiation Oncology, Akdeniz University, Turkey.
Reviewers:
(1) Michael Bordonaro, Geisinger Commonwealth School of Medicine, USA.
(2) Heba Gamal Abd El-Aziz Nasr, Al-Azhar University, Egypt.
(3) Saturnino Carmela, University of Basilicata, Italy.
Complete Peer review History: https://sdiarticle4.com/review-history/51655

Original Research Article

Received 01 August 2019
Accepted 03 October 2019
Published 12 October 2019

ABSTRACT

Background and Objective: Cancer is initiated due to abnormalities in the DNA of the affected cells leading to an extra mass of tissue termed a tumor. Breast cancer is the most public cancer amongst women world-wide. The present work is designed to investigate the ameliorating potential effects of grape seed proanthocyanidin extract (GSPE) in inhibition of Ehrlich cells growth and tumor development as a model for breast cancer. Also; the present work is designed to investigate the effect of GSPE on the changes in the levels of AFP, CEA, TNF-α and hematological alterations, and DNA damage examination on Ehrilch solid tumor (EST) bearing mice.
Materials and Methods: A total of 40 female mice were evenly distributed amongst four groups (G1, control group; G2, GSPE group; G3, EST group; IV, EST+GSPE group).
Results: Results revealed increased incidences of tumor growth in the untreated EST group, along with elevated levels of serum AFP, CEA, TNF-α, WBCs, platelets and DNA damage and an associated reduction in RBCs and Hb%. Treatment of EST with GSPE (EST+GSPE) modulates

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and improved these changes in CBC, tumor markers and DNA damage as compared to mice bearing EST.

Conclusion: This finding calls for more investigation on the benefits of grape seed proanthocyanidin extract (GSPE) as antineoplastic activity on other tumors as Ehrlich ascites carcinoma or cancer.

Keywords: Ehrlich solid tumour; GSPE; AFP; CEA; TNF-α; DNA damage; mouse.

1. INTRODUCTION

Cancer is initiated due to abnormalities in DNA of the affected cells leading to an extra mass of tissue termed a tumor. Cancer is reflected one of the primary reasons of death international, accounting for about 8.2 million deaths in 2012. Achievement of cancer chemotherapy is incomplete by drug induced contrary effects and multidrug conflict [1,2]. Consequently, there is an increasing interest in identifying antitumor agents of natural sources, which are active and produce fewer side effects than the conformist chemotherapeutic medications [3,4]. Breast cancer is the most public cancer amongst women world-wide (1.38 m new cases/year, 23% of all cancers). Graded as fifth cause of death (the first in women) from cancer overall (45800 deaths). In Egypt, it represents almost 37% of cancer in women (18% overall).

There are a numeral of in vivo experimental models based on experimental animals as well as the Ehrlich solid tumor (EST), derived from the mouse breast adenocarcinoma which is an aggressive and fast growing carcinoma capable of develop both in the solid or in the ascetic form depending whether inoculated subcutaneously or, intraperitoneously respectively [5,6]. Strong sign indicates that ROS play a significant role in the initiation in addition to the promotion phase of the carcinogenesis [7-10]. Most approaches therapies that used in cancer treatment is chemotherapy that is is unable to distinguish between the malignant and benign cells, the consequence of which can be manifest in multiple side effects [10-16]. Phytochemicals exist as natural compounds that have antioxidant, antiinflammatory and antitumor properties [17-21]. Several plant extracts or produces have been revealed to have important antioxidant action which may be a significant property of pharmaceutical plants related with the treatment of numerous ill-fated diseases [22-30].

Grape seed is amusing sources of the proanthocyanidins that contain many polyphenolic mixtures [31-33]. Grape seed proanthocyanidin extract (GSPE) exhibits important cytotoxicity on the way to human breast, lung and gastric adenocarcinoma cells [6,31]. Grape seeds contain several polyphenolic compounds such as proanthocyanidins (89%), dimmers (6.6%), trimers (5.0%), tetramers (2.9%) and oligomers (74.8%) [31,34,35]. Many antioxidant compounds were identified in the grape as phenolic acids (benzoic and hydroxyl cinnamic acids), anthocyanidins, stilbene derivatives, flavonols (quercetin and myricetin), and flavan-3-ols (catechin and epicatechin), The present work is designed to investigate the ameliorating potential of grape seed proanthocyanidin extract (GSPE) in inhibition of Ehrlich cells growth and tumor development. Also; the present work is designed to investigate the effect of GSPE on the changes in AFP, CEA, TNF-α and hematological alterations, and DNA damage examination on mice Ehrilch solid tumor.

2. MATERIALS AND METHODS

2.1 Chemical and Reagent

GSPE: GSPE (Gervital capsules; 150 mg) was gotten from MEPACO (Arab company for medicinal and pharmaceuticals plants - MEDIFOOD). Enshas El Raml, Sharkeya,– Egypt.

2.2 Transplantation of Tumor Cells and Induction of Ehrlich Solid Tumor (EST)

The Egyptian National Cancer Institute (NCI; Cairo University, Egypt) supplied the mice which had been injected with Ehrlich ascites carcinoma (EAC). These were utilized as the source of EAC cells. 0.2 ml of acitic fluid was aspirated from each EAC bearing mice and diluted with diluted with physiological saline.

Between 2.5 and 3 million EAC cells were injected beneath the skin on the left thigh of each mouse. The presence of the tumor was confirmed by scarfing a select number of mice and the Ehrlich solid tumor was exposed and its size was measured.

2.3 Animals

A total of 80 female CDI mice (aged between ten to twelve weeks old and weighing between 23-25 kg each) were performed for the experiments. They had been obtained from the breeding unit at the Egyptian Organization for Biological Products and Vaccines, Abbassia, Cairo. Free access to normal diet and water supplies was granted to all mice.

The experiments were conducted according to guidelines issued by the Ethical Committee of Faculty of Science at Tanta University and subject to approval by the Institutional Animal Care and Use Committee (IACUC-SCI-TU-0041). This paper is part of scientific collaborations between Prof. Dr. Ehab Tousson in Tanta University and the Menoufia University in Egypt and Qassim University in KSA.

2.4 Experimental Design and Animal Groups

The mice were equally divided into four groups:

Group 1: Control group in which mice did not received any treatment.

Group 2: GPSE group in which mice were received GPSE (50 mg/Kg body weight/ day, orally by stomach tube) for 2 weeks [6].

Group 3: Ehrlich solid tumor (EST) group; mice were injected with $2.5x10^6$ cells each to stimulate EST according to Aldubayan et al. [5].

Group 4: Post treated EST with GSPE (EST+GSPE); Ehrlich cells ($2.5x10^6$) subcutaneously injected mice will be kept for two weeks then treated with GSPE for another 2 weeks.

2.5 Tumor Sizes

Tumor sizes were determined in all mice, the radii of the developing tumors (EST) were measured every 3rd day from day 8 to day 14, using vernier calipers and the tumor volume was estimated using the formula: $V = 4/3\ r1\ 2\ r2$, where $r1$ and $r2$ represent the radii from two different sites.

The percentage of inhibition of tumor volume in animals =

(Tumor volume of Control on 14th Day – Tumor Volume of Treated on 14th day / Tumor volume of Control on 14th Day) x 100

2.6 Sample Collection

By the end of the experiment, mice were euthanized with intraperitoneal injection with sodium pentobarbital. Blood samples from each mouse were obtained from the vena cava and gathered in non-heparinised glass tubes before being left for thirty minutes to clot at room temperature prior to their being subject to 5000 rpm centrifugal for ten minutes [36]. Sera were separated and stored in aliquots at −80°C until required. In addition solid tumor of each mouse was removed, weighed and measure volume after labelling samples; they were kept at -20ºC until biochemical, histology and immunohistochemistry analysis.

2.7 Evaluation of Alpha Fetoprotein Tumour Marker

Alpha-fetoprotein (AFP) was evaluated via automated using the mini-VIDAS® AFP quantitative enzyme-linked fluorescent assay (ELFA) system (Biomerieux, Marcy-L'Etoile, France).

2.8 Determination of Carcinoembryonic Antigen

By using Mybiosource Mouse Carcinoembryonic Antigen (CEA) Elisa Kit (Mybiosource, San Diego, USA). This CEA enzyme linked immunosorbent assay applies a technique called a quantitative sandwich immunoassay.

2.9 Determination of Tumor Necrosis Factor Alpha (TNF-α) Level

The quantitative sandwich enzyme immunoassay technique using monoclonal antibody specific for TNF-α was employed according to instruction procedure of the kit purchased from R&D System (R&D systems, Minneapolis, USA).

2.10 Evaluating the Level of Tumour Necrosis Factor Alpha (TNF-α)

TNF-α-specific monoclonal antibodies were used to conduct a quantitative sandwich enzyme immunoassay according to the manufacturer's protocol (R&D systems, Minneapolis, USA).

2.11 Evaluating the Level of Anti-double Stranded DNA (Anti-dsDNA)

The level of dsDNA autoantibodies was quantified by plasma enzyme immunoassay in accordance with the kit supplier's protocol (Demeditec Diagnostics, Kiel, Germany).

2.12 Determination of Complete Blood Count (CBC)

Measurement of complete blood count (CBC) of each sample RBC, total and differential WBCs count, Platelets, Haemoglobin, mean corpuscle volume (MCV), mean corpuscle haemoglobin content (MCHC) was done by using Sysmex haematology analyser according to the method of El-Moghazy et al. [37]; El Atrash et al. [38].

2.13 Comet Assay

Comet assay (single cell gel electrophoresis, SCGE) was used to detect any prospective tumor tissue damage for DNA. It detects DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilized nuclear DNA [6].

2.14 Statistical Analysis

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 16). Data were presented as the mean± standard error of mean (SEM) and statistically analyzed by one-way ANOVA (Analysis of Variance) followed by the Least Significant Difference (LSD) tests. Significance at $P<0.05$ was considered statistically significant. LSD comparisons were performed to assess the significance of differences between groups.

3. RESULTS

3.1 Effect of GSPE on Tumor Volume in Ehrlich and Ehrlich+GSPE Groups

Fig. 1 shows the effect of treatment with GSPE on the growth and proliferation of subcutaneously injected Ehrlich cells by assessing their growth dependent change at tumor volume in different groups after 28 days of Ehrlich injection. The resulting data revealed a significant decrease in tumor volume at Ehrlich+GSPE group compared with Ehrlich group (Table 1).

Table 1. Effect of GSPE on mice Ehrlich solid tumor volume

	Ehrlich	Ehrlich+GSPE
Tumor volume in cm^3	1.39 ± 0.13	0.87* ± 0.41
% of change		37.40

*Values are expressed as Means±SEM; n=10 for each treatment group; Significance was denoted as * (P<0.05)*

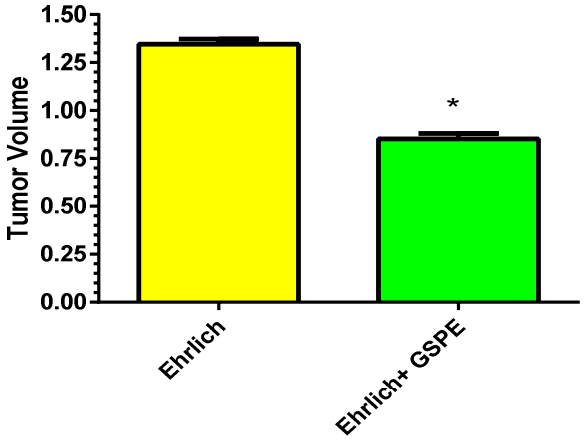

Fig. 1. Effect of GSPE on mice Ehrlich solid tumor volume
*Significance was denoted as * (P<0.05)*

3.2 Changes in Carcinoembryonic Antigen (CEA) Levels

A significant increase were detected in plasma CEA levels in Ehrlich group when compared with control group (Fig. 2). On the other hand; a significant decrease in plasma CEA levels in treated Ehrlich solid tumor with GSPE group (Ehrlich+GSPE) when compared with Ehrlich group (Fig. 2).

3.3 Changes in Serum Alpha Fetoprotein (AFP)

Fig. 3 shows a significant increase in AFP levels in Ehrlich group when compared with control group. On the other hand; a significant decrease in serum AFP levels in treated Ehrlich solid tumor with GSPE (Ehrlich + GSPE) groups when compared with Ehrlich group (Fig. 3).

3.4 Effect of GSPE on Plasma Tumor Necrosis Factor (TNF-α) Levels

Data on Fig. 4 revealed that TNF-α level was significantly increased in Ehrlich solid tumor group in comparison with the control or GSPE groups. Levels of TNF-α was insignificantly decreased in treated Ehrlich solid tumor with GSPE (Ehrlich+GSPE) as compared to their increased levels in Ehrlich solid tumor group (Fig. 4).

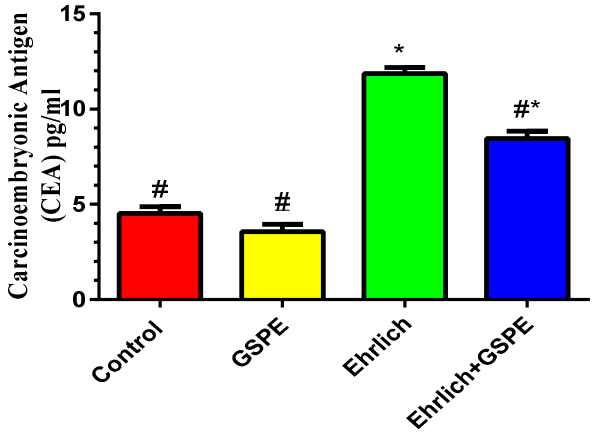

Fig. 2. Changes in carcinoembryonic antigen (CEA) levels in different groups
*Data are expressed as mean ± S.E.M of 6 observations. * and # significant difference compared to control and to Ehrlich group*

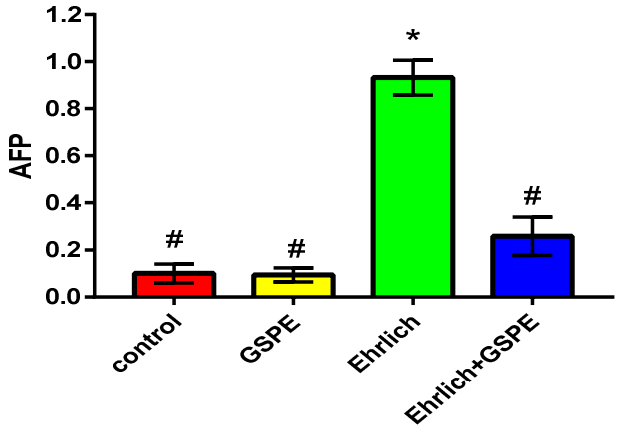

Fig. 3. Changes in serum AFP in different groups under study
** and # significant difference compared to control and to Ehrlich group*

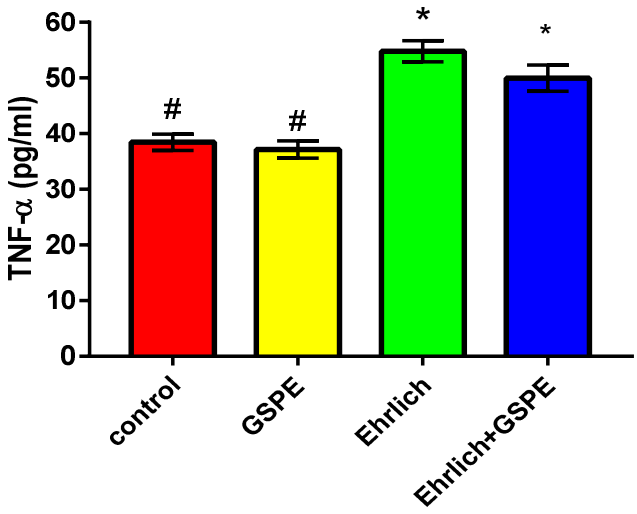

Fig. 4. Changes in plasma TNF-α levels in different groups
** and # significant difference compared to control and to Ehrlich group*

3.5 Effect on Hematological Parameters

Different treatment regimens used in the present study resulted in significant alterations of some hematological parameters compared to untreated tumor-bearing mice, while other parameters were not significantly altered. Hematocrit (HCT) level was non significantly reduced by the administration of GSPE compared to untreated EST tumor-bearing mice. No significant differences were observed between co-treated and post treated group.

Red blood cells (RBCs) were non-significantly elevated by the administration GSPE in treated groups compared to untreated EST tumor-bearing mice. Mice received GSPE in post-treated group showed a further non-significant reduction in RBCS level when compared to mice co-treated with GSPE. On the other hand significant reduction of platelet count (PT) in post treated group when compared to either untreated EST tumor-bearing mice where co-treated is non-significant. Also, Hemoglobin showed non-significant increase in treatment groups compared with EST bearing mice whereas WBCS level showed significant decrease in co-treatment and post treatment group compared with EST group (Table 2).

3.6 Effect of GSPE on DNA Damage

Elevation in DNA damage ($p<0.05$) in Ehrlich solid tumor (EST) were distinguished as manifest in extended tail length, tail DNA and tail moment. In contrast; treatments of EST with GSPE revealed significant ($p<0.05$) reductions in the level of DNA damage (Table 3 and Fig. 5).

4. DISCUSSION

Cancer is the most awful disease among people and characterized by unscheduled and uncontrolled cellular proliferation [2]. Cancer development involves conversion of normal cells to malignant cancer cells through the generation of genetic, epigenetic and other changes, so-called 'multi-step carcinogenesis. Ehrlich carcinoma has a similarity with human tumors which are the most sensitive to chemotherapy due to the fact that it is undifferentiated and has a rapid growth rate [39,40]. Grape is one of the world's largest fruit crops and one of the most commonly consumed antioxidants rich fruits in the world [32,41]. Grape seeds are increasingly being used to obtain functional food ingredients such as natural antioxidants and dietary supplements [35]. Grape seed proanthocyanidins (GSP) have become of high interest because of their biological properties, such as anti-inflammatory and anticancerigen properties with further investigation of interest due to proanthocyanidins potential use in cancer prevention in addition to its protective effects by reducing mitochondria damage and inhibiting cell apoptosis [42,43]. The current study is designed to investigate the ameliorating potential of GSPE in inhibition of Ehrlich cells growth and tumor development. In addition to the role of GSPE

against EST induced changes in AFP, CEA, TNF-α, and hematological alterations, and DNA damage.

Cancer development is associated with generation of reactive species (ROS) resulting in of DNA damage, mutations and chromosomal aberrations that subsequently leads to tissues disorganization and injuries [25]. Dinicola et al. [44] reported that; grape seed extract suppresses MDA-MB231 breast cancer cell migration and invasion. The present study suggested that; a significant decrease in tumor volume after GSPE treatment as compared to EST. Our results agree with Eldaim et al. [6] who reported that; co- and post treatment of EST with GSPE induced inhibition in EST tumor developments. Also; our results agree with Leon-Cabrera et al. [45] who demonstrated that Grape seed proanthocyanidin (GSPE) extract inhibited the proliferation, induced apoptosis, and reduced the secretion of inflammatory cytokines in the human esophageal squamous cancer cell line. Correspondingly with Guo et al. [46] who reported that; grape seed proanthocyanidin extract inhibits human esophageal squamous cancerous cell line ECA109 via the NF-*κ*B signaling pathway.

Tumour markers, e.g. serum Alpha-fetoprotein (AFP), are molecules present in the blood which show enhanced levels in individuals with certain types of cancer. AFP also sometimes called alpha-1-fetoprotein, alpha-fetoglobulin, or alpha fetal protein) is a protein that in humans is encoded by the AFP gene and AFP is one of tumor markers that can be detected mainly in case of liver cancer, hepatocellular carcinoma, liver metastasis and benign liver diseases [47]. The present study revealed significantly raised levels of serum AFP in the untreated EST group relative to the control, this outcome being ameliorated for the GSPE treated EST group. According to Choi and Kakar [48] the enhanced levels of serum AFP in mice with EST may be indicative of the inflammatory response. Our current results agree with Aldubayan et al. [5], who reported that; Ehrlich solid tumor induced elevations in AFP levels in female mice when compared with its level in control group. Our results were in line with that of Medhat et al. [49] who reported a significant increase in AFP levels in the EAC bearing mice. Shin and Moon [50] found that grape skin and seeds inhibits dimethylnitrosamine (DMN) induced liver injury in rats and subsequently inhibits the elevation of serum AST and ALT activities and serum level of AFP. Del Bas et al. [51] who reported that; Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1, SHP expression and reduced AFP in healthy rats.

CEA is a M, 180,000 glycoprotein that was first described by Gold and Freedman (19721) and has been most prominently used as a clinical marker for cancer of the gastrointestinal tract. The correlation between the level of cell-associated CEA and the degree of cellular differentiation has been equivocal. The present study shown increased in the levels of TNF-α in the untreated EST group relative to the control group contrariwise, treatments of EST with GSPE depletion the increased in CEA. Aldubayan et al. [5] studies the antineoplastic activity and curative role of avenanthramides against the growth of Ehrlich solid tumors in mice and reported that; CEA and AFP significantly increase in EST and decrease after treatments with Avenanthramides.

Table 2. Changes in complete blood picture in different groups

	G1	G2	G3	G4
WBCs(10^3/mL)	$4.40^{\#}$ ± 0.251	$6.84^{\#}$ ± 0.320	9.38^{*} ± 0.401	$7.26^{\#}$ ± 0.472
RBCs(10^6/mL)	$8.54^{\#}$±0.345	$10.03^{\#}$±0.544	7.548^{*}±0.350	$7.00^{\#}$±0.78
HGB (g/L)	$12.82^{\#}$±0.347	$14.42^{\#}$±0.517	10.5^{*}± 0.539	11.04±0.855
HCT (%)	$43.08^{\#}$±1.139	$47.34^{\#}$ ± 1.04	36.34^{*}±1.566	37.1^{*}±1.425
PT (10^3/mL)	$585.2^{\#}$±41.39	$780.5^{\#}$±23.3	759.2^{*}±48.55	$572.2^{\#*}$±39.74

Data are expressed as mean ± S.E.M of 10 observations. (*) significant difference compared to control group. (#) highly significant difference compared to EST group

Table 3. Comet assay parameters obtained by image analysis in cells of all groups after treatment experiment

Group	Tailed %	Untailed %	Tails length μm	Tail DNA%	Tail moment
Ehrlich	3	97	1.54±0.11 ^{c}	2.02	3.11
Ehrlich+GSPE	22	78	7.15±0.40 ^{b}	5.93	42.40

Different superscript letters in the same column of tail length showed significance difference at P< 0.05

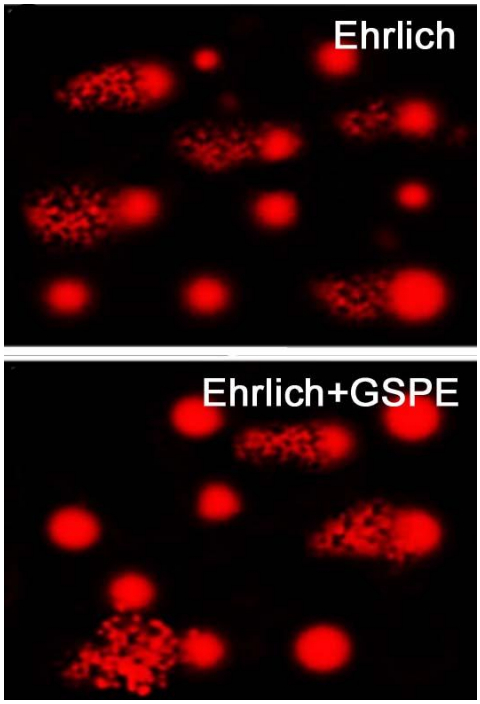

Fig. 5. Photomicrographs image of DNA damage in tumor in Ehrlich solid tumor and post treated Ehrlich solid tumor with GSPE, using a comet assay

According to Bachmann et al. [52] and Gueta et al. [53], cancer patients frequently develop cachexia, a syndrome arising from the effects of cytokines (e.g. TNF-α, IFN-γ) released by macrophages and typified by progressive loss of fat and muscle mass along with symptoms of anaemia, anorexia and extreme weakness. In particular, Warren et al. [54] cite the exceptionally pleiotropic cytokine TNF (tumour necrosis factor) as playing a key part in the host's immune defence, inflammation and homeostasis.

The present study revealed notably enhanced levels of plasma TNF-α in the untreated EST group relative to the control group, in complete agreement with a previous study by Aldubayan et al. [5]. However, the present results do not concur with those of Mansour et al. [55], who reported significantly reduced plasma levels of TNF-α and IL-10 in mice with EST relative to a control group.

Fadel et al. [56] who demonstrated infiltration of Ehrlich tumor cells in the internal organs, which may result from proliferation and migration of tumor cells leading to aggregations of inflammatory cells and degeneration of the mitochondria. Cancer is the uncontrolled growth and spread of abnormal cells, associated with dysregulation of apoptosis [57]. Apoptotic P53 stops the cell cycle at G_1 and G_2 in case of DNA damage, allowing DNA repairing proteins to activate [58-61]. If the damage is unrepairable Bax gene is activated leading to apoptosis [62]. The elevation in DNA damage in Ehrlich solid tumor (EST) in the current study were distinguished as manifest in extended tail length, tail DNA and tail moment, in distinction; the treatments of EST with GSPE revealed reduced in the DNA damage might be attributed to the grape seeds extract has a powerful antioxidant activity [63].

GSPE decreases the proliferation of colorectal cancer cells, enhances caspase-3 activity and subsequently increases apoptosis [64]. According to Aldubayan et al. [5], the Ehrlich tumour first arose spontaneously in the form of breast cancer in mice and has since become the most frequently used transplantable experimental cancer model. The ameliorative effect of GSPE against EST induced tumor DNA damage might due to its antioxidant action as it was designated that GSPE has a powerful antioxidant activity [31]. Finally, the results of the current study revealed that treatment of EST bearing mice with

GSPE reduced tumor volume, DNA damage AFP, CEA, and TNF-α.

4. CONCLUSION

This finding calls for more investigation on the benefits of grape seed proanthocyanidin extract (GSPE) as antineoplastic activity on other tumors as Ehrlich ascites carcinoma or cancer.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal ethic Committee approval has been collected and preserved by the author.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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