CHEMOTHERAPEUTIC POTENTIAL OF GRAPE SEED EXTRACT (VITIS VINIFERA) AGAINST CYCLOPHOSPHAMIDE INDUCED OXIDATIVE STRESS IN MICE

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ABSTRACT

Cyclophosphamide (CP) is one of the most potent chemotherapeutic antitumor drugs. Oxidative stress has been proven to be involved in cyclophosphamide-induced toxicity. Damage to normal tissues due to toxic metabolites limits the usage of CP efficiently for treating various cancers. Therefore, the present study was undertaken to examine the antioxidant potential of low and high dose (100 and 300 mg/kg body weight) of grape seed extract (GSE) against the toxicity of cyclophosphamide have been evaluated in adult Swiss albino mice. Cyclophosphamide treated animals revealed significant elevation of liver markere enzymes ; alanine aminotransferase (ALT) aspartate aminotransferase (AST) and alkaline phosphatase (ALP), while the activities of antioxidant enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR) were decreased in liver, colon and testis of mice. Furthermore, mice administered cyclophosphamide showed a marked increase in DNA tail length, percentage of DNA in tail as well as DNA tail moment (comet assay parameters) and GSE significantly attenuated them.

Key Words: Cyclophosphamide, Grape seed extract, Antioxidants, Liver, Colon, Testis.

INTRODUCTION

Cyclophosphamide (CP) is one of the important therapeutic chemotherapy drug used worldwide. CP is an alkylating cytotoxic cancer drug that depending on its dose and timing of

administration has been used as a chemotherapeutic and disease-modifying agent or to enhance immune responses [1, 2, 3, 4, 5, 6]. Reports dating from the 1980s have shown that under some conditions low-dose of CP can potentiate antitumor immunity in mouse models [4].

In fact CP is commonly used chemotherapeutic and immunosuppressive agent for the treatment of a wide range of neoplastic as well as some autoimmune diseases [7]. With increased success rate of cancer treatment, due in part to the aggressive use of high combination drug therapies, there has been growing concern about the long term side effects (carcinogenic) of these alkylating agents and other neoplastic drugs. There are several reports indicating the carcinogenic effects of CP in humans and animals [8,9]. An increased interest has been shown around the globe in rediscovering natural sources and food materials that could be helpful as therapeutic agents for the prevention of acute chemotherapeutic injuries.

It's well known that aspartate transaminase is similar to alanine transaminase in that it is another enzyme associated with liver parenchmal cells. It rose in acute liver damage, but is also present in red blood cells, cardiac and skeletal muscle and is therefore, not specific to liver. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage. Elevated AST levels are not specific for liver damage and AST has also been used as a cardiac marker [10,11]. While, ALT is an enzyme present in hepatocytes (liver cells).When a cell is damage, it releases this enzyme into the blood, where it is measured. ALT rises dramatically in acute liver damage, such as viral hepatitis or paracetamol overdose. Elevations are often measured in multiple of the upper limit of normal (ULM) [10,12]. However, alkaline phosphatase (ALP) is an enzyme in the cells lining the billary ducts of the liver. ALP levels in plasma will rise with large bile duct obstruction, intrahepatic cholestasis or infiltrative diseases of the liver. ALP is also present in bone and placental tissue [12].

In addition, thioredoxin reductase (TrxR), is the enzyme catalyze the NADPH-dependent reduction of thioredoxin and is noticed to play an important role in multiple cellular events related to carcinogenesis including cell proliferation, apoptosis, and cell signaling. This enzyme represents a promising target for the development of cytostatic agents [13].

Moreover, glutathione reductase (GR), is the major reductase catayez the reduction of oxidized glutathione in glutathione system. In addition, GR, is an enzyme that recycles a key cellular antioxidant molecule glutathione (GSH) from its oxidized form (GSSG) thus

maintaining cellular redox homeostasis [14]. Concerning, alpha- fetoprotein (AFP) is a glycoprotein normally produced in large quantities during embryonic life in the foetal yolk sac and liver [15]. Elevation of AFP level up to pathological range in adults correlates with the appearance of several malignancies such as hepatocellular carcinoma (HCC) and chronic liver disease [16].

Also, tumor necrosis factor alpha (TNF-α) is produced by macrophages and it plays an important role in tumor conditions [17, 18]. It has been reported that, TNF-α is an essential factor in tumor promotion [19]. It was found that, CP significantly increased TNF-α suggesting, CP preferentially affects macrophages functions [20, 21, 22]. Indeed, TNF-α plays a causal role in the development of liver injury [23]. Furthermore, TNF-α has been proven to play an important role in inflammation by mediating the proliferation and differentiation of immune cells and development of immune response [24]. TNF-α is one of the major inflammatory mediators secreted by activated macrophage and involved in many crucial events for the initiation of both acute and chronic inflammation, such as regulating the production of several cytokines, up regulation of adhesion molecule expression and activation of leukocyte – specific chemotactic cytokines [25].

On the other hand, natural products and herbal medicines have been used traditionally for various ailments to avoid any side-effects [26]. Phytomedicines become more popular due to its cultural, historical reasons and to meet primary health care requirements [27]. Natural compounds and indigenous plant based compounds could also have protective effect againt CP induced hepatotoxicities [28]. Grape seed extract (GSE) is a natural extract from the seeds of *Vitis vinifera.* It contains the most beneficial groups of plant flavonoids, proanthocyanidins oligomers. These flavonoids are potent antioxidants and exert many health-promoting effects [29]. Their effects include the ability to increase intracellular vitamin C levels, decrease capillary permeability, fragility, scavenge oxidants and free radicals. There is great evidence that GSE prevents oxidative injury by modulating the expression of antioxidant enzyme systems [30]. The oxidative DNA damage in the brain regions of aged rats was also modulated by GSE administration [31]. In addition, GSE has been shown to be protective against nitrosative/oxidative stress [32], and has exhibited superior antioxidant performance over vitamins C, E and beta-carotene in both *in vivo* and *in vitro* models [33]. It has been demonstrated that the activity of proanthocyanidins oligomers is approximately fifty times greater than that of vitamin C and vitamin E, in term of

antioxidant action [34]. Moreover, GSE has a significant cytotoxicity towards human breast, lung, gastric and colon adenocarcinoma cells, while enhancing the growth and viability of normal cells [35,36]. Moreover, GSE enhances anti-tumor effects of doxorubicin both *in vitro* and *in vivo* [37]. Furthermore , Kaur et al .[38] reported that GSE inhibit colorectal cell growth .These studies demonstrated that GSE is a potent scavenger of free radicals, bio-available and provide significant protection towards   multiple target organs against structurally diverse drug- and chemical induced toxic manifestation [33]. In view of the above findings, the present study is designed to evaluate the potential protective effects of orally administered GSE against cyclophosphamide-induced oxidative stress in mice. Thus, the current study was initiated to   determine whether CP could target TrxR *in vivo* as well as if it caused a preferential TrxR inhibition over other antioxidant enzymes, such as glutathione peroxidase, catalase and   superoxide dismutase. Besides liver function enzymes, inflammatory markers were evaluated. In addition, the chemo-preventive effects of GSE to attenuate CP induced oxidative stress in different mice organs were determined.

MATERIALS AND METHODS

1. Drugs

Grape seed extract (GSE) was obtained from the Division of Research, Development and Quality Control, Pharco Pharmaceuticals, Alexandria, Egypt. GSE was dissolved in distilled water just before use and was administrated by an oral gavage at two different dose levels: 100 and 300 mg/kg body weight (Low and High GSE therapeutic dose respectively) calculated according to Koga *et al*. [39] for 7 and 14 days.

2. Cyclophosphamide

Cyclophosphamide was injected intraperitoneally (IP) in a dose of 50 mg/kg body weight daily for 5 consecutive days [40]. Intraperitoneal injection of CP was performed due to it's rapidly clearance after 3-12 hours by urine [41].

Experiment Animals

Healthy male adult Swiss albino mice weighing between 20-25 g supplied from the animal house of National Research Center (Dokki, Giza, Egypt) were used for this study. Animals were housed in cages under proper environmental conditions at room temperature 22-24°C and 12h light/dark cycle and fed with a commercial pellet diet (Wadi El Kabda Co., Cairo, Egypt). The animals had free access to water. The animals were acclimatized to the laboratory conditions for one week before beginning the experiment. All the experiments

were designed and conducted according to the Ethical Committee of National Research Center.

Experimental Protocol

Animals were divided into six separated groups. Each group contains 10 mice

Group I: Control mice treated with normal saline. Group II: Mice intoxicated by IP injection of CP . Group III: Intoxicated mice treated with GSE (100 mg kg^{-1}b.wt. orally for 7 days consequently).Group IV: Intoxicated mice treated with GSE (300 mg kg^{-1}b.wt. orally for 7 days consequently). Groups V and VI: Intoxicated mice treated with GSE (100 and 300 mg kg^{-1}b.wt. respectively orally for 14 days consequently). At the end of experimental period the animals were fating and blood was obtained by cutting , the sublingual vein centrifuge the blood at 3000 rpm for 15 min. then serum was separated and storage at -80 ^{o}C, for biochemical analysis. Then mice sacrificed by cervical decapitation after overnight fasting. Liver, colon and testis tissues were immediately washed with ice-cold physiological saline and homogenized in 0.1M Tris-HCL buffer (pH 7.4) and aliquots were used for the assays.

Biochemical estimation

1-Serum biomarkers for liver function tests and total protein content

AST, ALT and ALP were measured by the method of Gella et al. [42], where the transfer of amino group from aspartate or alanine formed oxalacetate or pyruvate, respectively and the developed color was measured at 520 nm. Total protein content was assayed by the method of Bradford [43], where Coomassie Brilliant Blue dye reacted with Bradford reagent and gave a blue complex at 595 nm. Alkaline phosphatase, catalyzed in alkaline medium the transfer of phosphate group from 4 nitrophosphatase to 2-amino-2-methyl-1-propanol (AMP) and librated 4-nitrophenol. The developed color was measured at 510 nm [44].

2- Determination of antioxidant enzymes

Glutathione reductase activity was assayed in the liver, colon and testis tissues according to the method of Erden and Bor [45]. The oxidation of NADPH was followed at 340 nm and one unit of activity is defined as the oxidation of 1 nmole NADPH/min/ mg protein.

Thioredoxin Reductase was assayed according to the method of Arnér and Holmgren [46]. TrxR uses NADPH to reduce 5,5-dithiobis-(2-nitrobenzoicacid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) which is absorbed strongly at 405-414 nm.

3- Inflammatory markers

Tumor necrosis factor-α and α-fetoprotein, were quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits. These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intra assay precision and small amount of serum sample required to conduct the assay.

Comet assay (single cell gel electrophoresis, SCGE)

Cell suspension (100 μl) of liver and colon was mixed with 600 μl of low-melting agarose (0.8% in PBS). 100 μl of this mixture was spread on pre-coated slides. The coated slides were immersed in lyses buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. Staining with ethidium bromide 20 μg/ml at 4°C. The observation was with the samples still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope (With excitation filter 420-490nm [issue 510nm]).

The comets tails lengths were measured from the middle of the nucleus to the end of the tail with 40x increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of EtBrstained DNA using a 40X objective on a fluorescent microscope. Although any image analysis system may be suitable for the quantitation of SCGE data, we use a Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample [47].

Statistical analysis

Data were analyzed by comparing values for different treatment groups with the values for individual controls. Results are expressed as means ± SD. The significant differences among values were analyzed using analysis of variance (one way Anova) coupled with post-hoc and least significance difference (LSD). Anova at $p \leq 0.05$ using Co-stat program.

% change = (mean of control mice - mean of treated mice)/ mean of control mice× 100.

% improvement = mean of treated - mean of intoxicated mice / mean of control mice×100.

RESULTS

Effect of GSE on serum transaminases and alkaline phosphatase

CP was metabolized in the liver by its microsomal enzymes. CP intoxication could leads to abnormal biochemical changes which were reflected in the serum. Table 1 represents the activities of serum marker enzymes; AST, ALT, and ALP in control and experimental mice that reflects the tissue damage. In CP challenged mice, as compared to control, almost two and three fold increase in the activities of AST and ALT were observed in the CP intoxicated mice. Administration of GSE resulted in markedly decrease in the activities of marker enzymes. Figure 1 depicts the levels of AST and ALT in the liver of experimental animals. Decrease in the activities of these enzymes in CP intoxicated mice and significant normalization during treatment with GSE was observed.

Effect of GSE on antioxidant levels and total protein content

CP group recorded significant decrease in hepatic GR (53.33%) and TrxR (56.82%) while colon GR exhibited percentage reached to 18.95%, TrxR 3.91%. Also, testis GR recorded 24.0% and TrxR 69.23%. In addition, hepatic total protein content showed significant decrease in CP intoxicated mice by percentage reached to 5.13%. While, the percentage of reduction in total protein content reached to 29.76% in colon. However, the percentage decrease in total protein content recorded 35.45% in testis. Treatment with low and high doses of GSE ameliorated hepatic GR level by 22.86 and 44.76% respectively and TrxR level by 40.91 and 86.36%, respectively. While, GR of colon improved by 35.95, 63.40%, respectively and TrxR by 34.78 and 82.61%, respectively.

Effect of GSE on DNA damage

A significant increase in different comet assay parameters (TL, % DNA in tail and TM) has been shown in animals receiving cyclophosphamide compared with the negative control animals (figure 2 and 3). Therapeutic treatment with GSE significantly reduced CP induced DNA damage as indicated by reduction in different comet assay parameters (TL, % DNA in tail and TM) (figure 3). Photomicrographs of comets in the hepatocytes and colon cells stained with ethidium bromide in different experimental groups are illustrated in figure 4 and 5.

Table (1): Levels of AST, ALT and ALP   in sera of different experimental groups.

Parameters Groups	AST	ALT	ALP
	Mean ± SE (U/L)		
Control	17.53 ± 0.367[f]	22.79 ± 0.045[f]	17.38 ± 0.427[d]
Positive treatment Cyclophosphamide (Zero time)	34.54 ± 0.954[a]	66.93 ± 0.517[a]	33.39 ± 1.43[a]
1 week / Low dose GSE (100 mg/kg)	28.41 ± 0.656[b]	43.43 ± 2.708[b]	27.85 ± 1.08[b]
1 week / High dose GSE (300mg/kg)	19.16 ± 0.372[e]	38.63 ± 0.977[c]	26.21 ±2.04[b]
2 week / Low dose GSE (100 mg/kg)	24.46 ± 0.388[d]	38.71 ± 0.519[c]	23.18 ± 1.21[c]
2 week / High dose GSE (300mg/kg)	18.08 ±1.56[e]	28.73 ± 0.303[e]	18.73 ±1.17[d]

Data are means ± SD of ten mice in each group. Statistical analysis is carried out using one way analysis of variance (ANOVA), and Co-state computer program. Unshared superscript letters between groups are significant values at $p \leq 0.05$.

Table (2): Levels of hepatic antioxidants enzymes; GR and TrxR in different experimental groups.

Parameters Groups	GR n mole NADPH/min./mg protein	TrxR n mole NADPH/min/mg protein
	Mean ± SE	
Control	0.105 ± 0.013[c]	0.044 ± 0.004[c]
Positive treatment Cyclophosphamide (Zero time)	0.049 ± 0.002[d]	0.019 ± 0.0002[e]
1 week / Low dose (100mg/kg)	0.062 ± 0.002[d]	0.025 ± 0.001[e]
1 week / High dose (300mg/kg)	0.079± 0.015[d]	0.033 ± 0.002[d]
2 week / Low dose (100mg/kg)	0.073 ± 0.020[b]	0.037 ± 0.001[d]
2 week / High dose (300mg/kg)	0.096 ± 0.020[c]	0.057± 0.009[a]

*Values are presented as means ± SD (n=10 mice/group). Unshared superscript letters between groups are significant values at $p \leq 0.05$.

Table (3): Levels of colon antioxidants enzymes; GR and TrxR in different experimental groups

Parameters / Groups	GR n mole NADPH/min./mg protein	TrxR n mole NADPH/min/mg protein
	Mean ±SE	
Control	$0.153 \pm 0.065^{(bc)}$	$0.023 \pm 0.005^{(e)}$
Positive treatment Cyclophosphamide (Zero time)	$0.124 \pm 0.012^{(c)}$	$0.006 \pm 0.002^{(a)}$
1 week / Low dose (100 mg/kg)	$0.118 \pm 0.085^{(c)}$	$0.015 \pm 0.003^{(b)}$
1 week / High dose (300 mg/kg)	$0.241 \pm 0.037^{(a)}$	$0.022 \pm 0.003^{(b)}$
2 week / Low dose (100 mg/kg)	$0.179 \pm 0.003^{(ab)}$	$0.014 \pm 0.001^{(e)}$
2 week / High dose (300 mg/kg)	$0.221 \pm 0.104^{(d)}$	$0.025 \pm 0.002^{(d)}$

*Values are presented as means ± SD (n= 10 mice /group). Unshared superscript letters between groups are significant values at $p \le 0.05$.

Table (4): Levels of testis antioxidants enzymes; GR and TrxR in different experimental groups.

Parameters / Groups	GR n mole NADPH/min./mg protein	TrxR n mole NADPH/min/mg protein
	Mean ±SE	
Control	$0.025 \pm 0.0001^{(b}$	$0.039 \pm 0.000^{(e)}$
Positive treatment Cyclophosphamide (Zero time)	$0.011 \pm 0.001^{(c)}$	$0.012 \pm 0.000^{(a)}$
1 week / Low dose (100 mg/kg)	$0.019 \pm 0.001^{(a)}$	$0.031 \pm 0.001^{(b)}$
1 week / High dose (300 mg/kg)	$0.022 \pm 0.001^{(b)}$	$0.032 \pm 0.000^{(b)}$
2 week / Low dose (100 mg/kg)	$0.023 \pm 0.002^{(b)}$	$0.035 \pm 0.001^{(e)}$
2 week / High dose (300 mg/kg)	$0.028 \pm 0.000^{(b)}$	$0.037 \pm 0.006^{(d)}$

*Values are presented as means ± SD (n= 10 mice /group). Unshared superscript letters between groups are significant values at $p \le 0.05$.

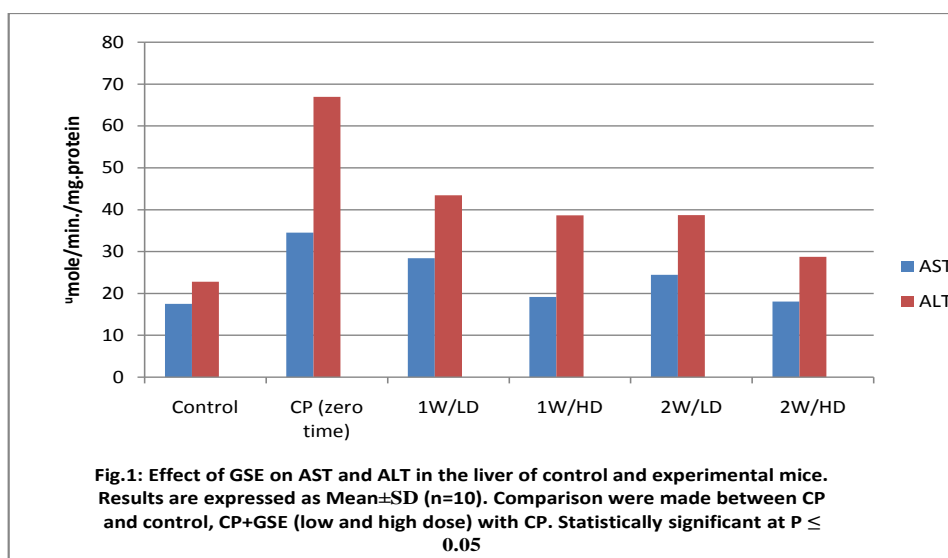**Table (5): Levels of TNF-α and AFP in liver tissues of different experimental groups.**

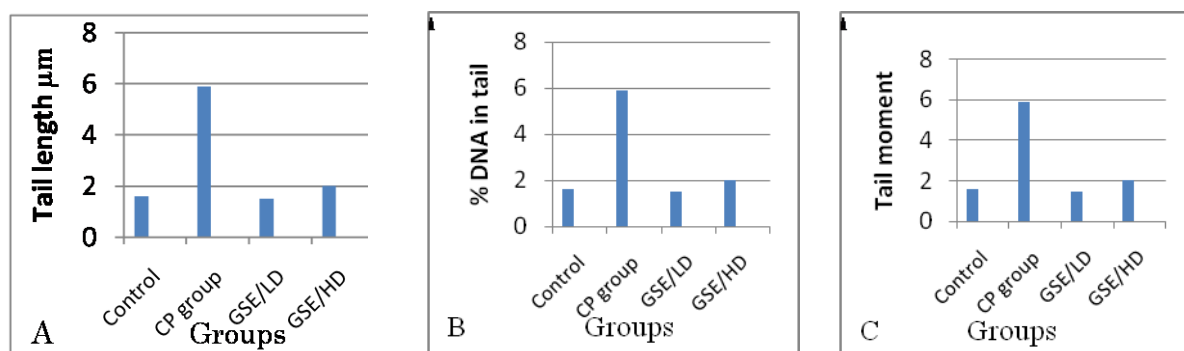| Parameters / Groups	TNF-α Pg/100mg	AFP ng/100mg
	Mean ±SE	
Control	15.67± 0.001[a]	0.389± 0.000[a]
Positive treatment Cyclophosphamide (ZERO)	25.41 ± 0.001[b]	1.38± 0.002[b]
Low dose (100 mg/kg)	17.37 ± 0.002[a]	0.68± 0.003[c]
High dose (300 mg/kg)	14.85 ± 0.001[b]	0.41± 0.004[b]

*Values are presented as means ± SD (n= 10 mice /group). Unshared superscript letters between groups are significant values at $p \leq 0.05$.

Table (6): Levels of TNF-α and AFP in colon tissues of different experimental groups.

| Parameters / Groups	TNF-α Pg/100mg	AFP ng/100mg
	Mean ±SE	
Control	20.88± 0.07[a]	0.447± 0.004[a]
Positive treatment Cyclophosphamide (ZERO)	28.57 ± 0.07[b]	2.33± 0.001[b]
Low dose (100 mg/kg)	25.07± 0.06[a]	1.88± 0.002[c]
High dose (300 mg/kg)	22.18± 0.05[b]	0.75± 0.006[b]

*Values are presented as means ± SD (n= 10 mice /group). Unshared superscript letters between groups are significant values at $p \leq 0.05$.

Fig.1: Effect of GSE on AST and ALT in the liver of control and experimental mice. Results are expressed as Mean±SD (n=10). Comparison were made between CP and control, CP+GSE (low and high dose) with CP. Statistically significant at P ≤ 0.05
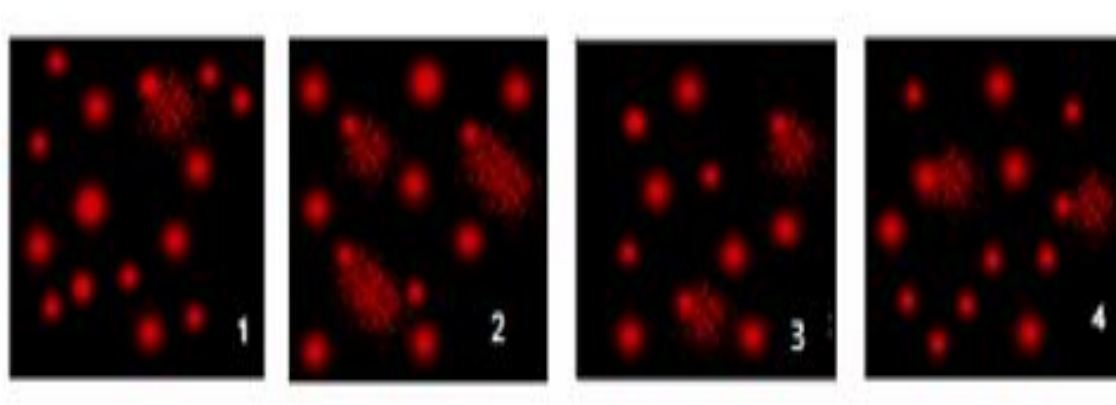
Figure 2: Comet assay parameters in the hepatic tissues of mice of different experimental groups. Results are given as mean ± S.D. for 5 mice.
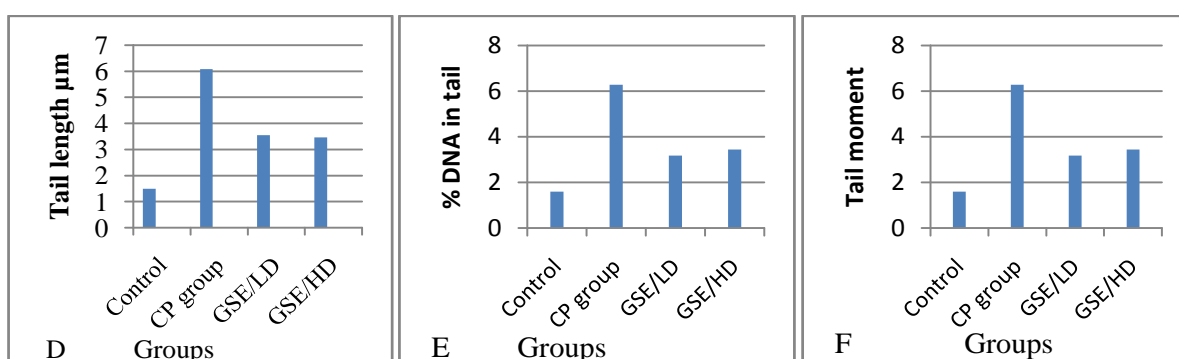
Figure 3: Comet assay parameters in the colon tissues of mice of different experimental groups. Results are given as mean ± S.D. for 5 mice.

Figure 4: Photomicrographs of comets in the hepatocytes stained with ethidium bromide in different experimental groups. Control (1), CP (2), low dose of GSE (3), high dose (4).
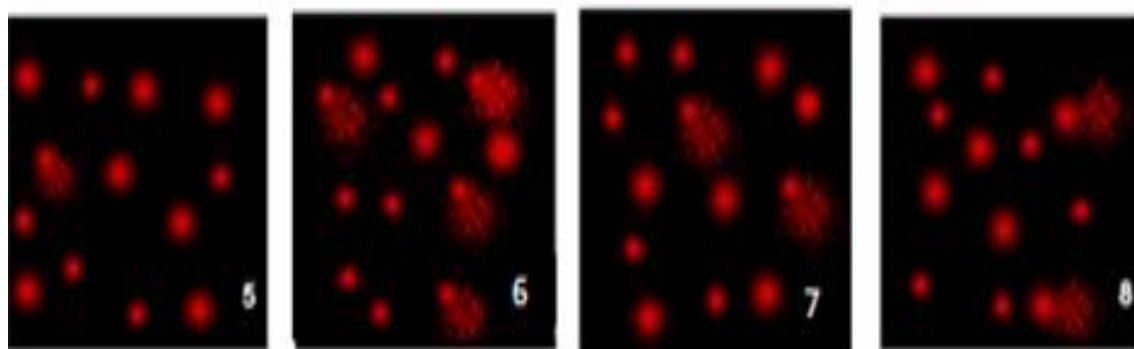
Figure 5: Photomicrographs of comets in the colon cells stained with ethidium bromide in different experimental groups. Control (5), CP (6), low dose of GSE (7), high dose (8).

DISCUSSION

Chemotherapy with CP can cause secondary tumors in humans by activating hepatic mixed function oxidases. Phosphoramide mustard, the major antineoplastic metabolite of CP, is an alkylating agent that induces a variety of changes in DNA [48,49], through its ability to form labile covalent DNA adducts and cross linkages [50].

Administration of chemotherapeutic drugs could leads to single nucleotide polymorphisms (SNPs) in chemotherapeutic drug metabolizing enzymes that are responsible for adverse drug reactions (ADR) like alopecia, nausea, vomiting etc. with abnormal liver functions [51]. Cytochrome P_{450} group of enzymes have extensive functions in liver that includes the detoxification of xenobiotics [52]. In the present study, elevation of serum marker enzymes in CP intoxication reflected the liver damage. Hepatopathy could leads to the leakage of marker enzymes; AST, ALT and ALP into the circulation confirming the extent of liver damage [53]. Also decreased levels in liver tissues and increased serum levels of both AST and ALT could be due to toxic compounds affecting the integrity of liver cells [54].

Prolonged destruction of hepatic cells results in more hepatic release that caused an elevation in serum ALP level [55]. Puiggros *et al.* [56] reported that grape seed extract prevents oxidative injury by modulating the expression of antioxidant enzyme systems. This could exert protection towards hepatocytes leading to the decline in serum marker enzymes, revealing the ameliorating effect of GSE. In the previous study, oral administration of GSE to normal control rats showed no histopathological changes [57]**.** In a parallel line with Özer Tehirli et al. [57] study, GSE could be considered as a safe and efficacious against CP induced hepatotoxicity .

Elevation of AFP level up to pathological range in adults correlates with the appearance of several malignancies such as HCC and chronic liver disease [58]. Increased AFP and TNF-α levels observed in our study were due to the consequences of CP intoxication. On the other hand, GSE down–regulated the concentration of these cytokines and reduced the severity of injury. This may be explained on the basis of GSE interfering with cancer cell growth and proliferation, as well as inducing cell death appears to be one of its greatest highlights, which may be contributing to some of the clinical benefits demonstrated by the extract Kundu et al [59].

The amelioration effect by the post-administration of GSE is probably due to its renowned anti-inflammatory and antioxidant potency. GSE is also reported to possess significant multi organ histological protection against various toxic insults [60, 61, 62, 63, 64]. In addition, Veluri *et al.* [65] reported that gallic acid which is considered as major active constituents of GSE, showed a very strong dose- and time-dependent growth inhibition as well as apoptotic death of human prostate cancer DU145 cells. The antioxidant activity of GSE may be explained on the basis of it trapping free radicals (hydroxyl, lipid free radicals, free iron molecules and lipid peroxides), delaying fat oxidation, inhibiting the major substance responsible for generating oxygen derived free radicals (xanthin oxidase) and reducing the concentration of H_2O_2 [66] that produced by the oxidative stress resulted from *O*-Nitrotoluene treatment.

In accordance with the present study Balu *et al.* [67] demonstrated that the antioxidant activity of grape extract was increased when the extract concentration increased. Besides, antioxidant/antiradical activity of grape seed extract, it was shown to possess many biological properties including the inhibition of DNA damage [68] and COX-2 gene expression [69]. Thus, it could be concluded that, GSE treatment showed ameliorating effects of precancerous stage in liver, colon and testis tissues induced by CP administration in dose- and time-dependent. While, these preliminary results appear promising and need further studies to elucidate the modulatory effects of GSE on early and late stages of liver, colon and testis cancer. Moreover, the present study confirmed the toxicity of CP as chemotherapeutic drug.

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