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Effect of Grape Seeds (*Vitis vinifera* L.) and Mandarin Peels (*Citrus reticulate* L.) Extracts on the Cardiotoxicity Induced by Cyclophosphamide in Rats

Hanan Kamal Mohamed[1*], Hanaa Mostafa Abd El-Fattah[1], Heba Barakat[1], Kawkab A. Ahmed[2] and Sahar Mousa Galal[1]

[1]Department of Biochemistry and Nutrition, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt.
[2]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. Author HMAEF designed the study, wrote the protocol and wrote the first draft of the manuscript. Author HKM managed the analyses of the study, performed the statistical analysis and managed the literature searches. Authors HB and SMG wrote the protocol, wrote the first draft of the manuscript and performed the statistical analysis. Author KAA made histopathological examination of liver and heart tissue. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The current study was developed to investigate the influence of grape seeds (GS) and mandarin peels (MP) extracts as powerful antioxidants on the cardiotoxicity induced by cyclophosphamide (CP) in rats.
Place of Study: Department of Biochemistry and Nutrition, Faculty of Women for Arts, Science and Education, Ain Shams University.
Methodology: Sixty adult male Sprague-Dawley rats were divided into 6 groups. Group (1): Rats were received distilled water daily orally for 6 weeks and injected interperitoneally (i.p) with saline

**Corresponding author: E-mail: hanankamal979@yahoo.com;*

(0.9 %) (2.5 ml / kg BW) as single dose at the end of the sixth week of experiment. Group (2): Rats were received distilled water orally and injected with single dose of cyclophosphamide which dissolved in saline (200 mg/kg BW. i.p.) at the end of the sixth week of experiment**.** Groups (3 and 4): Rats were received grape seeds extracts low and high doses (150 and 300 mg /kg BW), respectively daily orally for 6 weeks then injected with cyclophosphamide as group 2. Groups (5 and 6): Rats were received mandarin peels extracts low and high doses (150 and 300 mg /kg BW), respectively daily orally for 6 weeks then injected with cyclophosphamide as group 2.

Results: Our results documented that CP caused a significant increase in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK-P), creatine kinase (CK-MB), lactate dehydrogenase (LDH), creatine kinase (CK) enzymes activity and serum malondialdehyde (MDA) level. While total antioxidant capacity level (TAC) showed a significant decrease. On the other hand cardiac catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities and cardiac β cell lymphoma (Bcl-2) level showed a significant decrease in CP group while cardiac p53, caspase-3 and DNA fragmentation levels showed a significant increase in CP intoxicated group. Also, some histopathological changes were observed in liver and heart tissues in CP group. Oral administration of GS and MP caused an ameliorative effect in oxidative and apoptotic biomarkers, liver and heart function enzymes activity with an improvement of histopathological changes in liver and heart tissues.

Conclusion: Our data proved that the protective effect of grape seeds and mandarin peels in cyclophosphamide intoxicated group may be due to their antioxidant, anti-inflammatory and anti-apoptotic properties.

Keywords: Cyclophosphamide; cardiotoxicity; grape seeds; mandarin peels; apoptosis.

1. INTRODUCTION

The circulatory system contains the cardiovascular system that transports blood, and the lymphatic system which distributes lymph throughout the body, providing nutrients and oxygen that are needed, and then transporting the waste products and harmful chemicals away from them [1].

Cardiotoxicity can be defined in several ways including reduced left ventricular ejection fraction (LVEF), damage to cardiac cells and structure, conduction abnormalities, vascular abnormalities, in addition to other adverse effects that perturb normal cardiac function [2].Toxicity of cardiac is one of the life-threatening complications of cancer therapy. Systemic anticancer treatments may exert their own toxic effects or can aggravate the adverse effects of other drugs [3]. Cardiotoxicity is the most important adverse reaction of chemotherapy, leading to an important rise of morbidity and mortality [4].

Cyclophosphamide (CP) is a potent alkylating agent that is used broadly as anticancer against different types of human tumors and immunosuppressant. Using of CP had been associated with numerous toxic effects in different organs. CP induced acute cardiotoxicity which may range from endothelial injury, hemorrhagic myopericarditis, arrhythmias, congestive heart failure to fatal myocardial depression [5].

Cyclophosphamide was metabolized into two active compounds, phosphoramide and acrolein metabolite by hepatic microsomal P450 enzyme. Phosphoramide causes cytotoxicity and acrolein had toxic effects on normal cells. Acrolein activated reactive oxygen species (ROS) and induced peroxynitrite formation which extremely damages on the proteins, lipids and DNA in the cell. CP caused hepatotoxicity**,** genotoxicity**,** lung toxicity, nephrotoxicity and cardiotoxicity [6].

Moreover, CP-induced cardiotoxicity had been linked to depression of the activities of krebs cycle enzymes because of increase in inner mitochondrial membrane permeability to calcium leading to uncoupling of mitochondrial-linked ATP synthesis. Another important patho-mechanism for CP-induced toxicity is oxidative stress induced-activation of nuclear factor-κB (NF-κB) which results in numerous cytokines release. This transcription factor played a vital role in the regulation of genes including the inflammation process and cell proliferation [7].

Natural plants and its active constituents had been used in the many studies to improve toxicities in the different body systems that are induced by diverse toxicants. The safety, efficacy and the low price of the natural antioxidant

agents such as herbs and some plants in comparison to other therapeutic agents made them an excellent choice in the prevention and treatment of toxicities in consistent with World Health Organization (WHO). So, there is an inevitable desire for searching in the use of the natural antioxidant agents in treatment of toxicity and investigating its mechanism and efficacy [8].

The ameliorative effects of dietary natural compounds have drawn increasing attention; a variety of dietary antioxidant is often present in vegetables, seeds, and fruits. Furthermore, therapy of this dietary antioxidant is considered a common practice adopted in traditional and alternative medicine. Fruits provide a valid tool for the health benefits owing to their powerful antioxidant content [9].

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Polyphenolic compounds are ubiquitous in nature. They are categorized according to chemical structure as flavonoids (such as flavanols, flavonols, flavones, flavanones, isoflavones, and anthocyanidins). Many of which are found in fruits, vegetables, tea, coffee, beer and wine [10].

Grapes *(Vitis vinifera)* are one of the largest fruit crops worldwide. Grapes are good sources of dietary flavonoids, which are powerful antioxidant compounds. Grape seeds antioxidant power is much stronger than those of vitamin C and vitamin E and may include radical scavenging, quenching, and enzyme-inhibiting actions [11].

Grape seeds extract (GSE) contains a number of polyphenols, including procyanidins and proanthocyanidins, which are powerful free radical scavengers. Numerous pharmacological studies proved the anti-inflammatory, antioxidative, antitumor, antibacterial, and hepatoprotective properties of grape seed [9].

Citrus fruit is one of the most important fruits all over the world, due to health-related elements and valuable components which contains vitamins C, carotenoids, flavonoids, pectin, calcium, potassium…etc. Citrus fruits considered an expensive resource of soluble and insoluble fiber with various benefits such as removing the toxic effects in the body [12].

These citrus fruits are the precious resource of phytochemicals which are useful and play a role in physiological functions and metabolic change of human body. Citrus fruit has potential health benefits like antimicrobial, anti-inflammatory, antiviral and anticancer [13].

Mandarins *(Citrus Reticulate L.)* are a different group of thin-skinned, easy-peeling fruit. Mandarins are becoming increasingly popular with consumers, largely because of the ease with which they can be eaten as compared to other types of citrus that are more difficult to peel [14].

Mandarin has nutritive importance owing to its particular composition as flavonoids, especially polymethoxyflavones and flavanones (hesperidin, narirutin, tangeritin and naringin) which identified in pulp as well as in peel. In the by-products of citrus fruit after juice production, phenolic acids such as caffeic, *p*-coumaric, ferulic, and sinapic acids had also been identified. The two flavonoids, narirutin and hesperidin, were determined in mandarin peels extracts (MPE). The amount of hesperidin detected in the MPE was five times higher than that of narirutin [15].

The present study was conducted to assess the influence of grape seeds (GS) and mandarin peels (MP) extracts on cardiotoxicity induced by cyclophosphamide (CP) in rats.

2. MATERIALS AND METHODS

2.1 Chemicals

Cyclophosphamide (CP) was purchased from Sigma Company for Chemicals Cairo, Egypt. Ethanol (70%) was purchased from El-Gomhoria Company for Chemicals and Drugs and all other chemicals were of high analytical grade.

2.2 Plant Material

Grape seeds (*Vitis vinifera* L.) and mandarin fruit (*Citrus reticulate* L.) were purchased from Ministry of Agriculture, Giza, Egypt.

2.3 Experimental Animals

Adult male rats Sprague-Dawley strain weighing 150-180 g were obtained from animal breeding house of National Research Centre (NRC), Giza, Egypt. Animals were handled in this study in accordance with the principles of laboratory animal care" (NIH publication No. 85- 23, revised 1985). The experimental animals were housed individually in metallic cages with good ventilation and under conventional condition (22

± 3°C and natural light/dark cycle). All rats were received the standard commercial diet (obtained from NRC; Giza, Egypt) and water *ad libitum* for 7 days (acclimatization period) before starting the experiment.

2.4 Preparation of Grape Seeds (GS) and Mandarin Peels (MP) Ethanolic Extract

Two hundred grams of GS were grinded to obtain powder by an electric grinder and two hundred grams of MP were carefully washed with running water till being completely clean and manually peeled. The peels were dried by sunlight for seven days till obtain fixed weight. The dried peels were grinded to obtain powder by an electric grinder. The two powders were soaked separately in 70% ethanol for 72 h, and then the mixture of each was filtered through a cotton cloth for separation of debris particles. The residue of each after filtration was re-extracted twice under the same condition to ensure complete extraction. The extract of GS or MP were filtered and evaporated to semisolid mass by a rotary evaporator. The extract of GS or MP were placed in dark bottles and stored in refrigerator at -20°C.

2.5 Determination of Total Phenols and Total Flavonoids Content in Grape Seeds and Mandarin Peels

Total phenols were determined in GS and MP ethanolic extracts by using the Folin-Ciocalteu method, while total flavonoids were determined by aluminum chloride colorimetric method according to [16] and [17] respectively.

2.6 Determination of Main Phenolic Compounds in Grape Seeds and Mandarin Peels

The concentration of main phenolic compounds of GS and MP was determined by ultra- High Performance Liquid Chromatography (HPLC) teqnique according to [18].

2.7 Induction of Cardiotoxicity

Cardiotoxicity were induced in rats (i.p) injection with one gram of CP which dissolved in 10 ml saline immediately (200 mg/kg BW) before administration to rats at the sixth week of the experiment.

2.8 Experimental Design

Sixty adult male Sprague-Dawley strain rats were divided into six groups; each group consists of 10 rats. The two extracts of GS and MP were separately freshly suspended in distilled water and given to the rats orally.

Rats were treated as follows Group (1): Rats were received distilled water daily orally for 6 weeks and injected interperitoneally with saline (0.9 %) (2.5 ml / kg b.wt) as single dose at the end of the sixth week of experiment. Group (2): Rats were received distilled water orally and injected with single dose of cyclophosphamide which dissolved in saline (200 mg/kg b.wt. i.p.) at the end of the sixth week of experiment. Groups (3 and 4): Rats were received grape seeds extracts low and high doses (150 and 300 mg /kg b.wt) respectively daily orally for 6 weeks then injected with single dose of cyclophosphamide as group 2. Groups (5 and 6): Rats were received mandarin peels extracts low and high doses (150 and 300 mg /kg b.wt) respectively daily orally for 6 weeks then injected with single dose of cyclophosphamide as group 2.

2.9 Handling of Blood and Tissue Samples

At the end of the experimental period (6 weeks) and after overnight fasting, all rats were weighed and sacrificed. Blood samples were collected from cardiac portal vein into centrifuge tubes. Serum separation by centrifugation at 1500 rpm for 15 min, then kept at -20°C. Heart and liver were separated immediately and washed by saline solution (0.9%NaCl) then blotted on filter paper. Part of heart was stored at -20°C until used for biochemical analysis and parts of heart and liver were preserved in 15% formalin for histological examination.

2.10 Biochemical Assessment

2.10.1 Determination of some cardiac and hepatic function enzymes

Serum ALT, AST and ALK-P activities were determined using colorimetric method according to *Murray* [19 and 20] and *Belfield and Goldberg* [21] respectively. Serum LDH, CK-MB and CK enzymes activity was determined by kinetic method using spectrum diagnostic kit according to *Van der heiden* et al. [22], *Wurzburg* et al. [23] *and Young* [24] respectively.

2.10.2 Assessment of oxidative stress biomarkers & antioxidant status

Serum MDA and TAC levels were determined by colorimetric method using biodiagnostic kit according to *Ohkawa* et al. [25] and *Koracevic* et al*.* [26] respectively. Cardiac CAT, SOD and GPx enzymes activity was determined by colorimetric method using biodiagnostic kit according to *Aebi* [27]*, Nishikimi* et al*.* [28] and *Paglia and Valentine* [29] respectively.

2.10.3 Determination of apoptotic markers

Cardiac p53, caspase-3 and Bcl-2 protein level were determined by Elisa kit method from My Biosource, p53 were determined according to Findley et al. [30] while caspase-3 and Bcl-2 were determined according to Binabaj et al. [31]. DNA Fragmentation was determined by colorimetric method from My Biosource Eliza kit according to Sellins and Cohen [32].

2.11 Microscopic Examination of Liver and heart Tissues

Specimens of heart and liver from all animals were collected and fixed n formalin 15%. Washed, dehydrated, cleared and embedded in paraffin. Paraffin blocks were sectioned at 4–5 micron thickness and stained with hematoxylin and eosin for histopathological examination by a light microscope (Olympus BX50, Japan) under magnification X400 [33].

2.12 Statistical Analysis

The data were statistically analyzed by one-way (ANOVA) and Post Hoc Tukey. A difference was considered significant when *p* was less than or equal to 0.05. The data was analyzed using (SPSS) statistics version 16.0 according to [34].

3. RESULTS AND DISCUSSION

3.1 Total Phenols and Flavonoids Contents in Grape Seeds and Mandarin Peels

The results presented in (Table 1) indicated that the total phenols expressed as [mg of gallic acid equivalent (GAE) / g of ethanolic extract] and total flavonoids expressed as [mg of rutin equivalent (RE) / g ethanolic extract].

In consistent with our findings, Abdrabba and Hussein (2016) found that total phenolic content of pulps, seeds and peels of red grape were 11.65, 73.59 and 13.73 mg GAE/g extract, respectively. The phenolic compounds are broadly distributed inside grapes. The composition of phenolics depends upon whether the extraction is performed on whole grape pulp, skin, or seeds. The total extractable phenolics in grape are present at only about 10% or less in pulp, 60–70% in the seeds, and 28–35% in the skin. The phenol content of seeds may range from 5% to 8% by weight [35].

Tita et al. [36] found that total polyphenol content in GS was 211.21mgGAE/g dry extract. Extraction yields differ depending on the quality of the solvent and the working conditions. The obtained results attest to the fact that grape seeds contain significant amounts of polyphenols with strong antioxidant activity. .

In addition Brahmi et al. (2021) reported that total phenolic content (TPC) of grape seeds extract was (240.59 ± 7.77 mg GAE/g) and total flavonoids content (TFC) was (6.89 ± 0.23 mg QE/g) [37]. *Garcia-Jares* et al*. (2015)* noticed that the TPC were from 99 to 121 mg GAE/g in the GS of 11 Spain grape varieties [38]. Likewise, *Pantelić* et al*. (2016)* have determined that the TPC ranged from 38.02± 0.46 to 102.98 ± 0.58 mg GAE/g in the seeds of 13 grapevine varieties from Serbia [39]. The TPC measured in the extracts of ten GS varieties ranging between 34.628± 2.435 and 71.244 ± 0.762 mg GAE/g [40].

Hua et al. (2018) illustrated that the total phenolic contents of mandarin peels extract was 32.76 mg (GAE) [41]. Also, Zhang et al. showed that the total phenolic contents in the peels of *C. reticulata* Blanco oscillated from 29.38 to 51.14 mg GAE which were similar to present results [42].

In agreement with our results Safdar et al. showed that maximum polyphenols in ethanolic mandarin kinnow peel extracts were (29.75 + 0.23 mg GAE/g of extract). Results revealed that mandarin kinnow peel extract varied considerably as function of solvent concentration level [43].

The present data were confirmed by the results of Masoud and El-Hadidy [44] who found that total phenols of mandarin was (50.51±2.41mg/g GAE) and total flavonoids was (1.65±0.02 mg/g RE). Ghasemi et al. reported that total phenolic contents of *Citrus reticulate* varieties peel powder

was in the range of 104.2 to 172.1 mg/ g as GAE [45].

3.2 The Concentration of Main Phenolic Compounds of Grape Seeds and Mandarin Peels

Analysis of GS and MP extracts using HPLC detected the main active phenolic compounds. Figs. (1 and 2) indicate the percentage of main phenolic compounds in GS and MP extract.

3.3 Cardiac and Hepatic Function Enzymes in Experimental Groups

The LDH, AST and ALT are well known diagnostic parameters of cardiac injury, which heart failure, cardiotoxicty, myocarditis and myocardial infarction. The LDH is a specific enzyme released into the blood and cytoplasm during cardiotoxic dysfunction. AST and ALT enzymes are critical transaminases that are released as a result of cardiac metabolism. CK and CK-MB enzymes are specific biomarker that was determined in the heart failure [46].

Single dose of CP (200 mg/kg BW) resulted in cardiac damage as indicated by significant increase in serum ALT, AST, ALK-P, LDH, CK-MB and CK activities (P≤0.05) (Tables 2 and 3). These results were confirmed by microscopic examination of heart tissues which showed cytoplasmic vacuolization of cardiac myocytes, oedem in-between cardiac myocytes associated with mononuclear inflammatory cells infiltration [Fig. 4 (a and b)] and in liver tissue which showed kupffer cells activation, hepatocellular steatosis and fibroplasia around bile duct associated with mononuclear inflammatory cells infiltration in the portal triad Figs. 10 and 11.

Administration of GS and MP (low or high dose) with CP significantly *(P ≤ 0.05)* lowered the activities of cardiac enzymes when compared with CP group. The most significant ameliorating effect on serum AST and CK-MB were seen in high dose of MP while ALT, LDH, and CK were seen in the high dose of GS comparing to CP group. This indicated that treatment with GS and MP extracts caused an improvement of CP toxicity and these results were confirmed by microscopic examination of heart and liver tissue which cause improvement in heart and liver tissue.

Table 1. Total phenols and flavonoids content of grape seeds and mandarin peels ethanolic extracts

Active constituents	GS extract	MP extract
Total phenols (mg (GAE) / g of extract)	93.25±2.91 mg	24.50±8.08 mg
Total flavonoids (mg (RE) / g extract)	75.13±1.50 mg	2.89±0.19 mg

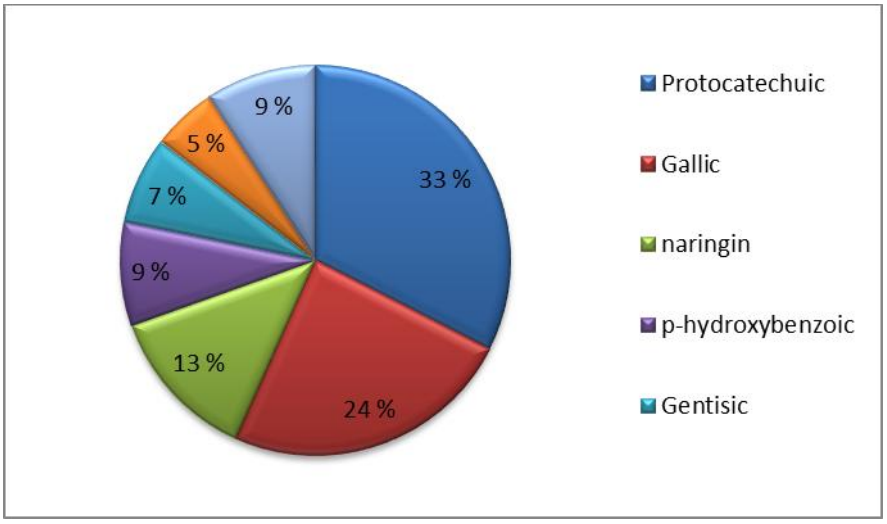

Fig. 1. Pie chart of the main active phenolic compounds of grape seeds extract

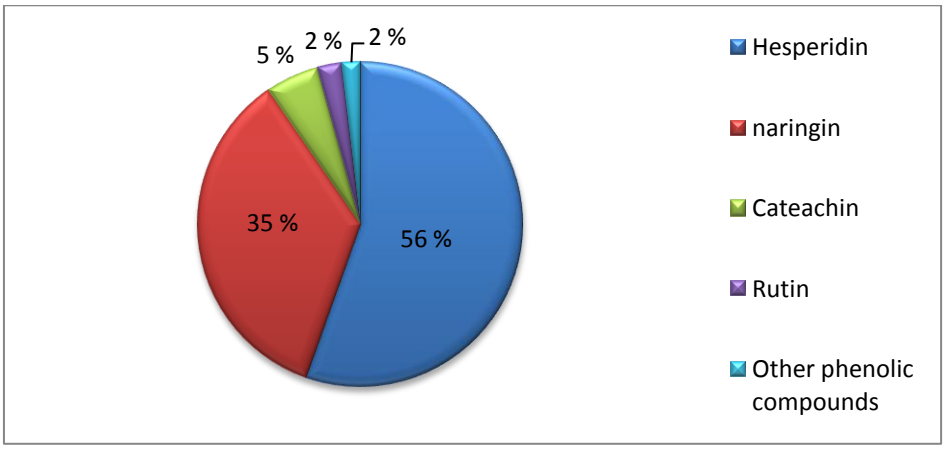

Fig. 2. Pie chart of the main active phenolic compounds of mandarin peels extract

In agreement with our findings Gellen et al. (2021) reported that there were a significant increase in serum LDH, AST, ALT, CK and CK-MB enzymes activity in CP intoxicated group as compared to normal control group [6]. Tesfaye et al.(2021) reported that there was a significant increase in plasma ALT and AST value in the CP-treated group compared to the normal control group (P<0.001).It is known that apart from cardiac injury, AST and ALT are also released in response to liver injury. However, AST tends to be more specific to heart injury, whereas ALT is more specific to liver damage [47].

Administration of CP (150 and 200 mg /kg / B.W) caused a significant increase in serum ALT, AST, CK-MB and LDH enzymes activity intoxicated rats when compared with normal control group [48,49,50].

Table 2. Serum alanine aminotransferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALK-P) enzymes activity in the experimental groups (mean ± S.D)

Parameter / Groups	ALT (U/L)	AST (U/L)	ALK-P (U/L)
G1: Normal Control(-ve)	41.70 ±1.40^{b}	203.68 ±1.48^{e}	153.12±2.39^{c}
G2: Intoxicated by CP(+ve)	79.11 ±2.22^{a}	328.24 ±2.52^{a}	182.71±2.52^{a}
G3: CP+GS (low dose)	42.39 ±1.72^{b}	224.62 ±3.99^{b}	163.33±.68^{b}
G4: CP+GS (high dose)	41.56 ±1.56^{b}	222.89 ±2.77^{b}	162.40±2.17^{b}
G5: CP+MP (low dose)	42.84 ±2.20^{b}	215.52 ±2.56^{c}	155.07±2.96^{c}
G6: CP+ MP (high dose)	42.35 ±2.52^{b}	211.92 ±1.44^{d}	163.86±1.64^{b}
LSD	1.78	2.34	1.96

There is no significant difference between means have the same letters in the same column n=10 rats, (P≤0.05)

Table 3. Serum lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB) and creatine kinase (CK) enzymes activity in the experimental groups (mean ± S.D)

Parameter / Group	LDH (U/L)	CK-MB (U/L)	CK (U/L)
G1: Normal control (-ve)	475.44±17.71	255.49±3.44^{d}	151.45±1.45^{f}
G2: intoxicated by CP	905.36±8.62^{a}	503.54±1.06^{a}	432.38±1.75^{a}
G3:CP + GS (low dose)	739.44±11.25^{b}	270.88±8.17^{b}	216.61±1.70^{b}
G4: CP +GS (high dose)	528.03±9.49^{d}	269.31±2.89^{b}	154.01±1.18^{e}
G5: CP + MP (low dose)	546.30±9.18^{c}	264.72±7.31^{c}	205.02±.26^{c}
G6: CP + MP (high dose)	531.42±2.32^{d}	258.22±1.80^{d}	156.81±.33^{d}
LSD	9.64	4.40	1.14

There is no significant difference between means have the same letters in the same column n=10 rats, (P≤0.05)

Cyclophosphamide is a cardiotoxic agent inducing a direct myocardial endothelial damage and destruction of myocardial cells. As a result, LDH and CK were increased in heart tissues and in the blood stream [51]. Omole et al. (2018) found that LDH and CK activity in CP group (50 mg /kg / BW, i.p.) as single dose was significantly increased by (43.28 + 2.38 U/L) and (50.48 + 5.38 U/L) respectively when compared with normal control (13.90 + 2.17 U/L) and (19.90 + 4.17 U/L) respectively. The increase in LDH and CK its specific and sensitive markers for heart muscle damage [52].

Lian et al. (2016) showed a significant increase in serum ALT and AST enzymes activity ($P < 0.05$) in cisplatin toxicated group as compared to normal control group. Grape seeds proanthocyanidin extract (GSPE) showed a clear reduction in cardiac enzymes activity ($P < 0.05$) in serum ALT and AST, respectively [53].

In agreement with our results, Abirami and Kanagavalli (2013) showed that the activity of serum enzymes LDH and CK significantly increased in doxorubicin (DOX) induced cardiotoxicity when compared to normal control group. The ethanolic extract of grape seeds (200 mg /kg / BW) was significantly ($P<0.05$) reduced the enzymes activity in serum when compared to DOX treated animal. In this study grape seeds extract prevent the leakage of marker enzymes by scavenging lipid peroxides and improve the GSH levels there by protecting integrity of membrane. The antioxidant enzymes, constituting the first line of defense mechanism to prevent and neutralize the reactive oxygen species (ROS) induced damage [54].

In addition Safdar et al. (2017) reported that DOX administration (2.5 mg /kg / BW i.p) significantly increased serum LDH, CK-MB and CK activity as compared to normal control group. Serum cardiac markers such as LDH, CK-MB and CK are the significant indicators of deviation in normal cardiac activity. Pre-treatment with mandarin kinnow peel extract (150 and 300 mg /kg / B.W) significantly minimize the effect of DOX administration on serum cardiac biomarkers level in different treatment groups. The cardioprotective potential of mandarin kinnow peel extract might be attributed to the presence of antioxidant phenolic compounds with free radical scavenging activity [43].

Donia et al. (2019) observed that there was a significant increase in serum LDH and CK activity in DOX-toxicated rats compared to normal control group. While, serum LDH and CK activities in groups of rats which administered DOX + hesperidin (HDN) (one of the active component of MP) was significantly reduced as compared to DOX group. The mechanism for the elevation of these markers seems owing to the oxidative damaging effect of DOX to cardiac tissue and the subsequent excessive release of these markers into circulation. On the other hand, oral administration of HDN to DOX-injected rats could reduce the elevated levels of LDH and CK which might be due to its antioxidant and ROS scavenging properties [55].

Hao et al. (2019) found that there was a significant increase in the activity of LDH and CK-MB enzymes in isoproterenol (ISO) toxicated group as compared to normal control group. Pretreatment with naringen (NG), (NG + ISO) could decrease ($P< 0.01$) the activity of this markers enzymes .An elevation of this marker enzyme due to high dose of ISO cause over production of free radicals and resulted in leakage of cardiac enzymes from cytosol into blood. Pretreatment with NG could decrease these enzymes due to anti-lipid peroxidation and cardio protective property [56].

3.4 Serum Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC) in Experimental Groups

Table (4) showed that administration of CP (200 mg/kg BW) was remarkably ($P \leq 0.05$) elevated the oxidative biomarkers, MDA and decreased serum TAC levels comparing to normal group. Co-administration of GS and MP with CP caused significant decline in serum MDA while TAC was significantly increased comparing to CP group. The supplementation with GS and MP in high dose caused the best improvement in the MDA and TAC levels compared with toxic group.

It has been reported that free radicals generated during treatment with CP causes membrane injury, which resulted to the loss of function and integrity of myocardial membrane [52].

In agreement with our results (Wei et al. (2012) and Cetik et al. (2015)) showed that CP in a dose (50, 100, 150 mg / kg / B.W) reduced TAC and increased MDA levels, which indicated that CP-induced oxidative stress and cardiotoxicity [57,58].

Table 4. Serum malondialdehyde (MDA) and Total antioxidant capacity (TAC) levels in the experimental groups (mean ± S.D)

Parameter / Group	MDA (nmol/ml)	TAC (mM/L)
G1: Normal control group(-ve)	1.34±.237^{e}	85.61±1.26^{a}
G2: Intoxicated by CP(+ve)	5.61±.368^{a}	24.00±1.30^{f}
G3: CP+ GS (low dose)	4.21±.618^{b}	62.08±1.80^{d}
G4: CP+ GS (high dose)	3.07±.578^{d}	73.40±2.03^{c}
G5: CP + MP (low dose)	3.70±.844^{c}	57.37±1.73^{e}
G6: CP + MP (high dose)	2.76±.310^{d}	75.99±1.15^{b}
LSD	0.479	1.417

There is no significant difference between means have the same letters in the same column n=10 rats, (P≤0.05)

In addition, *Omole* et al*. and Gunes* et al*.* showed that administration of CP (50 and 150 mg / kg / BW) significantly elevated the level of plasma MDA compared to the control group [52,50]. Moreover, the obtained results were in agreement with Gado et al*.* [59] who suggested that CP administration induced a significant increase in MDA level in heart causing an alteration in the cellular membrane structure and blocking cellular metabolism.

In addition, CP (200 mg /kg / BW, i.p.) significantly increased the level of serum MDA when compared with normal rats. In the cardiotoxicity induced by CP, the free radicals production was increased. These free radicals causes to deterioration the integrity and function of myocardial membrane. Besides, this is accompanied by vascular and endothelial damage in the myocardium [6,49,60].

The results of the present study were confirmed by the results of Al-Sowayan [61] who reported that rats injected with DOX showed a significant (P<0.05) increase the level of MDA compared to the control groups.GSPE (200 mg/kg/day) along with DOX significantly caused an improvement in MDA and this may be due to the polyphenolic content of GSPE which scavenge free radicals and act as good antioxidants against lipid peroxidation in the phospholipid bilayer [62].

Also, Lian et al. [53] showed that myocardial MDA level of lipid peroxidation was significantly increased in the cisplatin administered group, comparing to control group (P < 0.05), however the administration of GSPE reduced MDA level in heart tissues with a significant milder pathological change caused by cisplatin. These observations indicated that GSPE had protective effect on cisplatin induced oxidative stress in cardiac tissue.

Additionally, the current results were in concement with the results of Kwatra et al. [63] who found that there was a significant increase (P<0.001) in serum MDA level in DOX toxicated group compared with the normal control group. Though, pretreatment with NG at both doses (50 and 100 mg / kg / B.W) indicated a statistically significant (P<0.001) reduction in MDA level as compared with DOX-toxicated group.

Donia et al. [55] reported that serum MDA level was significantly higher in DOX group compared to normal control group. While, in DOX + HDN group, serum MDA was significantly diminished than that of DOX group**.** Oxidative stress acts an important role in DOX-induced cardiotoxicity by inducing lipid peroxidation which negatively affects heart. Oral administration of HDN to the DOX injected rats reduced the elevated MDA levels suggesting that the cardioprotective effects of HDN which may be due to its antioxidant action.

Hao et al. [56] reported that pretreatment with NG (50 mg/kg/ B.W) for 14 days significantly suppress the MDA level which increased in group of rats which toxicated with ISO as compared to control group.

3.5 Cardiac Antioxidant Enzymes Activity in Experimental Groups

Table 5 clarifies that antioxidant defense enzymes (CAT, SOD and GPx) activity were significantly (P ≤ 0.05) declined in response to group injected with CP compared to control group. Additionally, it was detected that cardiac CAT, SOD and GPx enzymes were significantly increased by treatment with GS and MP (low and high doses) comparing to CP group. The supplementation with high doses of GS and MP caused the best improvement in the cardiac CAT, SOD and GPx enzymes compared with toxic group.

Table 5. Cardiac Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes activity in the experimental groups (mean ± SE)

Parameter / Group	Catalase (U/g)	SOD (U/g)	GPx (mU/ml)
G1: Normal control group (-ve)	123.66±3.59[a]	12.58±1.12[a]	222.33±2.34[a]
G2: Intoxicated by CP(+ve)	63.62±1.27[f]	2.88±.097[e]	92.92±1.70[f]
G3:CP+GS (low dose)	101.60±2.09[e]	6.43±.266[d]	177.00±2.36[e]
G4: CP+GS (high dose)	111.89±1.98[c]	9.87±.468[b]	196.30±1.60[c]
G5: CP+ MP (low dose)	105.84±2.04[d]	8.22±.253[c]	182.63±.756[d]
G6: CP +MP (high dose)	117.35±1.79[b]	9.58±.143[b]	210.82±1.65[b]
LSD	2.01	0.469	1.63

There is no significant difference between means have the same letters in the same column n=10 rats, (P≤0.05).

Cyclophosphamide induced a significant decrease in SOD, CAT, GPx activities due to the promotion of $OH^{\cdot}$ radicals formation, and initiation and propagation of lipid peroxidation. Moreover, it was suggested that the decrease in the activities of antioxidant enzymes is a consequence of increased oxidative stress in the cardiac tissues due to the overproduction of active ROS [64,65,66,67,68].

Free radical scavenging enzymes are the first line of cellular defense against oxidative injury. SOD detoxifies the superoxide radicals to hydrogen peroxide and CAT dismutates H_2O_2 to water (H_2O) and O_2, while GPx converts glutathione to oxidize glutathione, and at the same time reduce H_2O_2 to H_2O, and lipid hydroperoxides (ROOH) to the corresponding stable alcohols [69].

In agreement with our results Omole et al. (2018) and Mansour et al. (2015) reported that there were a significant reduction in heart SOD, CAT and GPx enzymes activity in rats injected interperitonially with CP (50 and 200 mg /kg / B.W.) when compared with the control group [52,48].

In addition (Emeka et al. (2020) and Gellen et al. (2021)) reported that CP (200 mg /kg / B.W, i.p.) significantly decreased the activity of CAT and SOD compared to the control group in cardiac tissue ($P < 0.001$) [60,6].

Furthermore, Lian et al. (2016) indicated that SOD, CAT and GP_X activity in heart tissue significantly diminished in cisplatin group ($P < 0.05$). Results from this experiment showed that pretreatment with GSPE (400 mg / kg / B.W) significantly elevated the activity of SOD, CAT and GPx in cardiac tissue. Furthermore, the histopathological change caused by cisplatin was significant milder in the GSPE protective group. These observations indicated that GSPE had protective and therapeutic effects on cisplatin induced oxidative stress in cardiac tissue [53].

Also the study of Safdar et al. (2017) concluded that DOX administration to rats significantly reduced the myocardial SOD, CAT and GPx. However, pre-treatment with mandarin kinnow peel extract (300 mg / kg) significantly diminished the effect of DOX administration on cardiac antioxidant level in different treated groups owing to its polyphenolic content [43].

Moreover, the study of Kwatra et al. [63] showed that DOX caused a significant decrease ($P<0.001$) in the activities of heart SOD as well as CAT when compared with the normal control group. However, pretreatment with NR at both doses (50 and 100 mg / kg / B.W) restored the activities of SOD as well as CAT levels as compared with the DOX-toxicated group It might be due to its antioxidant property and ability to up regulate mRNA expression of the antioxidant enzymes, according to that the naringenin, the major metabolite of NR, possesses superoxide scavenging with antioxidant activities and it also enhances aortic dilation.

Hao et al. [56] reported that there were a significant reduction ($P< 0.01$) in cardiac SOD and CAT in ISO injected rats as compared to control group. Pretreatment with NG (50 mg/kg) for 14 days significantly improved the activity of these antioxidant enzymes.

3.6 Apoptotic Biomarkers in Experimental Groups

The current results showed that CP toxicity induced massive increase ($P \leq 0.05$) in cardiac

p53, caspase- 3 and DNA fragmentation levels. While, massive decrease (P ≤ 0.05) in the level of cardiac Bcl-2 when compared with control group. It was noticed from the results that pretreatment with GS and MP (low and high doses) to CP intoxicated rats caused a significant improvement (P ≤ 0.05) in heart p53, caspase-3, Bcl-2 and DNA fragmentation levels when compared with CP group (Table 6). The supplementation with high doses of GS and MP caused the best improvement in the heart p53, caspase- 3 and DNA fragmentation levels compared with toxic group.

Cyclophosphamide induced cardiomyopathy is closely associated with its ability to induce apoptosis in cardiac cells via DNA intercalation, activation of p53 protein and generation of ROS. CP increased the pro-apoptotic protein (Bax) expression and caspase-3 level in cardiac tissues while it decreased the anti-apoptotic protein (Bcl-2) expression leading to cardiomyocyte apoptosis. The increase in apoptosis markers has been closely linked to activation of NF-κB. As mentioned, CP-induced apoptosis via the intrinsic mitochondrial dependent apoptotic pathway as a consequence of ROS overproduction. CP significantly increases mRNA expression of p53, caspase-3 and DNA fragmentation and decreases the expression of Bcl-2 [70,7].

Interestingly, GS and MP extract restored the up regulation of p_{53} gene and down regulation in Bcl-2 gene to the normal values, suggesting that GS and MP may blocks apoptosis in cardiac myocytes induced by CP. Also, this may be due to that, GS and MP include (phenolic and flavonoids compounds) and antioxidants which decreased the rate of apoptosis caused by the toxic effects of CP [71].

The current results were in agreement with the results of Avci et al. (2016) who found that the levels of the heart DNA fragmentation and caspase-3 in the CP-treated group was significantly increased ($p < 0.001$) than those of the control and other treated groups [72]. On other hand immunohistochemical analysis of Bcl-2 staining showed that the percentage of Bcl-2 expression in the CP group was significantly decreased than that in the control and other treated groups. They added that, the rate of apoptotic cells was significantly higher in the tissues of rats which administered CP. Iqubal et al. (2019) showed that treatment with CP (200 mg / kg / BW) showed an elevation of cleaved caspase 3 expression, in the cardiac tissue ($P < 0.001$). Apoptosis is another consequence of cardiotoxicity and cleaved caspase 3 elevation is one of the important apoptotic markers [49].

Also, Liu et al. (2018) found that the rats of myocardial ischemia/reperfusion (I/R) models increased expressions of p53, Caspase-9, Caspase-3 and Bax in the ischemic group and reduced Bcl-2 expression and Bcl-2/Bax ratio than that in the control group ($p < 0.05$); Compared with the ischemic group, low and high doses of procyanidin decreased P53, Caspase-9, Caspase-3 and Bax expressions and increased Bcl-2 expression and Bcl-2/Bax ratio ($p < 0.05$) [73].

Table 6. Heart p53, Bcl-2, Caspase-3 and DNA fragmentation in the experimental groups (mean ± SE)

Parameter / Group	p_{53} (pg/ml)	Bcl-2 (ng/ml)	Caspase-3 (ng/ml)	DNA fragmentation
G1: Normal control(-ve)	39.84±1.32^{f}	194.46±2.38^{a}	1.79±0.099^{d}	1.56±.191^{e}
G2: Intoxicated by CP(+ve)	124.81±1.63^{a}	85.66±1.32^{f}	9.52±0.328^{a}	30.07±1.98^{a}
G3: CP +GS (low dose)	85.15±1.84^{b}	156.21±1.89^{d}	3.59±0.327^{b}	11.61±.842^{b}
G4: CP +GS (high dose)	51.32±1.32^{d}	178.72±1.19^{c}	2.79±0.167^{c}	2.53±.206^{d}
G5: CP + MP (low dose)	76.16±1.68^{c}	152.67±.741^{e}	3.61±0.829^{b}	9.86±.351^{c}
G6: CP +MP (high dose)	48.30±1.23^{e}	183.58±1.37^{b}	1.85±o.144^{d}	2.41±.195^{d}
LSD	1.36	1.41	0.358	0.810

There is no significant difference between means have the same letters in the same column n=10 rats, (P≤0.05).

In consistent with our findings Fu et al. [74] showed that Bax and Cleaved caspase-3 protein levels were much higher, while Bcl-2 level was much lower in middle cerebral artery occlusion (MCAO) mice than that in Sham mice. After administration of GSP (250 mg/kg), Bax and Cleaved caspase-3 protein levels were significantly decreased, while Bcl-2 level was significantly increased as compared with MCAO mice. These data indicated that GSP administration altered apoptosis-associated gene expression in mice.

Ruan et al. [75] reported that GSPE significantly elevated the expression of Bcl-2 and reduced the expression of Bax in myocardial tissue after myocardial infarction (MI). The Bcl-2 protein family is associated with the regulation of cell apoptosis. Bcl-2 plays an anti-apoptotic role, while Bax plays a pro-apoptotic role.

Our results were in agreement with the results of Rehman et al. (2014) and Liu et al. (2016) reported that p53 plays a critical role in the cellular apoptotic pathway leading from Cisplatin -DNA crosslinking to caspase-3 activation. In addition, DNA damage leads to phosphorylation of p53 in the liver. However, grape seed proanthocyanidin extract modulated the values of P53 in heart near to normal values [76,77]. Also Pallarès et al (2013) reported that GSPE considered as antioxidant, anti-inflammation and anti-atherosclerosis, procyanidins elicit the up-regulation of a sequence of antioxidant and detoxification enzymes that enhance cellular defences [78].

Selvaraj and Pugalendia. (2018) reported that the expressions of caspase-3 caspase-9 and Bax were up regulated in ISO-rats and down regulated expression of Bcl-2 and Bcl-xL. Administration of HDN to ISO-induced rats up regulated the expression of Bcl-2 and Bcl-xL and down regulated Bax, caspase-3 and caspase-9 expression. Administration of HDN increased the expression of the anti-apoptotic proteins and decreased the expression of the pro-apoptotic protein [79].

In addition Meng et al. [80] found a significant increase in the protein expression of p53 in the acute myocardial infarction (AMI) mice, compared with that in the control group. While, treatment with HDN an active component of mandarin peels extract (50 and 100 mg/kg) significantly decreased the protein expression of p53.

3.7 Microscopic Examination of Heart Tissues in Experimental Groups

The histopathological results of this study were strongly supported by the biochemical findings. It was clear from the microscopic examination of heart in Fig. (3) that normal control group showed normal cardiac myocytes, revealed by the normal structure of the tissue which consists of normal cardiac myocytes. On the other hand Fig. (4 a and b) show cardiac myocytes in the form of cytoplasmic vacuolization of cardiac myocytes, oedem in-between cardiac myocytes associated with mononuclear inflammatory cells infiltration in CP group.

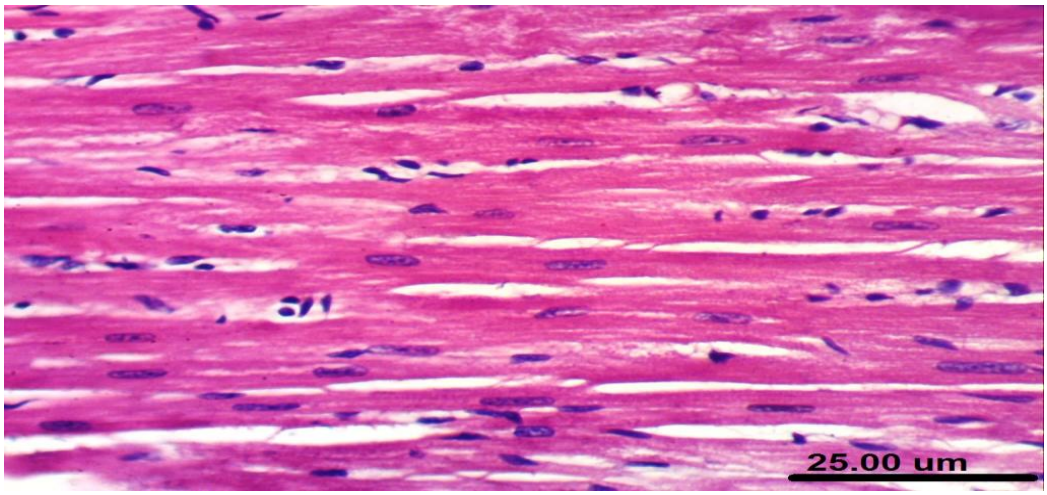

Fig. 3. Heart section of rat from group 1 showing normal cardiac myocytes (H & E X 400, scale bar 25 um)

Pretreatment with either GS or MP to intoxicated rats showed a great improvement in heart tissue. Figs. (5 and 7) show that CP intoxicated rats which administered low doses of GS and MP showed slight oedema in-between cardiac myocytes. While, Fig. (6) shows no histopathological alterations of CP intoxicated rats which treated with high dose of GS.

Moreover, Fig. (8) shows heart section of CP intoxicated rats which administered MP of high dose showed no histopathological alterations.

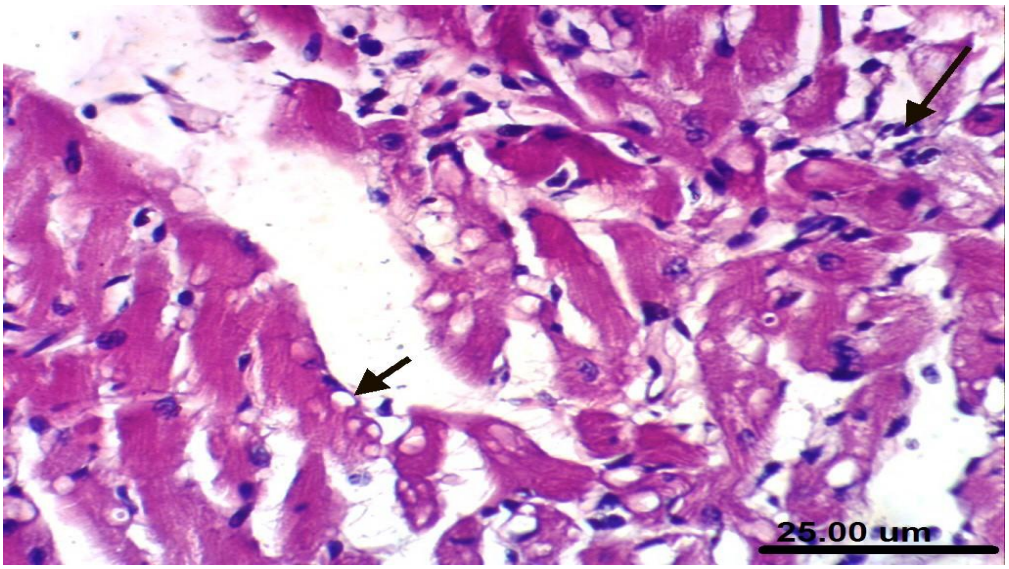

Fig. 4a. Heart section of rat from CP intoxicated group (group 2) showing cytoplasmic vacuolization of cardiac myocytes (arrow) (H & E X 400, scale bar 25 um)

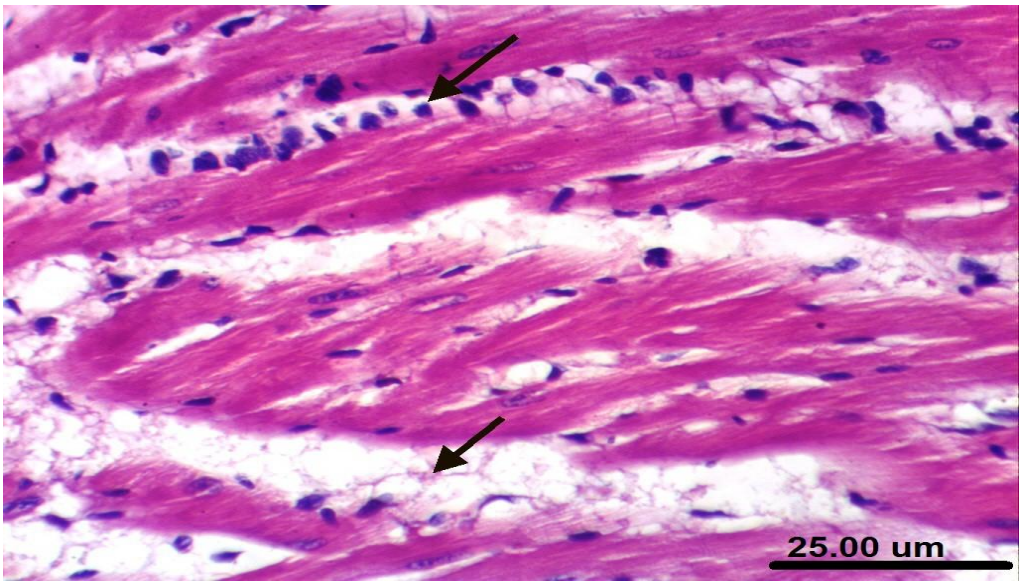

Fig. 4b. Heart section of rat from CP intoxicated group (group 2) showing oedem in-between cardiac myocytes (short arrow) associated with mononuclear inflammatory cells infiltration (long arrow) (H & E X 400, scale bar 25 um)

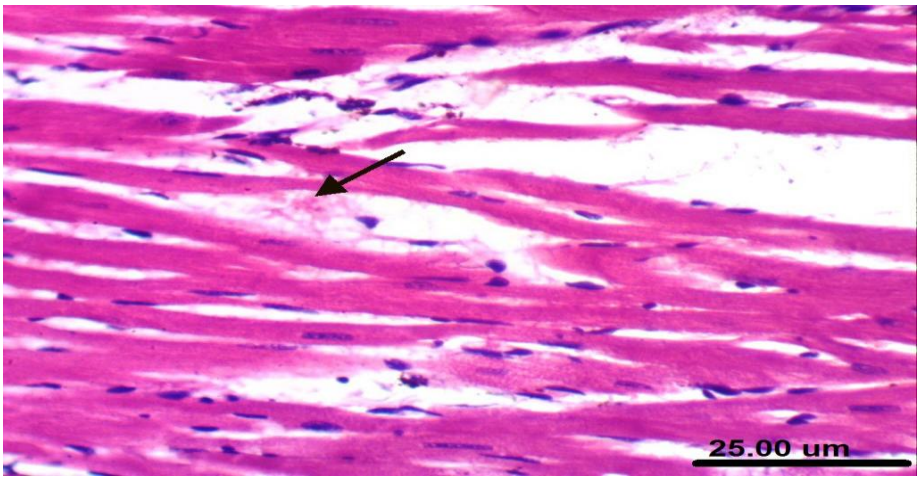

Fig. 5. Heart section of rat which administered low dose of GS (group 3) showing slight oedem in-between cardiac myocytes (arrow) (H & E X 400, scale bar 25 um)

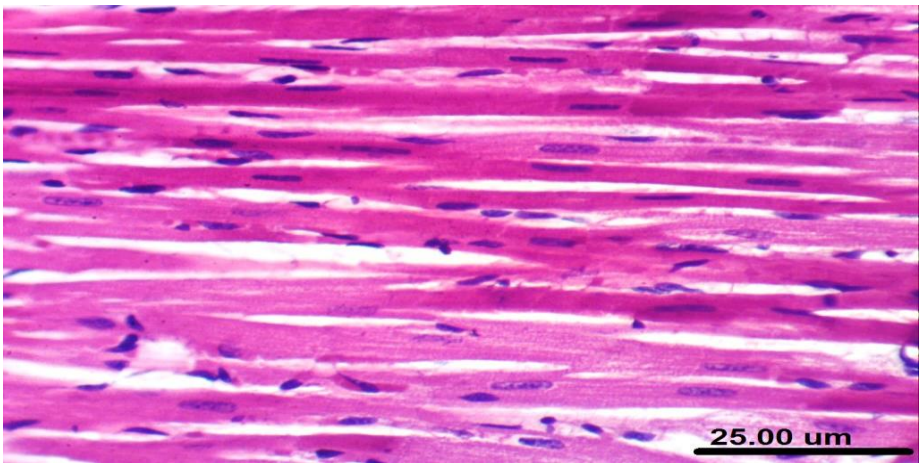

Fig. 6. Heart section of rat which administered high dose of GS (group 4) showing no histopathological alterations (H & E X 400, scale bar 25 um)

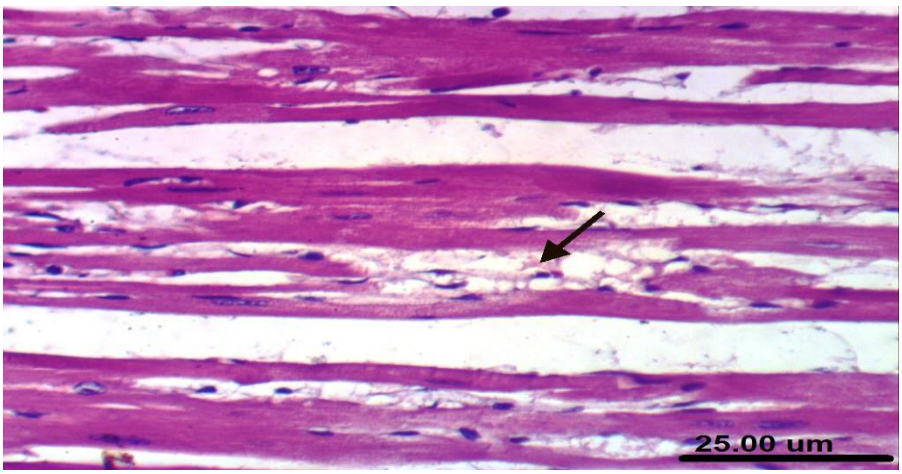

Fig. 7. Heart section of rat which administered low dose of MP (group 5) showing slight oedem in-between cardiac myocytes (arrow) (H & E X 400, scale bar 25 um)

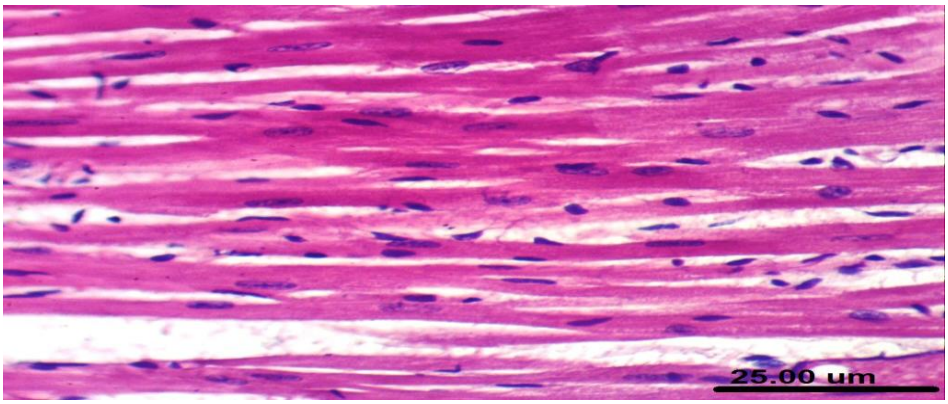

Fig. 8. Heart section of rat which administered high dose of MP (group 6) showing no histopathological alterations (H & E X 400, scale bar 25 um)

Our results go hand in hand with the results of Tesfaye et al. [47] who observed that the cardiac tissues in the normal control group showed normal morphological architecture. However, the cardiac tissues of rats administered with only CP (200mg/kg/BW. i.p) showed necrotic cardiocytes, haemorrhage and oedema. This may be a result of the formation of free radicals and oxidative stress induced by CP-metabolites including acrolein. Gellen et al. (2021) reported that administration of CP (200 mg/kg/BW. i.p) to rats caused endothelial dysregulation, degenerative areas, thickening in vascular wall [6]

The results of this study were in agreement with that of Omole et al. [52] who found that administration of CP (50 and 200 mg /kg / BW) produced massive change in the myocardium showing a varying degree of vacuolar changes in the cardiac muscle fibers, vacuolization of the cardiomyocytes and infiltration of inflammatory cells.

In addition, Gunes et al. [49] reported a regional increase of eosinophilic staining in cardiac muscle cells, shrinking and dark staining of the nuclei due to intense chromatin, and irregularity in nuclear borders with small degeneration foci of myocardial cells of rats receiving CP (200 mg /kg / BW) due to hemorrhage and edema.

Razmaraii et al. [81] reported that there were significant changes in DOX group including cytoplasmic vacuolization, interstitial edema. On the other hand, In GSE (100 mg/kg/24h, for 16 days) group the myocardial damage was dramatically attenuated, as compared to the DOX group. Therefore, it could be speculate that GSE led to cell preservation and decreased necrosis, cytoplasmic vacuolization and maintained a normal morphology and structure for the cardiac muscle.

Also, Safdar et al. [43] who showed that marked degeneration and cardiomyopathy occurred in myocardium of DOX administered group as evident by cytoplasmic vacuole formation and edema. Pretreatment of DOX intoxicated group with (150 mg / kg / BW) mandarin kinnow peels extract + DOX resulted in moderate changes to myocardium of rats. Rats group pretreated with (300 mg / kg / BW) mandarin kinnow peels extract for 60 days + DOX showed slight changes in myocardium like cytoplasmic vacuole formation.

3.8 Microscopic Examination of Liver Tissues in Experimental Groups

The current histopathological results were strongly supported by the biochemical findings. It was clear from the microscopic examination of liver in Fig. (9) that normal control group showed a normal histological structure of hepatic lobule. In contrast, liver of rats from CP intoxicated group showed Kupffer cells activation, hepatocellular steatosis as shown in Fig. (10) and fibroplasia around bile duct associated with mononuclear inflammatory cells infiltration in the portal triad as shown in Fig. (11).

While, in Fig. (12) CP intoxicated rats which administered low dose of GS showed focal hepatocellular necrosis associated with inflammatory cells infiltration. On the other hand, administration of the high dose of GS showed slight congestion of central vein and hepatic sinusoids Fig. (13).

Fig. 14 show liver section of CP intoxicated rats which administered low dose of MP showed slight hyperplasia of biliary epithelium and oedema in the portal triad. While, the liver sections of rats administered high dose of MP showed vacuolar degeneration of focal hepatocytes Fig. 15.

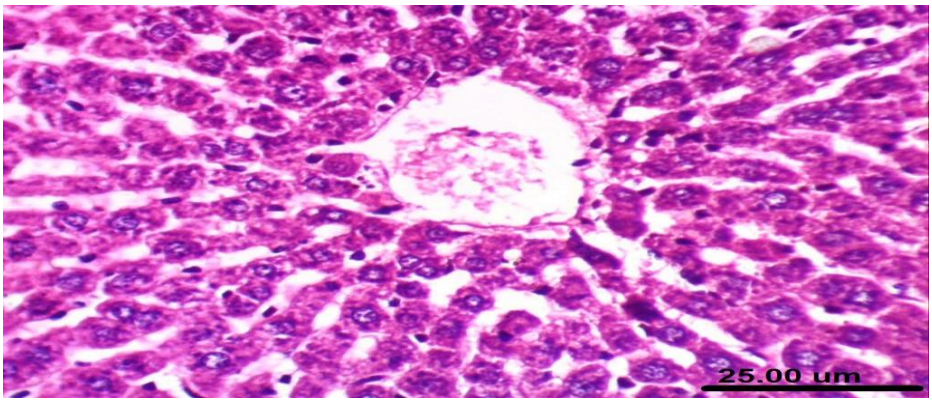

Fig. 9. Liver section of rat from group 1 showing the normal histological structure of hepatic lobule (H & E X 400, scale bar)

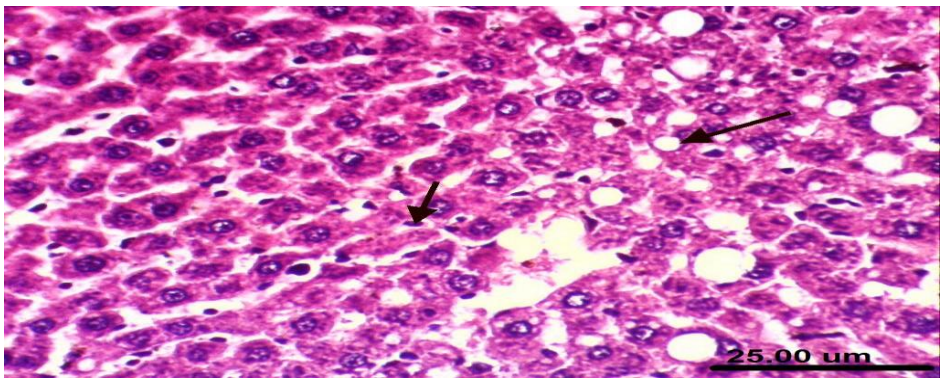

Fig. 10. Liver section of rat from CP intoxicated group (group 2) showing Kupffer cells activation (short arrow) and hepatocellular steatosis (long arrow) (H & E X 400, scale bar 25 um)

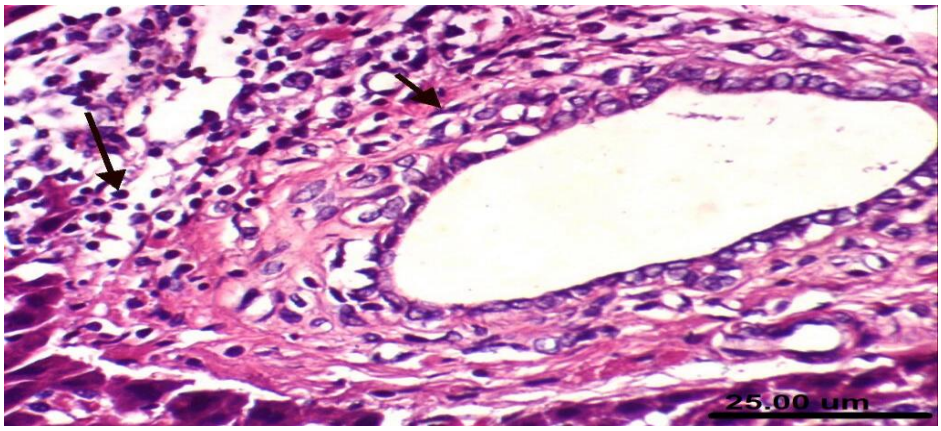

Fig. 11. Liver section of rat from CP intoxicated group (group 2) showing fibroplasia around bile duct (short arrow) associated with mononuclear inflammatory cells infiltration in the portal triad (H & E X 400, scale bar 25 um)

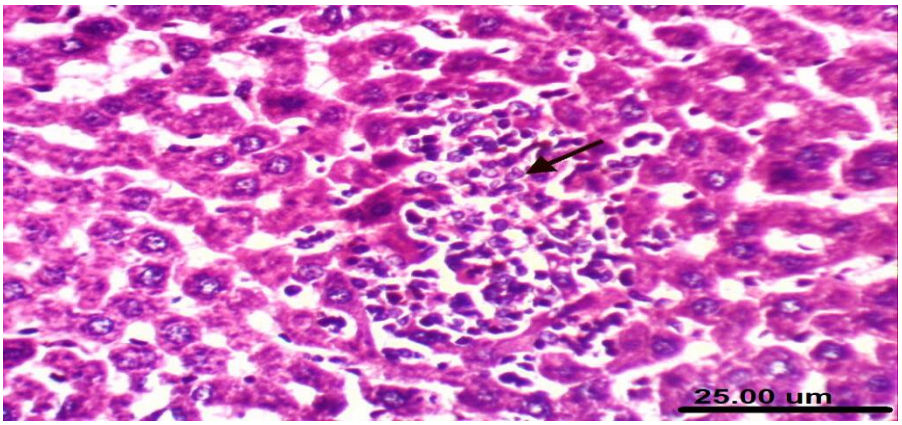

Fig. 12. Liver section of rat which administered low dose of GS (group 3) showing focal hepatocellular necrosis associated with inflammatory cells infiltration (arrow) (H & E X 400, scale bar 25 um)

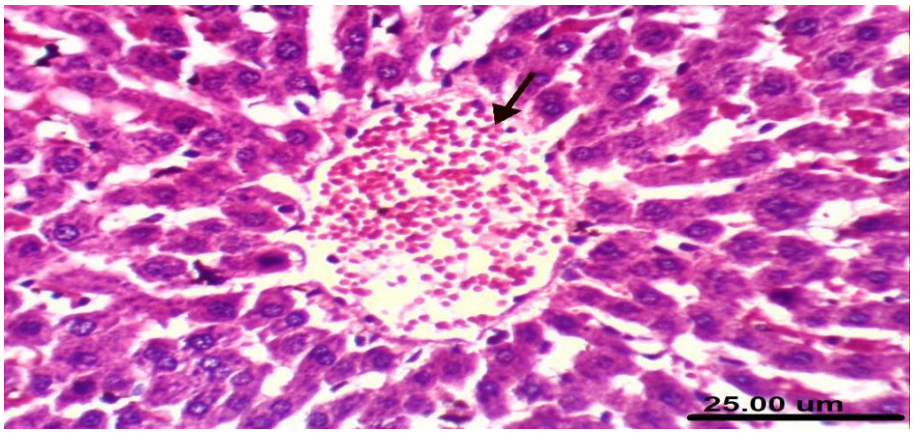

Fig. 13. Liver section of rat which administered high dose of GS (group 4) showing slight congestion of central vein and hepatic sinusoids (H & E X 400, scale bar 25 um)

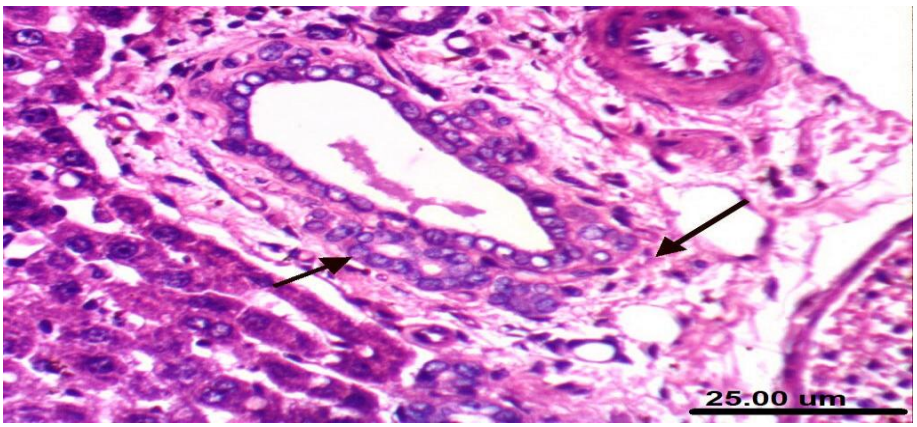

Fig. 14. Liver section of rat which administered low dose of MP (group 5) showing slight hyperplasia of biliary epithelium (short arrow) and oedema in the portal triad (long arrow) (H & E X 400, scale bar 25 um)

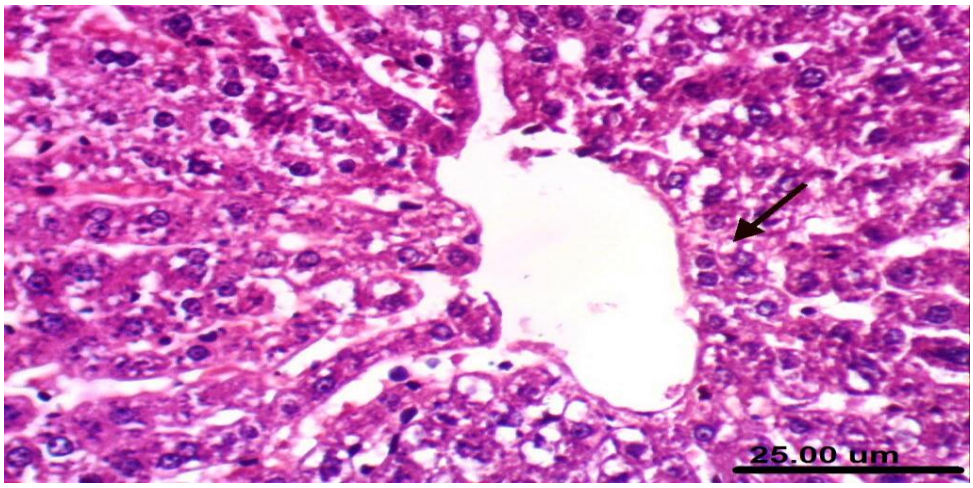

Fig. 15. Liver section of rat which administered high dose of MP (group 6) showing vacuolar degeneration of focal hepatocytes (H & E X 400, scale bar 25 um)

In agreement with our results El-Karim and El-Amrawi. (2019) found that CP-treated group showed severe hemorrhage with broadening of portal area with mononuclear inflammatory cell and fibroblast besides vacuolar degeneration of the majority of hepatocytes. Histopathological examination of liver, affirmed that CP caused liver damage which was evidenced by presence of several different hepatic lesions, which might be caused by cellular damaging potential of CP metabolites in relation to generation of ROS [82].

Also, Hassan and Al-Rawi [83] showed that liver section of the rats in the group treated with GSPE and injected with gibberellic acid induced oxidative stress and cellular alterations showed an improvement of the central vein structure and the portal region and reduction of inflammatory cells. This finding may be attributed to the inhibitory effect of GSPE on pro-inflammatory cytokines evidencing its anti-inflammatory effect. They concluded that, the hepatoprotective effect of GSPE in the development of liver may be related to the reduction of lipid peroxidation by its anti-oxidative activity and the ability to scavange reactive oxygen species.

Rabee and Bennasir [84] found that rats treated with HDN (100 and 200 mg/kg) and injected with CCl_4 (2 ml/kg/BW) as single dose revealed vaculation occurs, more eosinophilic infiltration, better viability, less damage, nuclei are healthier, less disruption of the lattice nature of hepatocytes and less damaged hepatocyte cell membrane were less than the positive control group. The group treated with HDN (200 mg/kg) faded vaculation and less infiltration by the inflammatory cells than HDN (100 mg/kg).

Also, Abdel-Sttar et al. [85] illustrated that HDN treated group showed normal central vein with slightly dilated congested blood sinusoids. Most hepatocytes were normal with acidophilic cytoplasm and vesicular nuclei, activation of Von Kupffer cells can be also observed. HDN modulated the severity of hepatic damage caused by CCl_4 leading to a further confirmation of its anti-fibrotic effect.

4. CONCLUSION

Our study concluded that, administration of single dose of CP (200 mg/ kg/ BW.i.p.) led to cardiotoxicity and hepatotoxicity. Pretreatment with GS or MP (low and high doses) to CP intoxicated rats protect the liver and heart tissues from damage due to their high content of phenols and flavonoids, which enhance the antioxidant, anti-apoptotic activities and protect against oxidative stress.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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