Protective Effects of Lemon Fruit Extract on Liver in Mice Males Treated with Cyclophosphamide

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Abstract: The protective effects of lemon fruit extracts (LFE) were evaluated against histological changes induced in liver of mice males treated with cyclophosphamide (CP). A total of thirty male mice were divided into six groups: Gr1 control group, Gr2 treated with LFE (10ml/kg b wt.), Gr3 treated with CP (10mg/kg b wt.), and Gr4 treated with CP (20mg/kg b wt.), Gr5 treated with LFE (10ml/kg) +CP (10mg/kg), Gr6 treated with LFE (10ml/kg) + CP (20mg/kg). Histological examination of the livers in Gr3 and Gr4 showed loss of hepatocytes architecture, blood sinusoids congestion, vacuolar degeneration, inflammatory cellular infiltration in between degenerated hepatocytes, formation of pyknotic nuclei and hepatocellular necrosis. Addition of lemon fruit extract to cyclophosphamide treated mice resulted in a marked improvement in liver tissue. Liver restoration of normal histological structure, marked reduction or disappearance of cytoplasmic vacuoles in most hepatocytes, less dilation of central and portal veins with mild-to-moderate inflammation in portal space. Marked reduction of sinusoids congestion were detected. Conclusion: Our results reveal that lemon fruit extract induces potent hepatoprotictive effects against CP.
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1.Introduction

Cancer chemotherapy has demonstrated an important role in the treatment of most solid tumors (Habibi *et al*., 2014). Cyclophosphamide, the alkylating agent is widely used as an anti-tumor and immunosuppressive agent (Bhattacharjee *et al*.,2014). It has been found to cause various side effects. The target organs are liver, lungs, heart, urinary bladder and reproductive system (Khan *et al*., 2014). It has also caused many toxic effects to normal cells in humans and experimental animals (Fraiser *et al*., 1991; Gokhale *et al*., 2003). These include pulmonary fibrosis, gastrointestinal bleeding, irreversible azospermia in man and haemorrhagic cystitis (Gilman and Rall, 1999). Moreover, CP-induced microvascular fatty changes in liver (Bissell and Ataya, 2001). CP has been found to produce secondary malignancies such as bladder malignancies and myeloprolifer-ativemalignancies (Khan *et al*., 2014). However, CP requires metabolic activation by the hepatic cytochrome P450 system (Gilman and Rall, 1999). Metabolic conversion of CP leads to the formation of cytotoxic metabolites, acrolein and phosphormide mustard (Nau *et al*., 1982). Phosphormide mustard is believed to have anti-tumor effects, whereas, acrolein may be responsible for CP-induced liver damage (Hongo *et al*., 1988). These metabolites caused inhibition of DNA, RNA and protein synthesis and rapid death of divided cells by modification and cross linkage of purine bases in DNA or alkylating nucleophilic sites in DNA, RNA and protein such as –COOH, - NH2, - SH and OH^2 (Khan *et al*., 2014) CP, induced cellular toxicity, genotoxicity and mutagenic effects (Ponticelli and Passerrini, 1991).

Previous studies reported that CP generated reactive oxygen species (ROS), like the hydroxyl radical, hydrogen peroxide and superoxide anion, and further suppresses the liver's antioxidant defense mechanisms (Stankiewicz *et al*., 2002; Bhattachary *et al*., 2003). Biological compounds with antioxidant properties intake can ameliorate the toxic effects of chemotherapy and may contribute to the protection of cells and tissues against the side – effects of ROS and other free radicals induced by antineoplastic drug such as cyclophosphamide (Weijl *et al*., 1997; Habibi *et al*., 2014). Lemon is a citrus fruits that contains several flavonoid compounds which are considered the most important antioxidant agents (Miyake *et al*., 1998; Minato *et al*., 2003). The anticarcinogenic, antitumor and anti- inflammatory activities of flavonoids have been refloated by Bracke *et al*. (1994) Schramm *et al*. (2003) Psotova *et al*. (2004). Furthermore, lemon fruit contains a high content of vitamin C which has been known to be effective as an anticlastogenic and antimutagenic agent (Castillo *et al*., 2000; Aly and Donya, 2002) and a strong antioxidant (Rao, 1997), as well as protects DNA from oxidative damage (Antunes and Takahashi, 1999). Therefore, the aim of this work was to study the possible protective effect of lemon fruit extract against histological changes in liver of male mice treated with cyclophosphamide.

2.Materials and methods

Materials

1. Cyclophosphamide: Has been known commercially as Endoxan, it was dissolved in saline solution and purchase from (Baxter Oncology, Halle, Germany).
2. Lemon fruit extract: The lemon fruits were obtained from Jeddah market, Saudi Arabia. Lemon fruits washed with distilled water, cut intosmall pieces, grinded in a mixer (Moulinex type 753) and then introduced for the tested animals.

Animals

Swiss albino male mice (*Musmusculus* 2n=40) MFI strain, 8 – 9 weeks old, weighted 30 ± 3g, which have been obtained from animal house at the King Fahad Medical Center at King Abdulaziz University in Jeddah. Animal were housed in polyplastic cages with steel wire tops in an air conditioned room (22 ± 1 °c, 45 – 75% relative humidity) with 12h light/12h dark cycles. Food and water were provided ad libitum.

Methods

Thirty male mice were divided into six groups (each containing five mice) as follows: Group one (Gr1) was treated with saline solution on intraperitoneal(i.p.) as a vehicle control, group two (Cr2) was treated with LFE by oral intubation (o.i.) (10ml / kg b wt.), group three (Gr3) was treated with CP (10mg / kg b wt.) (i.p.), group four (Gr4) was treated with CP (20mg / kg b wt.) (i.p.), group five (Gr5) was treated with [LFE 10ml / kg (o.i.) + CP 10mg / kg (i.p.)], group six (Gr6) was treated with [LFE 10ml / kg (o.i.) + CP 20mg / kg (i.p.)]

Animal treatment

CP was introduced by intraperitoneal injection (i.p.) as recommended by Anton (1997). LFE was given by oral intubation (o.i) as reported by Sakr *et al*. (2013). All groups were treated daily for five consecutive days (Naghshvar *et al*., 2012). The mice were killed 24h after the last dose.

Tissue procurement

Upon the injection, the mice were killed and dissected, the liver was removed and dried before weighing, any part of the liver was used for histological examination.

Histology

The tissues were fixed overnight in 10% buffered neutral formalin processed to paraffin wax, sectioned at 5mM, and stained with haematoxylin and eosin (H+E) (Mallory, 1900) for examination by light microscopy.

3. Results

The weights of the liver of the CP-treated mice were not different from those of the control ones.

- Control group (Gr1) and LFE group(Gr2):

Microscopic examination of the sections of Gr1 and GR2 (Fig.1a&b) and (Fig.2 a&b), respectively showed normal histological structure, like hepatic lobes containing cords of hepatocytes with sinusoids between there cords. The central vein and portal treads appeared normal.

- Cyclophosphamide treated groups (Gr3) and (Gr4) :

Results presented in (Fig.3 a&b) showed that treatment with the low dose of CP (Gr3) caused loss of hepatocytes architecture, central vein congestion, damage to endothelium of sinusoids with sinusoidal congestion, vacuolar degeneration, formation of pyknotic nuclei and hepatocellular necrosis. While the treatment with high dose of CP (Gr4) induced more degenerative changes in the liver, it appeared more susceptible (Fig.4 a&b). Abnormal histological structure, rupture of the epithelium causing internal bleeding, vacuolar degeneration, inflammatory cellular infiltration in between degenerated hepatocytes, formation of pyknotic nuclei in most hepatocytes or karyorrhexis and karyolysis nuclei in some other hepatocytes, and hepatocellular necrosis were detected. Moreover, increase of fat in the interstitial tissue, appearance of lipid droplets in many hepatocytes as well as on the wall of the central, portal vessels and dilated sinusoids with marked proliferation of kupffer cells were observed.

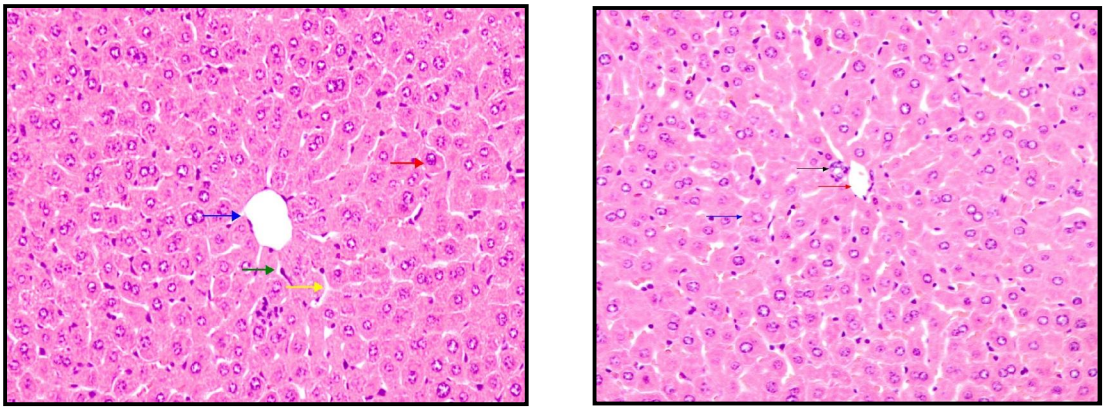
- LFE and CP treated groups (Gr5) and (Gr6):

The histoexamination of the animals treated with lemon fruit extract plus low dose of cyclophosphamide (Fig.5 a&b) and with high dose (Fig.6 a&b) revealed marked improvements in the liver tissues in both (Gr5) and (Gr6) respectively.

The liver resorted its normal histological structure, marked reduction or disappearance of cytoplasmic vacuole in most hepatocytes, less dilation of central and portal veins with mild-to-moderate inflammation in portal space. Marked reduction of sinusoids congestion, proliferation of binucleated hepatocytes and enlarged kupffer cells were also detected.

4. Discussion

Liver sections from the control group (Gr1) and lemon fruit extract treated mice (Gr2) showed normal hepatic lobules formed of hepatocytes radiating from central vein to the periphery of the lobules. In contrast, treated mice with CP induced degenerative changes in the liver. CP caused loss of hepatocyte architecture, blood sinusoids congestion, rupture of the epithelium causing internal bleeding, vacuolar degeneration, inflammatory cellular infiltration in between degenerated hepatocytes, formation of pyknotic nuclei and hepatocellular necrosis were detected. Histological examination revealed increase of fat in the interstitial tissue and appearance of lipid droplets in many hepatocytes and on the wall of the central and portal vessels. Arien *et al*. (1976) in this connection, reported that tissue toxicity appears in the histological sections as cell degeneration accompanied with vacuoles formation and fatty accumulation with necrosis in the tissue.

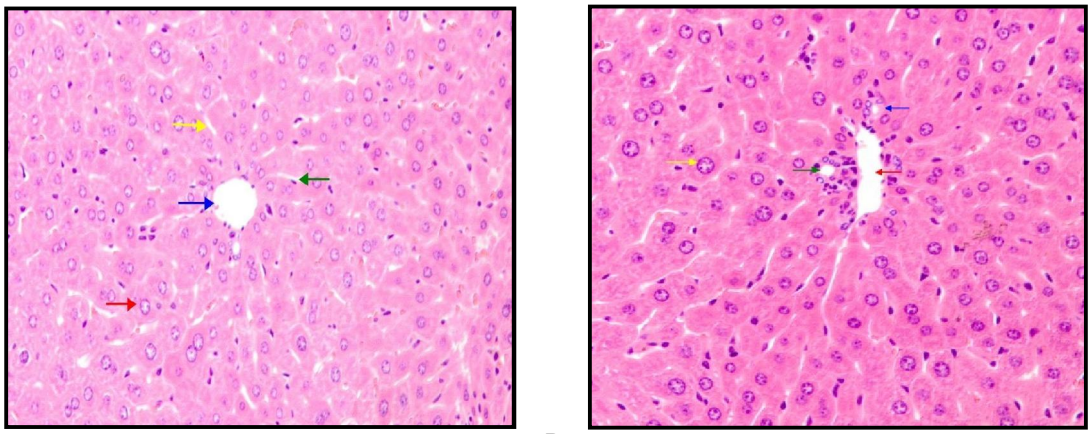


A B

Fig-(1) – control group (Gr1):

A: Transverse section in the liver of a mouse showing normal structure, normal central vein (blue arrow), normal hepatocyte with polygonal shape (red arrow) and normal sinusoidal space (yellow arrow), kupffer cells (green arrow). H+E (x200)

B: Transverse section in the liver of a mouse showing normal portal vein (red arrow), normal hepatic artery (blue arrow) and bile ductile (green arrow). H+E (x200)

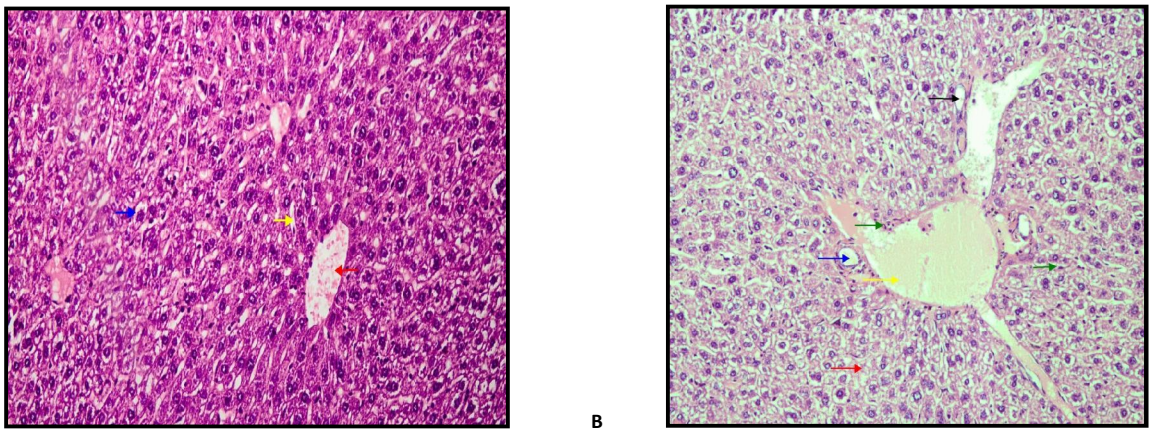


A B

Fig-(2) – lemon fruit extract 10ml/kg (Gr2):

A: Transverse section in the liver of a mouse showing normal structure same as control group. H+E (x200).

B: Transverse section in the liver of a mouse showing normal portal space same as control group. H+E (x200)

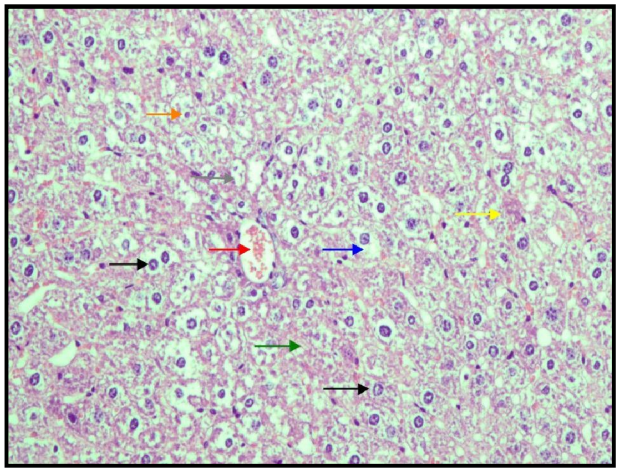


A B

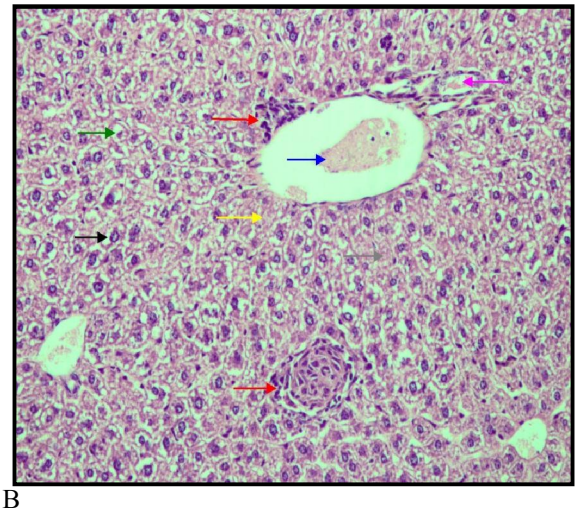
Fig-(3) – cyclophosphamide 10mg/kg group (Gr3):

A: Transverse section in the liver of a mouse showing central vein congestion (red arrow), dilated and congested sinusoidal space (yellow arrow), vacuolar degeneration (blue arrow). H+E (x200)

B: Transverse section in the liver of a mouse showing portal artery congestion (black arrow) and portal vein congestion (yellow arrow), and necrotic hepatocytes (red arrow). Many kupffer cells (green arrow). H+E (x200)

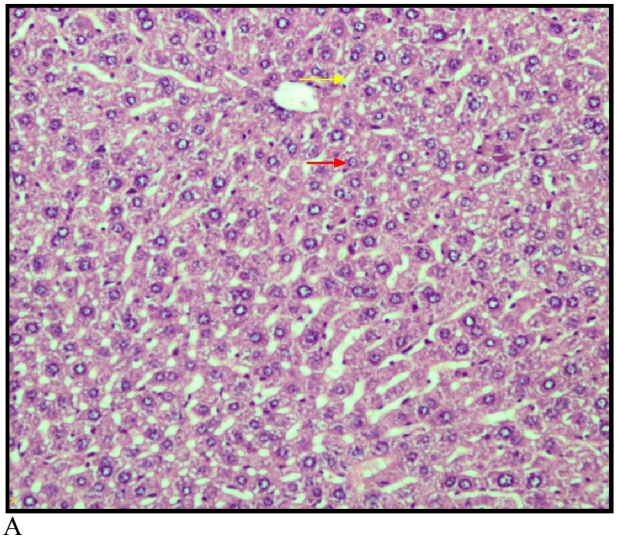


A



B

Fig-(4) – Cyclophosphamide (20mg/kg) group (Gr4):
A: Transverse section in the liver of a mouse showing loss of hepatocytes architecture, central vein congestion (red arrow), vacuolar degeneration (blue arrow), hepatocellular necrosis (green arrow), inflammatory cellular infiltration (yellow arrow), pyknotic nuclei (orang arrow) karyorrhexis nuclei (black arrow), karyolysis nuclei (gray arrow). H+E (x200).
B: Transverse section in the liver of a mouse showing abnormal portal space with moderate - to – sever inflammation (red arrow), portal vein congestion (blue arrow), necrotic hepatocytes (yellow arrow), formation of pyknotic nuclei (green arrow), karyorrhexis nuclei (black arrow), karyolysis nuclei (gray arrow), bile ductile (pink arrow). H+E (x200).



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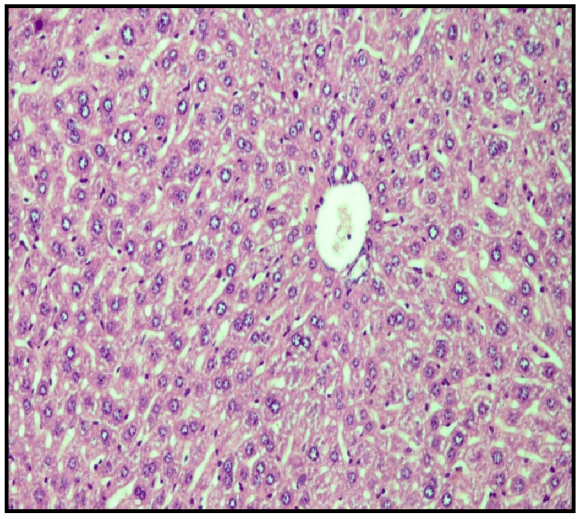
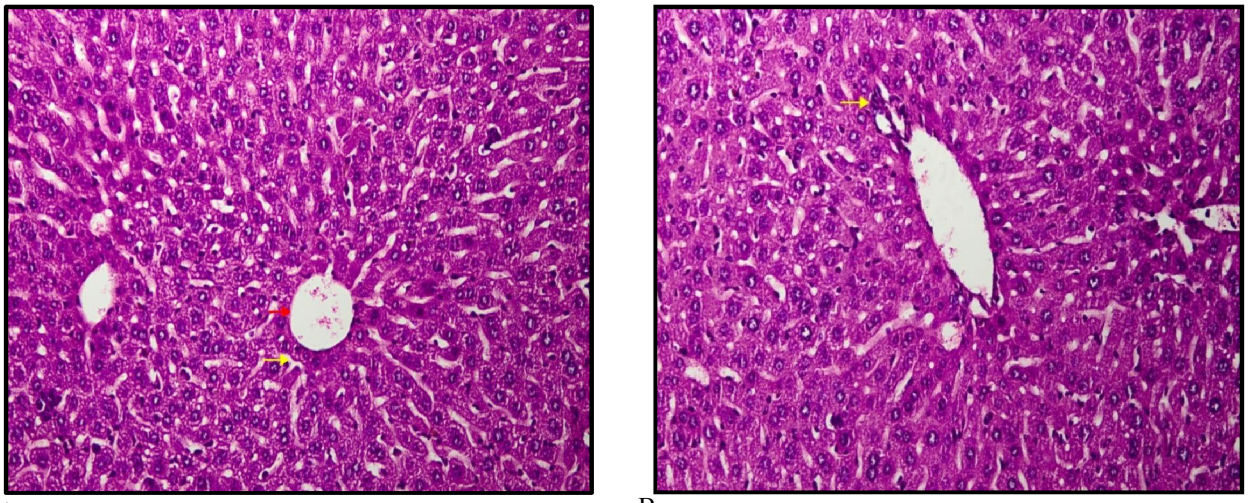


B

Fig-(5) – LFE (10ml/kg) + CP (1omg/kg) group (Gr5):
A: Transverse section in the liver of a mouse showing resorted of hepatocytes architecture, normal hepatocyte (red arrow), disappeared congest sinusoidal space (yellow arrow). H+E (x200).
B: Transverse section in the liver of a mouse showing a normal portal space. H+E (x200).



A B

Fig-(6) – LFF (10ml/kg) + CP (20mg/kg) group (Gr6):
A: Transverse section in the liver of a mouse showing semi normal histological structure, reduced of vacuolar formation, semi congested sinusoidal space (yellow arrow), semi normal central vein (red arrow). H+E (x200).
B: Transverse section in the liver of a mouse showing semi normal portal space with mild – to - moderate inflammation (yellow arrow), reduced of kupffer cells. H+E (x200).

Mollendroff (1973) referred to the formation of cytoplasmic vacuoles within the tissue to cellular defense mechanism against toxic substances to prevent them from interfering with the cellular metabolism. Vacuoles formation in the cytoplasm of hepatocytes represent the beginnings of autolysis.

Kissane (1975) reported that the hepatocytes degeneration could have attributed in the defect of cellular metabolism or inhibition of protein synthesis in hepatocytes. Burkitt *et al.*(1996) reported that disturbance in metabolism and especially in fatty acids metabolism in hepatocytes caused fat accumulation within cells which in turn souble in alcohol used in the prepration of histological section causing the appearance of vacuoles.

On the other hand, Curran and Crocrer (2005) explained the appearance of fatty vacuoles to reduction in the synthesis of low-density lipoproteins, which is responsible for the transport of triglycerides out the liver.

Our results are in agreement with those of Nepomnyashchikh *et al.* (2010) who found that male rats treated with a single dose of CP showed hemodynamic disorders in all animals: unevenly plethoric sinusoids lumen was seen in some cases. This sinusoids continued numerous mononuclear cells (mainly Kupffer cells) and necrobiotic hepatocytes.

Shokrzadeh *et al.* (2014) reported that treatment of male mice with a single dose of CP, caused dilated and congested sinusoidal space, lymphocyte between hepatocytes, small portal space with modrate-to-severe inflammation and necrotic small hepatocyte. CP-induced histological changes in mice liver like fatty infiltration and central vein congestion (Khan *et al*., 2014).

Previous results referred the cellular toxicity to the effect on glutathione (GSH) levels in different organs especially liver, kidney and serum. The glutathione antioxidant system plays a vital role in cellular defense against reactive free radicals and other oxidant species (Meister, 1988).

This was confirmed by Michel *et al.* (1992) who concluded the decrease in glutathione (GSH) level by 20% lead to inhibition in the cell defense mechanism against the toxic drugs which lead to cell injury and death. GHS and its enzymatic system proved to be important in alleviating cellular toxicity (Bompart, 1990).

CP, reduces glutathione levels in the liver of mice and effects the liver enzymes activities such as glutathione peroxidase (GPX), glutathione-s-transfers(GST), Catalase(CAT) and superoxide dismutase (SOD), which play an important role in the defense mechanisms against oxidative stress (Miyake *et al.*,1998, Bhattacharjee *et al*., 2014).

Based on previous results, the damage in liver tissues could be a tribute to the low level of glutathione and oxidative effect of CP. Since, CP produces a high level of active free radicals and reactive oxygen species (ROS) such as hydroxyl radical and hydrogen, superoxide anion peroxide (H_2O_2) in the liver (Stankiewiez *et al.,*2002, Battacharya *et al.,*2003) which destroy the cell

membranes and suppresse the liver's antioxidant defense mechanisms (Cutter, 1992, Miyake *et al*., 1998).

The strong alkylating properties of CP was also reported by Battacharjee *et al.* (2004) and Khan *et al*. (2014). Acrolein can alkylate nucleophilic sites in DNA, RNA and protein like OH2-SH-NH2 and COOH which cause cellular toxicity and genotoxicity effects (Ponticelli and Passerrini, 1991).

The histological examination of concomitant treatment of CP with LFE showed that these treatment could not prevent hepatotoxicity completely. In addition, LFE improved the hepatocytes architecture and reduced the focal inflammation, hepatocytes necrosis, congestion in blood vessels and sinusoids, the cytoplasmic vacuoles, the numbers of lymphocytes and most of hepatocytes and its nuclei appeared normal.

The protective effect of lemon fruit extract against the histological damages caused in the liver by the treatment with cyclophosphamide may be attributed to vital elements of lemon fruit such as flavonoids and phenolic compounds present in LFE which is known as bioflavonoids or vitamin P and hesperidin and eriocitrin.

Erioitrin had potent antioxidative activity than the other citrus flavonoid compounds (Miyake *et al*., 1998). Eriocitrin administration showed protection against oxidative stress in rat livers and scavenged free radicles and prevented the formation of superoxide and hydroperoxide (Minato *et al*., 2003).

The lemon flavonoids eriocitrin and hesperidin may function to increase the concentration of antioxidative enzymes like catalase and glutathione in liver tissue (Miyake *et al*., 1998).

Lemon juice was considered as potent antioxidant as it contains citrate, flavonoids, vitamin E, vitamin C (Miyake *et al*., 1998, Minato *et al.,*2003) and limonids (Yu *et al*., 2005).

As reported, the ability of vitamin C as an anticlastogeneic and antimutageneic agent (Anderson *et al.*, 1995) and as strong antioxidant agent (Rao, 1997) and all the aforementioned vital elements work as scavengers for free radicals and prevent it from destroying cells and tissues.

In conclusion, the finding of our study indicate that cyclophosphamide can adversely damage the liver tissue, while lemon fruit extract co-administration could effectively prevent these adverse effects and protect the liver.

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