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Ameliorative Effect of Fennel Oil on Cyclophosphamide Induced Hepatotoxicity in Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author SAS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SYS and RHB managed the analyses of the study. Author RHB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Cyclophosphamide (CPA) is an alkylating agent widely used as an anticancer and immunosuppressive drug. Fennel (*Foeniculum vulgare Mill)* essential oil is a traditional medicine used against many diseases. The present work studied the effect of fennel oil against liver damage induced by the anticancer drug, cyclophosphamide (CPA) in albino rats. Animals were divided into 4 groups: group1: control, group2: orally given fennel oil (1 ml/kg body weight once a week for six weeks), group3: treated with CPA (15 mg/kg body weight once a week for six weeks) and group4: treated with CPA and fennel oil. The liver removed for histological and immunohistochemical preparation. Blood was collected and sera were prepared for biochemical analysis. The results revealed that CPA caused histological alterations in the liver including degeneration of hepatic cells, cytoplasmic vacuolation, fatty infiltration and congestion of blood vessels. Immunohistochemical observations revealed that both cell proliferation marker Ki67 and apoptotic marker caspase-3 were increased. Biochemical results revealed increase in the liver enzymes

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activity ALT and AST. Treating rats with CPA and fennel oil caused an improvement in the histological structure of the liver and decreased Ki67 and caspase-3. Moreover, ALT and AST activity appeared with normal value. It is concluded that administration of fennel oil exhibited ameliorative effect against CPA-induced hepatic toxicity in albino rats. This effect may be attributed to the antioxidant property of fennel oil.

Keywords: Cyclophosphamide; fennel oil; liver; apoptosis; antioxidants.

1. INTRODUCTION

Cyclophosphamide (CPA) is a nitrogen mustard alkylating agent, an anticancer, anti-neoplastic agents. The main use of cyclophosphamide is with other chemotherapy agents in the treatment of lymphomas, myloma some forms of brain cancer, leukemia, mycosis, neuroblastoma, retinoblastoma, breast cancer [1]. Oral cyclophosphamide is rapidly absorbed and then converted by mixed-function oxidase enzymes (cytochrome P450 system) in the liver to active metabolites [2]. Cardiotoxicity is a major problem with people treated with higher dose regimens [3]. In addition, CPA was found to affect male reproduction. Nicolini et al. [4] showed that the biological actions of cyclophosphamide are dose-dependent. At higher doses, it is associated with increased cytotoxicity and immunosuppression, while at low, continuous doses, it shows immunostimulatory and antiangiogenic properties.

Anton [5] reported that CPA induced many histopathological changes in the liver of mice after a single i.p dose. CPA has been reported to produce genotoxicity and oxidative stress in mice [6] and early lung injury in rats [7].

Medicinal plants contain phytochemicals and numerous chemical compounds, which are used in treatment of different diseases. Fennel plant (*Foeniculum vulgare*) is a medicinal plant belongs to the family Apiaceae (*Umbelliferae*) [8]. This herb is traditionally used as treatment for colic, wind, irritable bowel, kidneys, spleen, liver, lungs, suppressing appetite, breast enlargement, promoting menstruation, improving digestive system, milk flow and increasing urine flow [9].

Essential oils of fennel have hepatoprotective effects [10] as well as anti-inflammatory, and antioxidant activities [11]. Many phytochemical studies have been conducted to study the chemical composition of the essential oil of fennel from different origins and have shown that the major components are phenylpropanoid derivatives and monoterpenoids [12]. The present work investigated the effect of fennel oil on cyclophosphamide-induced hepatic toxicity in albino rats.

2. MATERIALS AND METHODS

2.1 Chemical and Plant Used

2.1.1 Endoxan (cyclophosphamide)

Endoxan was obtained as tablets from Baxter Oncology Halle, Germany.

Each tablet contains 50 mg cyclophosphamide. Endoxan was dissolved in distilled water and orally given by gastric tube at a dose level of 15 mg/kg body weight once a week for six weeks [13].

2.1.2 Fennel oil

Fennel essential oil (FEO) was purchased from a local market at shebin El-Kom, Menufyia Governement (El Masry Everline company). Fennel essential oil was given at a dose level of 1 ml/kg body weight once a week for six weeks [14].

2.2 Experimental Animals

Forty eight healthy adult male albino rats (Rattus norvigicus), three months age weighting 150±10 g were purchased from experimental rat house localized in Helwan. Animals were kept in plastic cages (each contained six animals) in the animal house for two weeks before the experimental work. Animals were kept at 25 ± 2℃ with relative humidity of 50-60% and on 12 h light/ 12 h dark cycle. They received a standard diet composed of 50% barley, 20% yellow corn, 20% dry milk, 10% different vegetables and tap water. The study and all procedures were approved by the Animal Care and Bioethics Committee, Menoufia University, Egypt (Approval No. MNSH175). Animals were divided into four groups.

Group 1 (Control group): Animals of this group (12 rats) were served as control group and were given standard diet and tap water.

Group 2 (Fennel oil group): Animals of this group (12 rats) were orally given fennel oil at a dose level of 1 ml/kg body weight once a week for six weeks.

Group 3 (CPA group): Animals of this group (12 rats) were orally treated with endoxan at a dose of 15 mg/kg body weight once a week for six weeks. 6 animals were selected after 3 weeks and another 6 were selected after 6 weeks.

Group 4 (CPA+Fennel oil group): Animals were given endoxan and then after two hours they were given fennel oil, with the same doses of group 2 and 3.

2.3 Histological Studies

For histological study, liver immediately removed after 3 and 6 weeks, and fixed in 10% formalin for 24 hours. Specimens were dehydrated in ascending series of ethyl alcohol, cleared in two changes of xylene, infiltrated in three changes of molten paraffin (melting point 58-60℃) and then embedded in molten paraffin blocks. Paraffin sections (5 micron thickness) were sectioned using a rotary microtome and mounted on clean glass slides. Sections were stained with Ehrlich's hematoxylin and counter stained with eosin for histological examination.

2.4 Immunohistochemical Studies

For Immunostaining methods of ki67 and Caspase-3, slides were deparaffinized in xylene and rehydrated in a series of graded alcohol concentrations. Then rinsed in phosphate-buffered saline (PBS) containing 0.1% tween-20. Antigen retrival was performed by placing slides in sodium citrate solution (PH 6.0) at 90℃. Avidin (0.001% in PBS) and biotin (0.001% in PBS) were blocked in each section by using Avidin/biotin blocking solutions, where sections were incubated and rinsed with PBS between steps. Sections were incubated with monoclonal primary rat antibodies (Neo Markers, Cat.#Ms-113-P, Fremont, CA,USA), at appropriate dilution (1:200) in antibody diluent, directed against rat Ki-67 or Caspase (each antibody was used separately to react on different slides) at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated in peroxidase blocking solution (3%H_2O_2 in PBS) at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated in biotinylated secondary antibody in PBS at room temperature. Slides were washed in PBS-Tween 20. For detection, sections were incubated in horse radish peroxidase (HRP)-streptavidin solution at room temperature. Slides were washed in PBS-Tween 20. Sections were incubated in peroxidase substrate solution "3,3-diaminobenzidine tetrahydrochloride (DAP)" until adequate color was developed. Slides were washed in PBS-Tween 20. Sections were counterstained with hematoxylin, dehydrated through garded alcohol series, clear in xylene and mounted with DPX [15].

2.5 Biochemical Analysis

For biochemical analysis, blood samples were collected in clean centrifuge tubes. Blood samples left to clot in room temperature and then serum separated by centrifugation at 3000 rpm for 20 minutes. The collected serum stored at -18 -20℃ until analysis. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated according to the method of Reitman and Frankel [16].

2.6 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). The significance of differences means was evaluated by using independent sample t test. All statistical analysis was performed using SPSS statistical version 16 software package.

3. RESULTS

3.1 Histological Observations

The liver of control animal is formed of hepatic lobules which are made up of radiating plates, cords, or strands of cell forming a network around central vein. The strands are alternating with narrow blood sinusoids also radically extending along the liver lobule. The sinusoids are narrow blood space with irregular boundaries composed essentially of only a single layer of fenestrated endothelial cells in addition to large irregularly shaped cells of the mono nuclear type (The von kuffer cells) which are known to be activity phagocytic cells seen in (Fig. 1-A). The bile ductules appeared rounded or oblonged in shape according to the plane of sectioning. It is

bounded by a layer of cuboidal cells encircled by a thin sheath of connective tissue. The portal vein is wide in size, being either empty or containing a few blood cells, whereas the hepatic artery branches are much narrower and are devoid of any blood cells (Fig. 1-B). Liver of animals treated once a week with fennel oil for three and six weeks showed nearly normal histological structure (Fig. 1-C).

Liver of animals examined after three weeks of treatment with CPA exhibited a distinct histological change when compared with control group. After three weeks of treatment with cyclophosphamide the liver lost the arrangement of the cells. In addition, central veins were congested with blood (Fig. 2-A), and after six weeks of treatment with cyclophosphamide the hepatic cells were damaged and lost their characteristic appearance, the hepatocytes showed cytoplasmic vacuolation and congested blood vessels (Fig. 2-B). Leucocytic infiltration (Fig. 2-C), and fatty infiltration was observed (Fig. 2-D).

Liver of rats treated with CPA and fennel oil for 3 weeks showed an improvement in the histological appearance of the liver with few leucocytic infiltration (Fig. 3A). After 6 weeks of treatment with cyclophosphamide and fennel oil, the histological picture of the liver appeared better than liver of animals treated only with cyclophosphamide. The hepatocytic tissue appeared normal and hepatocyte were restored the normal appearance with normal cytoplasm and nuclei (Fig. 3-B).

3.2 Immunohistochemical Results

3.2.1 The expression of Ki-67

In control group, Ki-67 was expressed in the nuclei of the hepatocytes as brown color. On the other hand, the negative nuclei of the hepatocytes are stained blue with hematoxylin (Fig. 4-A). Animals given fennel oil for six weeks showed expression of Ki-67 in the nuclei of the hepatocytes nearly similar to control group (Fig. 4-B). After six weeks of treatment with CPA an increase in expression of Ki-67 immunoreactivity were observed in most of the nuclei of the hepatocytes (Fig. 4-C). Liver sections obtained from rats treated with CPA followed by fennel oil for six weeks, showed a decrease in expression of Ki-67 immunoreactivity in the nuclei of hepatocytes (Fig. 4-D).

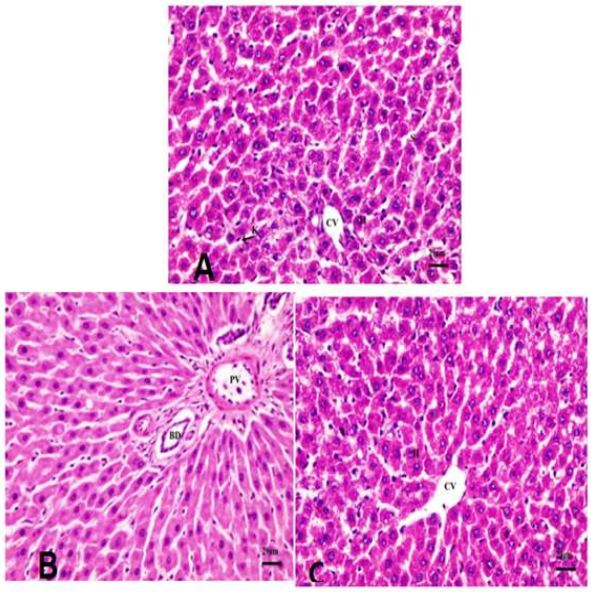

Fig. 1. A) A photomicrograph obtained from central area of liver of a control rat showing central vein (CV), hepatocyte (H), kuffer cells (K) and sinusoid (S), (H&E). B) A photomicrograph obtained from portal area of liver of a control rat showing portal vein (PV) and bile duct (BD), (H&E). C) A Photomicrograph obtained from liver of a rat treated with fennel oil for six weeks showing normal structure of hepatocyte (H) and central vein (CV), (H&E). D) A Photomicrograph obtained from liver of a rat treated with CPA for three weeks showing congested and enlarged central vein, (H&E)

The results in Fig. 5 showed the percentage area of Ki-67 positive nuclei of the hepatocytes in the different experimental animals after six weeks. The percentage area of Ki-67 positive nuclei of the hepatocytes showed a significant increase ($P<0.05$) in rats treated with CPA when compared with control groups. Treatment of rats with CPA followed by fennel oil for six weeks, resulted in a significant decrease of Ki-67 positive nuclei of the hepatocytes when compared with CPA groups.

3.2.2 The expression of caspase-3

In control group, caspase-3 was expressed in cytoplasm of few hepatic cells as brown color. The cytoplasm of hepatic cells stained weakly (Fig. 6-A). Animals given fennel oil for six weeks showed expression of caspase-3 in cytoplasm of hepatocytes nearly similar to control group (Fig. 6-B). After six weeks of treatment with CPA an increase in expression of caspase-3 immunoreactivity was observed in of cytoplasm of most of hepatic cells (Fig. 6-C). Liver sections obtained from rats treated with CPA followed by fennel oil for six weeks, showed a decrease in expression of caspase-3 immunoreactivity in the cytoplasm of hepatic cells (Fig. 6-D). The data in Fig. 7 showed the percentage area of caspase-3 expression in cytoplasm of hepatic cells in the different experimental animals after six weeks. The obtained data showed there were no significant differences between control groups and fennel oil groups. The percentage area of caspase-3 expression in cytoplasm of hepatic cells showed a significant increase ($P<0.05$) in rats treated with CPA for six weeks when compared with control groups. Treating rats with CPA followed by fennel oil for six weeks, resulted in a significant decrease of caspase-3 expression in cytoplasm of hepatic cells when compared with CPA groups.

3.3 Biochemical Results

The change in serum ALT and AST activity is seen in Figs. 8 & 9. Treating rats with fennel oil showed non-significant difference in serum ALT and AST activity in compared with animals of control group in all treatment periods. On the other hand, there was a significant increase in serum ALT and AST activity in animals treated with CPA for three and six weeks. Animals treated with CPA and fennel oil for three and six weeks revealed a significant decrease in ALT and AST activity in comparison with CPA group.

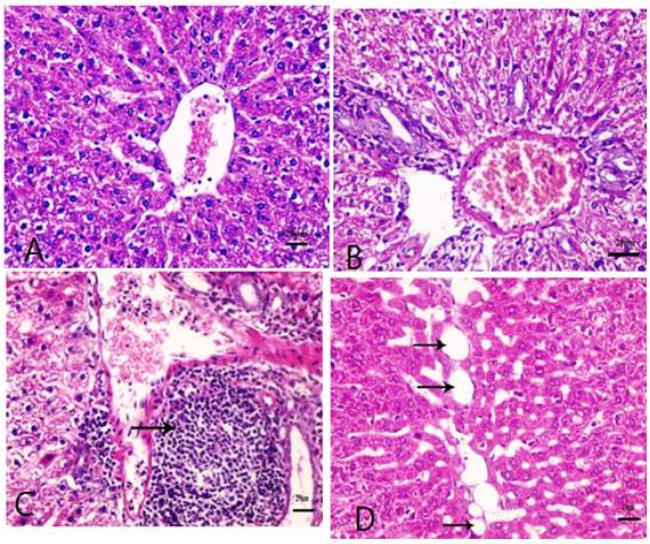

Fig. 2. A) A Photomicrograph obtained from liver of a rat treated with CPA for three weeks showing congested and enlarged central vein, (H&E). B) A Photomicrograph obtained from liver of a rat treated with CPA for six weeks showing congested blood vessel and cytoplasmic vaculation, (H&E). C) A Photomicrograph obtained from liver of a rat treated with CPA for six weeks showing leucocytic infiltration (arrow) and congested blood vessels, (H&E). D) A Photomicrograph obtained from liver of a rat treated with CPA for six weeks showing fatty infiltration (arrows), (H&E)

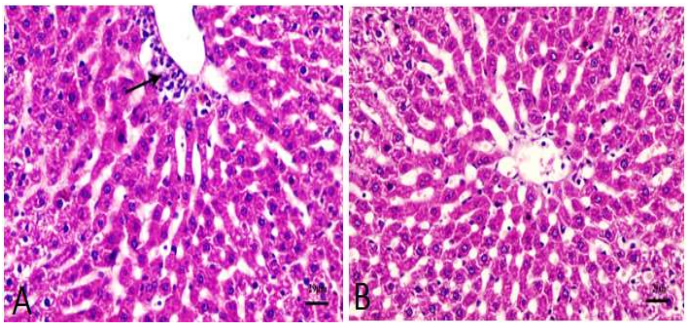

Fig. 3. A) A Photomicrograph obtained from liver of a rat treated with CPA followed by fennel oil for three weeks showing few leucocytic infiltrations (arrow),(H&E). B) A Photomicrograph obtained from liver of a rat treated with CPA followed by fennel oil for six weeks showing restoration of normal appearance of cytoplasm and nuclei in hepatocyte, (H&E)

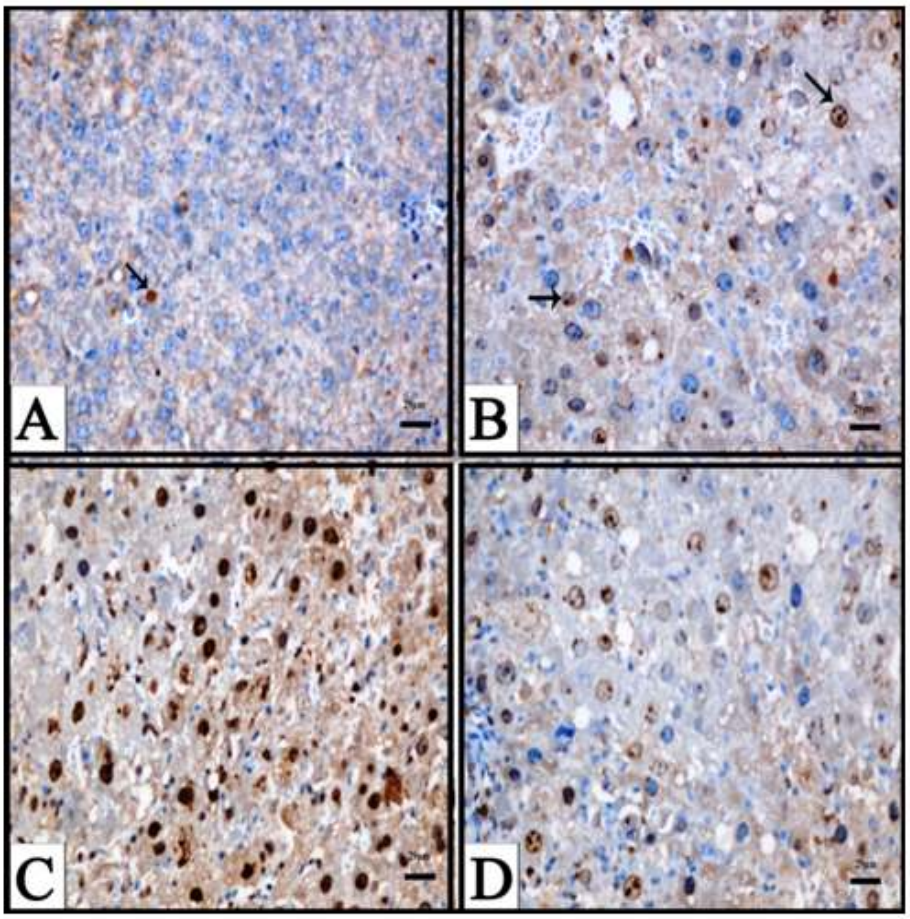

Fig. 4. A) A Photomicrograph obtained from liver of a control rat showing expression of Ki-67 in nuclei of hepatocytes as brown color. B) A Photomicrograph obtained from liver of a rat treated with fennel oil for six weeks showing normal expression of Ki-67. C) A Photomicrograph obtained from liver of a rat treated with CPA for six weeks showed an increase in expression of Ki-67 in most nuclei of hepatocytes. D) A Photomicrograph obtained from liver of a rat treated with CPA followed by fennel oil showed a decrease in expression of Ki-67 in the nuclei of hepatocytes, (Ki-67 immunostaning, counter stained with hematoxylin)

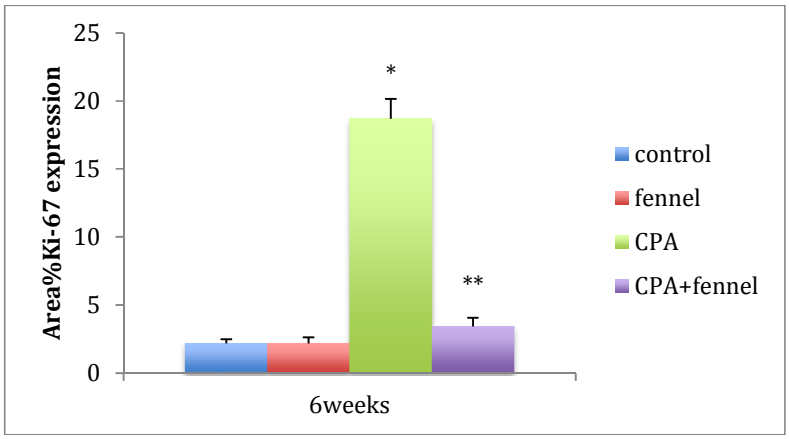

Fig. 5. The percentage area (Mean area% ±D.S) of Ki-67 expression in rat liver of different experimental groups

(*): significant at P < 0.05 compared with control group; (**): significant compared with CP group

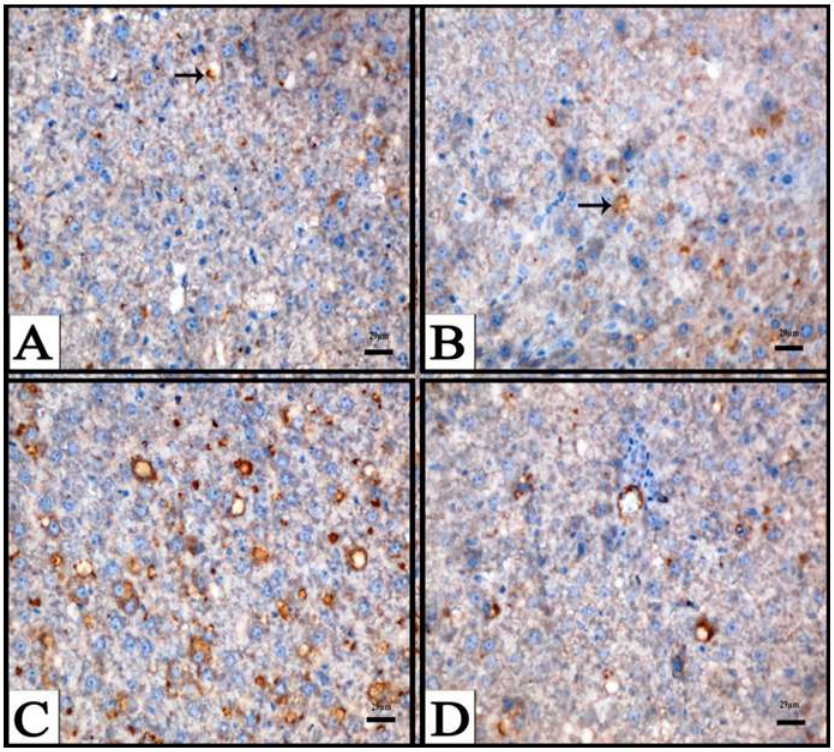

Fig. 6. A) A Photomicrograph obtained from liver of a control rat showing expression of caspase-3 in cytoplasm of hepatocytes as brown color (arrow), (caspase-3 immunostaning, counter stained with hematoxylin). B) A Photomicrograph obtained from liver of a rat treated with fennel oil for six weeks showing expression of caspase-3 in cytoplasm of the hepatocytes (arrow), (caspase-3 immunostaining, counter stained with hematoxylin). C) A Photomicrograph obtained from liver of a rat treated with CPA for six weeks showed an increase in expression of caspase-3 in most cytoplasm of hepatocytes, (caspase-3 immunostaining, counter stained with hematoxylin). D) A Photomicrograph obtained from liver of a rat treated with CPA followed by fennel oil for six weeks showed a decrease in expression of caspase-3 in the cytoplasm of hepatocytes, (caspase-3 immunostaning, counter stained with hematoxylin)

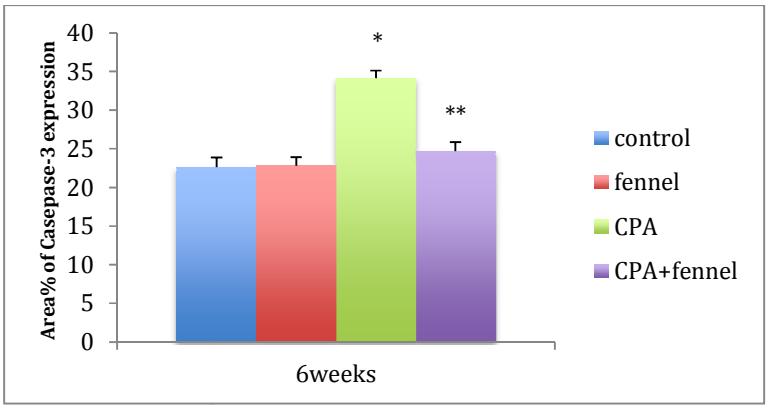

Fig. 7. The percentage area (Mean area% ±(D.S) of Caspase-3 expression in rat liver of different experimental groups

(*): significant at P < 0.05 compared with control group; (**): Significant compared with CPA group

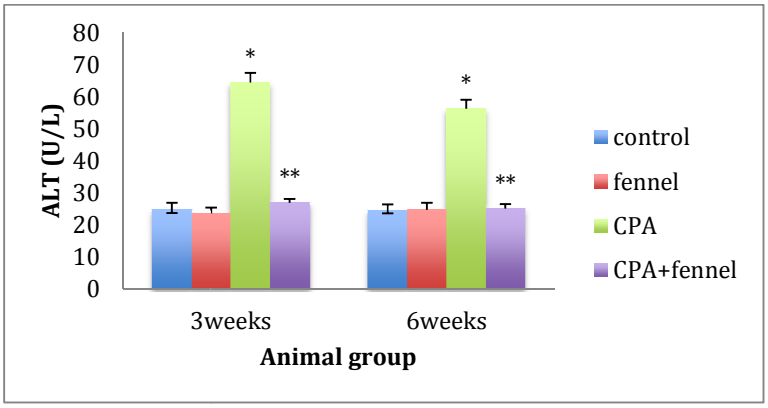

Fig. 8. Effect of different treatments on serum ALT activity (U/L) after three and six weeks of treatment

(*): significant at P < 0.05 compared with control group; (**): significant compared with CPA group

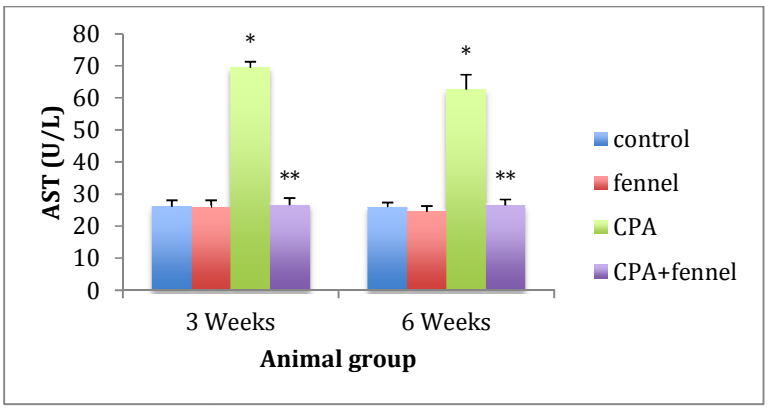

Fig. 9. Effect of different treatments on serum AST activity (U/L) after three and six weeks of treatment

(*): significant at P < 0.05 compared with control group; (**): significant compared with CPA group

4. DISCUSSION AND CONCLUSION

Cyclophosphamide is an anticancer drug used for treatment of several neoplasms. On the other hand it showed several adverse effects including reproductive and hepatic toxicity in humans and experimental animals. CPA itself is devoid of alkylating activity and must first undergo bioactivation by hepatic microsomal cytochrome P450 mixed function oxidase system [17,18]. The main alkylating metabolite, phosphoramide mustard, is responsible for the therapeutic activity. However, another metabolite, acrolein, causes the inactivation of microsomal enzymes and results in increased ROS generation and lipid peroxidation in several tissues [19]. The bioactivation of CPA in hepatocytes targets liver to its primary cytotoxic attack, resulting in CPA-induced hepatoxicity and decreased liver weight [20]. Consuming CPA often follows anorexia, nausea and vomiting. Its major side effects include reduced blood cells, elevated concentrations of uric acid, decreased gonad function, causing amenorrhea, azoospermia, and oligospermia [21,22].

In the present study, results revealed that treating rats with CPA induced many histological alterations in the liver and these alterations were more prominent in animals treated for six weeks. Histological alterations in the liver include leucocytic infiltration, congestion of blood vessels and cytoplasmic vacuolation of the hepatocytes. Similarly, Sakr et al. [23] reported that CPA caused many histopathological alterations in the liver of mice included leucocytic infiltrations, congestion of blood vessels, dilation of sinusoids, cytoplasmic vacuolization of the hepatocytes, activation of Kupffer cells, and apoptosis. Gustafsson et al. [24] attributed this liver injury to accumulation of high level of the hepatotoxic 4-hydroxylate cyclophosphamide metabolite. The hepatic effect of CPA was observed in different animals by many investigators [25-27]. Zhang et al. [28] indicated that activation of CPA by hepatic cytochrome p450, yielding cytotoxic nitrogen mustards capable of reacting with DNA molecules to form cross links and lead to cell apoptosis and/or necrosis.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are liver enzymes and they have the function of transferring the amino group from alpha amino acids to alpha-keto acids. A large amount of ALT and AST is released in the blood mostly during liver cell damage [29]. Thus, detection of the serum level of ALT and AST allows monitoring liver cell damage. Significant increase in ALT and AST levels of sera of CPA treated rats was recorded in the present study. The same results were also observed after CPA treatment in albino rats [30]. Sondhi and Gupta [31] reported that treatment of white Zealand rabbits with CPA for five days revealed a significant increase in serum ALT and AST levels.

Immunohistochemical observations revealed increase in expression of both Ki67 and caspase-3. Antigen KI-67 is a nuclear protein that is associated with cellular proliferation. Furthermore, it is associated with ribosomal RNA transcription [32]. Inactivation of antigen KI-67 leads to inhibition of ribosomal RNA synthesis [33]. Caspase-3 is a marker of the early phase of apoptosis [34] and is essential for certain processes associated with the formation of apoptotic bodies [35]. Similar to our results Sultan and Ayman [36] reported that CPA treated rats showed significant increase in expression of the apoptotic marker casepase-3 in liver.

It was reported that CPA-induced toxicity is due to its oxidative stress. In this concern, Selvakumar et al. [37] reported that CPA treatment resulted in elevated MDA levels because of the excessive generation of free radicals. Manda and Bhatia, [38] reported that fifteen days oral administration of CPA induced depletion in the levels of glutathione peroxidase, catalase and super oxide dismutase. Reduction of these enzymes was also recorded in various tissues as a result of CPA treatment [39,40].

The current study showed that treating rats with CPA followed by fennel oil revealed nearly normal appearance of hepatic tissues with a decrease in the activity of ALT and AST. In agreement with these results, Sheweita et al. [41] reported that essential oils extracted from fennel alleviated hepatotoxicity of CPA in mice through assessment of hepatotoxicity biomarkers (AST, ALT, ALP) and histopathology of liver tissue. Özbek et al. [42] showed that oil extracted from F. vulgare has a protective effect against the toxicity induced by carbon tetrachloride in rat livers. Al-Amoudi [43] reported that fennel oil ameliorated valporic acid-induced histological alterations in liver and kidney of rats. Sameeh et al. [44] reported that treating rats with chlorpyrifos with conjugation with fennel oil caused a statistically significant decrease in ALT and AST levels when compared with chloropyrifos groups. Animals treated with CPA

and fennel oil showed a decrease in expression of Ki-67 and Caspase-3 in liver. These results indicated the antiproliferative and antiapoptotic effects of fennel oil.

Fennel extracts including essential oil showed antioxidant activity. Mohamad et al. [45] demonstrated that fennel oil acts like antioxidants due to its ability to inhibit lipid peroxidation. Fennel essential oil has physiologic antioxidant activities including the radical scavenging effect, inhibition of hydrogen peroxides H_2O_2 and Fe chelating activities where it can minimize free radical which initiate the chain reactions of lipid peroxidation [46].

It has been reported that fennel oil contains different compounds including trans-anethole (1-methoxy-4-(1- propenyl) benzene or para-propenylanisole), fenchone and estragole [47]. It is concluded that administration of fennel oil exhibited ameliorative effects against CPA-induced hepatotoxicity in male rats. This effect of fennel oil might be due to induction of antioxidant defense systems by one or more of its constituents.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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