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Original Research Article

Fenugreek down-regulating caspase-3 and VEGF expression as a therapeutic agent in acetaminophen-induced hepatotoxicity in male rats

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Abstract

People especially youth are used to consume overdoses from pain relief drugs to kill pain. Acetaminophen (AC) that has the commercial name Panadol is the more famous drug. AC overdose (750 mg/kg) can cause hepatotoxicity with oxidative stress as one of the possible mechanisms mediating the event. The present study aimed to explore the therapeutic effects of 200 mg/kg of fenugreek (FEN) on AC-induced hepatotoxicity, for seven consecutive days. Treatment with FEN prevented the AC-induced hepatotoxicity, by increased protein, albumin, and decrease liver function levels. These biochemical analyses authenticated with the histological improvement in the hepatic tissue. The improvement of hepatic histological picture come in accordance with the reducing expression of caspase-3 and vascular endothelial growth factor (VEGF) in hepatic cytoplasm when compared with AC-treated animals. In conclusion, FEN has a protective role against AC-induced hepatotoxicity by enabling hepatocyte regeneration and reducing inflammatory activity.

Keywords: Acetaminophen, Antioxidant, Fenugreek, Hepatotoxicity, Oxidative stress

INTRODUCTION

Acetaminophen (AC) or acetaminophen toxicity is one of the major causes of poisoning worldwide (Gunnell et al., 2000), and its overdose is commonly associated with hepatic damages (Nelson, 1995) AC toxicity is mediated by the activity of its reactive metabolite known as N-acetyl-p-benzoquinoneimine (NAPQI), which is detoxified by intracellular glutathione (GSH) (Borne, 1995). Therefore, an overdose of AC will saturate the conjugation pathways of GSH and cause depletion of cellular GSH. This subsequently led to the reduction capacity of GSH to detoxify NAPQI. Increased level of NAPQI mediates oxidative damage, and thus enhances cellular injury and organ dysfunction, including renal damage (Hart et al., 1994). Experimental studies suggested that AC-induced hepatorenal injury is mediated by oxidative damage (Das et al., 2010; Kheradpezhouh et al., 2010). Therefore, the alternative treatments for AC toxicity could be achieved using a natural compound with antioxidant activity.

Fenugreek (FEN) leaves of the Leguminosae family; are consumed widely in African, European, and Asian countries as a leafy vegetable or as beverage; and it's scientific name is *Trigonella foenum*-Graceum. FEN is a rich source of calcium, iron, β- carotene, thiamine, niacin, vitamin C and vitamin K (Sharma, 1986). FEN seeds rich in polyphenolic flavonoids and contain lysine and L-tryptophan rich proteins, mucilaginous fibre, coumarin, fenugreekine, nicotinic acid, phytic acid, scopoletin, trigonelline, and other rare chemical constituents such as saponins. Saponins are a large group which composed of

diosgenin, yamogenin, gitogenin, tigogenin, sapogenins, and neotigogens, which are thought to account for many of its significant therapeutic effects (Billaud and Adrian, 2001; Ibrahim, 2015). FEN is used worldwide as a culinary spice as well as a medicinal herb to soothe the stomach and help maintain blood sugar levels. The seeds are rich in protein (20-30%) and contain about 50% fiber, 25% soothing mucilage, 6-10% lipid, 44-59% carbohydrate (Kaviarasan et al., 2007; Das, 2014). In comparison with other legumes, fenugreek seeds contain higher ratios of minerals such as Ca, P, Fe, Zn and Mn as demonstrated by Mishkinsky et al. (1974).

Apoptosis is a controlled physiological cell death process that occurs as a result of normal cellular differentiation and development. Defects in apoptosis contribute for many diseases, including cancer, autoimmune and neurodegenerative disorders. This process is mediated by a complex mechanism involving intracellular proteases, the caspases, activators and inhibitors of these cell death proteases (Reed, 2000; Persad et al., 2004). Vascular endothelial growth factor (VEGF) is an inducer of angiogenesis, which is a hallmark of various ischemic and inflammatory diseases. It has been reported that macrophages and lymphocytes stimulate angiogenesis via the release of angiogenic factors, including VEGF (Reinders et al., 2003; Zhang et al., 2014). With respect to mechanism of action, VEGF is endothelial cell-specific; it stimulates endothelial proliferation, increases vascular permeability and changes the gene expression of endothelial cells (Kraft et al., 1999).

The present study designed to elucidate the potential protective effects of FEN against AC-induced hepatotoxicity. The effects were determined by measuring the levels of serum AST, ALT, and ALP (indicator of liver function), total protein and albumin (indicator for protein profile), and by hepatic histopathological, and by immuno-histochemical studies of caspase-3 (apoptosis marker) and VEGF (inflammation marker).

MATERIAL AND METHODS

Animals and experimental doses

Male *Rattus rattus* rats (110±10 g) were obtained from the Egyptian Organization for Vaccine and Biological Preparations, Helwan, Egypt; and used in the present experiment. Twenty-eight animals were housed in solid plastic cages with a wire mesh lid, 4 rats/rectangular cage. They were housed in a controlled room temperature and a 12 h light/dark cycle. Animals were fed mouse pellet and fresh water *ad libitum* for a week prior to experiments. Rats were randomly divided into four groups containing 7 animals each and all treatments were given daily for seven days. AC and FEN were administered orally. Rats in group I served as the control group and were administered distilled water only. Rats belonging to group II received 1000 mg/kg FEN, according to Joshi et al. (2015). Rats of group III received 750 mg/kg AC, according to Sathishkumar and Baskar (2014). In Group IV, rats were treated with 750 mg/kg AC and 1000 mg/kg of FEN. On Day 8, all animals were anaesthetized with sodium phenobarbital to minimize suffering and then scarified. All animal procedures are in accordance with the recommendations of the Canadian committee for care and use of animals (Canadian Council on Animal Care [CCAC], 1993).

Source of Drugs

Fenugreek tablets (610 mg) were purchased from General Nutrition Corporation (GNC), USA. Acetaminophen tablets (500 mg) were purchased from GlaxoSmithKline (GSK) Company, United Kingdom.

Biochemical Investigation

Blood was collected by heart puncture when sacrificed, allowed to clot, and then sera were separated and stored at 20°C for spectrophotometrically measurement of serum markers of liver injury. Activities of AST, ALT and ALP were determined according to Reitman and Frankel (1957) and Bowers and McComb (1966). The values were expressed as units/liter (U/l). Total protein was determined according to the method of Peters (1968) and albumin was determined by the method of Doumas and Giggs (1971). Total protein and albumin values expresses as g/dl.

Histological and Electron Microscope Preparations

Pieces of liver were immediately removed after sacrifice, rinsed with normal saline, fixed in 10% formalin solution, dehydrated in ascending series of alcohol, cleared in xylene and finally embedded in paraffin wax. Sections of 4µm thickness were cut using rotary microtome and mounted on clean slides, subsequently stained with hematoxylin and eosin (H-E), and observed under a light microscope, according to the method of Bancroft and Gamble (2002). For electron microscopic studies, fresh small pieces of liver were fixed in 3% glutaraldhyde-formalhyde for 5h then in (0.2 M) Na cacodylate for 2h at 4°C, then washed in phosphate buffer pH 7.2 for 30min and post fixed in 1% osmic acid (2% OsO_4+ 0.3 M of Na cacodylate) for 2h at 4°C. Then tissue pieces washed in phosphate buffer (pH 7.2) for 30min at 4°C. Samples were dehydrated through ascending grades of ethanol and embedded in epoxy resin in an oven at 60°C for 14h to produce a firm block. Semithin sections were stained

Table 1. Biochemical analysis of liver function and protein profile in control and experimental groups

Groups	C	FEN	AC	AC+FEN
AST (u/L)	58.41 ± 0.58	54.66 ± 0.23	173.30 ± 5.93^{abd}	67.91 ± 2.60^{c}
ALT (u/L)	50.99 ± 0.32	48.54 ± 0.65	178.41 ± 3.76^{abd}	55.21 ± 0.54^{c}
ALP (u/L)	62.04 ± 0.74	61.06 ± 1.20	155.51 ± 1.62^{abd}	64.89 ± 1.64^{c}
Total protein (g/dl)	7.24 ± 0.35	7.32 ± 0.40	5.79 ± 0.30^{ab}	6.75 ± 0.20^{c}
Albumin (g/dl)	4.68 ± 0.26	4.90 ± 0.30	3.51 ± 0.20^{ab}	5.22 ± 0.30^{c}

Values are mean±SE. Superscript letters denote the significance at ($p<0.05$); a: Significant to C, b: significance to FEN, c: Significant to AC, and d: significant to AC+FEN

with toluidine blue stain. Specimens prepared in Faculty of Science, Ain Shams University.

Immunohistochemical studies

Immunohistochemistry is the process of localizing proteins in tissues by exploiting the principle of antibodies binding specifically to antigens. The visualization of the antibody is commonly accomplished by conjugating an enzyme to the antibody. This can produce a color changing reaction. The advantage of this method is the ability to show exactly where a given protein is located. The expression of caspase3 (orb153764), and VEGF (bs-1957R) from Biocompare Company, South San Francisco, USA) in liver sections was determined immunohistochemically in formalin-fixed, paraffin-embedded tissue. Blocks were cut into 4 mm thick sections mounted on glass slides, and incubated at 4°C overnight. Sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. To improve the quality of staining, microwave oven-based antigen retrieval was performed. Slides were probed with either anti-caspase-3 (1:100), or anti-VEGF (1:200). Sections were washed 3 times with PBS for 10 min each and incubated with biotin-labeled anti-mouse IgG for 1 h at room temperature. After washing, sections were then stained with a 4-chloro-1-naphthol, which gives a blue-grey color according to the manufactured instructions. Positive control slides showed no staining when the primary antibody was omitted.

Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA), SPSS version (16) was used for comparison between groups followed by post-hoc Duncan test. Data were expressed as mean ± SE and $P<0.05$ showed statistically significance.

RESULTS

Liver Function and Protein Profile Analysis

Table (1) showed the effect of FEN on serum liver functions in AC-induced hepatotoxic rats for 7 days. AC administration caused liver dysfunction which revealed by the significant increase in AST, ALT, and ALP levels that recorded 173.3, 178.41, and 155.51 respectively. FEN administration inhibited these elevations which reached 67.91, 55.21, and 64.89 for AST, ALT, and ALP respectively. AC administration caused inhibition in total protein (5.79) and albumin (3.51) serum levels which restored as a result of FEN administration; returning near to the normal values as reported in table (1).

Histopathological Observations

Sections of liver from rat control and rat treated with FEN revealed normal lobular architecture with normal hepatic cells radiating from the central vein. Hepatocytes appeared with well-defined nuclei and preserved cytoplasm (Figure 1 and 2). AC induced marked histopathological lesion which was characterized by hyaline material filed portal vein, fibroblasts distribution, and hydropic change (Figure 3), central vein stuffed with RBCs, apoptotic nuclei, activated kupffer cells and vacuolated blood sinusoids (Figure 4), hypertrophies with fibroses arterial wall, and hyperplasia giant bile duct (Figure 5). Hepatic tissue from AC+FEN treated rats revealed histological improvement appeared as radial arrangement of hepatocytes (Figure 6).

Ultrastructure Studies

Histological investigation of the semi-thin liver sections from control and FEN treated rats showed normal histological pattern with radiating hepatocytes (Figure 7 and 8). Liver sections from rat treated with AC revealed dilated congested central vein, some hepato-cytes appeared without nuclei, and degenerative area as

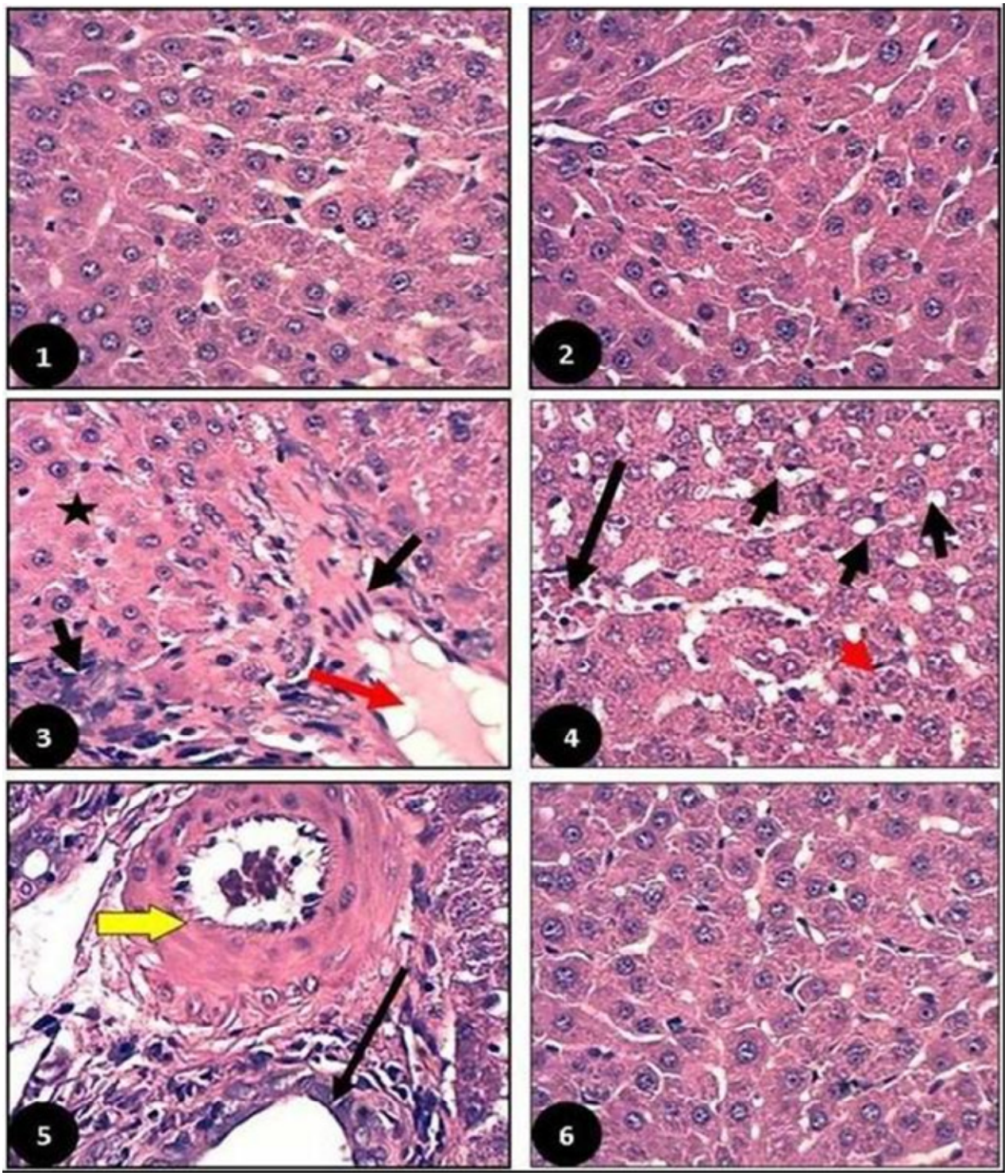

Figure 1. Photomicrograph of normal liver section from control rat showing the radiating cords of hepatocytes (H-E, X400).
Figure 2. Photomicrograph of positive control liver section from FEN administered rat showing radiating normal cords of hepatocytes and abundant blood sinusoids (H-E, X400).
Figure 3. Photomicrograph liver section from AC administered rat showing hyaline material filed portal vein (red arrow), fibroblasts around (black arrows), and hydropic change (star), (H-E, X400).
Figure 4. Photomicrograph liver section from AC administered rat showing central vein stuffed with RBCs (long arrow), apoptotic cells (red head) activated kupffer cells and vacuolated blood sinusoids (heads), (H-E, X400).
Figure 5. Photomicrograph liver section from AC administered rat showing hypertrophies with fibroses arterial wall (yellow arrow), and hyperplasia giant bile duct (long arrow), (H-E , X400).
Figure 6. Photomicrograph liver section from AC+ FEN administered rat showing relatively normal reforms of hepatic tissues (H-E, X400).

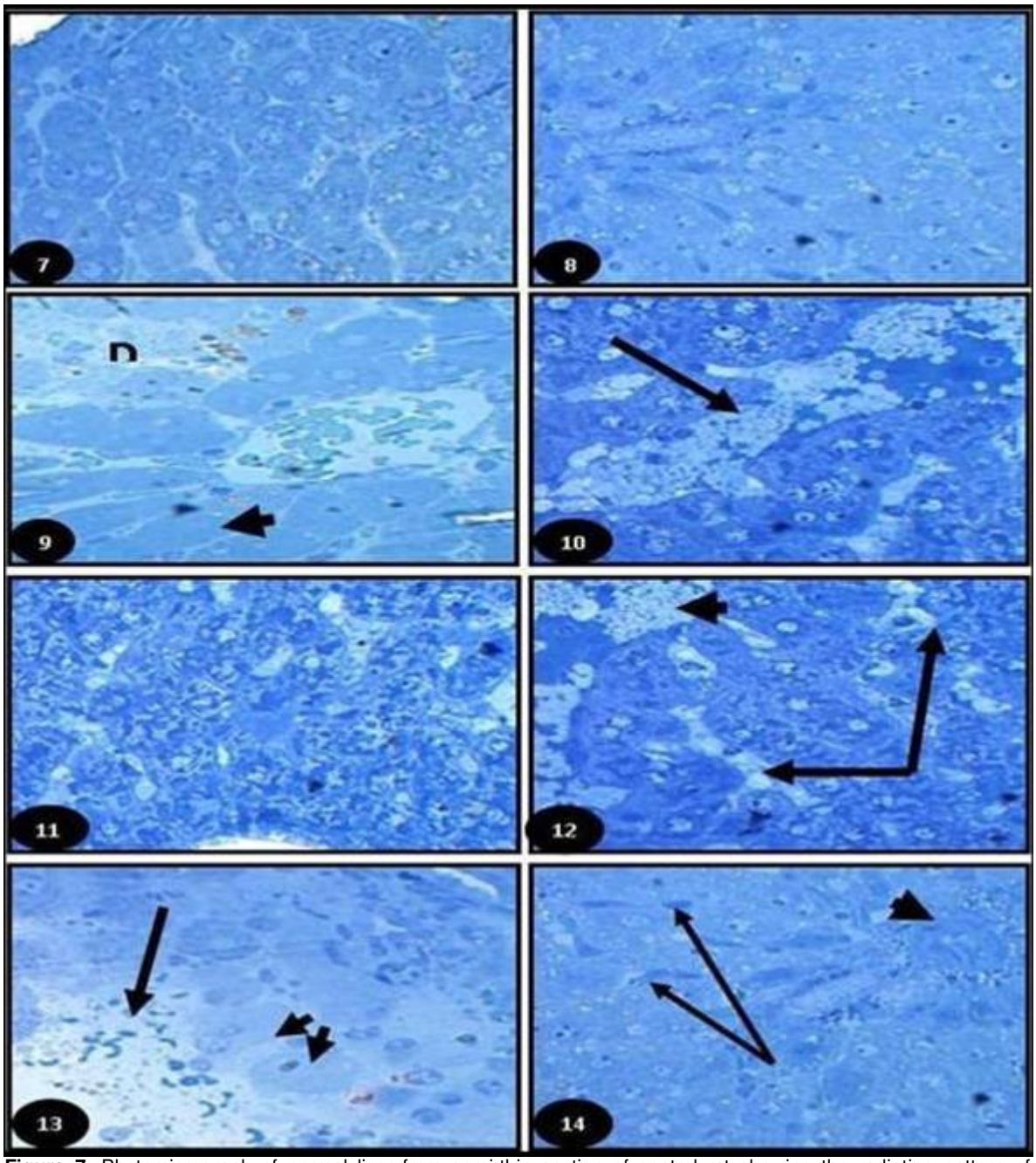

Figure 7. Photomicrograph of normal liver from semi-thin section of control rat showing the radiating pattern of hepatocytes with large nucleus. (Toluidine blue, X400)

Figure 8. Photomicrograph of positive liver from semi-thin section of **FEN** administered rat showing the radiating normal arrangements hepatocytes, (Toluidine blue, X400)

Figure 9. Photomicrograph liver section from **AC** administered rat illustrating, dilated congested central vein, some hepatocytes appeared without nuclei (head arrows), and degenerative area (D). (Toluidine blue, X400).

Figure 10. Photomicrograph liver section from **AC** administered rat showing dilated portal space filled with hyaline material (arrow) (Toluidine blue, X400).

Figure 11. Photomicrograph liver section from **AC** administered rat manifesting loss of normal arrangement of hepatic tissues, with apoptotic nuclei all over hepatoctes. (Toluidine blue, X400).

Figure 12. Photomicrograph liver section from **AC** administered rat showing dilated blood sinusoid (arrows), vacuolated cytoplasm and accumulate fat vacuoles (head arrows) ,(Toluidine blue, X400).

Figure 13. Photomicrograph liver section from **AC** administered rat showing necrotic area filled with RBCs (arrow) and loss of hepatocyts integrity (head arrows), (Toluidine blue, X400).

Figure 14. Photomicrograph of positive control of liver from **AC+ FEN**, administered rat showing relatively normal hepatocytes with blocked central vein (head arrows) and hemorrhaged blood sinusoids (arrows), (Toluidine blue, X400).

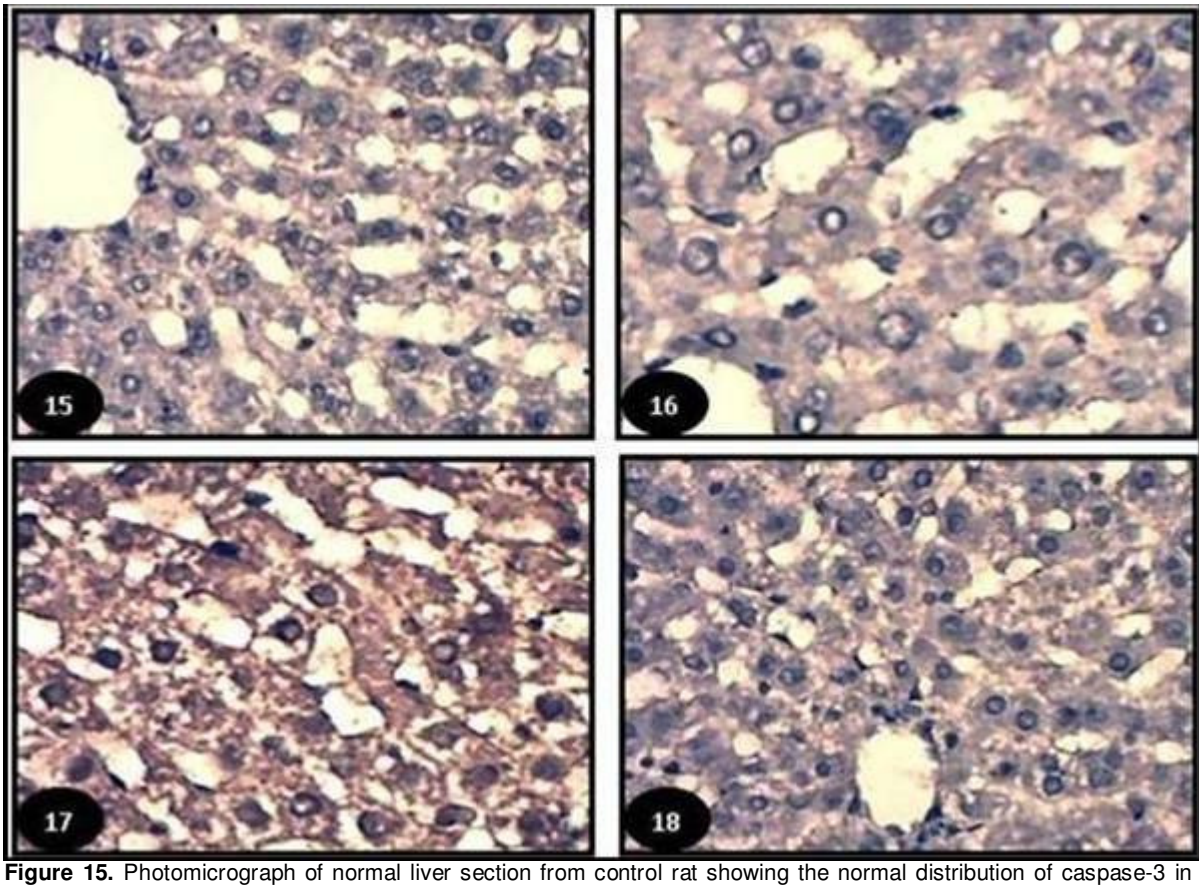

Figure 15. Photomicrograph of normal liver section from control rat showing the normal distribution of caspase-3 in the cytoplasm of hepatocytes (Caspase-3 immunostaining, x400).
Figure 16. Photomicrograph of control liver from **FEN** administered rat showing the normal distribution of caspase-3 in the cytoplasm of hepatocytes (Caspase-3 Immunostaining, X400).
Figure 17. Photomicrograph liver section from **AC** administered rat showing increase in caspase-3 distribution in the hepatic condensed chromatin of the nuclei which showing strong positive immunoreaction (Caspase-3 immunostaining, x400).
Figure 18. Photomicrograph of positive control of liver from **AC+FEN** administered rat showing relatively near to normal distribution of caspase-3 in hepatic tissue (Caspase-3 immunostaining, x400).

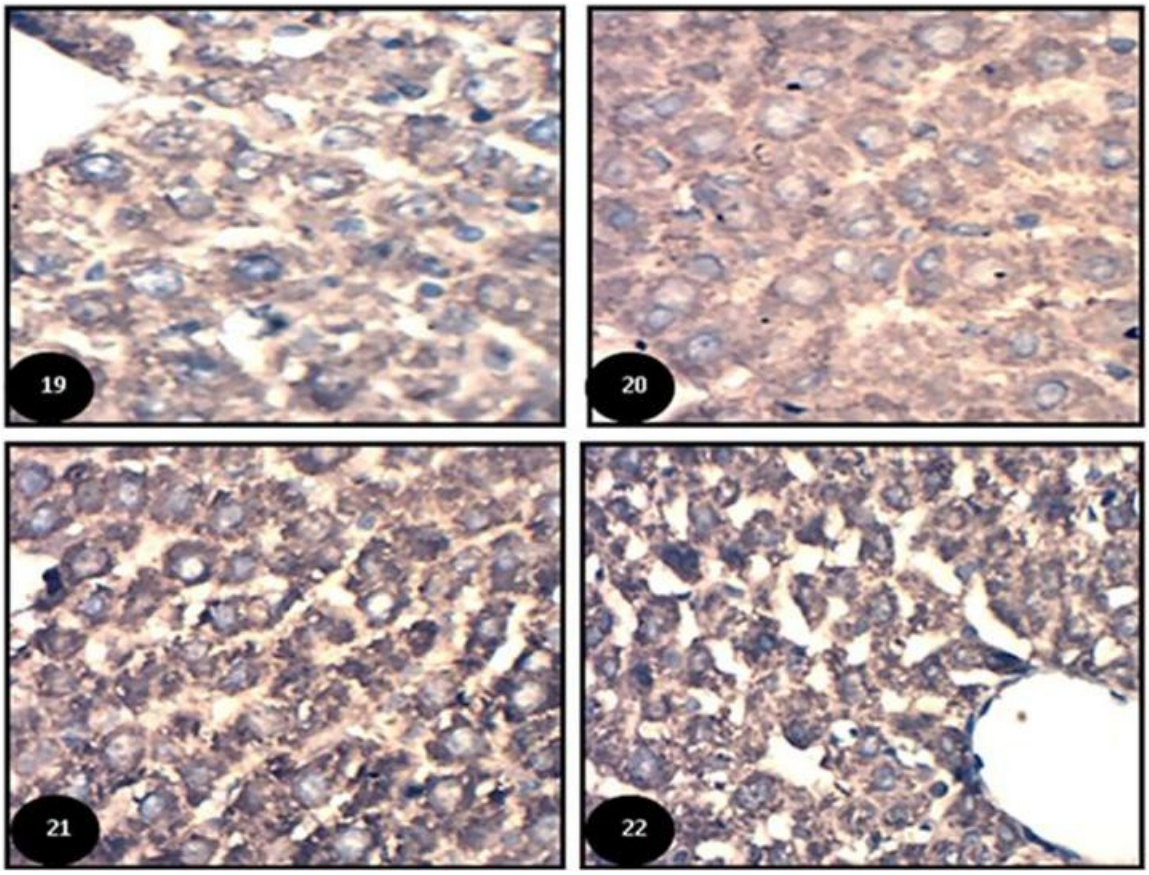

Figure 19. Photomicrograph of control liver section from control showing the normal distribution of VEGF in hepatocytes (VEGF immunostaining, x400)
Figure 20. Photomicrograph of positive control liver from **FEN** administered rat showing the relatively normal distribution of VEGF in hepatocytes (VEGF immunostaining, x400)
Figure 21. Photomicrograph liver section from **AC** administered rat showing intense staining for VEGF in hepatocytes cytoplasm (VEGF immunostaining, x400).
Figure 22. Photomicrograph of liver section from **AC+ FEN** administered rat showing relatively near to normal distribution of VEGF in hepatic cells (VEGF immunostaining, x400)

appeared in Figure 9, dilated portal space filled with hyaline material (Figure 10), loss of normal arrangement of hepatic tissues, with apoptotic nuclei all over hepatoctes (Figure 11), dilated blood sinusoid, vacuolated cytoplasm and accumulate fat vacuoles (Figure 12), necrotic area filled with RBCs and loss of hepatocyts integrity (Figure 13). Liver sections from rats treated with AC+FEN showed relatively normal hepatocytes with blocked central vein and hemorrhaged blood sinusoids (Figure 14).

Immunohistochemical Investigation

Caspase-3 expression

Sections of liver from control and FEN treated rats stained immunohistochemically for caspase-3 delineated the normal distribution in the cytoplasm of hepatocytes (Figure 15 and 16). On the other hand section in liver from rats received AC, showing increase in caspase-3 distribution in the hepatic condensed chromatin nuclei which showed strong positive immunoreaction (Figure 17). Liver sections of rats treated with AC+FEN showed normal distribution of caspase-3 in hepatic tissue (Figure 18).

VEGF expression

Sections of liver from control and FEN treated rats stained for VEGF revealed normal distribution in hepatocytes (Figure 19 and 20). An intense staining for VEGF was observed in hepatocytes cytoplasm (Figure 21) as a result of AC administration. VEGF immunolabeling was restricted to hepatocytes and was undetectable in non-parenchymal cells, including bile duct epithelial cells and myofibroblasts. Sections from rat liver treated with AC+FEN revealed near to normal distribution of VEGF in hepatic cells (Figure 22).

DISCUSSION

The liver is one of the vital organs of the animal body and plays a central role in transforming and clearing the chemicals, but it is susceptible to toxicity from other agents. Certain medicinal agents, like AC, when taken in overdoses or sometimes even within therapeutic ranges, may damage the liver. AC an over the counter drug is used as antipyretic and analgesic which can lead to hepatic failure (Yahya et al., 2013). Liver function enzymes (AST, ALTand ALP) increased significantly; while, total protein and albumin reported low levels in rats treated with AC. These increases of liver enzymes and decrease in total protein and albumin are indication of the hepatotoxic effect of AC. High doses of AC caused significant inhibition in the antioxidant activity; such as catalase and peroxidase that are responsible for the elimination of ROS, by catalyzing the conversion of hydrogen peroxides to water. The decreased activity of these antioxidants may lead to increased use of superoxides and hydrogen peroxides, which in fact leads to the production of hydroxyl radicals causing tissue damage (Kinalski et al., 2000; Madkour and Abdel-Daim, 2013).

Decrease in total serum protein may be associated with the decrease in the number of hepatocytes, which in turn may result in the decreased hepatic capacity to synthesize protein and consequently decrease liver weight (Anantha et al., 2012). It is well established that AC induces hepatotoxicity by metabolic activation; therefore it selectively causes injury to hepatocytes maintaining semi-normal metabolic function (Yahya et al., 2013). In therapeutic dose AC is converted by drug metabolizing enzyme to water soluble metabolites and secreted in the urine (Hinson et al., 2010). Saturated and excess AC is oxidatively metabolized by hepatic P-450 system to a toxic metabolite namely NAPQI. This is normally detoxified by GSH with both oxidative scavenger and redox regulation capacities (Chen et al., 2009). Normally GSH is a major antioxidant system and a crucial component of host defense which is responsible for scavenging reactive free radicals to prevent liver injury (Yahya et al., 2013). The toxic dose of AC caused the GSH depletion which results in the accumulation of NAPQI and subsequently then covalently binds to the cystinyl sulfhydryl groups of cellular proteins results in the generation of reactive oxygen species (ROS) hydrogen peroxide, superoxide anion and hydroxyl ion (Subramanian et al., 2013). The cell membrane is affected, induce lipid peroxidation and also cause hepatic necrosis.

FEN treatment showed a negative effect on this increase restoring liver function efficiency. This data come in accordance with (Mannangatti and Neelamegachari, 2015) who reported that extract of FEN leaves caused decreased levels reached near to normal of liver function enzymes. Mohammadzadeh et al. (2015) revealed that treatment of 1000 mg/kg FEN to AC-treated rats prevented the increase in the amount of hydrogen peroxide and MDA and the decrease in catalase and peroxidase activities in the liver of rats. This may led to the improving of liver function and protein profile by restoring normal hepatic physiology. The restoration of total protein and albumin might be attributed to the antioxidant properties of FEN which improves organ functions (Khalil, 2004). Flavonoid content of FEN may be responsible for the antioxidant enzyme activation and subsequent scavenging of free radical species (Belguith-Hadriche et al., 2010; Sakr and Abo-El-Yazid, 2012).

Histopathological examination of rat liver treated with AC showed significant hepatotoxicity; this come in accordance with Anantha et al. (2012) who reported hypertrophy and necrosis of hepatocytes and shrinkage of the central veins. There was extensive infiltration of the lymphocytes and Kupffer cells around the central vein and loss of cellular boundaries. Liver tissue of rats treated with AC revealed congestion of the portal vein with mild to moderate periportal area infiltration with some inflammatory cells mainly macrophages and lymphocytes (Madkour and Abdel-Daim, 2013).

Although remarkably safe of AC at therapeutic doses, excessive amounts cause centrilobular hepatic necrosis, leading to acute liver failure. The hepatotoxicity of paracetamol has been attributed to the formation of a highly reactive toxic electrophile, NAPQI by cytochrome P-450 (Madkour and Abdel-Daim, 2013). NAPQI is initially detoxified by conjugation with reduced glutathione (GSH) and excretion in urine (Vermeulen et al., 1992). When the rate of NAPQI formation exceeds the rate of detoxification by GSH, it oxidises tissue macromolecules such as lipid or -SH group of proteins. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death.

The present study reported an improvement in the histological picture of the hepatic tissue as a result of FEN administration, that alleviate the toxic damage of AC. Zargar (2014) showed that the FEN hydroalcoholic extract partially reduces liver cirrhosis in rats by inhibiting the activity of certain enzymes, mainly xanthine oxidase, and scavenging free radicals. The pharmacologically active principles present in extract may act by inhibiting the activity of some specific enzymes and/or ameliorating oxidative stress. The improvement of histological picture as a result of FEN treatment may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This come in accordance with Khalil (2004); Eidi et al. (2007); Sakr and Abo-El-Yazid (2012); Das (2014) who reported that the restoring of serum levels of transaminases coincidence with the healing of hepatic parenchyma and the regeneration of hepatocytes due to FEN treatment.

The present investigation reported the presence of apoptotic nuclei as appeared in the histological pictures of liver as a result of AC administration; that come in

accordance with the increase of caspase-3 expression in the hepatic tissue. Apoptosis is characterized morphologically by cell shrinkage, cytoplasmic membrane blebbing, nuclear fragmentation and condensation, and internucleosomal DNA cleavage. In liver, apoptosis plays a prominent part in the pathogenesis of toxic liver injury due to a variety of agents, viral hepatitis, and hepatocellular carcinomas (Kanzler and Galle, 2000). AC can also cause alterations in membrane permeability transition and release pro-apoptotic factors into the cytosol with the breakdown of mitochondrial membrane potential (MMP). These are accompanied by caspase-3, caspase-8, and caspase-9 activation, DNA fragmentation and apoptotic/necrotic cell death (Boulares et al., 2002; Cover et al., 2005; Kon et al., 2004; Yuan et al., 2010; Ramachandran et al., 2011). A concept has emerged that mitochondrial dysfunction and damage is a central event responsible for liver injury caused by AC.

In the present study the expression of VEGF increased in hepatic tissue cells from AC treated rats, which mediate immuno-inflammatory responses and angiogenesis, VEGF has a specific role in inflammation (Zhang et al., 2014). The inflammation-promoting effect of VEGF occurs mainly in the initial stages of the inflammatory cascade rather secondary to the T lymphocyte-mediated activation reaction, suggesting that VEGF is locally produced immediately (Conti et al. 2007). Fallsehr et al. (2005) delineated that VEGF action on endothelial cells and macrophages activated nuclear factor κB, which thereafter induced the synthesis of inflammatory cytokines and chemokines.

However, this is the first study showed that FEN administration reversed AC-induced elevation of caspase-3 and VEGF expression. This may be related to FEN antioxidant and stabilizing activities.

CONCLUSION

The present investigation delineated that histopathological, and immunohistochemical damage of the hepatic tissue; is attributed to the administration of overdoses of AC. Therefore, medical care must be conducted by doctors who must provide awareness through workshops and health bulletins to prevent misuse of AC. This will lead to maintain human health. To our knowledge, this is the first investigation, which has assessed the association between FEN administration and caspase-3 and VEGF expression in the hepatic tissue. Present data concluded that the immunomodulatory mechanism of action of FEN in inhibition caspase-3 expression to enable the hepatocyte to regenerate; and in decrease of VEGF expression to alleviate inflammation due to AC administration. These results may open up a new way for the treatment of liver diseases, by adding FEN to our daily diets.

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