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Effect of Barley, Green Tea and Doxorubicin against N-dimethylnitrosamine Induced Hepatorenal Toxicity in Rats

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ABSTRACT

Background: Hepatorenal toxicity is the development of renal dysfunction with liver problems. Objectives: The aim of the present study is to investigate the effects of the green tea, barley and doxorubicin alone or their combination on some changes produced by experimentally induced hepatorenal toxicity by N-dimethylnitrosamine (DMN) in rats. Methods: Hepatorenal toxicity was induced in rats by administration of DMN (0.25 mg/day/rat) orally daily for 6 weeks. Group1: given saline orally and served as normal control. Group2: received DMN for 6 weeks and served as hepatorenal toxicity control. Group3-5: given green tea extract (1 g/ kg) and barley extract (625 mg/ kg), orally for 4 weeks. Group 6-8: all rats given DMN for 6 weeks then treated with green tea, barley, doxorubicin or the combination of green tea, barley and doxorubicin for 4 weeks. Results: DMN provoked kidney and liver apoptosis as evidenced by elevation of kidney and liver functions, decrease in Ca serum level, elevation of tumor marker alpha-fetoprotein (AFP), ferritin and lactate dehydrogenase (LDH). DMN group had higher kidney nitric oxide (NO) and malondialdehyde (MDA) contents and lower superoxide dismutase (SOD), as well as it induced inflammation, apoptosis and caspase-3 expression. Green, barley and their combination improved the biochemical and histopathological changes induced by DMN. Conclusion: This study indicates the therapeutic effect of green tea, barley against DMN-induced kidney and liver apoptosis. Interestingly, our results exhibited that doxorubicin conjugated with green tea and barley increased renohepatoprotection activity and may decrease systemic toxicity of doxorubicin when administered in high dose.

Key words: Hepatorenal toxicity, Alpha-fetoprotein, Ferritin, Lactate dehydrogenase, Caspase-3, Rat.

Introduction

Hepatocellular apoptosis, a cardinal feature of liver diseases, characterized by liver fibrogenesis (Canbay *et al*.,2004) while Renal apoptosis plays role in an active mode of cell death. Although apoptosis is responsible for inflammatory and immune cells regulation, fibroblast numbers, and vascular homeostasis, it induces kidney injury, especially parenchymal renal cell and tubular cells (Gines *et al*., 1993). Several insults provoke renal apoptosis, including ischemia, toxic chemicals, radiation, and ureteral obstruction (Zhou *et al*., 2010). Excessive apoptosis cause atrophy, fibrosis and organ dysfunction (Hung and Chow, 2004).

Dimethylnitrosamine (DMN) may be released in discharges of some industrial processes as rubber manufacturing, leather tanning, pesticide manufacturing, food processing and dye manufacturing (Atsdr,1989).DMN may also be formed during the treatment of drinking-water (Pancholy, 1978).

DMN is a potent hepatotoxin and mutagen (Haggerty and Holsapple, 1990). It has been used as a model hepatotoxin and liver injury (Pritchard and Butler, 1989). DMN alkylates numerous cellular components including DNA, RNA, protein, and phospholipids in most organs, inducing its toxicity to yield reactive electophiles leading to hepatocyte apoptosis (Horn *et al*., 2000) and DNA damage that was directly related to cell death (Ray *et al*., 2004). DMN is metabolized in the proximal tubules by cytochrome P-450 monooxygenase (Towbin *et al*., 1979). So liver and kidney are the primary target organs for DMN-induced carcinogenesis (Khodos and Ray, 2009).

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Green tea has health benefits for multiple disorders that attracted attention, in the scientific and in consumer communities. Green tea"natural secret for a healthier life" is obtained from the plant Camellia sinensis (Theaceae family) by minimal oxidation during processing. The main constituent found in green tea is Epigallocatechin-3-gallate (EGCG). This component is responsible anticancer, antioxidant, antidiabetic, antiobesity, antihypertensive activity (Rani *et al*., 2014). Salama *et al*. (2014) showed protective effects of green tea aganist acute renal failure. Besides, EGCG prevents the ethanol induced hepatotoxicity and inhibits the development of the fatty liver and renal failure (Yun *et al*., 2007).

Barley (*Hordeum vulgare* L.) belongs to the grass family, Poaceae (Gramineae). It is the fourth most important cereal crop after wheat, maize and rice and is among the top ten crop plants in the world (Akar *et al*., 2012). Fastnaught et at., (1996) reported that the insoluble fiber found in barley helps the regulation of bowl function and also may help decreasing the risk for cancers; in addition, barley contains antioxidants, that reduce the rate of oxidative damage by scavenging free radicals. Brennan and Cleary (2005) demonstrated that soluble fiber, such as β-glucan, may be beneficial in regulating sugar, insulin, and cholesterol responses to foods. Barley inhibits hydrogen peroxide (H2O2) by provoking oxidative damage to DNA and apoptosis (Jeong *et al*., 2009). Barley also has hepatoprotective and hypolipidemic effects (Belal, 2011).

Therefore, this study aimed to investigate the effects of green tea, barley and their combination with doxorubicin against DMN-induced hepatorenal toxicity in rats targeting oxidative stress and apoptotic events that could be a mainstay of therapy

Materials and Methods

Animals:

Male wister rats (100-120 g) were used. Animals were housed for at least one week in the laboratory room prior to testing adaptation. They were kept under standard housing conditions (room temperature 24-27° C) with alternating 12 hours light and dark cycles and were allowed free access to food and water ad libitum. Animals received human care in compliance with the guidelines of the animal care and use committee of National Research Centre, Egypt. Experiments were performed according to the National Regulation of Animal Welfare and Institutional Animal Ethical Committee.

Chemicals:

N-Dimethylnitrosamine was purchased from Sigma Aldrish.

Plants:

70% alcoholic extract of Green tea leaves and70% alcoholic extract of Barley seeds were obtained from horticulture department of the ministry of agriculture and the plants were identified by Mrs. Terase Labib Taxonomist of orman garden in Giza. Egypt.

Drugs:

Doxorubicin was purchased from Sigma.

Experimental design:

Rats were randomly divided into 8 groups each of 8 rats. Hepatorenal toxicity was induced by oral administration of DMN (0.25 mg/ day/rat) for 6 weeks and treatment was carried out as follows: The first group received only saline orally and served as normal group. The second group received DMN orally daily for 6 weeks and served as positive control (Nagaase *et al*., 1983).The third group received green tea extract (1 g/ kg) orally for 4weeks (Roomi *et al*.,2005).The fourth group received barley extract (625 mg/ kg) orally for 4weeks (Hong and Jai Maeng, 2004).The fifth, sixth & seventh groups received DMN then the treatment by green tea extract and barley orally as well as Doxorubicin (2mg/kg) intraperitoneal for 4weeks (Haas *et al*.,2002).The eighth group received DMN then the treatment by combination of green tea, barley and Doxorubicin for 4 weeks. At the end of the experiment animals were blood samples and sacrificed were collected.

Methods:

Preparation of blood sample and tissue homogenate:

Blood samples were withdrawn from the retro-orbital vein of each animal, under light anesthesia by diethyl ether, according to the method of (Cocchetto and Bjornsson, 1983). Blood was allowed to coagulate and then centrifuged at 3000 rpm for 15min. immediately after blood sampling, animals were sacrificed by cervical dislocation and the kidneys and liver were rapidly removed, washed in ice-cooled saline, plotted dry and weighed. One kidney was homogenized, using a homogenizer (Medical instruments, MPW-120, Poland), with ice-cooled saline to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5 min. at 4°C in a cooling centrifuge to remove cell debris (Laborzentrifugen, 2k15, Sigma, Germany).

Biochemical markers:

Creatinine, uric acid and urea levels were determined in serum according to (Bowers and Wong, 1980), (Barham and Trinder, 1972) and (Batton and Crouch, 1977). Aspartate amino transferase (AST), Alanine amino transeferase (ALT) and Calcium levels were determined in serum using Biodiagnostic kits, Egypt. Alpha-Fetoprotein (AFP), Ferritin and lactate dehydrogenase (LDH) levels were measured in serum according to White *et al*., (1986), Abelev, (1974) and Dito, (1979). Tissue and nitric oxide (NO), malondialdehyde (MDA) and superoxide dismutase (SOD) contents were determined using Biodiagnostic kits, Egypt.

Histopathological studies:

Other kidney and liver specimens were separated from the animal immediately after killing and opened along convex side to ensure complete fixation. All the specimens were fixed in 10% formal saline for at least 72 h then washed with water and processed to obtain 4-6 Mm section. They were stained with hematoxilin-eosin for histopathological examination (Drury and Wallington, 1980).

Immunohistochemical analysis

Immunohistochemical staining of anti-caspase-3 antibodies was performed by streptoavidin–biotin. Fourmicrometer thick sections were deparaffinized and incubated with fresh 0.3 % hydrogen peroxide in methanol for 30 min at room temperature. The specimens were then incubated with anti-caspase-3 antibody as the primer antibody at a 1:100 dilution. The specimens were counterstained with H&E. Negative controls were prepared by substituting normal mouse serum for each primary antibody.

Statistical analysis:

Data were expressed as mean ± S.E. Analysis was done using ANOVA followed by the LSD test for multiple comparisons. Difference was considered significant at 0.05 level of probability using Graph pad prism program.

Results and Discussion

Effects of treatment with green tea, barley and doxorubicin and their combination on serum kidney function level in DMN-induced hepatorenal toxicity in rats

Induction of hepatorenal toxicity by DMN produced a significant increase in serum creatinine, urea and uric acid levels to 131.38%, 202% and 520.51%, respectively after 6 weeks of induction, as compared with normal control group. Treatment with green tea and barley did not affect serum creatinine, urea and uric acid levels in normal rats as compared with normal control group (Table 1).

Treatment with green tea or barley for 4 weeks after induction of hepatorenal toxicity significantly decreased serum creatinine levels to 69.07% and 78.08%, respectively; decreased serum urea levels to 73.88% and 67.51%, respectively and decreased serum uric acid levels to 74.51% and 64.61%, respectively as compared to DMN treated group (Table 1).

Treatment with doxorubicin alone and the combination of barley, green tea and doxorubicin for 4 weeks after induction of hepatorenal toxicity significantly decreased serum creatinine levels to 87.63% and 79.61%, respectively; decreased serum urea levels to 89.24% and 68.99% respectively; and decreased serum uric acid to 99.59% and 73.76%, respectively as compared to DMN treated group (Table 1).

Table 1: Effects of 4 weeks treatment with barley, green tea, doxorubicin and their combination on kidney function in normal and DMN-induced hepatorenal toxicity on rats

Parameter Groups	Creatinine (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)
Control negative saline	4.43 ± 0.11	22 ± 3.6	7.41 ± 0.48
DMN (0.25 mg/day/ml)	5.82 ± 0.1 *	44.44 ± 0.14*	38.57 ± 0.21*
Green tea(1 g/Kg)	4.16 ± 0.079@	21.95± 0.27@	7.21 ± 0.57 @
Barley(625 mg/Kg)	4.14 ± 0.15 @	22.35± 1.05 @	7.29 ± 0.12 @
DMN(0.25 mg/day/ml) + Green Tea(1 g/Kg)	4.02± 0.19 @	34.70 ± 0.5 *@	28.74 ± 0.22 *@
DMN (0.25 mg/day/ml) + Barley(625 mg/Kg)	4.3 ± 0.14@	30± 0.39 *@	24.92 ± 2.88*@
DMN(0.25 mg/day/ml) +Doxorubicin(2mg/kg)	5.1 ± 0.112 *@	39.66± 0.39 *@	38.41 ± 0.16 *
DMN+ Doxorubicin (2 mg/kg) +Green tea 1g/Kg) + Barley (625 mg/Kg)	4.06 ± 0.06 @	34.50± 1.99 *@	28.33 ± 0.26 *@

DMN: N-dimethylnitrosamine
*Hepatorenal toxicity was induced by administration of N-dimethylnitrosamine (DMN) (0.25 mg/ day/rat) orally for 6 weeks. Data were expressed as mean ± SE (n=8). Statistical analysis was carried out by one-way ANOVA followed by LSD multiple comparisons test.*Significance from normal control (saline) at P< 0.05. @Significance from DMN control at P<0.05.*

Effects of treatment with green tea, barley and doxorubicin and their combination on serum liver function and calcium levels in DMN-induced hepatorenal toxicity in rats

Induction of hepatorenal toxicity by DMN produced a significant increase in serum levels of AST and ALT, decrease in calcium level to 497.35%, 515.38% and 74.76%, respectively after 6 weeks of induction, as compared to normal control group. Treatment with green tea and barley did not affect serum AST, ALT and calcium levels in normal rats as compared to normal control group (Table 2).

Table 2: Effects of 4 weeks treatment with barley, green tea and doxorubicin and their combination on liver function and calcium in normal and DMN-induced hepatorenal toxicity on rats

Parameter Groups	Aspartate aminotransferase AST (U/L)	Alanine aminotransferase ALT (U/L)	Calcium (mg/dl)
Control negative saline	22.6 ± 0.47	18.2 ± 0.26	10.62 ± 0.14
DMN (0.25 mg/day/ml)	112.4 ± 0.473 *	93.8 ± 0.41 *	7.94 ± 0.036 *
Green tea(1 g/Kg)	23.184 ± 0.06 @	19.44 ± 0.10 @	10.3± 0.075 @
Barley(625 mg/Kg)	22.04 ± 0.076 @	19.08 ± 0.026 @	10.6 ± 0.035 @
DMN(0.25 mg/day/ml)+ Green Tea(1 g/Kg)	84.6 ± 0.36 *@	42.4 ± 0.36 *@	8.74 ± 0.05 *@
DMN (0.25 mg/day/ml) + Barley(625 mg/Kg)	73 ± 0.25 *@	28.6 ± 0.37 *@	9.04 ± 0.027 *@
DMN(0.25 mg/day/ml)+Doxorubicin(2mg/kg)	51.8 ± 0.25 *@	62.6 ± 0.36 *@	8.54 ± 0.091 *@
DMN+Doxorubicin(2mg/kg) + Green tea(1 g/Kg) + Barley (625 mg/Kg)	32 ± 0.41 *@	51.4 ± 0.35 *@	9.24 ± 0.057 * @

DMN: N-dimethylnitrosamine
*Hepatorenal toxicity was induced by administration of N-dimethylnitrosamine (DMN) (0.25 mg/ day/rat) orally for 6 weeks. Data were expressed as mean ± SE (n=8). Statistical analysis was carried out by one-way ANOVA followed by LSD multiple comparisons test.*Significance from normal control (saline) at P< 0.05. @Significance from DMN control at P<0.05.*

Treatment with green tea or barley for 4 weeks after induction of hepatorenal toxicity significantly decreased serum AST levels to 75.27% and 64.95%, respectively; decreased serum ALT levels to 45.20% and 30.49%, respectively and increased serum calcium levels to 110.08% and 113.83%, respectively as compared to DMN treated group (Table 2).

Treatment with doxorubicin alone and the combination of barley, green tea and doxorubicin for 4 weeks after induction of hepatorenal toxicity significantly decreased serum AST levels to 46.09% and 61.78%, respectively; decreased serum ALT levels to 66.74% and 82.11%, respectively and increased serum calcium levels to 107.56% and 108.20%, respectively as compared to DMN treated group (Table 2).

Effects of treatment with green tea, barley and doxorubicin and their combination on serum tumor marker levels in DMN-induced hepatorenal toxicity in rats

Induction of hepatorenal toxicity by DMN produced a significant increase in serum alpha-fetoprotein (AFP), ferritin and lactate dehydrogenase (LDH) levels to 133.24%, 126.73% and 565.85%, respectively after 6 weeks of induction, as compared to normal control group. Treatment with green tea and barley did not change serum AFP, Ferritin and LDH levels in normal rats as compared to normal control group (Table 3).

Treatment with green tea or barley for 4 weeks after induction of hepatorenal toxicity decreased serum AFP levels to 47.11% and 47.35%, respectively decreased serum ferritin levels to 88.58% and 86.07 %, respectively and decreased serum LDH levels to 32.46% and 26.52%, respectively as compared to DMN treated group (Table 3).

Treatment with doxorubicin alone and the combination of barley, green tea and doxorubicin for 4 weeks after induction of hepatorenal toxicity significantly decreased serum AFP levels to 86.90 % and 96.13%, respectively; decreased serum ferritin levels to 94.55% and 96.04%, respectively and decreased serum LDH levels to 84.88% and 94.05%, respectively as compared to DMN treated group (Table 3).

Table 3: Effects of 4 weeks treatment with barley, green tea and doxorubicin and their combination on serum tumor marker in normal and DMN-induced hepatorenal toxicity on rats

Parameter Groups	α-Fetoprotien (ng/ml)	Ferritin (ng/ml)	LDH (U/L)
Control negative saline	95.09 ± 0.77	33.3 ± 0.09	386.66 ± 1.18
DMN (0.25 mg/day/ml)	126.7 ± 1.62 *	42.2 ± 0.53*	2187.92 ± 2.45 *
Green tea(1 gm/Kg)	94.58 ± 1.01@	33.4 ± 0.89 @	382.177 ± 0.19 @
Barley(625 mg/Kg)	94.82± 0.48@	34.3 ± 0.75@	381.078 ± 0.67 @
DMN(0.25 mg/day/ml) + Green Tea(1 gm/Kg)	93.9 ± 0.35 @	37.38 ± 0.44*@	710.26 ±1.350 *@
DMN (0.25 mg/day/ml) + Barley(625 mg/Kg)	94.2 ± 0.78@	36.32± 0.08*@	580.3± 0.077 *@
DMN(0.25 mg/day/ml) +Doxorubicin(2mg/kg)	110.1± 0.6 *@	39.9 ± 2.41*@	1857.053 ± 1.53 *@
DMN+Doxorubicin(2mg/kg) + Green tea(1 gm/Kg) + Barley (625 mg/Kg)	105.84 ± 0.15 *@	38.32± 0.14*@	1746.5 ± 0.23 *@

DMN: N-dimethylnitrosamine
*Hepatorenal toxicity was induced by administration of N-dimethylnitrosamine (DMN) (0.25 mg/ day/rat) orally for 6 weeks. Data were expressed as mean ± SE (n=8). Statistical analysis was carried out by one-way ANOVA followed by LSD multiple comparisons test.*Significance from normal control (saline) at P< 0.05. @Significance from DMN control at P<0.05.*

Effects of treatment with green tea, barley and doxorubicin and their combination on tissue contents of NO, MDA and SOD in DMN-induced hepatorenal toxicity in rats

Induction of hepatorenal toxicity by DMN produced a significant increase in tissue NO and MDA levels to 472 % and 213%, respectively and as well as decrease tissue SOD level to 17.21% after 6 weeks of induction, as compared to normal control group.

Treatment with green tea and barley did not change tissue NO, MDA and SOD levels in normal rats as compared to normal control group (Figure 1, 2 & 3).

Treatment with green tea or barley for 4 weeks after induction of hepatorenal toxicity decreased tissue NO levels to 36.74% and 36.31%, respectively, decreased tissue MDA levels to 68.12% and 63.88 %, respectively and increased tissue SOD levels to 272.96% and 332.27 %,respectively as compared to DMN treated group (Figure 1,2&3).

Treatment with doxorubicin alone and the combination of barley, green tea and doxorubicin for 4 weeks after induction of hepatorenal toxicity significantly decreased tissue NO levels to 76.27% and 61.11%, respectively; decreased tissue MDA levels to 68.66% and 98.93%, respectively and increased tissue SOD levels to 162.88% and 234.14%, respectively as compared to DMN treated group (Figure 1,2 & 3).

Histopathology study

Histopathology study is representative to hematoxylin and eosin-stained liver sections in different groups (magnification x40). (A) Normal control: showing normal histological structure of the glomeruli and tubules at the cortex, (B1) DMN : showing focal inflammatory cells infiltration surrounding the congested blood vessel and glomeruli with degeneration in tubules epithelium, (B2) DMN: showing focal hemorrhage, (C)green tea: showing focal inflammatory cells aggregation in cortico-medullary junction, (D) barley: showing normal histological structure, (E) green tea treated group: showing congestion in cortical blood vessels with degeneration in tubules lining epithelium, (F) barley treated group: showing normal histological structure, (G) doxorubicin treated group: showing degeneration in the epithelial cells lining the tubules at the cortex, (H) combination of doxorubicin, green tea and barley treated group: showing congestion in cortical blood vessels with degeneration in tubules lining epithelium. (Figure 4).

Histopathology study is representative to hematoxylin and eosin-stained kidney sections in different groups (magnification x40). (A) Normal control: showing normal histological structure of the glomeruli and tubules at the cortex, (B1) DMN : showing focal inflammatory cells infiltration surrounding the congested blood vessel and glomeruli with degeneration in tubules epithelium, (B2) DMN: showing focal hemorrhage, (C)green tea: showing focal inflammatory cells aggregation in cortico-medullary junction, (D) barley: showing normal histological structure, (E) green tea treated group: showing congestion in

cortical blood vessels with degeneration in tubules lining epithelium, (F) barley treated group: showing normal histological structure, (G) doxorubicin treated group: showing degeneration in the epithelial cells lining the tubules at the cortex, (H) combination of doxorubicin, green tea and barley treated group: showing congestion in cortical blood vessels with degeneration in tubules lining epithelium.(Figure 5).

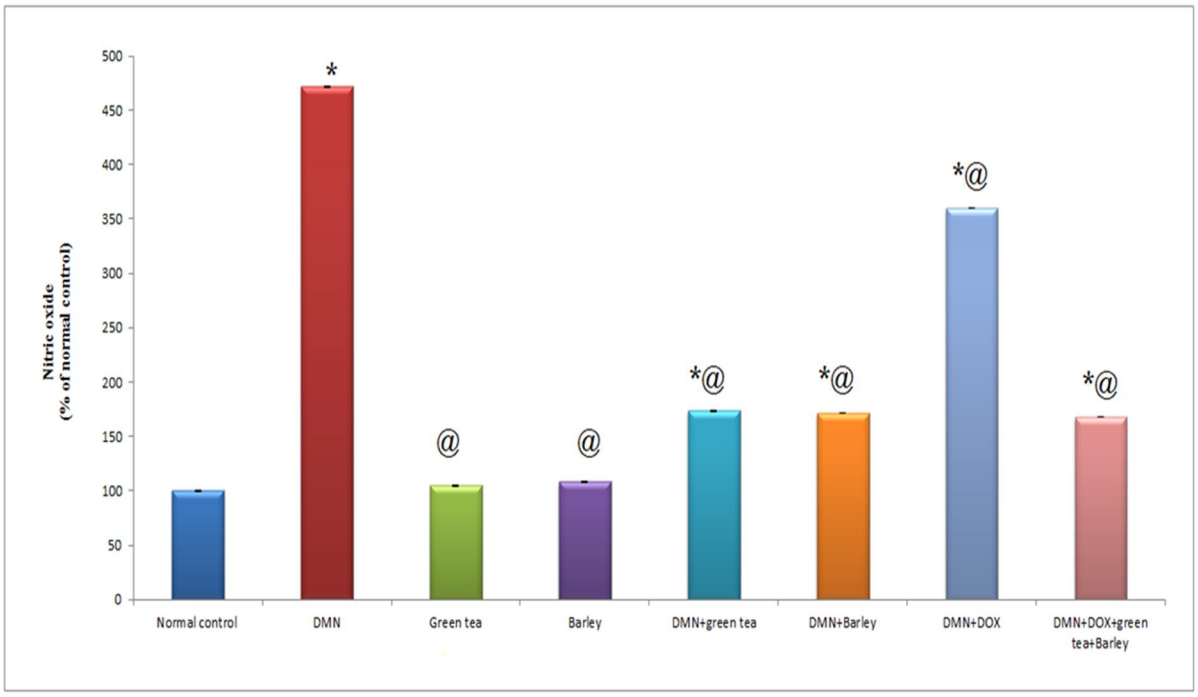

Fig. 1: Effects of treatment with green tea and barley and doxorubicin and their combination on tissue nitric oxide (NO) content in DMN-induced in DMN-induced hepatorenal toxicity in rats.

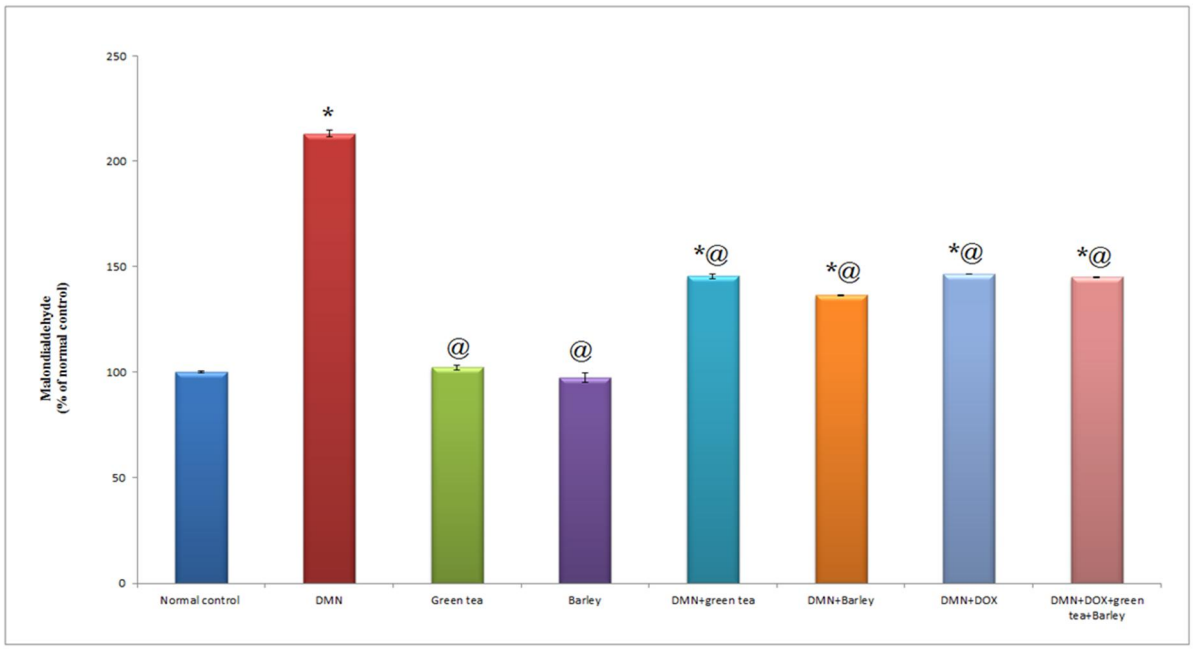

Fig. 2: Effects of treatment with green tea and barley and doxorubicin and their combination on tissue Malondialdehyde (MDA) content in DMN-induced in DMN-induced hepatorenal toxicity in rats

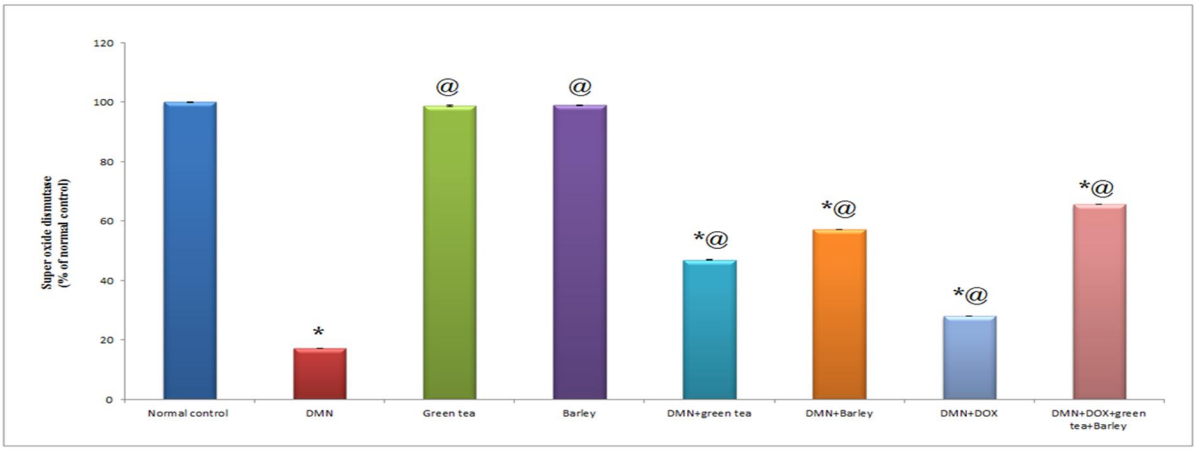

Fig. 3: Effects of treatment with green tea and barley and doxorubicin and their combination on tissue superoxide dismutase (SOD) content in DMN-induced in DMN-induced hepatorenal toxicity in rats

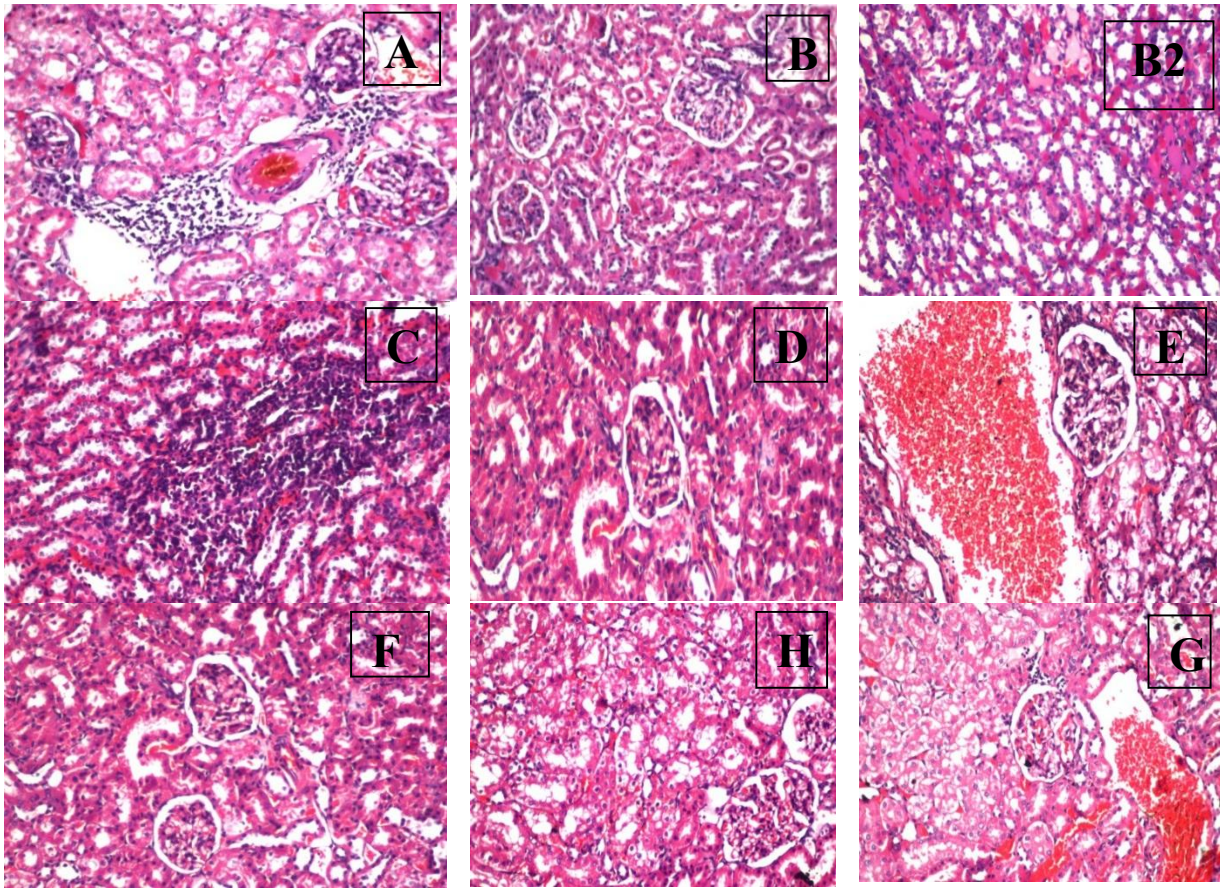

Fig. 4: Photomicrographs that are representative to hematoxylin and eosin-stained kidneysections in different groups (magnification x40). (A) Normal control: showing normal histological structure of the glomeruli and tubules at the cortex, (B1) DMN : showing focal inflammatory cells infiltration surrounding the congested blood vessel and glomeruli with degeneration in tubules epithelium, (B2) DMN: showing focal hemorrhage, (C)green tea: showing focal inflammatory cells aggregation in cortico-medullary junction, (D) barley: showing normal histological structure, (E) green tea treated group: showing congestion in cortical blood vessels with degeneration in tubules lining epithelium, (F) barley treated group: showing normal histological structure, (G) doxorubicin treated group: showing degeneration in the epithelial cells lining the tubules at the cortex, (H) combination of doxorubicin, green tea and barley treated group: showing congestion in cortical blood vessels with degeneration in tubules lining epithelium.

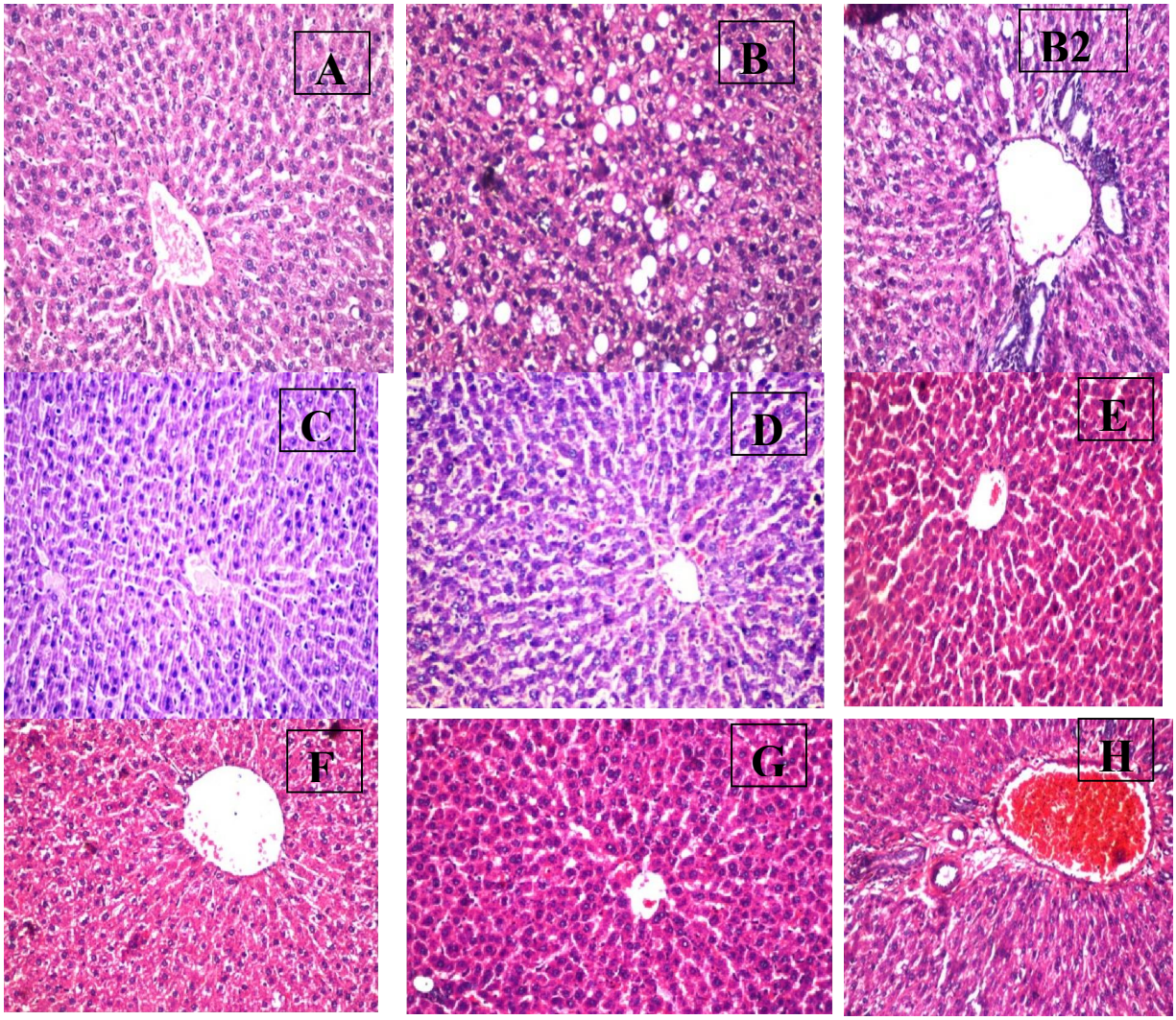

Fig. 5: Photomicrographs that are representative to hematoxylin and eosin-stained liver sections in different groups (magnification x40). (A) Normal control: showing normal histological structure of the central vein and surrounding hepatocyte in hepatic parenchyma, (B1) DMN : showing fatty change in hepatocytes and rupture of some hepatocytes, (B2) DMN: showing dilatation of portal vein with multiple newly formed bile ductles in portal area(C)green tea: showing normal histological structure of the central vein and surrounding hepatocyte in hepatic parenchyma, (D) barley: showing normal histological structure of the central vein and surrounding hepatocyte in hepatic parenchyma, (E) green tea treated group: showing normal histological structure of the central vein and surrounding hepatocyte in hepatic parenchyma, (F) barley treated group: showing normal histological structure of the central vein and surrounding hepatocyte in hepatic parenchyma, (G) doxorubicin treated group: showing normal histological structure of the central vein and surrounding hepatocyte in hepatic parenchyma, (H) combination of doxorubicin, green tea and barley treated group: showing congestion in portal vein.

Immunohistochemistry

Immunohistochemistry of caspase-3 localization in rats' kidney.

There was a significant increase in caspase activity in DMN group localized intracellular and inflammatory cells (B), compared in control negative group (A). Control negative and other groups showed decrease in caspase immuno-reactivity. In this micrograph, caspase immunoreactivity appears as dark brown staining. (A)Normal control, (B) DMN, (C) green tea, (D) barley, (E) green tea treated group, (F) barley treated group, (G) doxorubicin treated group and (H) combination of doxorubicin, green tea and barley treated group (magnification x 80) (Figure 6).

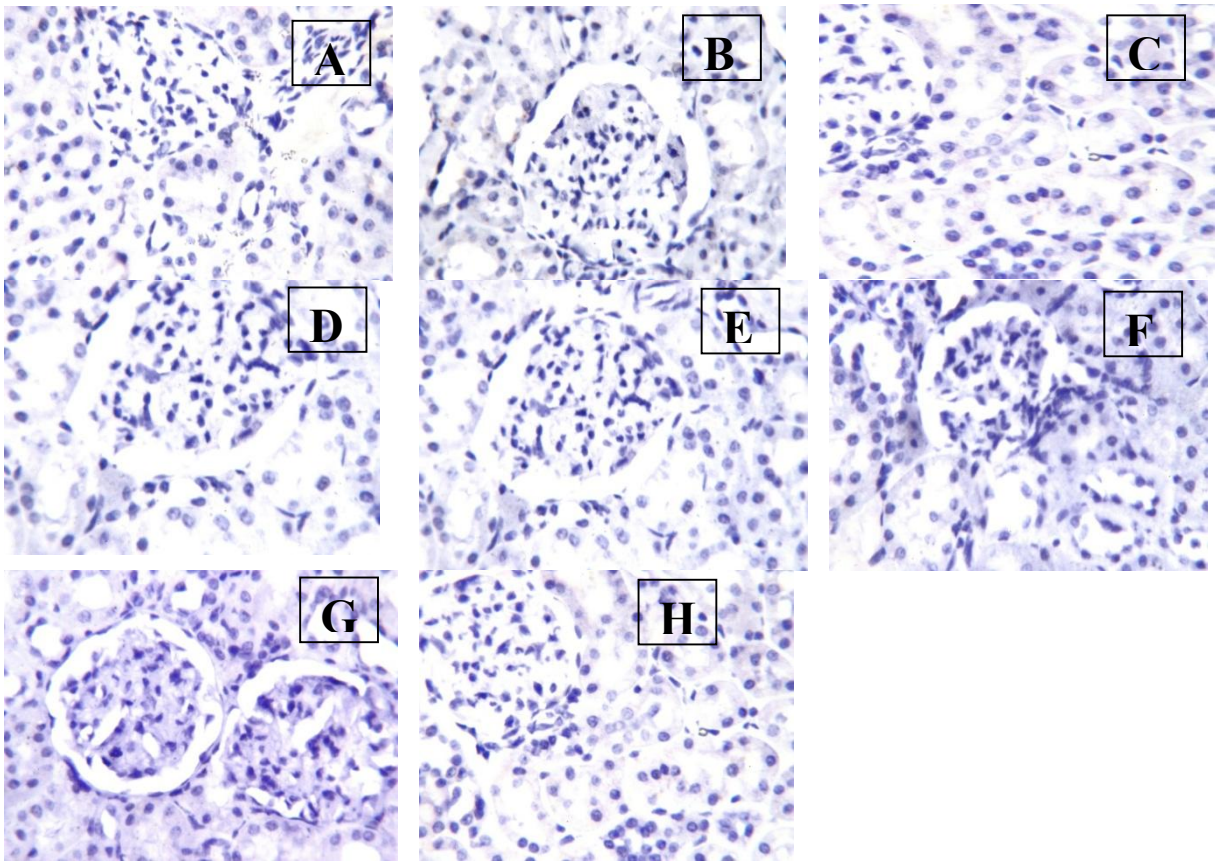

Fig. 6: immunohistochemistry of caspase localization in rats' kidney.
There was a significant increase in caspase activity in DMN group localized intracellular and inflammatory cells (B), compared in control negative group (A). Control negative and other groups showed decrease in caspase immuno-reactivity. In this micrograph, caspase immunoreactivity appears as dark brown staining. (A)Normal control, (B) DMN, (C) green tea, (D) barley, (E) green tea treated group, (F) barley treated group, (G) doxorubicin treated group and (H) combination of doxorubicin, green tea and barley treated group (magnification x 80).

Immunohistochemistry of caspase-3 localization in rats' liver.

There was a significant increase in caspase activity in DMN group localized intracellular and inflammatory cells (B), compared in control negative group (A). Control negative and other groups showed decrease in caspase immuno-reactivity. In this micrograph, caspase immunoreactivity appears as dark brown staining. (A)Normal control, (B) DMN, (C) green tea, (D) barley, (E) green tea treated group, (F) barley treated group, (G) doxorubicin treated group and (H) combination of doxorubicin, green tea and barley treated group (magnification x 80) (Figure 7).

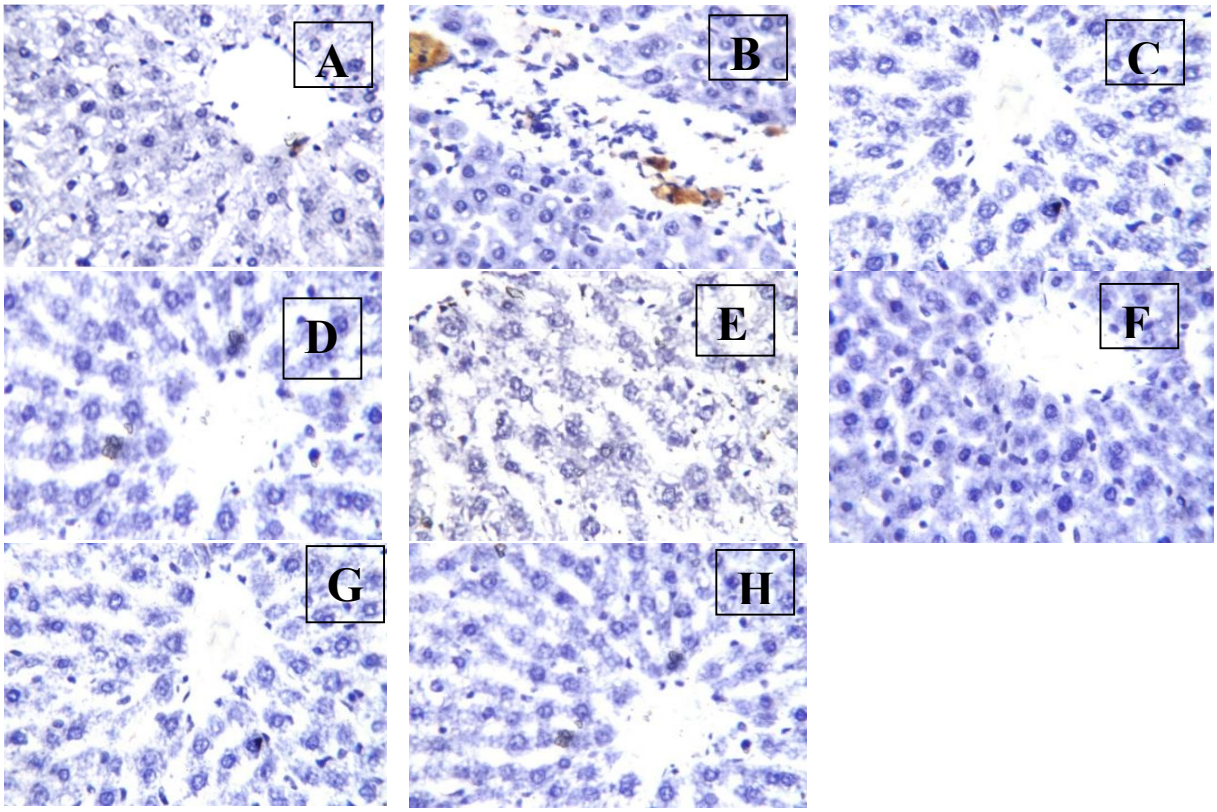

Fig. 7: immunohistochemistry of caspase localization in rats' liver.
There was a significant increase in caspase activity in DMN group localized intracellular and inflammatory cells (B), compared in control negative group (A). Control negative and other groups showed decrease in caspase immuno-reactivity. In this micrograph, caspase immunoreactivity appears as dark brown staining. (A)Normal control, (B) DMN, (C) green tea, (D) barley, (E) green tea treated group, (F) barley treated group, (G) doxorubicin treated group and (H) combination of doxorubicin, green tea and barley treated group (magnification x 80)

Discussion

In the present study, N-dimethylnitrosamine (DMN),at a dose of 0.25 mg/kg, induced-hepato renal toxicity as evidenced by a significant elevation of serum creatinine , urea and uric acid and an elevation of aspartate aminotransferase (AST) and alanine aminotranseferase (ALT) as liver function. These injuries were associated by an elevation of oxidative stress biomarkers such as tissue superoxide dismutase(SOD), nitric oxide (NO) and malondialdehyde (MDA) as well as tumor markers as alpha-fetoprotein, serum ferritin and lactate dehydrogenase (LDH). The present research work was aimed appraising the efficacy of 70% ethanol in the extraction of potent antioxidants from the leaves of green tea and the seeds of barley, commonly grown in Egypt.

Creatinine, urea and uric acid serum levels, in this work, were elevated in DMN treated rats as compared normal control. These results are in a line with Usunomena *et al*., (2016). Marked increase in serum urea and creatinine noticed in this study in DMN administered rats may be an indication of functional renal damage as the kidney is unable to excrete them. Elevated creatinine concentration is associated with abnormal renal function, especially as it relates to glomerular function (Bishop *et al*., 2005).

In current study, rats administered DMN-alone showed a significant increase in serum AST and ALT levels compared to control. These results are in line with Usunobun *et al*., (2015) who observed that the abnormal high level of serum AST and ALT indicates liver dysfunction and thus damaged structural integrity of the liver. In addition, DMN is a potent hepatotoxin that can cause liver fibrosis (George *et al*., 2001).

DMN treatment showed a significant increase in serum calcium level compared to control. This decrease supported by our immunohistopathological study that demonstrated caspase-3 expression induced

apoptosis. Previous studies have shown that DMN causes Ca dysregulation, and DNA fragmentation as well as apoptotic and necrotic cell death (Ray *et al*., 1993). George *et al*. (2006) reported a significant decrease of serum calcium levels in patients with liver fibrosis. Another study of (Nakano *et al*., 1996) exhibited reduced serum calcium levels due to decrease of serum albumin levels because albumin is responsible for the 47% of serum calcium bounding to it. Furthermore, Masuda *et al*. (1989) showed, in patients with liver cirrhosis, decrease in Dihydroxyvitamin D that is responsible for the retention and resorption of calcium ions by the kidney tubules. Moreover Liu *et al*., (2012) reported that the expression of caspase-3 was found to be significantly increased following 2 or 4 weeks of DMN treatment inducing liver fibrosis.

Our results showed that rats administered DMN treatment associated with expression of some proteins and enzymes evidenced by increased serum alpha-fetoprotein, ferritin and LDH levels compared to control. Previous study observed the alteration of LDH isoenzyme pattern in DMN induced liver fibrosis (George and Chandrakasan, 2000). AFP is a single polypeptide chain glycoprotein, produced by the liver and abnormal in 80% of patients with carcinoma (Jonson, 2001). Ye *et al*. (2016) found high level of ferritin in liver damage induced by DMN in rats. In the present study, DMN produced a significant increase in kidney NO and MDA contents as well as decrease in SOD content as compared with normal control group. DMN-treated rats exhibited an impaired oxidative balance (Rubiolo *et al*., 2008).These results suggest that DMN initiate lipid peroxidation and generate free radicals in renal tubules. An increase in lipid peroxides associated with serious damage to cell membranes, slow down of several enzymes and cell death (Pompella *et al*., 1991). Moreover treatment exhibited significant decreases SOD, CAT and GSH (Hong *et al*., 2010).

The current investigation showed that the treatment with green tea or barley on normal rats did not change serum kidney and liver functions, serum Ca level, markers of oxidative stress and tumor markers levels after 4 weeks of their administration as compared with normal control group.

In this study the beneficial effect of green tea and barley against DMN-induced hepatorenal toxicity. Green tea reduced the level of creatinine, urea and uric acid. These data confirmed with (Hassanein *et al*., 2012) who found the values of serum uric acid, urea nitrogen and creatinine decreased in group which treated with water extract of green tea treatment. Rehman *et al*., (2013) showed that green tea polyphenols markedly diminished cyclosporine-induced renal damage and improved renal function. Similarly, barley treatment, in present work, after induction of hepatorenal toxicity resulted in an improvement of kidney function as indicated by reduction of serum creatinine, urea and uric acid levels. Dietary fiber content in barley improves the level of kidney function (Rampton *et al*., 1984).

Administration of green tea or barley, in present work, after induction of hepatorenal toxicity resulted in an improvement of liver function as indicated by reduction of serum AST and ALT levels. Both enzymes AST and ALT are special liver enzymes that, in addition to being more sensitive to liver toxicity and histopathologic changes compared to other enzymes are measurable in less time (Balint *et al*., 1997). Takato *et al*. (2013) reported that green tea group showed significant reductions in ALT and AST levels and prescribed it for NAFLD patients. The supplementation with barley bran has significantly ameliorated the liver enzymes in hypercholesterolemic rats (El Rabey *et al*., 2013).

Administration of green tea and barley, in present work, after induction of hepatorenal toxicity and cancer resulted in a decrease of MDA and NO contents as well as showed an increase of SOD activity. It is well understood that green tea polyphenols have a beneficial property on oxidative stress of renal tissues (Asadi *et al*., 2013). Previous work demonstrated barley seed is effective in the treatment of liver disorders, due to phenolic compound (El Rabey *et al*., 2013).In addition barley decreased the level of lipid peroxide and increased the level of antioxidant GST and catalase (Jeong *et al*.,2009).

Administration of green tea and barley, in present work, after induction of hepatorenal toxicity exhibited an increase of serum calcium level. This result may be explained by improvement of renal tubule and liver and downregulation of caspase-3 induced apoptosis as shown in our immunohistochemical study. Caspase inhibitors are highly effective in preventing apoptotic cell death (Kaushal *et al*., 2001).

The current work, the treatment with green tea and barley, for 4 weeks after DMN induction, significantly decreased serum LDH, AFP and ferritin levels as compared with DMN group. Fetouh and Ibrahim, (2013) observed that green tea resulted in significant decrease in serum LDH activity when compared with cyclosporins group. Previous investigation exhibited that

barley bran restored LDH to the normal conditions in hypercholesterolemic rats (Rajanandh *et al*., 2013).

In current study, rats treated with doxorubicin after 6 weeks of induction of toxicity showed a significant decrease in kidney and liver functions, tumor markers, oxidative stress and caspase-3 expression as well as produced increase in calcium serum level as compared to DMN treated rats. In addition our results exhibited that doxorubicin conjugated with green tea and barley increased renohepato protection activity and may decrease systemic toxicity of doxorubicin when administered in higher doses than we use. In another study Luigi *et al*., (2005) revealed that lactosaminated albumin decreased systemic toxicity of doxorubicin when used to inhibit hepatocellular carcinoma.

Conclusion

It could be concluded that green tea and barley and their combination succeeded in lowering kidney and liver toxicity in rats due to the direct curative effect on the glomerular, tubular and liver structures through the scavenging of ROS. Furthermore green tea, barley and their combination reduced tumor markers as AFP, Ferritin and LDH as well as decreased kidney and liver apoptosis and inhibited caspase-3 expression. These results suggest green tea, barley and their combination may block early events of apoptosis induced by DMN via inhibiting the caspase-3 expression.

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