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Potential Recruiting and Hepatoprotective Effects of Ellagic Acid in D-Galactosamine-Induced Liver Damage in Rats

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MC, HMK and AA conceived and designed the study, analysed data and wrote the manuscript. JHA and DV collected data. All the authors contributed to all experimentation levels.

Key words
D-GaIN, Ellagic acid, Hepatotoxicity, TEM, Rats.

ABSTRACT

The present study aims to investigate the therapeutic and protective effects of ellagic acid (EA) on the toxicity of the liver induced by D-Galactosamine (D-GaIN) in rats. With this in mind, the rats were categorized into five groups. The study groups were given saline, 0.2 % dimethyl sulfoxide, D-GaIN, EA plus D-GaIN and D-GaIN plus EA, respectively. In the group given D-GaIN, the following transmission electron microscopic and light microscopic results were found: degenerative changes in the liver tissue, significant decreased in the number of activated Bcl-2, while increased in the number of Bax and caspase-3-positive hepatocytes, a significantly increase in levels of the activities of biochemistry markers (serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP)). In contrast, in the groups given D-GaIN and EA, a decrease in the damage of the liver tissue, a significant decrease activated Bax and caspase-3-positive hepatocytes, while increase in the number of Bcl-2 positive hepatocytes, a decrease in the biochemistry markers levels were found. Group 4, given EA before D-GaIN, showed better results when compared to Group 5, given EA after D-GaIN, in terms of histopathological and biochemical values. In conclusion, EA might play an important role in repairing D-GaIN-induced liver damage both as a protective and a therapeutic agent.

INTRODUCTION

The liver, also known as the major detoxifying organ in the body, has a crucial part in transforming and clearing chemicals from systemic circulation, which makes it vulnerable to the toxicity caused by chemotherapeutic agents. A number of medicinal agents have been reported to exacerbate the present damage in the liver when they are taken in quantities that exceed the tolerable dose. We also know of some chemical agents notorious for causing liver damage, such as alcohol, aflatoxin, antibiotics, carbon tetrachloride (CCI_4), acetaminophen, chlorinated hydrocarbons, peroxidized oils, D-galactosamine (D-GaIN), and chemotherapeutics (Morio *et al*., 2001; Han, 2002). Of these, D-GaIN is one of the most frequently used agents in causing experimental liver damage. This agent is reported to cause specific damage, that is, liver damage, with no effects on the other tissue or organs, which could be accounted for by the fact that the liver cells contain high levels of galactokinase and galactose-1-p uridiltransferase (Keppler and Decker, 1969). Once D-GaIN is metabolised, the uredines in the liver cells get depleted, resulting in the interruption of the protein synthesis and transcription process (Keppler *et al*., 1970). However, an interruption occurring in the transcription process of the liver cells make them extremely sensitive to the cytokines. Furthermore, D-GaIN causes mitochondrial dysfunction by raising caspase-3 and free radicals levels in the liver cells (Quintero *et al*., 2002).

Phenolic phytochemicals are capable of quenching free radicals and preventing cellular damage thanks to their phenolic rings and hydroxyl substituents, so that they can function as effective antioxidants. Polyphenolic compounds are generally found in widely consumed fruits, vegetables and derived products such as wine and tea. Several studies have reported plant-derived polyphenolic antioxidants to exhibit anti-inflammatory, anti-mutagenic, anti-carcinogenic, anti-viral and anti-oxidant activities (Priydarsini *et al*., 2002). Ellagic acid (EA) (2,3,7,8-tetrahydroxy[1]-benzopyrano [5,4,3-cde] [1]benzopyran-5,10-dione) is a phenolic constituent that exists in such fruits and nuts as raspberries, strawberries, walnuts, longan seed, mango kernel (Soong and Barlow, 2004) and pomegranate (Wang *et al*., 2004). EA possesses

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many biological activities, including potent antioxidant (Hassoun *et al*., 1997), anti-mutagen (Loarca-Piña *et al*., 1998) and anticancer (Whitley *et al*., 2003) properties. Even though the molecular mechanism of EA remains largely unknown, its potent scavenging action against -OH and O_2^- might account for these effects (Priyadarsini *et al*., 2002).

Based on the above information, the present study aims to investigate the therapeutic and protective effects of EA on D-GaIN-induced liver damage in rats.

MATERIALS AND METHODS

Chemicals

EA and D-GaIN was supplied by Sigma Chemicals Company, St. Louis, MO, USA, with the remaining chemicals and biochemicals obtained from local firms. Bax and Bcl-2 were purchased from Abcam, Germany, Caspase-3 (clone CPP32; NeoMarkers, USA).

Animals

Thirty five Sprague Dawley male rats weighing 180 to 240 gr were used in this study. The rats were kept in controlled laboratory conditions in which they were fed with pellet and tap water. They were categorized into 5 groups randomly, each containing 7 rats. Rats were kept at daylight and dark for 12 h with a temperature of 22±2 °C, along with humidity of 45-50% in automated controlled rooms. All the animal procedures followed in this study gained approval from the Animal Welfare Committee at Anadolu University (Ethical Committee No: 2014-22).

EA was suspended in DMSO and administered to the animals by gavage at a single dose of 20 mg/kg. D-GaIN was suspended in saline and injected intraperitoneally (*i.p.*) to the animals at a single dose of 750 mg/kg. The dose and administration period were selected in line with the previous studies (Table I) (Shi *et al*., 2008; Zhou *et al*., 2008; Rosillo *et al*., 2011).

Table I.- The details of the experimental groups.

Experimental groups	Day 0	Day 1	Day 2
1	Saline (0.5 mL)	Sacrified	
2	DMSO (0.5 mL)	Sacrified	
3	D-GaIN (750 mg/kg)	Sacrified	
4	Ellagic acid (20 mg/kg)	D-GaIN (750 mg/kg)	Sacrified
5	D-GaIN (750 mg/kg)	Ellagic acid (20 mg/kg)	Sacrified

Experimental design

The animals were randomly divided into 5 groups of 7 rats in each.

Histopathological investigations

The liver was cut into small pieces and fixed in Bouin's solution. Following dehydration in an ascending series of ethanol (70, 90, 96, 100%), the tissue samples were cleared in xylene, and then embedded in paraffin and sliced in 5-6 m sections. Later on, the sectioned samples were stained with Haematoxylin-Eosin (H-E) and Masson's trichrome (Masson), thus revealing collagen.

Immunohistochemistry

Sections of liver tissues were deparaffinized and rehydrated routinely. Antigen retrieval by citrate buffer (pH 6.0) was done by heating the sections in a microwave at 700 W for 10 min. After blocking with 3 mL/L H_2O_2 and swine serum, sections were incubated with the primary antibodies, directed against Bcl-2 (Abcam), Bax (Abcam) and caspase-3 (Thermo) at dilutions of Ultravisionquanto detection system (Thermo Scientific), respectively.

TEM evaluation

The liver specimens were cut in small pieces ($1mm^3$) and fixed in gluteraldehyde. Specimens were washed in 0.1 M phosphate buffer at 4°C, then post fixed in 1% osmium tetroxide. Specimens were dehydrated, then embedded in Epon resin. Ultrathin sections (50 nm) were cut, mounted on copper grids and stained with uranyl acetate and lead citrate. Specimens were examined and photographed with transmission electron microscope (TEM JEM 1200 EXII).

Biochemical assays

Serums were obtained from the blood samples of the rats used in the experiment for 10 minutes at 3000 rpm. The samples were then analyzed to determine serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) with the help of an automated biochemical auto-analyzer (HITACHI-917).

Statistical analysis

A package software version of SPSS 12.0 for windows was used when assessing the data that were obtained in the present study. The difference observed for serum ALT, AST and ALP levels in the groups were assessed via one-way ANOVA. The numerical value (p) for the difference was deemed as significant if it was $p<0.05$.

RESULTS

EA prevents liver degeneration caused by D-GalN

Hematoxylin-eosin stained samples were observed under light microscope (Leica DM6000 B). Structure of liver tissues collected from control group (Group 1) were

found to be normal. Liver structures of 0.2 % DMSO administrated rats (Group 2) were moderately impaired. However, liver structures of the rats given 750 mg/kg D-GaIN (Group 3) were severely impaired. Among the features observed in these damaged liver tissues necrotic cells with pycnotic nuclei and eosinophilic cytoplasm were remarkably detected. Vacuolization and karyolysis in hepatocytes accompanied with asymmetry in cellular cordon were recorded in these damaged tissues. The liver samples of D-GaIN after 24 h EA administrated group 4 compared to Group 5, which was given EA after 24 h D-GaIN administration, were better protected against hepatotoxicity than those in Group 5. Furthermore, the results from Group 5 were remarkably similar to those of control group (Fig. 1).

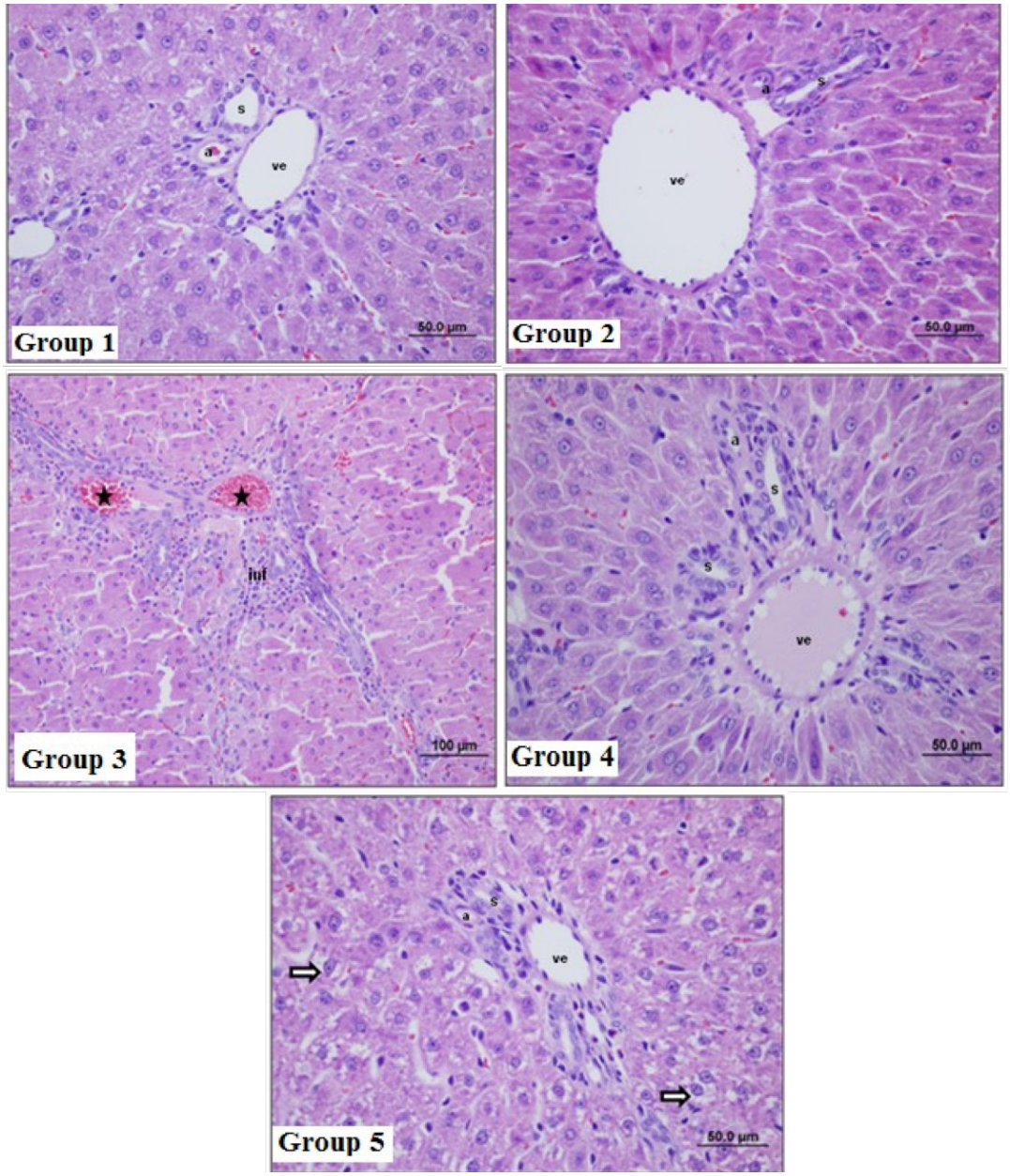

Fig. 1. Histological structure of rat liver. Group 1, the control, saline treatment; Group 2, 0.2% DMSO treated liver tissues; Group 3, tissues treated with GalN; Group 4, EA administered liver tissues before GalN treatment; Group 5, tissues administered with GalN before EA. (inf, inflammatory; *, vascular congestion; →, cellular damage; ve, venul; a, arteriole; s, bile duct 50 µm (x40) HE, haematoxylin and eosin stained rat liver tissues.

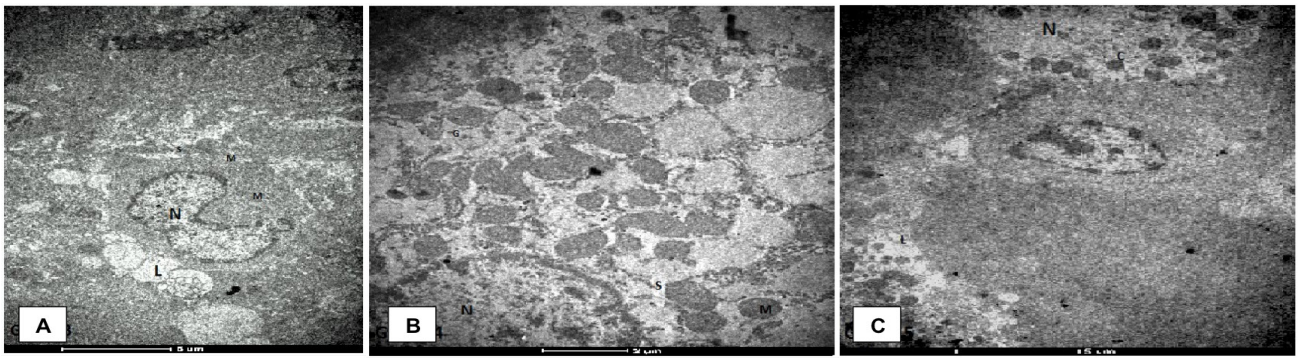

Fig. 2. Transmission electron micrographs showing morphological changes in rat liver cells. A, Group 3-GalN treated tissue showing the nucleus (N) compressed by large lipid droplets (L). The cytoplasm shows fewer rER and glycogen rosettes in-between high electron-dense mitochondria (m) and dilated sER (S) (4200x); B, Group 4-Tissues EA administered before GalN treatment with showing less lipid droplets (L), less electrondensity of mitochondria (m), less dilated sER (S) and condensed glycogen rosettes (G) compared to group 3 (8200x); C, Group 5- GalN before EA administered liver tissue with showing fewer lipid droplets (L) and ring shaped chromatin condensation © compared to D-galactosamine group (4200x).

The liver sections of the D-GaIN group examined by TEM showed many lipid droplets of different sizes compressing the nuclei and causing irregularity of their nuclear membrane. The cytoplasm showed fewer rough endoplasmic reticulum (rER) and glycogen rosettes in-between high electron-dense mitochondria and dilated smooth endoplasmic reticulum (sER), compared to the control group (Fig. 2).

Pretreatment with EA showed a noticeable improvement in D-GaIN-induced liver damage as there were fewer lipid droplets, less electron-density of mitochondria and less dilated sER in TEM examination (Fig. 2), compared to D-GaIN group.

EA prevents D-GalN-induced hepatocyte apoptosis

Liver specimens taken from all the study groups were immunohistochemically stained in order to determine concentration and intensity of Bcl-2, Bax and caspase-3 antigens. In group 3 number of Bcl-2 and Bax positive hepatocytes was decreased and increased, respectively, while the number of caspase-3 positive hepatocytes significantly decreased when compared to that of the control and DMSO groups which was of statistical significance ($p<0.05$). On the other hand, Bcl-2 and Bax positive hepatocytes were increased and reduced in Groups 4 and 5, respectively, while the number of caspase-3 positive hepatocytes showed a significant increase compared to that of Group 3 (Figs. 3-6), which was of statistical significance ($p<0.05$). Our immunohistochemical findings showed that EA pretreatment seems to provide a better protection than EA post treatment.

Pre and Post EA treatment prevent biochemical changes which causes D-GaIN

Serum ALT, AST and ALP levels showed no change of statistical significance in Group 2 when compared to the control group ($p>0.05$). As for Group 3 serum ALT, AST and ALP levels were found to have dramatically increased when compared to the control group ($p<0.001$). As to Group 4 and Group 5, serum ALT, AST and ALP levels showed a considerable decrease when compared to Group 3 ($p<0.001$) (Table II).

Table II.- Serum ALT, AST and ALP levels (Means ± SD) of the blood samples of rats.

Group	Serum ALT (IU/L) n=7	Serum AST (IU/L) n=7	Serum ALP (IU/L) n=7
1	43.88 ± 3.81	78.78 ± 5.47	160.10 ± 9.57
2	55.80 ± 2.71	81.42 ± 3.02	164.73 ± 17.80
3	648.09 ± 70.33^{ab}	551.93 ± 51.83^{ab}	237.99 ± 27.11^{ab}
4	152.88 ± 9.37^{abc}	158.52 ± 11.51^{abc}	171.58 ± 17.13^{c}
5	167.17 ± 11.43^{abc}	178.28 ± 19.85^{abc}	165.08 ± 22.52^{c}
P	p<0.001	p<0.001	*p<0.001*

Groups: 1, Saline (0.5 ml); 2, DMSO (0.5 ml); 3, DGalN (750 mg/kg); 4, Ellagic acid (20 mg/kg)+DGalN (750 mg/kg); 5, DGalN (750 mg/kg)+Ellagic acid (20 mg/kg). $^{a}p<0.001$ compared to control, $^{b}p<0.001$ compared to Group 2, $^{c}p<0.001$ compared to Group 3.

DISCUSSION

In spite of tremendous strides in the modern medicine, there are not much drugs available for the treatment of liver disorders (Bhandarkar and Khan, 2004). Liver diseases remain one of the serious health problems. Among the

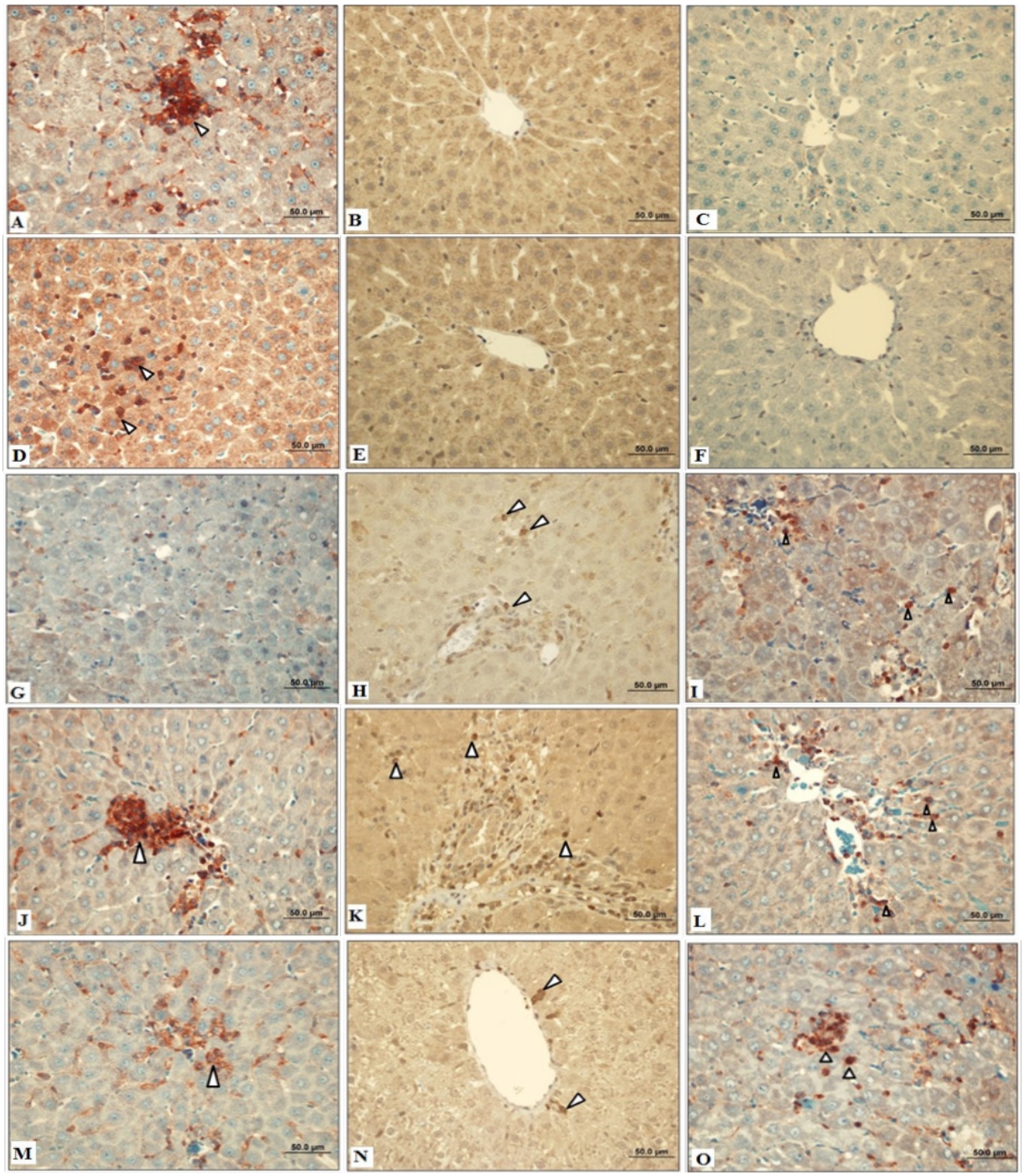

Fig. 3. Immunohistochemically-stained liver specimens of the rats in different study groups. Activated caspase-3 and Bax in control group and 0.2 % DMSO-treated showing almost negligible staining normal hepatocytes (B, C, E and F) while Bcl-2 protein expression in control group showing less intense staining normal hepatocyte as shown by arrows (A and D). Caspase-3 and Bax protein expression in D-GaIN-treated group showing more intense staining of hepatocytes and diffused staining as shown by the arrows (H and I) while Activated Bcl-2 in D-GaIN-treated group showing almost negligible staining normal hepatocytes as shown (G). Activated Caspase-3 and Bax immunostaining of liver treated with EA before D-GaIN and D-GaIN before EA showing less intense staining of hepatocytes as shown by arrows (K, L, N and O) while Bcl-2 protein expression in EA before D-GaIN and D-GaIN before EA groups showing less intense staining of hepatocytes as shown by arrow (J and M) (bar: 50µm).

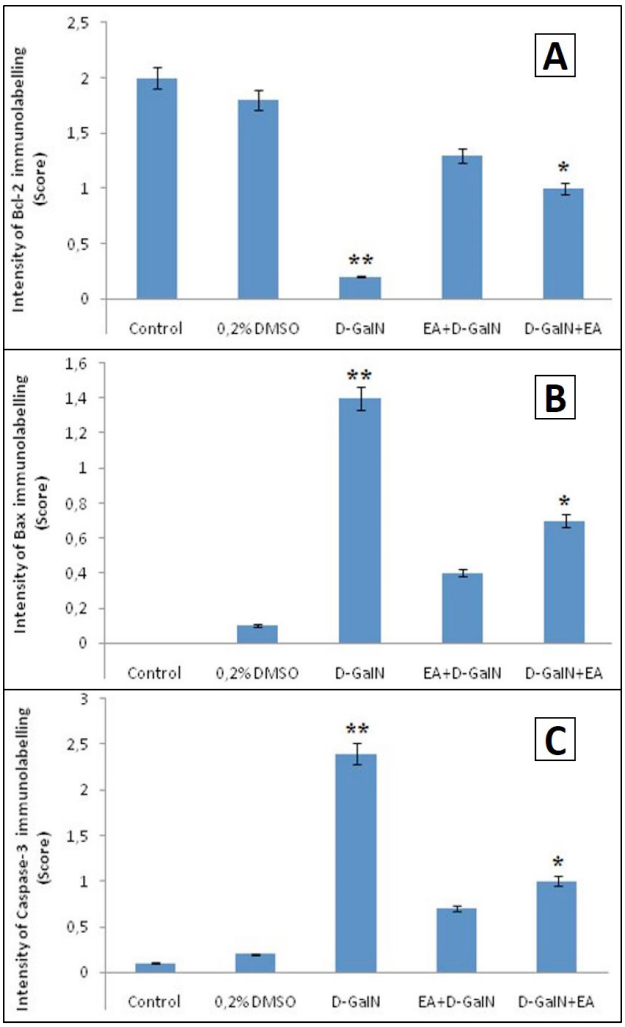

Fig. 4. Intensity of immunolabelling score of activated Bcl-2 (A), caspase-3 (B) and Bax (C) positive hepatocytes in the groups. $^{**}p<0.001$ and $^{*}p<0.05$compared to control (group 1) and 0.2% DMSO groups (group 2).

numerous models for experimental liver damage, D-GaIN-induced liver damage is similar to human virtual hepatitis in its morphological and functional features (Keppler *et al*., 1968).

The liver damage induced by D-GaIN, generally reflects a disturbance of liver cell metabolism, which leads to characteristic changes in serum enzyme activity. The increased levels of ALT, AST and ALP may be interpreted as a result of liver cell destruction or a change in membrane permeability. These enzymes are characteristic of liver damage; therefore, their release into the serum confirms GaIN-induced liver damage. Significant increases in ALT, AST and ALP activity were observed in the GaIN-intoxicated rats, consistent with previous reports (Mourelle and Meza, 1989; Lim *et al*., 1999). Lim *et al*. (1999) reported that 400 mg/kg D-GaIN administrated activities of serum ALT, AST, LDH and γ-GT were increased significantly. Blood samples for serum ALT activity and liver histology were collected at 24 h, the peak of injury after a single dose of 500 mg/kg D-GaIN. D-GaIN increased serum AST and ALT significantly. D-GaIN caused panlobular focal necrosis and periportal inflammation, which was accompanied by an inflammatory infiltrate of predominantly polymorph nuclear cells with a few lymphocytes and swollen macrophages (Robert *et al*., 1999). Shi *et al*. (2008) reported that mice intoxicated with 750 mg/kg D-GaIN developed severe hepatocellular injuries with a significant elevation in serum AST and ALT activities when compared to normal control group. Shi *et al*. (2008) reported that photomicrograph of 750 mg/kg D-GaIN-intoxicated mice liver section showed vacuolization of hepatocytes, sinusoidal dilation and congestion, infiltration of cells, loss of cell boundaries and ballooning degeneration, loss of architecture and cell necrosis.

D-GaIN inhibits mRNA, and protein synthesis in hepatocytes increased the sensitivity to TNF-alpha released from Kupffer cells, which activate signaling pathways leading to cellular death. Cells first begin to die via apoptosis. Consequently, inflammatory cells enter the liver parenchyma, and areas of necrosis develop (Stachlewitz *et al*., 1999). Although D-GaIN has been known as a hepatotoxin causing necrosis, it has also been reported to induce apoptosis in the liver of rats. Some researchers claimed that D-GaIN could induce apoptotic or necrotic cellular death according to dose and time of administration (Tsutsui *et al*., 1997). Sun *et al*. (2003) showed that 24 h after intraperitoneal administration of D-GaIN (1 g/kg body weight) to rats, the activity of caspase-3 in the liver increased significantly compared with that in the control group given saline. Also, Catal and Bolkent (2008) reported that in the group given D-GaIN, apoptotic cells with caspase-3 activity, which are liver injury markers induced by D-GaIN, increased. Our results showed that the injection of 750 mg/kg D-GaIN caused degenerative changes in the liver tissue, significant increase in the number of activated Bax and caspase-3-positive hepatocytes while significant decrease in the number of activated Bcl-2 positive hepatocytes, a an significantly increase in levels of the activities of ALT, AST and ALP (Figs. 3, 4; Table II).

Various agents have been attempted for protection and/or prevention of the side effects of many chemotherapeutics. One kind of these chemo preventive agents are flavonoids, which are found in almost all food categories with fruits and vegetables being the main source. Flavonoids have

many functions such as phenolic antioxidants, scavengers of free radicals, chelating agents, and modifiers of various enzymatic and biological reactions. EA is a naturally occurring plant polyphenol (Soong and Barlow, 2004) that exhibits antioxidative properties both *in vivo* (Hassoun *et al*., 1997, 2004) and *in vitro* (Seeram *et al*., 2005). In fact, EA has been shown to exert a potent scavenging action on both O_2 and ·OH, as well as lipid peroxidation (Iino *et al*., 2001). EA has been reported to be associated with various remedial properties such as anticancer, antidiabetic, atherosclerosis, hepatoprotective and antimicrobial activities (Vattem and Shetty, 2005). Kim *et al*. (2001) have suggested that the significant increase in the activities of hepatic marker enzymes such as AST, ALT, ALP and LDH manifested by cyclosporin induced hepatocellular damage. Administration of EA significantly decreased the activities of AST, ALT, ALP and LDH levels (Priydarsini *et al*., 2002). Thresiama and Kuttan (1996) induced liver fibrosis in rats by using CCl_4 and studied the antifibrotic activity of ellagic acid. Liver histopathology showed reduction in necrosis, inflammation and fibrous connective tissue indicating antifibrotic activity. This drug was given orally in two dose ranges of 20 and 100 mol/kg. There was a significant reduction in liver lipid peroxide, hydroxyproline and transaminases values at both doses. The present study has also found similar results to the above-mentioned studies, liver damage induced by 750 mg/kg D-GaIN, were determined to have been reduced in the groups given EA (Fig. 1). Likewise, the decreased serum ALT, AST, ALP levels and in the number activated Bax and caspase-3 hepatocytes due to liver damage also had increased while increase in the number of activated Bcl-2 hepatocytes (Figs. 3, 4; Table II). Histopathological observations further confirmed the membrane stabilizing effect of EA in D-GaIN challenged rats. Hepatocyte necrosis induced by D-GaIN was largely prevented by treatment of EA. The changes from EA pre-treated and post-treated rats showed significant hepatoprotective effects of EA against D-GaIN-induced liver injury in rats. The biochemical index and histopathological appearance from EA (20 mg/kg) pretreated rats were close to normal groups (Fig. 1).

CONCLUSION

Data obtained from the current study suggest that EA found to be more effective in curing liver damage in pretreated than in post treated groups.

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Conflict of interest statement

The authors report no conflicts of interest in this work.

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