HEPATO-PROTECTIVE AND THERAPEUTIC EFFECT OF ELLAGIC ACID ON D-GALACTOSAMINE-INDUCED LIVER DAMAGE

Jama Hussein ALI

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	Name-Surname	Signature
Member (Thesis Supervisor):	Prof. Dr. Hatice Mehtap KUTLU	**.....**
Member	**:** Assoc. Prof. Dr. Adnan AYHANCI	**……...**
Member	**:** Assoc. Prof. Dr. Filiz SUSUZ	**……..**

The Board of Directors of Anadolu University Graduate School of Sciences approved in …… by decision No……

Institute Director

ABSTRACT

Master of Science Thesis

HEPATO-PROTECTIVE AND THERAPEUTIC EFFECT OF ELLAGIC ACID ON D-GALACTOSAMINE-INDUCED LIVER DAMAGE

Jama Hussein ALI

Anadolu University

Graduate School of Sciences

Department of Advanced Technologies

Supervisor: Prof. Dr. Hatice Mehtap KUTLU

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Polyphenol-rich dietary foodstuffs, consumed as an integral part of vegetables, fruits, and beverages have attracted attention due to their antioxidant properties. Ellagic acid (EA), a polyphenolic compound widely distributed in fruits and nuts, has been reported to scavenge free radicals and protect cellular membranes. This study examined the protective mechanism of EA against D-galactosamine (D-GalN)-induced liver damage. A little is known about the influence of EA on D-GalN toxicity in vivo. D-GalN, a well-known hepatotoxicant, causes severe hepatocellular inflammatory damage and clinical features similar to those of human viral hepatitis in experimental animals. A little is known about the influence of EA on D-GalN toxicity in vivo. Thirty-five healthy male Wistar rats were equally divided into five groups.

First and second groups were used as controls. The control groups received 0.5 ml of normal saline and 0.2% (0.5 ml of DMSO), respectively. The third group received D-GalN (700 mg/kg). Fourth and fifth groups were recieved D-GalN (700 mg/kg) plus EA (25 mg/kg) and EA (25 mg/kg) plus D-GalN (700 mg/kg), respectively. The rats were killed at the end of the treatment period. The level of ALT, AST, ALP, and LDH were determined in liver tissue. Administration of D-GalN increased the level of these liver enzymes when compared to the control group. The administration of EA to D-GalN-treated rats decreased the ALT, AST, ALP, and LDH levels in these samples. D-GalN caused marked damages in the histopathological status of liver tissues. These damages were ameliorated by EA administration. In conclusion, EA may be used in combination with D-GalN in chemotherapy to improve D-GalN-induced liver damages.

Key words: Ellagic acid, D-Galactosamine, Hepatoprotective effect, Liver damage, antioxidant

ÖZET

Yüksek Lisans Tezi

FARELERDE D-GALAKTOZAMİN NEDENİ KARACİĞER HASARI ÜZERİNE ELLAJİK ASİT'İN KORUYUCU VE İYİLEŞTİRİCİ ETKİLERİ

Jama Hussein ALI

Anadolu Üniversitesi

Fen Bilimleri Enstitüsü

İleri Teknolojiler Anabilim Dalı

Danışman: Prof. Dr. Hatice Mehtap KUTLU

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Sebze, meyve ve içeceklerin bir parçası olarak tüketilen polifenol bakımından zengin diyet gıda maddeleri antioksidan özellikleri dolayısıyla dikkat çekmiştir. Meyve ve kuruyemişlerde yaygın olarak görülen bir polifenolik bileşen olan ellajik asidin (EA) serbest radikalleri temizlediği ve hücre zarlarını koruduğu rapor edilmiştir. Bu çalışma, D-galaktosamin (D-GalN) kaynaklı karaciğer hasarına karşı elajik asidin koruma mekanizmasını incelemektedir. Bilinen bir hepatotoksin olan D-GalN, kobay hayvanlarda şiddetli hepatoselüler inflamatuvar hasara ve insanlarda görülen viral hepatite benzer klinik özelliklere neden olmaktadır. Canlı organizmada

ellajik asidin D-GalN üzerindeki etkisine ilişkin çok az şey bilinmektedir. Otuz beş sağlıklı erkek Wistar sıçanı eşit olarak beş gruba ayrılmıştır. Birinci ve ikinci gruplar kontrol grubu olarak kullanılmıştır. Kontrol gruplarına sırasıyla 0,5 ml normal tuz ve %0,2 (0,5 ml) DMSO verilmiştir. Üçüncü gruba D-GalN (700 mg/kg) verilmiştir. Dördünce ve beşinci gruba ise sırasıyla D-GalN (700 mg/kg) ile ellajik asit (25 mg/kg) ve elajik asit (25 mg/kg) ile D-GalN (700 mg/kg) verilmiştir. Sıçanlar tedavi süresinin sonunca öldürülmüştür. Karaciğer dokusunda ALT, AST, ALP ve LDH seviyeleri tespit edilmiştir. D-GalN verilmesi, kontrol grubu ile karşılaştırıldığında bu karaciğer enzimlerinin düzeyini artırmıştır. D-GalN ile tedavi edilen sıçanlara elajik asit verilmesi, bu numunelerdeki ALT, AST, ALP ve LDH düzeylerini düşürmüştür. D-GalN, karaciğer dokularının histopatolojik durumunda belirgin hasarlara yol açmıştır. Bu hasarlar, ellajik asit verilerek iyileştirilmiştir. Sonuç olarak, D-GalN kaynaklı karaciğer hasarlarını iyileştirmek için kemoterapide D-GalN ile birlikte ellajik asit kullanılabilir.

Anahtar Kelimeler: Ellajik asit, D-Galaktozamin, hepatoprotektif etkisi, karaçığer hasarı, antioksıdan

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LIST OF SYMBOLS AND ABBREVIATIONS

mg : Miligram

kg : Kilogram

nm : Nanometer

ml : Mililitre

µg : Microgram

µl : Microlitre

mol : Mole

ºC : Centigrade

% : Percent

b.wt : Body weight

DMSO : Dimethyl sulfoxide

EDTA : Ethylenediaminetetraacetic acid

H_2O_2 : Hydrogen peroxide

OSO_4 : Osmium tetraoxide

BDMA : Benzyldimethylamine

DDSA : Dodecenylsuccinic anhydride

NADPH : Nicotinamide adenine dinucleotide phosphate

NADH : Reduced Nicotinamide adenine dinucleotide

NAD^+ : Oxidized Nicotinamide adenine dinucleotide

PBS : Phosphate Buffered Saline

PPM : Parts per million

RPM : Revolutions per minute

SPSS : Statistical Package for the Social Sciences

1. INTRODUCTION

1.1. Ellagic Acid

Nowadays there is a significant consideration for the role of diet in human health and special attention of plant polyphenols. Plant polyphenols play an important role in human nutrition and are implicated in numerous biological properties including antioxidant, anti-inflammatory, anticancer and antiatherosclerotic activities. Among these phytochemicals, ellagic acid (EA), a dimeric derivative of the gallic acid, occurs in fruits and nuts in either its free form, as EA-glycosides, or bound as ellagitannins (ETs) (Seeram et al., 2004). Table (1.1) is illustrated the total EA quantities of diverse fruits and plants (Zhang et al., 2014). EA was discovered by Braconnot in 1831 (Malini et al., 2011). EA (Fig 1.1) is a molecule with a molecular weight of 302 gmol-1. It is a molecule that is highly thermostable (melting point of 350°C) (Sepúlveda et al., 2011). EA is insoluble at pH<7 but remains soluble at physiological pH (Losso et al., 2004).

EA is mainly found in fruits and nuts such as raspberries, strawberries, walnuts, grapes, and black currants. Prior studies revealed that EA is one of the common dietary polyphenols that abundantly found in distilled beverages. The highest levels of EA are found in raspberries. According to the German national food consumption survey, the fruits and vegetables that each German national eats is measured to contain 5.2 mg/ day of EA (Ohan, 2002). EA is found also in muscadine grapes at average concentrations of 5.6 mg per 100 g of fresh muscadine grape wine and 10.2 mg per 100 g of fresh muscadine grape juice and has been associated with the formation of insoluble sediments during muscadine wine production (Losso et al., 2004). Several factors are necessary the protection of EA. These factors include DNA binding abilities, combating of the production of Reactive Oxygen Species (ROS), scavenging of ROS, and safeguarding of DNA from alkylating injuries. It seems that hydroxyl and lactone groups are significant to the structure of EA. It is suggested that EA is strongly responsible to the all aforementioned biological protections. Free radical studies of EA show that EA has better protection compared to vitamin E against oxidative stress (Ohan, 2002). EA inhibits tumor growth caused

by chemical carcinogens in an animal model. EA induces cell cycle arrest by inducing G0/G1 arrest, and inhibits overall cell cycle causing apoptosis in tumor cells (Li et al., 2005). Ghudhaib et al. (2010) reported that EA has anti-typhoid protection against animals. Additionally, this study has revealed that regular consumption of fruits which are rich in EA strongly mitigated the effect of Salmonella typhimurium on animals. Other accumulated studies also illustrated that EA has a positive effect on malaria, especially, plasmodium falciparum species.

Referring the previous works of literature that exhibited that EA has better protection than Vitamin E against oxidative stress induced hepatotoxicity. Yet, D-galactosamine (D-GalN) induced hepatotoxicity is sparsely reported in the literature and the protection offered by antioxidants especially EA is not well understood. So the present study was conducted whether EA, the above naturally occurring antioxidant could protect D-GalN induced rat liver damage. It was determined that D-GalN is a strong hepatotoxicant in rats when administrated by intraperitoneal (IP). Protection offered by EA was evaluated by determining the activities of Serum Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), and Lactate Dehydrogenase (LDH) which are hepatotoxic parameters, were therefore examined simultaneously to the histological studies of rat liver tissues as additional study for the effects of natural antioxidant of EA.

Table 1.1. Total EA concentrations of different plants (Zhang et al., 2014).

Fruits and plants	Total EA	Reference
Bananas	2*	6,7
Pear	4*	6,7
Tangerine	4*	6,7
Pineapples	6*	6,7
Plum	7*	6,7
Strawberry	31-78*	6-9
Pecan nut	33*	8
Walnut	59*	8
Raspberry	>150*	7,9
Cloudberry	>160*	7,9
Arctic Bramble	>160*	7,9
Strawberry cultivar	6-34.1**	10
Pongamia pinnata	1.5* (bark)	11
	0.1* (leaves)	
	0.4* (seeds)	
Geraniaceae	397*	12
Muscadine grape cultivars	587-1900* (skin)	13

*, mg/100 g dry weight; **, mg/100 g frozen weight.

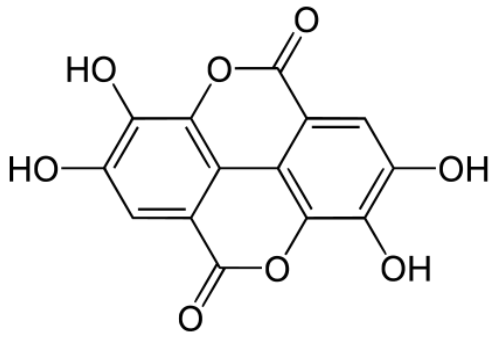


Fig 1.1 Molecular structure of EA.

1.1.1. Chemistry of Ellagic Acid

The massive structural diversity of the tannins depends on specific structural and chemical properties. Tannins can be divided into hydrolysable tannins and non-hydrolysable tannins by treating hot water or sometimes with tannases. Thus, the term „hydrolysable tannins' includes both the gallotannins and the ETs (Khanbabaee & van Ree, 2001). Landete, (2011) suggested that ETs contain an intricate class of polyphenols characterized by one or more hexahydroxydiphenoyl (HHDP) moieties esterified to a sugar, frequently glucose.

ETs are found a variety of different typical plant families. These compounds display an enormous structural variability due to the manifold possible sites for the linkage of HHDP residues with the glucose moiety, and particularly by their strong tendency to form dimeric and oligomeric derivatives (Niemetz & Gross, 2005). ETs are generally labile both in solution and in the field, undergoing hydrolysis and polymerization reactions. Clifford & Scalbert, (2000) study vividly confirmed that when ET reacts with acids or bases, ester bonds are hydrolyzed and the HHDP group rearranges into poorly water soluble EA. Landete, (2011) acknowledged that the polymerization reaction leads to the covalent attachment of ETs to the cell wall components. Different derivatives of EA present in plants formed through methylation, glycosylation and metoxylation of its hydroxyl groups. EA is mainly found in the plant vacuole, either in its free forms as EA or EA derivatives or else bound as water-soluble ETs. ETs and EA study clarified that the antioxidant efficiency of ETs and EA is directly associated with their degree of hydroxylation and decreases with the presence of a sugar moiety.

1.1.2. Biological Properties and Functionality of Ellagic Acid

A wide variety of studies indicated that EA has antimutagenic, antioxidant and anti-inflammatory activity in microbial and experimental animals. EA has been found to has a good anti-cancer effect; it suggested that EA combats the metabolism of various environmental toxins and as well as prevent the commencement of carcinogenesis induced by these chemicals. Additionally, EA was acknowledged to have antimutagenic activity and inhibits the binding of carcinogens to the DNA

(Vattem & Shetty, 2005). EA prevents the formation of various tumors, this mechanism of action can be possible because compounds such as ETs and EA, unequivocally interact with the cells walls or sites with the facility to complex proteins, preventing the proliferation of metastatic cells (Sepúlveda et al., 2011). EA could inhibit the activation of aflatoxin B1 in some Salmonella strains, which may result to inhibit the mutagenicity and carcinogenicity produced aflatoxin B1 (Soni et al., 1997). Vattem & Shetty, (2005) suggested that EA inhibited the DNA binding metabolism of several carcinogens, for instance, EA inhibits the binding of N-nitrosobenzylmethylamine (NBMA) in rat esophagus and the binding of benzo(a)pyrene metabolites in human epithelial cells. In vitro and in vivo studies demonstrated that EA exhibited hepatoprotective activity against CCl_4 (Singh et al. 1999). Furthermore, EA considerably enhanced the glutathione S- transferase (GST) enzyme activity, the GST isozyme levels and the Glutathione (GSH) levels which are detoxification parameters and necessary to a variety of cancerous cells. EA was also found to extensively reduce the number of bone marrow cells with chromosomal aberrations and chromosomal fragments as efficiently as alpha-tocopherol. Likewise, the administration of EA inhibited radiation-induced DNA strand breaks in rat lymphocytes (Vattem & Shetty, 2005). Incubation of EA at 1 to 100 μmol/L cancer cells dose-dependently suppressed cell growth. The findings of this study also suggested that EA could inhibit cancer growth by regulating factors that promote cancer metastasis like matrix metalloproteinases, vascular endothelial growth factor expression, and by inducing apoptosis (Losso et al., 2004). EA reduced the cellular damage induced by radiation, H_2O_2 and mitomycin C in the bone marrow cells of mice. The antitumor-promoting action of EA may be linked in part by inducing a redox modification of protein kinase C (PKC) which serves as a receptor for tumor promoters. EA has also inhibited the xenobiotic metabolizing enzymes like NADPH: quinone reductase and GST genes which can detoxify carcinogens and reduce carcinogen-induced mutagenesis and tumorigenesis. EA combats the final carcinogenic metabolites of several polycyclic aromatic hydrocarbons (PAH) (Vattem & Shetty, 2005).

Certain studies have revealed that EA is a good inhibitor of DNA topoisomerases which are played an important role in carcinogenesis. It seems that 3, 3′-hydroxyl groups and the lactone groups are essential elements for the topoisomerase mechanism of inhibition (Vattem & Shetty, 2005). Pteleopsis hylodendron extracts, presented substances such as EA derivates with antioxidant properties since these substances were considerably able to eradicate free radicals such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH). EA was tested in the reduction of the incidence of molecules such as N-nitrosometylbenzylamine (NMBA), which explains one of its characteristics as chemoprotective (Sepúlveda et al., 2011). It is important to underline the capability of ETs and EA as antiviral and antimicrobial agents. It has also been recommended that EA has the ability to hinder the growth of pathogens in humans, possibly coupled with protein in bacteria walls, such as that of Bacillus, Staphylococcus, and Salmonella. Fruit extracts from pomegranate (Punica granatum), which are rich in ETs and EA, the derived substances from these compounds were caused the inhibition of methicillin-resistant Staphylococcus aureus strains. These biological properties have been attributed to the ETs ability to inhibit gyrase activity which is associated with the cleavage of the DNA strand during the replication process.

It is shown that this bacterium does not have the ability to resist this type of antibacterial action. Also, different concentrations of EA against the human immunodeficiency virus have been tested, and it demonstrated the ability to inhibit the viral growth and spread due to the ability of EA to attach the HIV proteins. Moreover, viruses do not have the ability to replicate themselves in the presence of ETs; because they inhibit the integrase enzyme involved in the insertion of viral genomic material to host cell. This is particular in the case of retroviruses, including HIV, were these very specific phytochemicals also inhibit reverse transcriptase activity. Bioassays, which carried out using EA derivative compounds, isolated from the bark of Elaeocarpus parvifolius showed potent antiparasitic properties. These compounds were evaluated for the ability to inhibit Babesia gibsoni. One of the important biological properties of EA is that it showed the ability to inhibit radiation-induced PKC activity in cytosolic and particulate fractions of mouse liver cells. It was

shown that EA at a dose of 10 mM inhibits 60% activity of PKC in non-irradiated and radiated cells. Recently revealed biological activities of EA are those related with prevention of eye, kidney, heart and joints of upper and lower extremities damage, which are caused by high blood glucose levels. This protective action is due to the ability of EA to inhibit the enzyme aldolase reductase, which is responsible for the production of proteoglycan type compounds in small blood vessels, causing blindness, kidney damage, paralysis, heart attacks and loss of limbs associated with the two types of diabetes. Furthermore, EA increases the insulin activity and has several inflammatory effects and oxidative stress reduction (Sepúlveda et al., 2011).

1.1.3 Bioavailability of Ellagic Acid

The bioavailability of EA has not been sufficiently investigated and particularly have never been widely studied in vivo. Most of the bioavailability studies of EA are limited to rats and mice (Seeram et al., 2004). The effects of ETs and EA as antioxidants, and against various diseases, such as atherosclerosis and cancer, have been studied in several in vitro studies. But yet, the number of in vivo studies designed to investigate the metabolism of these compounds by the cells is very limited (Larrosa et al., 2012). When mice were provided 600 mg/kg b.wt of ETs (from raspberries or pomegranates, by gavage), EA (0.05% of dose) was excreted in the urine as a result of absorption and metabolism of ETs. Though, nearly no EA was recovered from the blood or tissues of mice fed for 1 week on a diet containing 1% EA. Following oral administration of EA to rat, 10% of the dose was excreted and found in the urine and feces of the mice. The low concentrations of free EA in plasma indicated that it has low solubility in water and its extensive metabolic transformation and degradation prior to absorption. Moreover, EA has been reported to bind permanently to DNA and cellular proteins, which might responsible its limited transcellular absorption. It has been anticipated that the gut microflora metabolize insoluble EA. Since ETs are more soluble than EA, the in vivo action of physiological pH or enzymatic action by gut microflora could cause them to break down to release EA units (Seeram et al., 2004).

1.1.4 Hepatoprotective Effect of Ellagic Acid

Treating liver diseases with herbal drugs has a long tradition in India, China, and Japan. Nearly 170 phytoconstituents isolated from 110 plants belonging to 55 families have been reported to possess liver protective activity. About 600 commercial herbal formulations which were claimed hepatoprotective activity are being sold all over the globe. EA is an active ingredient from such few plants (Girish et al., 2009). EA has been shown to possess numerous anticarcinogenic and antimutagenic properties towards a variety of different carcinogens, including nitrosamines, azoxymethane, mycotoxins, and PAH. Dietary administration of 400 p.p.m. EA for 16 weeks inhibited the incidence of hepatocellular neoplasms induced in rats by N-2-fluorenylacetamide by 70%. EA has chemoprotective properties towards several carcinogens and inhibits NMBA-induced tumors in rat liver and esophagus (Ann et al., 1996). Moreover, EA has protective effects on ethanol-induced toxicity through the regulation of NO and Transforming Growth Factor-β1 (TGF-β1) production in liver cells (Sohn et al., 2013).

Devipriya et al. (2008) found that oral administration of EA protects the liver from alcohol toxicity by diminishing the hepatic marker enzymes, lipid peroxidation markers, and the lipid metabolism of the liver. According to a data, which reveals the protective effect of EA against oxidative damage induced $AlCl_3$ in the liver shows that EA decreased the lipid peroxidation in the liver by alleviating the Malonaldehyde (MDA) levels in $AlCl_3$ treated animals. Moreover, it enhances the antioxidant level of the liver by stimulating GSH-Px and GSH activities in the liver. Prior studies observed that EA might enhance the Vitamin E level, particularly in the liver. Vitamin E can take a good role to the regulation of H_2O_2 production in mitochondria. The study indicated that overproduction of mitochondrial ROS might lead to extensive tissue damages (Özkaya, 2010). Additional study of EA to cisplatin-treated animals reduced the MDA levels. A possible clarification for the enhancement of MDA concentration may be decreased the formation of antioxidants in cisplatin-induced tissues, which in view of increased activity of ROS allows a consequent increase in MDA production Additional study which is conducted by Yüce et al. (2007) also reported that cisplatin-treated animals reduced the MDA levels in the

liver. They assumed that a possible clarification for the enhancement of MDA level might be decreased the formation of antioxidants in tissues that are induced on cisplatin, which in view of increased activity of ROS allows a consequent increase in MDA production.

1.1.5 Antioxidant Nature of Ellagic Acid

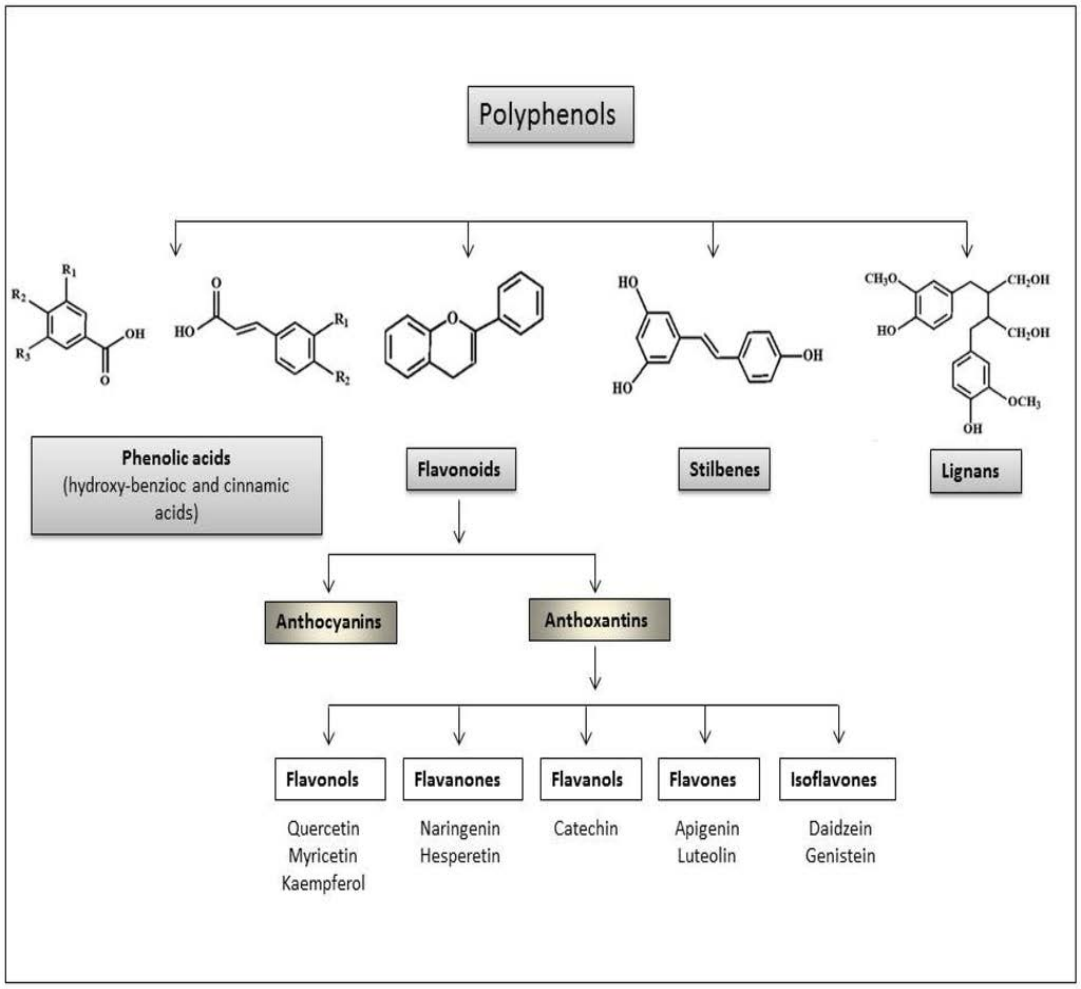
ROS and reactive nitrogen species (RNS) are produced inside cells causing damage to many important biomolecules that have been correlated in several human diseases. They are always kept in balance by endogenous antioxidants, but under diseased conditions, the balance is altered, leading to oxidative stress. Thus, EA is a good dietary antioxidant which is capable of scavenging free radicals that could prevent diseased conditions. There is a strong correlation between the consumption of phenolic fruits abundantly and incidence of coronary heart diseases (CHD), this has been largely related to the antioxidant action of EA. EA demonstrated cardioprotective properties against CCl_4-induced liver tissues. Furthermore, it is found that EA has better protection than vitamin E against oxidative stress. The recently published study reported that the experiential cytogenetic protection against H_2O_2 by EA could be through scavenging reactive free radicals (Ohan, 2002). Özkaya, (2010) found that EA enhances the antioxidant response of the cell by directly scavenging free radicals or by up-regulating the pathways in the cell that might result in cellular antioxidant enzyme responses. EA scavenges both the superoxide anion O_2 and the hydroxyl radical ($^{\cdot}OH$), as well as lipid peroxidation. A prior study, which was observed the effect of EA on cisplatin-induced animals indicated that EA scavenged the free radicals (O_2^- and $^{\cdot}OH$), and suppressed oxidative DNA damage (Yüce et al., 2007). EA was found to scavenge ROS and RNS such as OH radicals, peroxyl radicals, NO_2 radicals, and peroxynitrite with rate equal to those of many recognized antioxidants such as vitamin E and vitamin C. EA has low solubility in water, but the solubility of EA increases in organic solvents and it suggests that EA can act as a good lipophilic antioxidant. This property along with its ability to scavenge free radicals makes it a good candidate for the chain-breaking antioxidant (Ohan, 2002).

1.1.6. Dietary Polyphenols

Polyphenols are one of the most significant antioxidants. Scalbert et al. (2005) found that daily intake of polyphenols could be as high as 1 g/d, which i is approximately 10 times higher than the intake of vitamin C and 100 times higher that the intakes of vitamin E and carotenoids. Their chief sources are fruits, beverages, vegetables, cereals, dry legumes, chocolate, tea, coffee, and red wine. The core factor responsible for the availability of less research on polyphenols is the diversity and the complication of their chemical structure (Archivio et al., 2007). Polyphenols are getting growing interest from consumers and food industries for numerous motives. Various studies suggested the relation between the consumption of polyphenol-rich foods and the prevention of diseases. Regular consumption of polyphenols prevent cancers, stroke and CHD, for instance, tea protects against cancers and CHD whereas soy protects breast cancer and osteoporosis. The second reason is related to the chemical nature of polyphenols. Polyphenols are the most abundant antioxidants in our diet, they scavenge free radicals and they prevent various diseases associated with the effect of free radicals, such as cancers, cardiovascular diseases, inflammation and others (Scalbert & Williamson, 2000). Phenolics are differentiated by having at least one aromatic ring with one or more hydroxyl groups attached. More than 8000 phenolic structures have been reported and they are widely dispersed throughout the plant kingdom many occur in food. Phenolics range from simple, low molecular weight, single-aromatic-ring compounds to the large and complex tannins and derived polyphenols (Crozier et al., 2009).

The major groups of polyphenols are flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans (Fig 1.2) (Archivio et al., 2007).

Fig 1.2. Classification and structure of the main polyphenol classes (Câmara et al., 2013).



Phenolic acids are abundant in foods. The most normally encountered are caffeic acid and, to a minor amount, ferulic acid. One of the major food sources of ferulic acid is wheat bran. Caffeic acid is found in many fruits and vegetables and in coffee. Other phenolic acid derivatives are hydrolyzable tannins and normally found in blackberry, raspberry, strawberry, and wine. Flavonoids are the richest polyphenols in our diets. The most commonly encountered are flavones, flavonols, isoflavones, anthocyanins, flavanols, proanthocyanidins and flavanones. Some of these flavonoids are restricted to certain types of food. The main source of isoflavones is soy, which contains approximately 1 mg of genistein and daidzein/g dry bean. Citrus fruits are the common source of flavanones. Flavones are less familiar and were found in sweet red pepper and celery. The main flavanols are catechins. They are very rich in tea. Proanthocyanidins are found in plants as polymeric flavanols. They are responsible

for the astringency of food. General sources are grapes, red wine, tea, and chocolate. Anthocyanins are commonly found in red fruits such as cherries, strawberries, raspberries, grapes, and red currants (Scalbert & Williamson, 2000).

1.2. Apoptosis

Apoptosis is derived from the Greek word for a natural process of leaves falling from trees or petals from flowers, it is a separate form of programmed cell death (Wu, 2001). Cell death, especially apoptosis, is possibly one of the most extensively studied subjects among cell biologists. comprehending apoptosis in disease conditions is very important as it not merely offers insights into the pathogenesis of a disease but may also give hints on how the disease can be treated (Wong, 2011). The enhanced understanding of apoptotic signaling pathways and the cloning and characterization of proapoptotic or antiapoptotic genes have fascinated enormous interest to this process and elevated the possibility that therapeutic strategies which change apoptotic pathways may be useful in the treatment of cancer, infectious diseases, degenerative syndromes and other pathological conditions (Wu, 2001).

Apoptosis is an extremely planned and genetically controlled type of cell death, necessary for embryonic development to ensure appropriate organogenesis (Guicciardi & Gores, 2005). There is an extensive variety of stimuli and conditions, both physiological and pathological, that can elicit apoptosis, not all cells will inevitably die in response to the same stimulus. Irradiation or drugs used for cancer chemotherapy results in DNA damage in some cells, which can result in apoptotic death through a p53-dependent pathway. Some hormones, such as corticosteroids, may result in apoptotic death in some cells (e.g., thymocytes) even though other cells are unaffected or even not stimulated (Elmore, 2007). It is described by a number of different morphological changes, such as chromatin condensation and marginalization, cell shrinkage, and plasma membrane blebbing, which are accompanied by biochemical features such as DNA fragmentation, membrane alterations, and degradation of specific cellular proteins, as a result of the enormous activation of a large number of intracellular proteases and endonucleases. In the final stages, the dying cell is fragmented into membrane-bound vesicles containing relatively intact organelles and chromatin residues called „„apoptotic bodies'' which

are swiftly engulfed by neighbouring cells and professional phagocytes, such as resident macrophages. Apparently, the most significant characteristic of apoptosis is its exceedingly regulated occurrence. Apoptosis occurs through specific molecular pathways that are under the firm control of an intricate network of proteins and their inhibitors. The apoptotic control occurs generally at checkpoints in the pathways that can also potentially be used as targets for therapeutic interventions of apoptosis (Guicciardi & Gores, 2005).

Apoptosis is always different from necrosis; there are mainly two processes that can occur separately or simultaneously. Generally, low doses of different stimuli such as heat, radiation, hypoxia, and anticancer drugs can cause apoptosis but these same stimuli can result in necrosis at higher doses. lastly, apoptosis is an energy-dependent process that engages the activation of a group of caspases and an intricate cascade of events that link the initiating stimuli to the final demise of the cell (Elmore, 2007).

1.2.1. Mechanisms of Apoptosis

Apoptosis can be caused by different stimuli, but there are mainly two main molecular pathways: the death receptor pathway (the extrinsic pathway) and the mitochondrial pathway (the intrinsic pathway). Both the intrinsic and extrinsic pathways have an extensive variety of intracellular proteases like caspases and endonucleases. The extrinsic pathway begins at the cell membrane following the engagement of a family of cytokine receptors named death receptors (for instance, tumour necrosis factor receptor 1 (TNF-R1), Fas/CD95, and TNF-associated apoptosis inducing ligand receptors 1 and 2 (TRAIL-R1 and TRAIL-R2)) by their similar ligands (TNF-α, Fas ligand (FasL)/CD95L, TRAIL). Ligand/receptor binding stimulates several proteins (that is, procaspase-8 and -10) at the intracellular domain of the receptor to form an intricate generally referred to as Death Inducing Signaling Complex (DISC). The signal generated at the DISC results in cell death which, depending on the cell type, may or may not need the involvement of mitochondria for its execution (Fig 1.3). The intrinsic pathway is elicited by various extracellular or intracellular signals, such as gamma irradiations, oxidative stress, toxins, endoplasmic reticulum stress-inducing factors, growth factor deprivation, or some

chemotherapeutic drugs which stimulate mitochondrial dysfunction. Hence, the structure of organelles and membrane permeability are altered, and mitochondrial proteins are released into the cytosol, along with pro-apoptogenic factors such as cytochrome C (Fig 1.4). A group of proteases, in particular, the caspases, play central roles as executors of the cell death programme (Guicciardi & Gores, 2005).

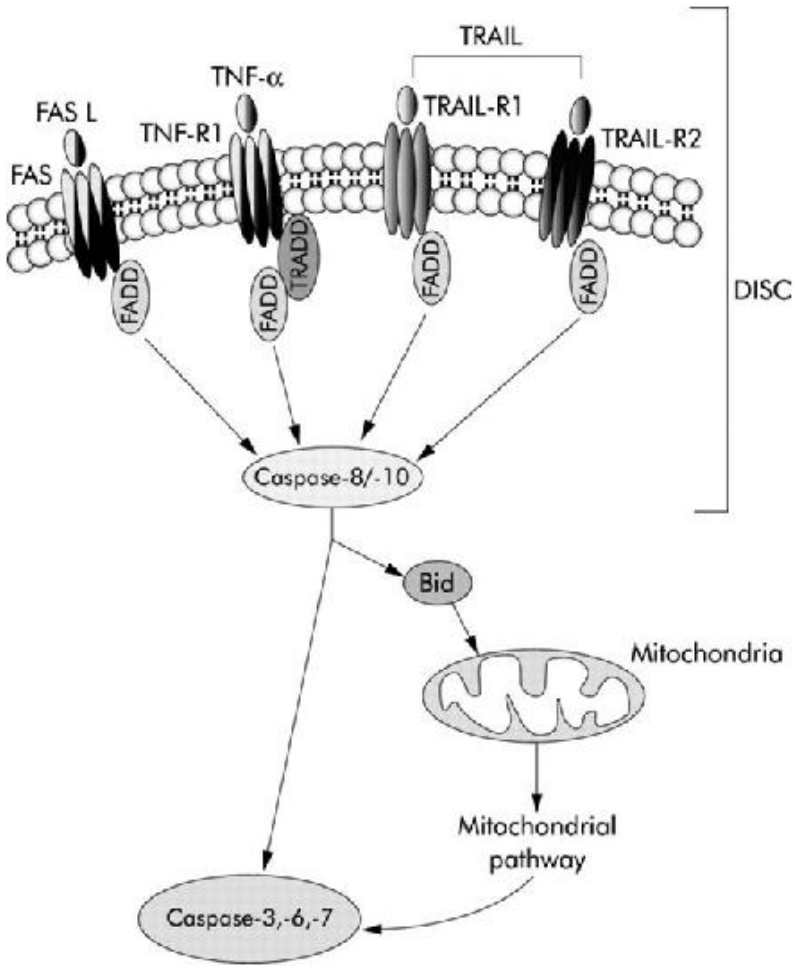


Fig 1.3. Death receptor mediated (extrinsic) pathway of apoptosis (Guicciardi & Gores, 2005).

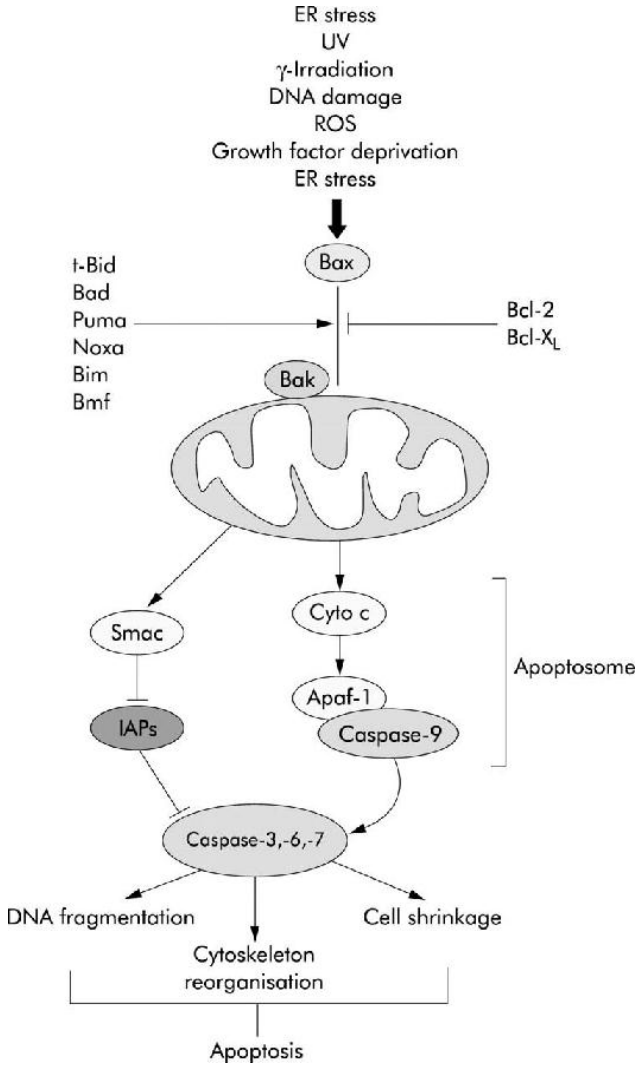


Fig 1.4. Mitochondria mediated (intrinsic) pathway of apoptosis (Guicciardi & Gores, 2005).

1.2.2. The Role of Caspases in Apoptosis

A group of proteases, specifically the caspases, play a good role as executors of the cell death programme. To date, 14 mammalian caspases are recognized. Caspases, which are mainly involved in apoptosis, are (caspases-2, -3, -6, -7, -8, -9, -10, and -12). These caspases are divided into upstream caspases (initiator caspases) and downstream caspases (effector caspases). Upstream caspases (-2, -8, -9, -10) are activated following binding to adaptors (such as FADD or apoptosis associated factor 1), which can make self-association and autocatalytic activation. On the other hand, downstream caspases (-3, -6, and -7) have not the capability to self-associate and

need cleavage by initiator caspases for their activation. Activated downstream caspases are able to perform nuclear degradation, altering of cell's cytoskeleton, and membrane blebbing. The capacity to both disrupted apoptosis, however, may be therapeutically very valuable (Guicciardi & Gores, 2005).

1.2.3. The Role of BCL-2 Family in Apoptosis

The Bcl-2 family of proteins comprises both pro- and anti-apoptotic members, they are intracellular proteins, which involve in the regulation of apoptosis and possibly they are the most significant controlling proteins of the intrinsic pathway. Currently, the mammalian Bcl-2 family classified at least 20 members within four conserved regions, named Bcl-2 homology (BH) 1–4 domains. The family can be further classified into three major subclasses, which depends on their homology functions. The first subclass includes the anti-apoptotic proteins Bcl-2, Bcl-XL, and Mcl-1, which share the highest homology in all of the four conserved BH 1–4 domains. Mostly they are found in mitochondrial but some have also been found on the endoplasmic reticulum and nuclear membrane. The second and third subclasses include only pro-apoptotic proteins: the so-called multi-domain Bax-like proteins, such as Bax and Bak, and the BH3 only proteins, such as Bid, Bad, Bim, Noxa, and Puma. Both multi-domain and BH3 proteins are only essential for apoptosis (Guicciardi & Gores, 2005).

1.2.4. Anticancer Effects and Mechanisms of Ellagic Acid

Accumulated studies supported that eating a diet rich in fruits and vegetables can prevent and reduce the incidences of various forms of cancer. Research on pomegranate juice in prostate cancer patients has shown benefits on significant markers of disease progression. ETs and EA, inhibit prostate cancer cell growth through cell cycle arrest and stimulation of apoptosis. Additionally, they inhibit the activation of inflammatory pathways including but not limited to the nuclear factor-kappa B (NF-kB) pathway. Inhibition of angiogenesis has also been demonstrated both in vitro and in vivo for prostate cancer (Heber, 2008). Zhang et al. (2014) suggested that EA has preventive and therapeutic effects against several types of cancers. They claimed that EA has in vitro and in vivo chemopreventive properties,

anti-cancer carcinogenic properties, antiproliferative and pro-apoptotic actions, and effects on subcellular signaling pathways. EA has indeed been shown to inhibit carcinogenesis in both in vitro and in vivo studies, e.g. 12-O-tetradecanoylphorbol-13- acetate (TPA)-induced tumor promotion in mouse skin. This study supports that detoxification of Benzo(a)pyrene Diolepoxide (BPDE) by EA is a likely mechanism by which EA could prevent cancer. EA can inhibit cancers by handling various strategies like stimulating of detoxifying enzymes, altering cancer cell cycle, acting as antioxidants, and is also able to reduce carcinogen directly prior to reaching its DNA target (Huetz et al, 2005).

EA considerably reduces the proliferation and stimulates the apoptosis of human osteogenic sarcoma (HOS), human pancreatic adenocarcinoma cell lines (MIA PaCa-2 and PANC-1), and human pancreatic cancer cell lines (MIA PaCa-2 and HPAF-II cells). It can achieve these effects by decreasing NF-kB activity, enhancing the mitochondrial death pathway, which is related to loss of mitochondrial membrane potential ($\Delta\psi m$), cytochrome C release, and caspase-3 activation. Other studies support that EA inhibits the expression of Bcl- 2, cyclin D1, CDK2, and CDK6 whereas it stimulates the expression of Bax protein in cancerous tissues (Zhang et al., 2014). EA can stop the proliferation of ovarian carcinoma cell lines by arresting the G1 phase, increasing the activation of p53 and p21, and decreasing the activation of cyclins D1 and E. EA can also induce caspase-3-mediated apoptosis by inducing the Bax/Bcl-2 ratio. A prior study in the human nasopharyngeal carcinoma cell line (NPC-BM1) shown that EA reduces cell viability by reducing human telomerase activities. Additionally, EA can inhibit the activation of caspase-3, PKC, NF-kB, and c-Myc and induces the tumor suppressor protein p21 and TGF-β1. It is also suggested that EA stimulates cancer cell death by blocking energy metabolism (Zhang et al., 2014).

EA has protection effect on DNA damage both in vitro and in vivo. it is acknowledged that EA activates the genes and proteins which are involved in DNA repair mechanisms. On the other hand, EA down-regulates mitogen-activated protein kinase, which is a key cell-signaling pathway. Furthermore, EA supposedly possesses

anti-inflammatory properties. Investigation of the effect of EA on the colon cancer of the Wistar albino rats found that EA demonstrates anti-inflammatory property by inhibiting nitric oxide synthase, COX-2, TNF-α, and IL-6 through the inhibition of NF-kB. There are wide varieties of studies that show that EA inhibits cancer metastasis. EA also stimulates anti-angiogenic effects via the VEGFR-2 signaling pathway in several cancers particularly the breast cancer. The structure-based interaction between EA and VEGFR-2 was examined. EA can form hydrogen bonds and aromatic interactions within the ATP-binding region of the VEGFR-2 kinase unit and thus extensively inhibit a series of VEGF-induced angiogenesis processes. The main functions and effective dose of EA on different cancer types are summarized in Table 1.2 (Zhang et al., 2014).The main functions and effective dose of EA on different cancer types are summarized in Table 1.2.

Table 1.2. Effects of EA in different cancer cell lines or xenografted animals (Zhang et al., 2014).

Cancer type	Cell line/animal	Effective concentration	Biological effects
Breast cancer	Cell line: MDA-MB-231	2.5-20 μM	Inhibits cancer cell proliferation and migration by downregulating VEGF-induced angiogenesis, VEGF-2 tyrosine kinase activity and its downstream MAPK, and PI3K/Akt pathways
	Animal: Female ACI rats	400 ppm	Downregulates 17β estradiol by reducing 17β-hydroxysteroid dehyrdogenase and reduces mammary tumor incidence
Osteogenic sarcoma	Cell line: ATCC CRL1343	4-100 μg/mL (IC_{50} =6.5 μg/mL)	Induces apoptosis by upregulating Bax and activating caspase-3
Pancreatic cancer	Cell line: MIA PaCa-2, and PANC-1	10-50 mM	Stimulates the mitochondrial pathway of apoptosis associated with mitochondrial depolarization, cytochrome C release, and downstream caspase activation
Ovarian carcinoma	Cell line: ES-2 and PA-1	10-100 μM	Elevates p53 and Cip1/p21, decreases cyclin D1 and E levels, and induces caspase-3-mediated apoptosis by increasing the Bax/Bcl-2 ratio
Nasopharyng- eal carcinoma	Cell line: NPC-BM1	50-200 μM	Reduces cancer cell viability by increasing caspase-3 activity, downregulating Bcl-2, and decreasing telomerase activity
Lymphoma	Animal: Dalton's Lymphoma bearing mice	40-80 mg/kg body weight	Prevents cancer progression and increases life span of DL mice by downregulating the PKC signaling pathway and induces cancer cell death by blocking energy metabolism
Prostate cancer	Cell line: PLS10(rat)	80-200 μM (IC_{50} =100 μM)	Inhibits invasive potential through action on the activity of proteases, such as collagenase/gelatinase and collagenase IV

1.3. Biology of Liver

The liver is the largest gland in the human body, weighing around 3 pounds comprising about 1/50 of the adult body weight. It is found on the right side of the body, under the diaphragm and at the back of the ribs 5 through 10 (Allen,2002; Ishibashi et al., 2009). The liver is one of the most significant and complicated organs in the human body. It's crucial function is to control the substances absorbed from the digestive system before distributing to the circulatory system. An entire loss of liver function may cause death within 1 minute, indicating the liver's significance (Allen, 2002).

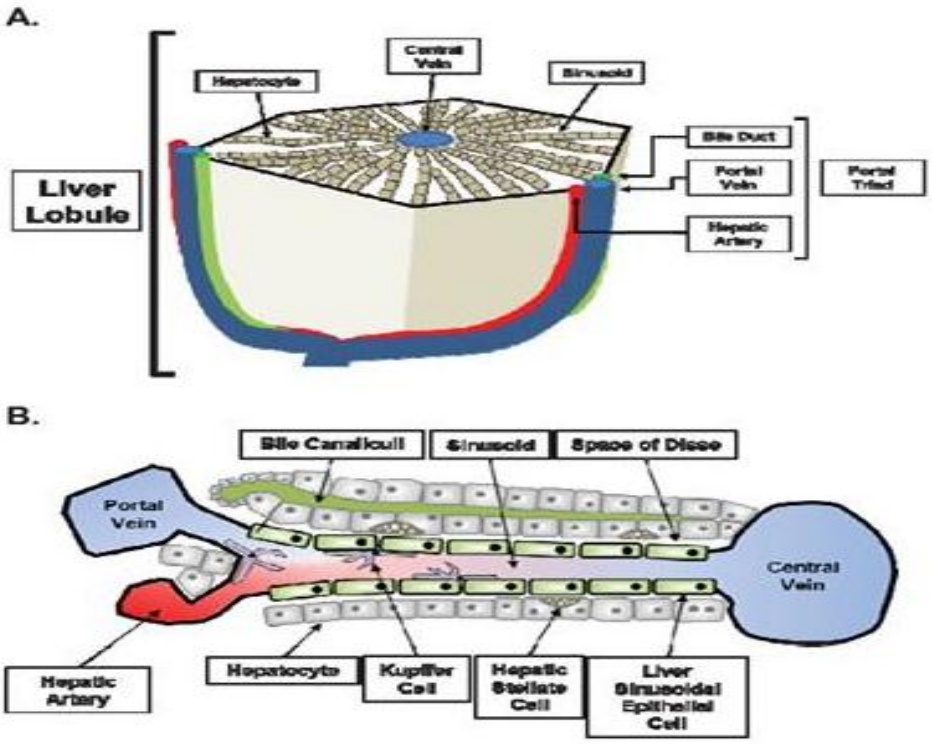


Fig 1.5. Diagrams depicting liver architecture, cell types, blood oxygen content and bile.

A. Model describing the basic structure of liver lobule. B. Model of cellular structure and blood and bile flow of the liver sinusoid from the portal triad (left side) to the central vein (right side) (Correnti, 2014).

The liver has over 500 vital metabolic and regulatory functions. One of the most important functions of the liver is the maintenance of blood glucose and it's a major target of insulin regulation. In the fasting state, when glucose is low, stored glycogen can be quickly converted back into glucose and if glucose demand surpasses glycogen is stored. It can synthesize glucose from other sources including amino acids in a process called gluconeogenesis. In the fed state when glucose is in abundant supply, insulin activates the liver to store excess glucose in the form of glycogen and suppresses liver glucose synthesis. When glycogen stores are full, the liver converts excess glucose to triglyceride, which can be transported to adipose tissue for storage (Correnti, 2014). The liver receives 80% of its blood supply from the gut through the portal vein, which is abundant in bacterial products, environment toxins, and food antigens. The remaining 20% is from vascularization by the hepatic artery (Gao et al., 2008).

The liver has a significant barrier function, keeping many soluble factors from entering the circulation which can cause inflammation. Thus, the liver plays an important role in regulating inflammation. Kupffer Cells (KCs) in the liver have significant capability to remove circulating inflammatory bacterial products including lipopolysaccharide (LPS) and inhibit helper T cell responses to LPS. LPS is an inflammatory molecule that triggers TNF-α production through binding and activating to Toll-like Receptor 4 (TLR4) in KC, which can in turn activate T cells. Additionally, the liver can also limit inflammation through its role in removing activated T cells from circulation. Liver resection stimulates hepatocyte replication which stops when liver mass is repaired, in rats it is normally in the second week after surgery. This property of the liver is clinically important as it makes the liver donation possible and forms the basis of resection as a treatment for liver cancer. Prior studies demonstrated that hepatocyte replication can occur repeatedly and considerable resection studies show that only 10 percent of the liver is required to maintain the organism to function and completely regenerate their original liver. Furthermore, the liver also carries out many other functions, including cholesterol and bile salt synthesis, storage of vitamins and minerals. It removes nitrogen from the blood, altering it into urea which can be excreted safely by the kidneys and removes

damaged or aged red blood cells from circulation. The hemoglobin from red blood cells is broken down to bilirubin which is normally removed by the liver. Accumulation of bilirubin in the serum is an indication of liver damage and is the cause of yellowing of the skin and eyes characteristic of jaundice (Correnti, 2014).

1.3.1. Liver Cell Types

The liver is comprised of many various cell types which are divided into parenchymal cells (hepatocytes) and non-parenchymal cells (other liver cell types). It has been anticipated that the hepatocyte population accounts for approximately 78% of the liver tissue volume, whereas non-parenchymal cells constitute about 6.3% in which about 2.8% are endothelial cells, 2.1% KCs, and 1.4% hepatic stellate cells (Ito cells). The extracellular space represents approximately 16% of the liver tissue volume (Ishibashi et al., 2009).

Hepatocytes have significant synthetic and metabolic abilities, they are arranged in plates which are only a single cell thick. Blood flowing toward the hepatic vein passes both the surface of the hepatocyte plates and toxins and nutrients in the blood are removed by the hepatocytes. KCs are macrophages that are found sinusoids. These cells assist removing of blood cells particularly red blood cells and bacteria. They also break down heme (in hemoglobin) into bilirubin, which then becomes one of the major pigments of bile. Sinusoidal endothelial cells have large bi-directional pores that proteins and other substances created by the liver can also be passed back into the blood. Bile duct epithelial cells produce bile salts which act as a detergent and breaks fats down into smaller components so they can be digested in the small intestine. Hepatic stellate cells (Ito cells) are found in the space of Disse. They are crucial to repairing the liver cells because when the liver is injured, the Ito cells are altered into cells that produce collagen, which leads to liver fibrosis (Allen, 2002).

1.3.2. Liver Function Tests

The liver is a complex organ, which has a variety of biochemical, synthetic, excretory, and defense functions. Blood tests used for primary assessment of liver disease include measuring levels of serum aminotransferases (ALT and AST), ALP,

and LDH. The pattern abnormal elevation of these enzymes normally points whether the disease is acute or chronic and whether cirrhosis and hepatic failure are present. Serum enzyme levels fluctuate widely from normal to moderately abnormal. Noticeable elevation of aminotransferases in the appropriate clinical context shows acute cell necrosis caused by viral infection, drugs, toxins, alcohol, or Ischemia (Hyder, 2013). Normal ranges are based on distributions from "healthy" volunteers. The upper limit of normal (ULN) is defined as the mean + 2 SD, which implies that 2.5% of the liver tests from these healthy persons exceed the ULN (Verslype, 2004).

1.3.2.1. Serum Aminotransferase

The aminotransferases are sensitive indicators of liver cell injury, particularly acute hepatocellular diseases, for instance, hepatitis (Hyder, 2013). Aminotransferases are good indicators of hepatocellular damage. ALT and AST are found in high concentration in hepatocytes, where they catalyze the transfer of alpha-amino groups from alanine and aspartate to the alpha-keto group of ketoglutaric acid, to produce pyruvic acid and oxaloacetic acid, respectively. They enter the circulation when hepatocytes are injured (Kew, 2000). Sensitivity and specificity of ALT for the detection of the liver disease is around 83 %, whereas the sensitivity and the specificity of AST are significantly lower than (70%) (Verslype, 2004). ALT is more specific than AST because it occurs completely in the liver, whereas AST occurs also in heart, skeletal muscle, kidney, brain, pancreas, and blood cells. ALT is limited to the cytoplasm, but AST is found in both mitochondria and cytoplasm. Although these aminotransferases are sensitive indicators of hepatic damage, neither alone is an ideal marker (Kew, 2000). In general, ALT is the most sensitive testing in acute and obstructive liver diseases, whereas AST is more sensitive in chronic liver diseases. AST and ALT are eliminated without degradation. Both are found in bile, but there may be other routes of elimination. The half-life of ALT in serum is 50 hours while AST has a half-life of 12 hours. The transaminases serve as liver function tests which reflect necrosis of the hepatic cell (Wolf, 1999).

Table 1.3. The typical range of elevated aminotransferase levels (AST and ALT) in various liver diseases (Limdi, 2003).

Mild Elevation (<3-fold)	**Moderate Elevation (<3-20 fold)**	**Severe Elevation (>20-fold)**
• Fatty liver • Nonalcoholic steatohepatitis • Chronic viral hepatitis	• Acute viral hepatitis • Chronic viral hepatitis • Alcoholic hepatitis • Autoimmune	• Viral hepatitis • Drug-induced hepatitis • Ischemic hepatitis

1.3.2.2. AST/ALT Ratio

The ratio of AST to ALT has more clinical utility than assessing individual elevated levels (Gowda et al., 2009). The AST to ALT ratio is useful diagnostically in patients with the alcoholic liver disease, 70% of whom have a ratio >2 (Limdi, 2003). In most patients with acute liver injury, the ratio is 1 or less. More subtle and specific tests of hepatocellular damage will undeniably become available in due course, but in the meantime measurement of serum aminotransferase concentrations best fulfills this purpose (Kew, 2000).

1.3.2.3. Alkaline Phosphatase

ALP comprises a family of enzymes located in the liver, bone, placenta, and intestine that hydrolyze phosphate esters at alkaline pH (Limdi, 2003). ALP act on a large variety of physiologic and non-physiologic substances. Although the exact natural substrate of ALP in the body is not recognized, it is related with calcification and mineralization process in bone and probably in lipid transport in the intestine (Srivastava & Chosdol, 2007). The serum ALP activity is mostly from the liver with 50% contributed by bone. Normal serum ALP is approximately 41 to 133U/L (Gowda et al., 2009).

ALP is bound to cell membranes or microsomes. An augment in serum ALP activity is usually associated with either physiologic causes or pathologic lesions of liver or bone. Physiologic causes include ALP derived from pregnancy, normal bone growth in children, and the intestinal ALP isoenzyme present in serum of healthy individuals with blood type O and B. Increased serum ALP-linked with the liver disease is caused by intra- or extrahepatic cholestasis and some destruction of hepatic cell membranes. The increase of serum ALP is observed in patients who have some form of extrahepatic or intrahepatic bile duct obstruction. Cholestasis stimulates the synthesis of ALP by the bile ductule cells, providing more ALP, which finally enters

the blood (Wolf, 1999). The degree of elevation of AP levels does not help differentiate between extrahepatic and intrahepatic cholestasis (Limdi, 2003).

1.3.2.4. Lactate Dehydrogenase

Even though LDH is not widely considered to be a liver function test, the increase of serum LDH occurs in different types of liver disease, particularly liver cancer malignancies. The increase of LDH may occur in liver injuries caused viruses, alcohols, and drugs. However, Serum LDH is not considered a specific test of liver function since damage to other organs is commonly associated with serum LDH elevations. Thus, when serum LDH is elevated as a result of hepatic disease, the most likely diagnosis is hepatic malignancy (Wolf, 1999). LDH is a common sensitive marker of solid neoplasm and many studies demonstrated increased LDH activity in different types of tumors. Jayakumar et al. (2012) proposed that LDH is a common sensitive marker of solid neoplasm and increased LDH activity is shown in different types of tumors.

1.4. D-Glactosamine

D-GalN causes panlobular focal hepatocyte necrosis, which resembles drug-induced hepatitis. The mechanism by which D-GalN causes liver injury is ambiguous. Biochemical studies theorized that D-GalN caused liver injury by exhausting the uridine pool in hepatocytes, causing inhibition of messenger RNA (mRNA), and protein synthesis. It is found that the toxicity of D-GalN was caused by the inability of the cell to synthesize vital cell membrane components, resulting in hepatocyte death. A variety of different studies was supported that addition of uridine supplement prevented D-GalN toxicity in vivo and in vitro. Certainly, in the mouse, liver injury after D-GalN is observed only when a low dose of endotoxin is also administered, causing stimulation of KCs and TNF-α release. Conversely, D-GalN causes hepatotoxicity in the rat without additional endotoxin. This difference may be the fact that D-GalN causes endotoxemia in rats but not in mice (Stachlewitz et al., 1999). D-GalN can cause hepatic damage within a period of 24 h. Numerous studies have suggested that D-GalN causes alteration of liver enzymes (Vasanth Raj et al., 2010).

A more recent study acknowledges that D-GalN changes gut permeability and increases bacterial translocation leading to endotoxemia, which is responsible for the hepatic effect. D-GalN-induced endotoxemia occurs in rats but not in mice. A prior study of D-GalN-induced hepatotoxicity found that there is an extreme inter-animal variability in the degree of the induced toxicity, and it was noticeably found that the toxic response was correlated with an incapability to fully metabolize the dosed compound. Hence, rats showing liver damage also demonstrated much urinary D-GalN, whereas urinary D-GalN was mainly absent in rats where liver damage was not apparent. Additionally, it was suggested that ATP depletion could be a key factor in D-GalN toxicity as a result of the need to re-synthesize UDP-Glucose which is depleted by the metabolism of D-GalN (Coen et al., 2007). A recently published study also reported that D-GalN induces apoptosis and necrosis in hepatocytes by increasing oxidative stress, for instance, the intracellular production of H_2O_2 (Watanabe et al., 2006).

2. MATERIALS AND METHODS

2.1. Material

2.1.1. Animal Experiments

Healthy male Wistar rats, about 2-2.5 months and weighing 200–250g were obtained from the Anadolu University Animal House. The animals were housed in a small polycarbonate cage at an ambient temperature of (22 ± 2 ° C) and humidity of (45-50%), on a 12 h light/dark cycle, and a standard pellet diet and distilled water were available without restriction. Animals used a protocol which was approved by the National Institutes of Health and Anadolu University Animal Experiment Research Center. After being acclimatized for 1 week, the rats were randomized and divided into 5 groups of 7 animals in each group.

2.2. Method

2.2.1. Chemicals

EA was supplied by Sigma Chemicals Company, St Louis, MO, USA (Yüce et al., 2007). D-GalN was also purchased by Sigma Chemicals Company, St Louis, MO, USA (Coen et al., 2007). All other chemicals and biochemicals were of analytical grade obtained from local firms. The organic solvents were distilled before use.

2.2.2. Experimental Design

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

Group I: Control rats were administrated 0.5 ml of normal saline through the IP.

Group II: Control rats were administrated 0.2% (0.5ml of DMSO) through the IP.

Group III: Rats were administrated D-GalN (750 mg/kg body weight) through the IP.

Group IV: Rats were administrated EA (20 mg/kg body weight) through gavage followed by administration of D-GalN (750 mg/kg body weight) after 24h through the IP.

Group V: Rats were administrated D-GalN (750 mg/kg body weight) through IP followed by administration of EA (20 mg/kg body weight) after 24h through the gavage.

At the end of the experimental regimen, animals in different groups were killed by cervical decapitation. Blood samples were collected by heart puncture and serum was separated by centrifugation and used for various biochemical estimations. The liver was dissected out, washed in ice-cold saline, blotted dry and weighed. Then homogenate was prepared in phosphate buffer 0.1 M, pH 7.4 and used for the biochemical analysis.

2.2.3. Anesthesia and Surgical Applications

All experimental work will be carried out using a sterile environment and sterile surgical instruments. Animals were anesthetized using with Ketamine / Rompun (50 + 10 mg / kg) and intra-cardiac blood samples were collected using 1/5 EDTA tubes. Blood was counted using Hemavet 850 brand of mouse calibration device, where the rest of blood were centrifuged using Eppendorf Centrifuge 5804 R for 10 minutes at 3000 RPM. In centrifugation AST, ALT, LDH, and ALP serums samples were obtained for measurements. Serum samples were transferred into polyethylene tubes will be kept in the freezer for biochemical analysis -80˚C. ALT, AST, ALP, and LDH measurements will be made using HITACHI-917 autoanalyzer with commercial kits.

2.2.4. Histopathological Studies

The liver samples fixed for 48 h in 10% formalin were dehydrated by passing successfully in different mixtures of ethyl alcohol and water, cleaned in xylene and embedded in paraffin. Sections of the liver (5–6 μM thick) were prepared and then stained with hematoxylin and eosin dye (H&E) and mounted in the neutral deparaffinated xylene (DPX) medium for microscopic observations.

The preparation of liver samples

The liver samples which are taken from the rats were washed carefully with normal saline and then they were divided into small sections and fixed 10 % of buffered formalin.

Paraffin processing and sectioning of liver samples

- Tissue Fixation
- 10 % formaldehyde (Neutral buffered formalin)
- Tissue processing
- Tissue embedding (Blocking)

Studying of Fine Structural Changes with Transmission Electron Microscope (TEM)

Electron Microscope and evaluation apoptosis are considered to be the most valuable method ("gold standard"). It is the method that morphological changes can be observed most accurately. It will be utilized for the determination of the fine structure (mitochondria, lysosomes, endoplasmic reticulum, organelles, etc.) of the cells. Moreover, subcellular details can also be examined (for example, the state of the mitochondria, such as the degradation of the cell membrane or nucleus membrane). At electron microscope studies, nucleus fragmentation can be monitored clearly, compared to normal cells, cytoplasmic shrinkage, chromatin condensation and fragmentation can be monitored at apoptotic cells.

TEM Tissues to follow,

- **Fixative Preparation**: Is the first fixative buffered glutaraldehyde.
  Phosphate Buffer;
  By mixing Na_2HPO_4 and KH_2PO_4, the buffer solution was prepared. According to the structure and size of the cells to be studied, the cells will be fixed during detection at +4 ºC between 4-24 hours.

- **Washing**: Cells will be washed with buffer solution to remove fixative from fixed cells (3x15 min.)

- **Second Fixation (Osmium Tetroxide):** Washed cells taken into 1 percent OSO_4 and rotated at rotator for 2h and ensured that they are fixated. After this, they will be ablated with a buffer with 3x15 min.

- **Dehydration:** Made with ethyl alcohol**.**
- **Become Transparent:**

  Propylene 30 min. X2

  Resin+ propylene oxide (1/1)

Cell at this mixture will be rotated for 2h at rotator.

- **Blocking:** Blocking operation is performed with freshly prepared resin. Polymerization process will be carried out for 48h at 60 ºC.

Preparation of Resin Solution:

Araldite CY212 20 ml,

DDSA 20ml,

BDMA 0.6 ml,

Dibutylphthalete 1 ml,

- **Painting of Sections:** Preparation of uranyl acetate paint;
- **Preparation of Lead Citrate Paint:**

A Mixture

B Mixture

It will be mixed by mixing 0.5 ml of A mixture to B mixture and strongly shaking it. (It should be between pH 12-13). It will be put inside a clean falcon and stored at dark and at +4 ºC. Painted sections will be studied at microscope and photographs will be taken.

2.2.5. Biochemical Determination

ALT, AST, ALP, and LDH Serum Measurement

The activities of AST, ALT, ALP, and LDH were measured in serum by using the diagnostic kits of Sigma Diagnostics, according to standard procedures using commercially available kits.

1. ALT Serum Measuring Principle

Serum ALT levels (U/L) was determined using (Boolabo, Maizy, and France) commercial kits with CRONY AIRONE 200 RA autoanalyzer.

Measuring Principle: The method was developed by Wrobles on Ladue and then optimized by Henry and Bergmeyer. The complete schematic reaction is as follows:

ALT

Oxoglutarate + L-alanine $\rightleftharpoons$ Glutamate + Pyruvate

The rate of conversion of the NADH to NAD + can be determined by monitoring the decrease in absorbance colorimetrically at 340 nm. This rate of conversion from the reduced cofactor to the cofactor is a function of the activity of ALT.

2. AST Serum Measuring Principle

Serum AST levels (U/L) was determined using (Boolabo, Maizy, and France) commercial kits with CRONY AIRONE 200 RA autoanalyzer.

Measuring Principle: The method was developed by Wrobles on Ladue and then optimized by Henry et al. The complete schematic reaction is as follows:

AST

L-Aspartate + 2-Oxoglutarate ⇌ Oxaloacetate + L-Glutamate

The rate of conversion of the NADH to NAD + can be determined by monitoring the decrease in absorbance colorimetrically at 340 nm. This rate of conversion from the reduced cofactor to the cofactor is a function of the activity of AST.

3. LDH Serum Measuring Principle

Serum LDH levels (U/L) was determined using (Boolabo, Maizy, and France) commercial kits with CRONY AIRONE 200 RA autoanalyzer.

Measuring Principle: The method was developed by Henry et al. The complete schematic reaction is as follows:

LDH

Pyruvate + NADH ⇌ L-Lactate + NAD+

The rate of conversion of the NADH to NAD + can be determined by monitoring the decrease in absorbance colorimetrically at 340 nm. This rate of conversion from the reduced cofactor to the cofactor is a function of the activity of LDH.

4. ALP Serum Measuring Principle

Serum LDH levels (U/L) was determined using (Boolabo, Maizy, and France) commercial kits with CRONY AIRONE 200 RA autoanalyzer.

Measuring Principle: The method was developed by Lowry, Bessey, and Brock. Then, it was developed by Hausmen et al. In the presence of magnesium and zinc ions; p-nitrophenyl phosphate is hydrolyzed to form p-nitrophenol and phosphate. The complete schematic reaction is as follows:

ALP

P-nitrophenyl phosphate + H_2O -------→ P-nitrophenol + Phosphate

(Colorless) **Mg++; pH 10.3** (Yellow)

The rate of conversion of the p-nitrophenyl phosphate to the colored p-nitrophenol is proportional to the ALP activity. The p-nitrophenyl phosphate product with yellow color is monitored spectrophotometrically at 405 nm.

2.2.6. Statistical Analysis.

All values were presented as mean ± S.E.M. Differences were considered to be significant at $P < 0.05$. Data from the blood serum measurements of ALT, AST, ALP, and LDH were analyzed and statistical analyses were performed using one-way analysis of variance (ANOVA) by "SPSS 12.0 for windows".

3. FINDINGS

3.1. Determination of biochemical parameters

All rats survived the experimental period until sacrifice. The intoxication of rats with D-GalN 700 mg/kg b.wt significantly altered the biochemical parameters when compared with the normal control rats (p<0.001, Table 3.1). A significant increase in the levels of AST, ALT, ALP and LDH levels were observed intoxicant groups. Treatment with EA 20 mg/kg b.wt significantly decreased the level of AST, ALT, ALP and LDH levels towards normal. All these significant changes in the levels of biochemical parameters refer to the effect of EA in protecting the liver by restoring the altered levels in rats.

Table 3.1. Effect of EA on the biochemical parameters of D-GalN intoxicated rats

Groups	**ALT (U/L)**	**AST (U/L)**	**LDH (U/L)**	**ALP (U/L)**
1	43.884±3.8055	78.775± 5.4732	576.71±49.398	160.10±9.5713
2	60.088±5.3096	81.987±5.3925	592.28±42.413	195.29±18.989
3	648.09±70.327**	551.93±51.828***	956.28±83.039**	237.98±27.107**
4	152.87±9.3734**,#≠	158.521±11.505**,#≠	664.14±47.726*,#	171.57±17.1326*,#≠
5	167.16±11.427**,#≠	178.27±19.853**,#≠	640.85±141.51*,#	165.08±22.516*,#≠

The values are the means ± S.E.M. of 7 mice per group.

* (p <0.05), ** (p <0.001), significantly different from the control groups.

#≠ (p<0.001), ≠ (p<0.01), significantly different from the vehicle-treated D-GalN groups.

3.1.1. Evaluation of serum ALT enzyme activity of experimental groups

The serum ALT activities were 43.8843±3.80550U/L and 60.0886±5.30966 in control groups. After D-GalN injection, the serum ALT activities increased to approximately 11 to 15-fold that in control groups. These increases were significantly attenuated by 20 mg/kg of EA. D-GalN caused significant increases in the ALT level of the liver ($p<0.001$) when compared to the control groups. Significant decreases ($p<0.001$) observed in the EA + D-GalN and D-GalN + EA groups compared to the D-GalN alone group with respect to ALT levels. ALT levels of EA + D-GalN and D-GalN + EA groups slightly increased ($p<0.05$) when compared to the control groups.

3.1.2. Evaluation of serum AST enzyme activity of experimental groups

The serum AST activities increased 7-fold after D-GalN injection. This increase was significantly attenuated by 20 mg/kg of EA. D-GalN caused significant increases in the AST level of the liver ($p<0.001$) when compared to the control groups. Significant decreases ($p<0.001$) observed in the EA + D-GalN and D-GalN + EA groups compared to the D-GalN alone group with respect to AST levels. AST levels of EA + D-GalN and D-GalN + EA groups slightly increased ($p<0.05$) when compared to the control groups.

3.1.3. Evaluation of serum LDH enzyme activity of experimental groups

The serum LDH activities increased approximately 1.6-fold after D-GalN injection. This increase was significantly attenuated by 20 mg/kg of EA. D-GalN caused significant increases in the LDH level of the liver ($p<0.001$) when compared to the control groups. Significant decreases ($p<0.01$) observed in the EA + D-GalN and D-GalN + EA groups compared to the D-GalN alone group with respect to LDH levels. LDH levels of EA + D-GalN and D-GalN + EA groups slightly increased ($p<0.05$) when compared to the control groups.

3.1.4. Evaluation of serum ALP enzyme activity of experimental groups

The serum ALP activities increased approximately 1.2 to 1.5-fold after D-GalN injection. These increases were significantly attenuated by 20 mg/kg of EA. D-GalN caused significant increases in the ALP level of the liver ($p<0.01$) when compared to the control groups. Significant decreases ($p<0.01$) observed in the EA +

D-GalN and D-GalN + EA groups compared to the D-GalN alone group with respect to ALP levels. LDH levels of EA + D-GalN and D-GalN + EA groups slightly increased (p <0.05) when compared to the control groups.

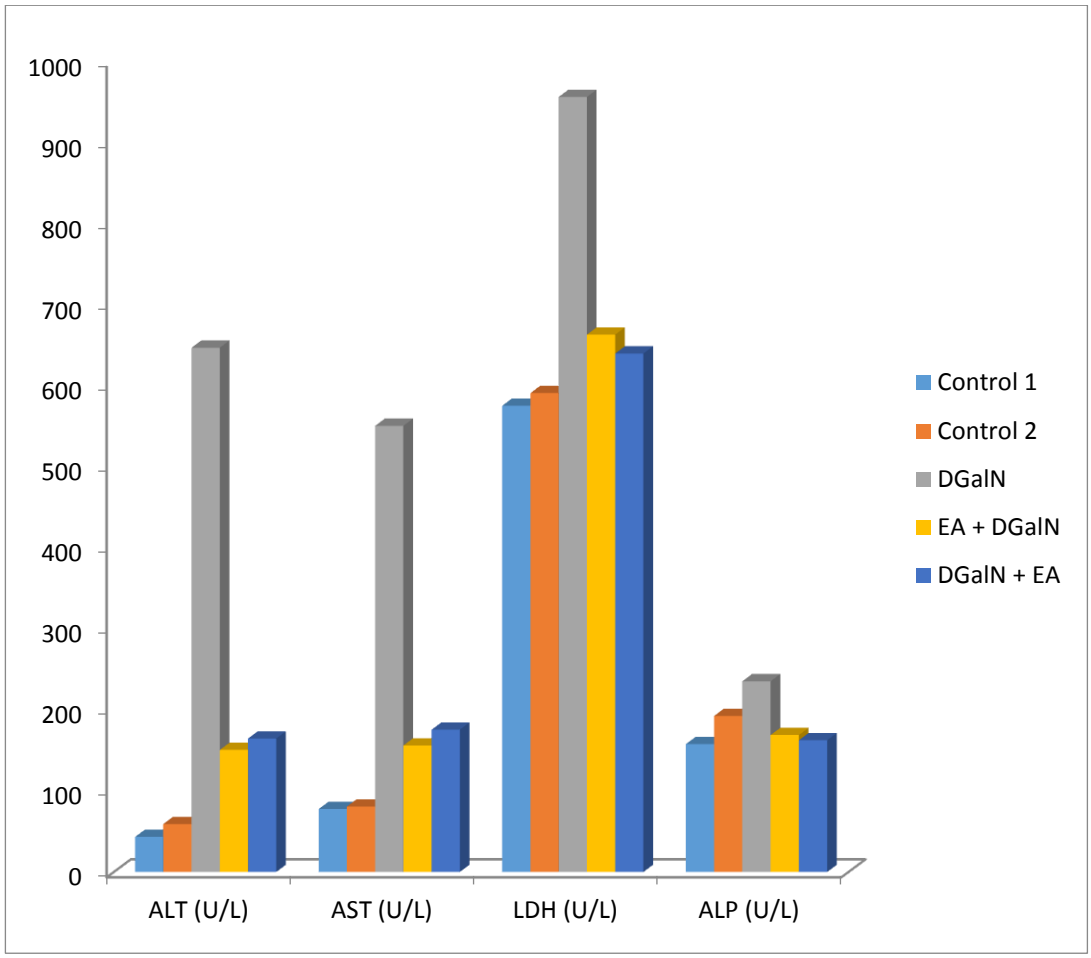


Fig 3.1. ALT, AST, LDH, and ALP levels in different experimental groups.

* p<0.05 (comparing of the differences between control groups and vehicle-treated groups), ** p<0.001 (comparing of the significant differences between control groups and vehicle-treated groups), ## p<0.001 (comparing of the significant differences between vehicle-treated groups), ≠ p<0.01 (comparing of the differences between vehicle-treated groups).

3.2. Evaluation of Histological Studies

3.2.1. Evaluation of Liver Samples by Light Microscope

The histological features shown in Fig 3.2 indicate a normal liver lobular architecture and cell structure in the livers of control animals. In control groups, cells maintained their original sinusoidal and hepatic morphology. However, livers exposed to D-GalN (Fig 3.3) showed broad hemorrhagic necrosis, extensive areas of portal inflammation complete damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis, congestion of hepatic sinusoids and a moderate increase in inflammatory cell infiltration. These pathological alterations were attenuated in animals receiving EA treatments (Fig 3.4, 3.5). The disorganization caused by the D-GalN treatment was somewhat (Fig 3.3) to very obviously (Fig 3.4, 3.5) reduced in the sections from the rats given EA. Liver sections of D-GalN +EA and EA + D-GalN groups showed pronounced modulated effects when compared to D-GalN treated groups. EA at a dose of 20 mg/kg b.wt showed good improvement in hepatocytes as compared to that of D-GalN treated animals. There was slight congestion and mild vacuolization observed in EA+ D-GalN and D-GalN + EA treated groups.

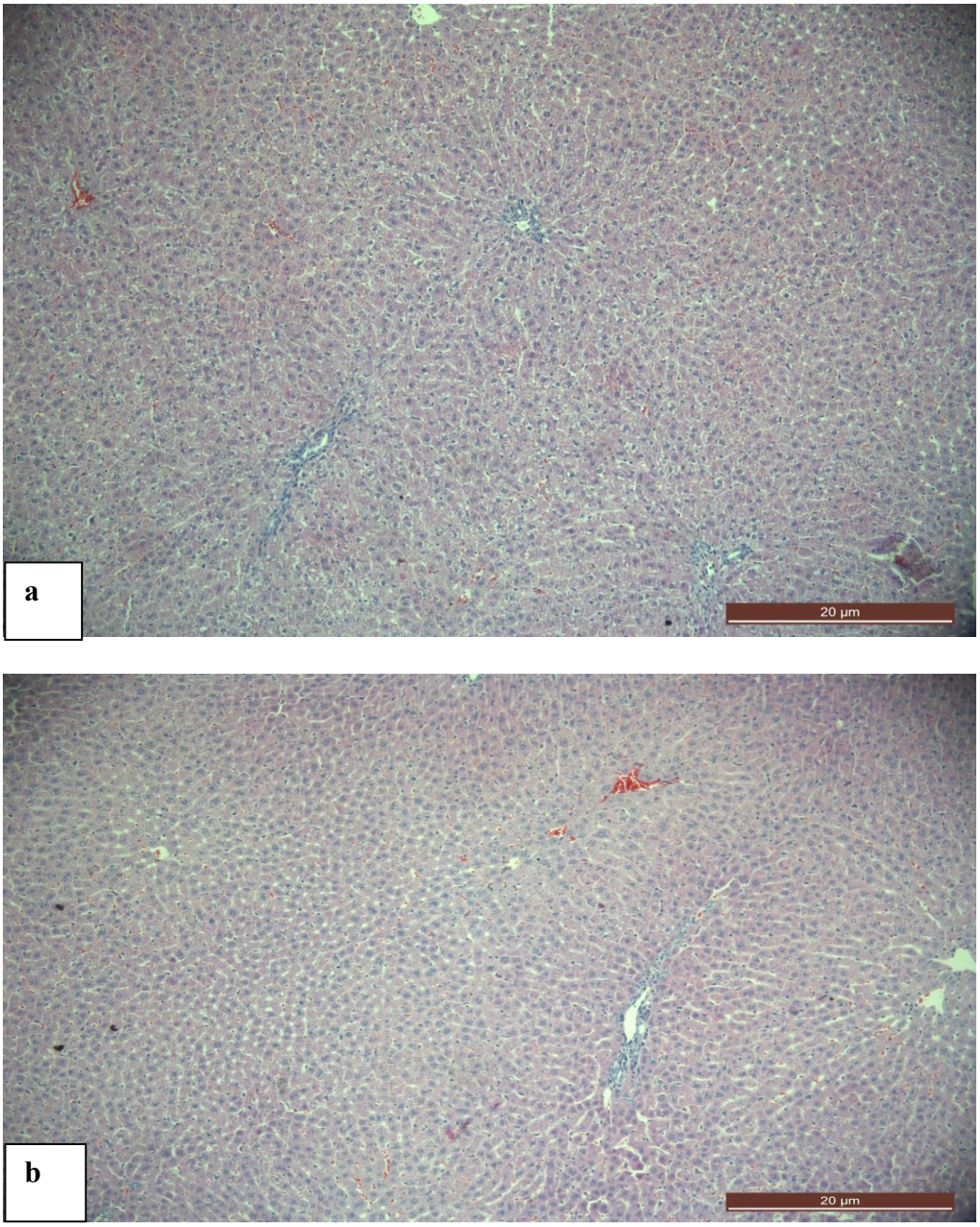


Fig 3.2. Light microscopic examination of the control groups (a, b) showing normal liver lobular architecture and cell structure (10X, HE).

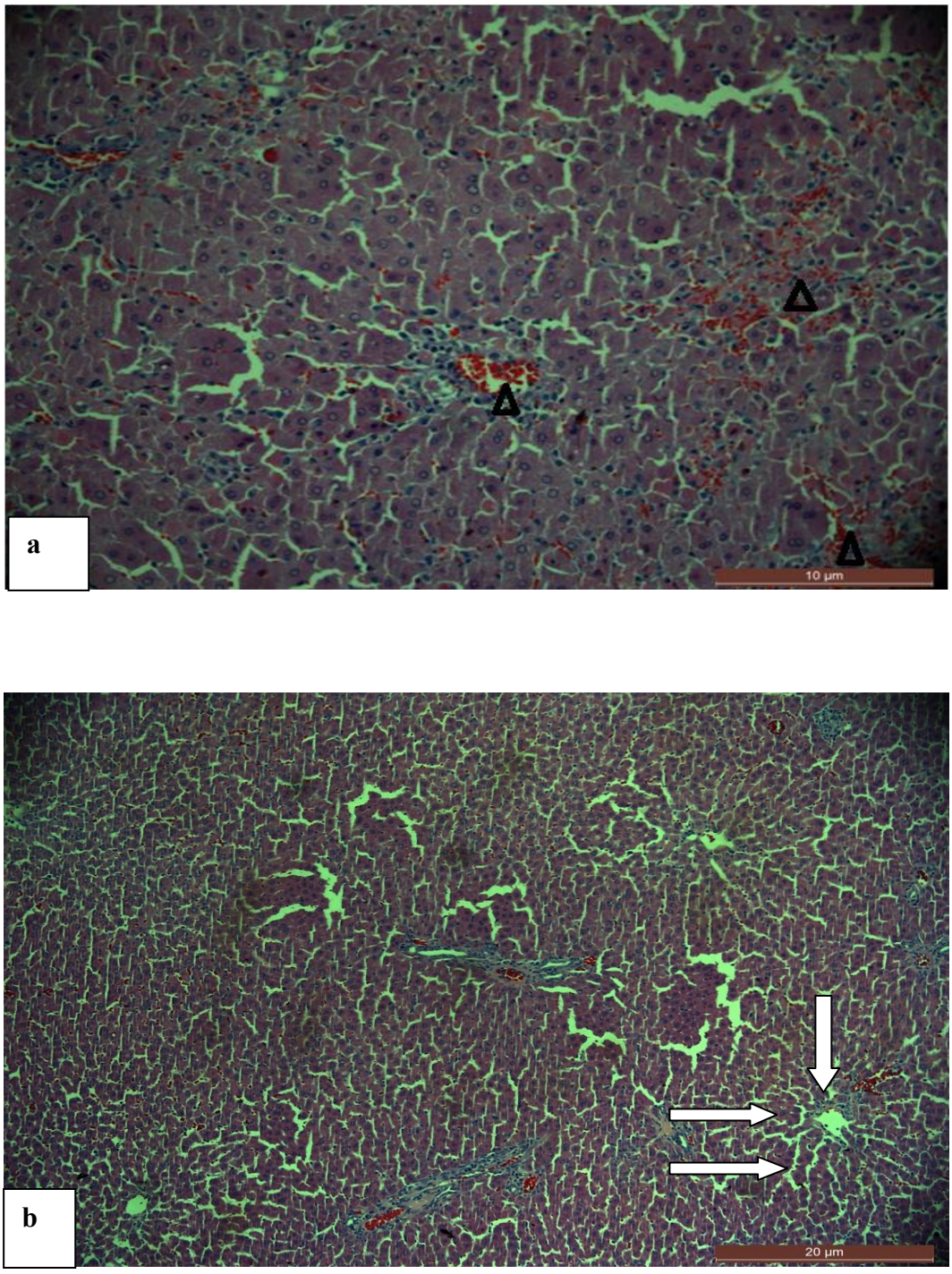


Fig 3.3. Light microscopic examination of D-GalN treated liver tissues (a, b). Head of the arrows indicating extensive portal inflammation and arrows showing damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis and congestion of hepatic sinusoids (a-b, 10X, 20X, HE).

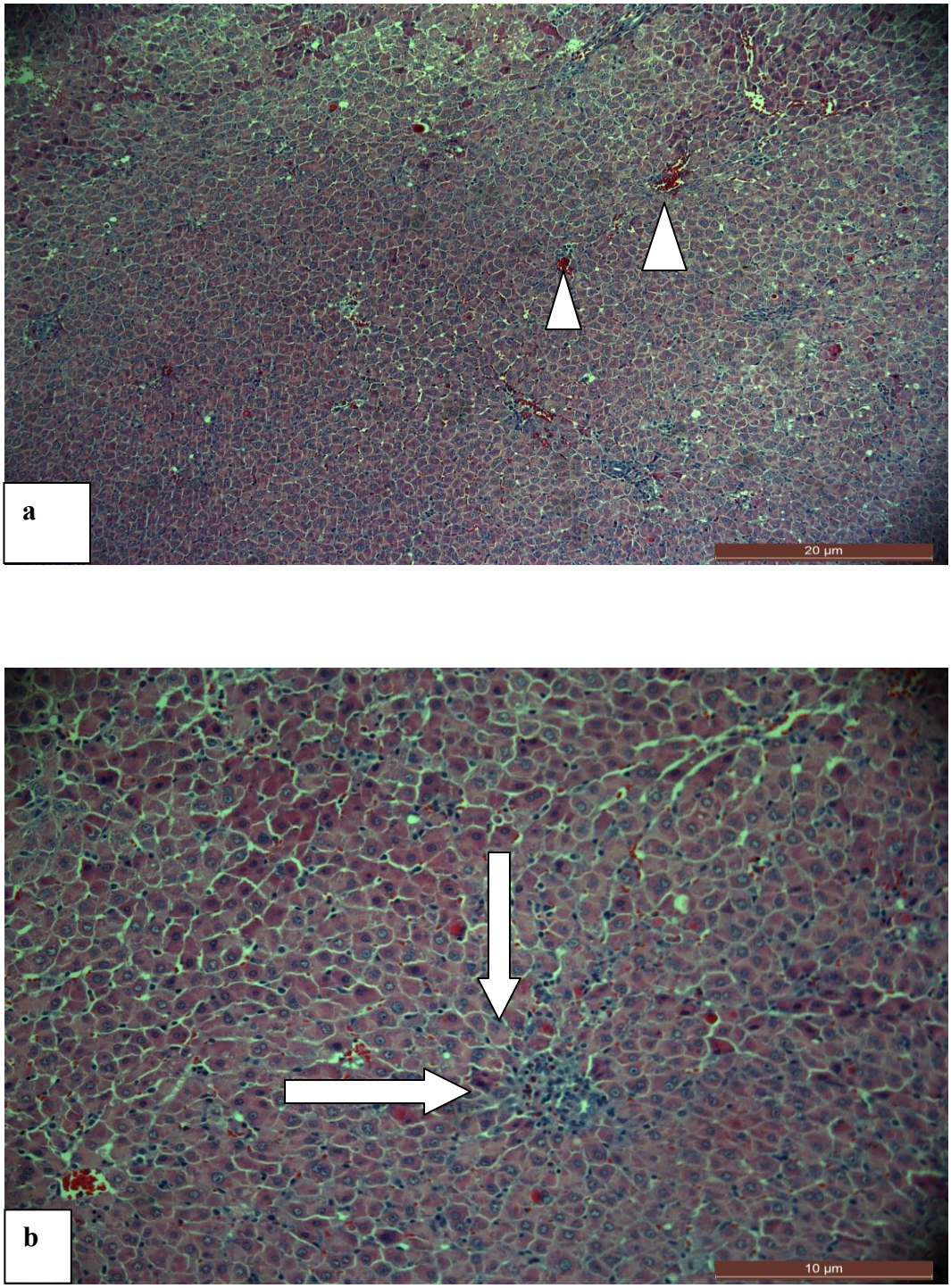


Fig 3.4. Light microscopic examination of EA + D-GalN treated liver tissues (a, b). Head of the arrows indicating less portal inflammation and arrows showing hepatocytes maintaining near normal architecture with mild hepatocellular vacuolization, less focal hepatic necrosis and mild congestion of hepatic sinusoids (a-b, 10X, 20X, HE).

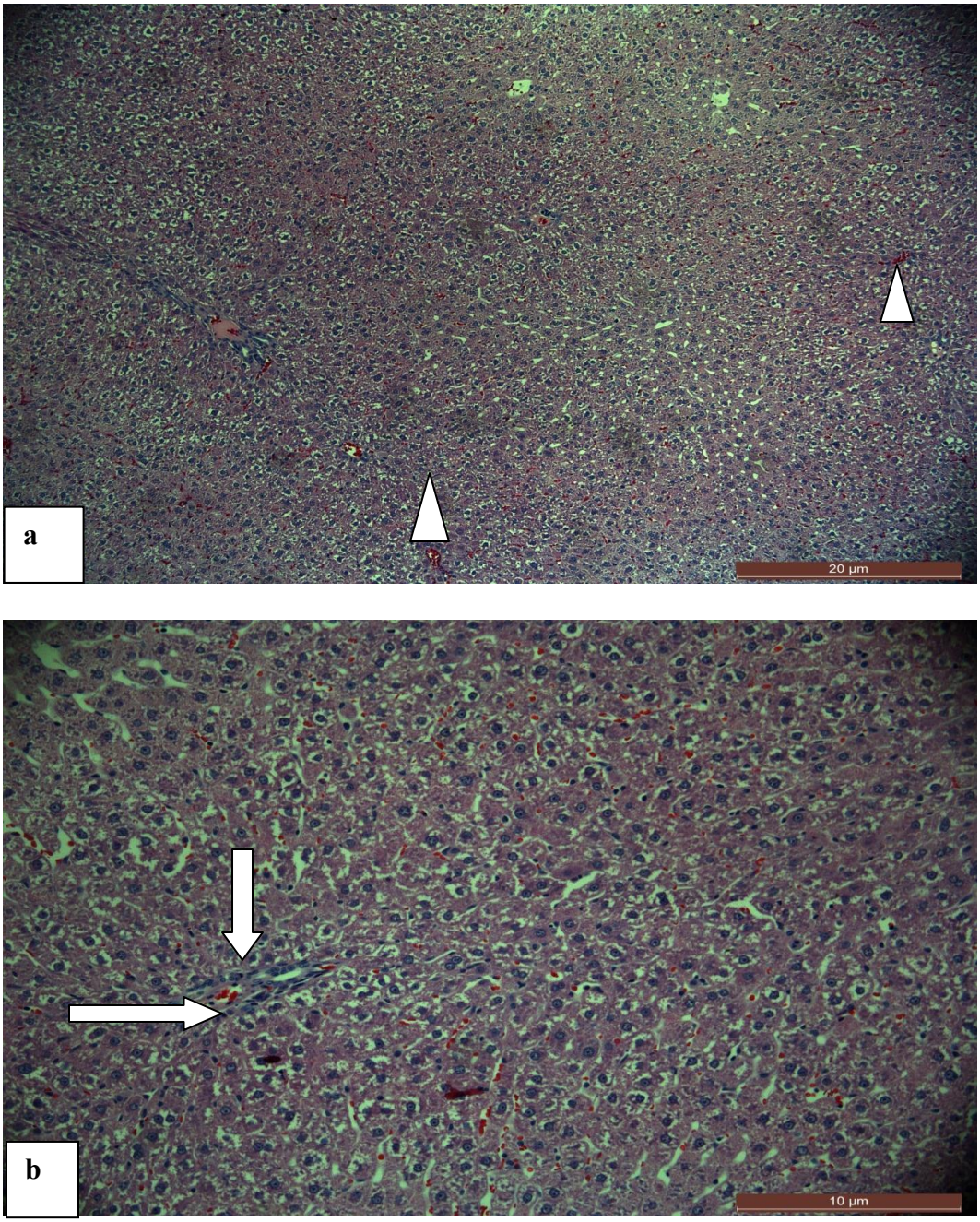


Fig 3.5. Light microscopic examination of D-GalN + EA treated liver tissues (a, b). Head of the arrows indicating less portal inflammation and arrows showing hepatocytes maintaining near normal architecture with mild hepatocellular vacuolization, less focal hepatic necrosis and mild congestion of hepatic sinusoids (a-b, 10X, 20X, HE).

3.2.2. Evaluation of Liver Samples by Transmission Electron Microscope (TEM)

In order to observe the effects of EA on D-GalN administrated rat liver samples, all animal groups were investigated by Transmission Electron microscope (TEM). The hepatocytes of liver tissues of control groups treated by normal saline and DMSO were observed small uniform nuclei, granular cytoplasm, and normal structure of cells and organelles (Fig 3.6). The hepatocytes of D-GalN treated rats were detected multiple irregular shaped nuclei close to each other with an irregular cytoplasm and slightly shrunken cell changes, mitochondrial with melting cristae, and damaged endoplasmic reticulum (Fig 3.7). EA administrated groups (EA + DGalN and DGalN + EA) were examined TEM and the liver cells were observed and nuclear membrane and chromatin became evident, the damage of mitochondrial cristae and endoplasmic reticulum were restored, regular nuclear membrane structure and accumulation of glycogen inside cells were also observed (Fig 3.8, 3.9).

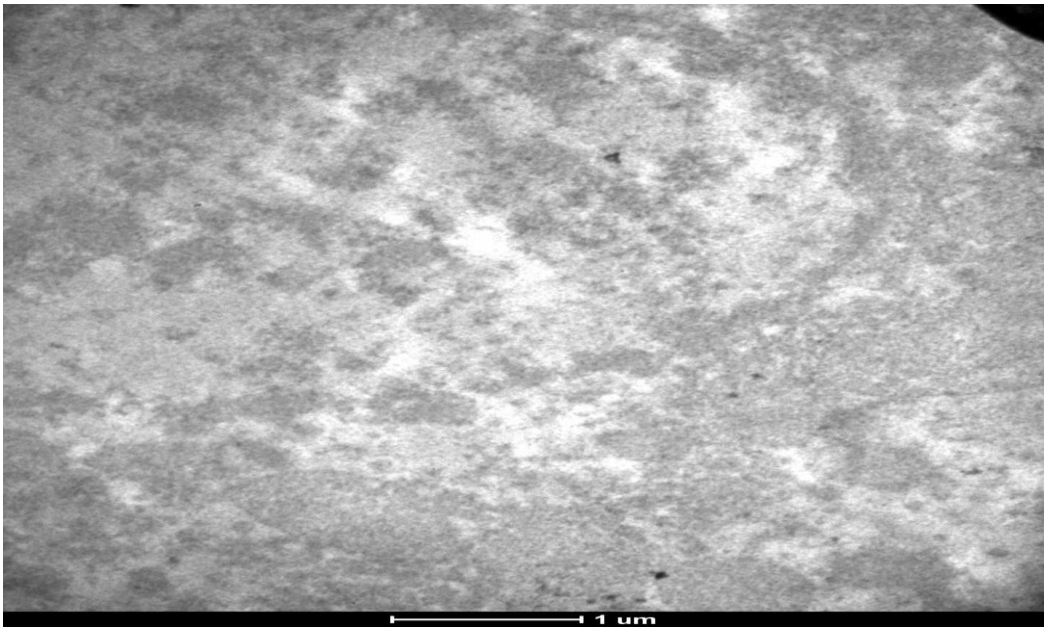


Fig 3.6. Transmission Electron Microscope (TEM) examination of the liver tissue of the rats of the control groups showing normal structure of cells and organelles, cytoplasmic granules, and small and uniform nuclear hepatocytes (16500X).

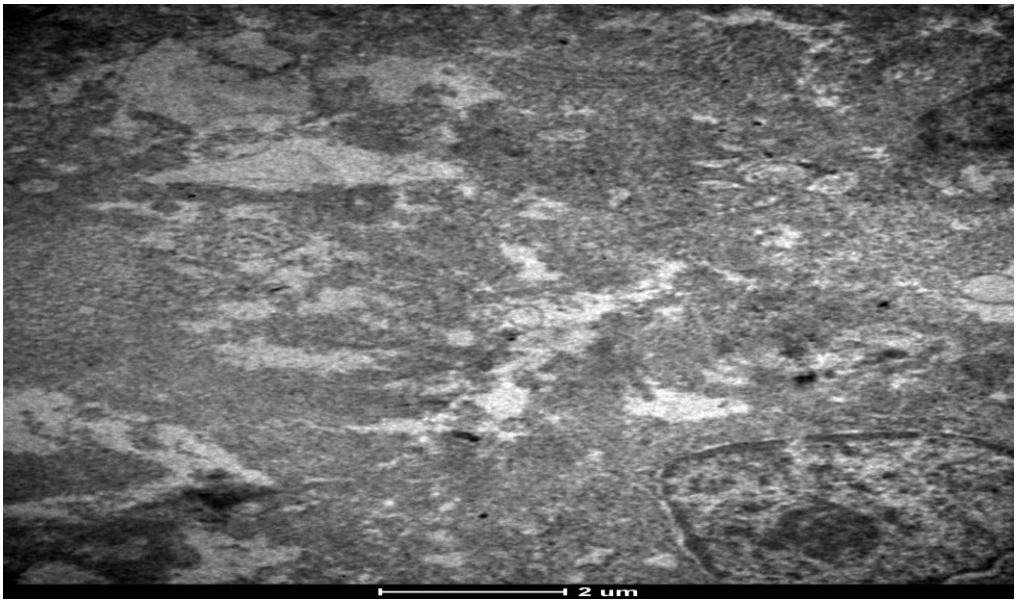


Fig 3.7. TEM examination of the liver tissue of the rats of D-GalN treated rats showing multiple irregular shaped nuclei close to each other with an irregular cytoplasm and slightly shrunken cell changes, loss of mitochondria cristae, and damaged endoplasmic reticulum (8200X).

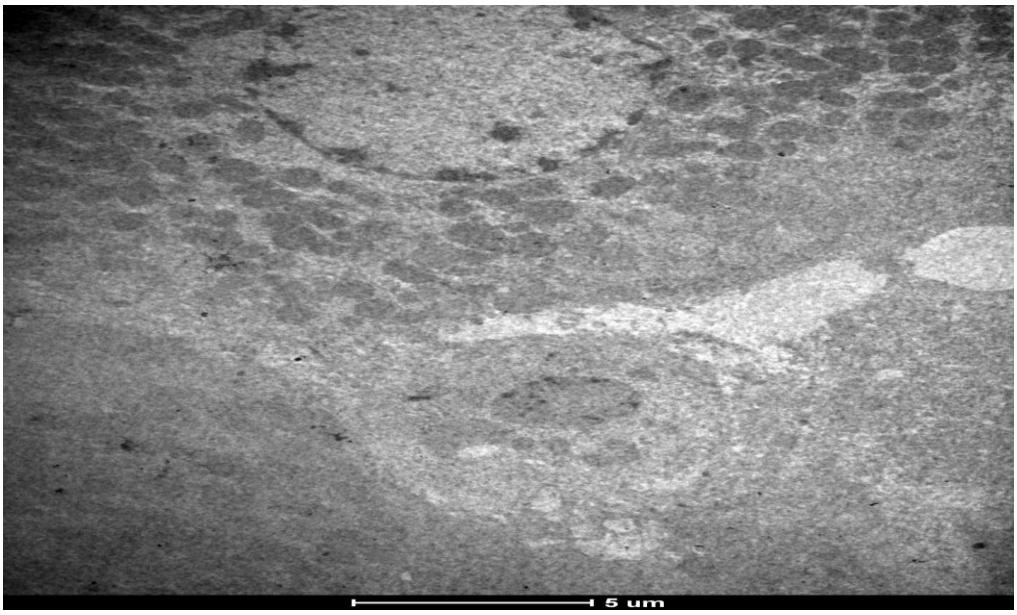


Fig 3.8. TEM examination of the liver tissue of the rats of the pretreated EA (EA + D-GalN) showing protection of mitochondrial cristae and endoplasmic reticulum, regular nuclear membrane structure and accumulation of glycogen inside the cells (4200X).

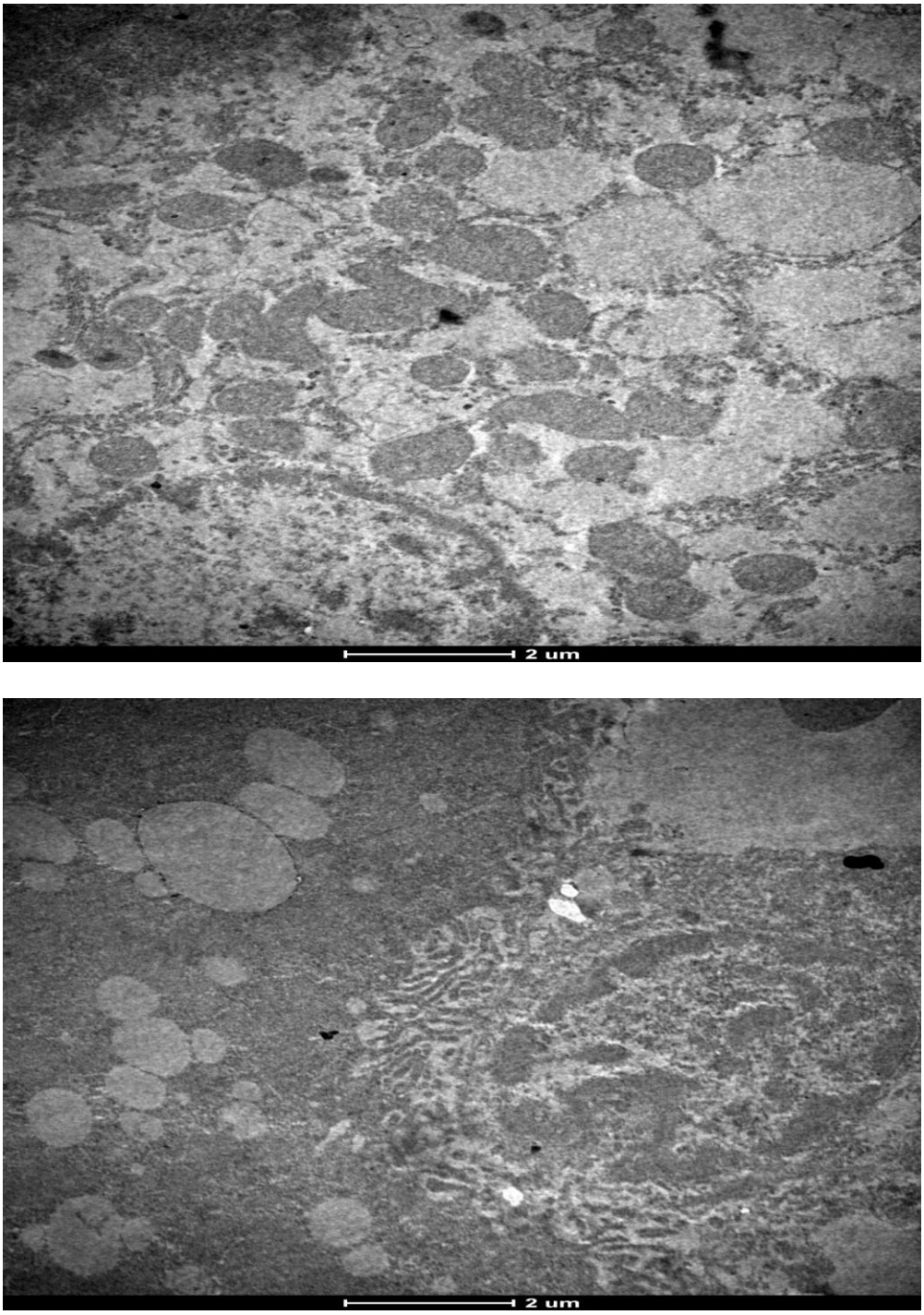


Fig 3.9. TEM examination of the liver tissue (a, b) of the rats of the post treated EA (D-GalN + EA) showing protection of mitochondrial cristae and endoplasmic reticulum, regular nuclear membrane structure and accumulation of glycogen inside the cells (8200X).

4. DISCUSSIONS AND CONCLUSIONS

The liver is a complex organ and plays a key role in supporting the process of detoxification, lipid metabolism, hormone production, decomposition of red blood cells, and synthesis of plasma protein (Sohn et al., 2013). Hepatic injury is induced by viruses, alcohol or hepatotoxic agents and results in enormous hepatocyte death. Only about 10% of patients can be rescued on hepatic medical drugs. Hence, there is a critical need for effective therapy against hepatic injuries (Kim et al., 2010). The current study investigated the hepatoprotective effects of EA at the dose level of 25 mg/kg on experimentally induced hepatic toxicity by using a single dose of 750 mg/kg D-GalN and on in vivo liver rat model. To the best of our knowledge, this is one of the first studies to report on the potential protective effect of EA against D-GalN-induced acute hepatic injury.

The use of dietary antioxidants is an important preventive method to minimize the pathological and toxic effects associated with xenobiotics (Celik et al., 2013). EA, a type of polyphenol compound that widely exists in herbs and numerous types of fruits and nuts, has recently gained increasing attention, it has been well established that EA exhibits anticancer, antimutagen and antimicrobial functions, as well as others. Abundant studies concerning the antimutagen and anticancer effects of EA were performed in the 1970s (Zhang et al., 2014). Accumulated studies confirm that EA has a strong correlation in liver protection. New study, which was designed to examine the effect of EA on liver marker enzymes, lipid peroxidation, and tissue lipid levels during alcohol-induced toxicity states that oral administration of EA protects the system from alcohol toxicity by decreasing the liver marker enzymes and lipid peroxidative markers and modulating the lipid metabolism in a dose-dependent manner (Devipriya et al., 2008). It was reported that EA decreased the liver enzymes upon CCl_4-induced toxicity due to its antioxidant effects (Gümüş et al., 2011). According to a data, which reveals the protective effect of EA against oxidative damage induced $AlCl_3$ in the liver shows that EA decreased the lipid peroxidation in the liver by alleviating the MDA levels in $AlCl_3$ treated animals. Also, it enhances the antioxidant level of the liver by stimulating GSH-Px activities and GSH levels in

liver. It was also, observed that addition of EA improved the Vitamin E level, particularly in the liver. Vitamin E can directly regulate H_2O_2 production in mitochondria and acknowledged that the overproduction of mitochondrial ROS is the first step that leads to tissue damage that is observed in Vitamin E deficiency syndromes (Özkaya, 2010). Additional study of EA to cisplatin-treated animals reduced the MDA levels (Yüce et al., 2007). Moreover, EA has protective effects on ethanol-induced toxicity through the regulation of NO and TGF-β1 production in liver cells (Sohn et al., 2013).

EA is good dietary antioxidant gifted of scavenging free radicals that could prevent diseased conditions. It is found that EA has better protection than vitamin E against oxidative stress. A recently published study reported that the experiential cytogenetic protection against H_2O_2 by EA could be through scavenging reactive free radicals (Ohan, 2002). It is found that phenolic antioxidant such as EA improves the antioxidant response of the cell not only by themselves acting as redox modulators by virtue of their free radical scavenging activity but they are also able to up-regulate pathways in the cell that can replenish the need for reducing equivalents that hold up cellular antioxidant enzyme response (Özkaya, 2010). EA has been revealed to exert a potent scavenging action on both the superoxide anion O_2 and the OH radical, as well as lipid peroxidation. A prior study, which was observed the effect of EA on cisplatin-treated animals reduced the MDA levels, increased the GSH levels in liver and heart tissues in comparison to the cisplatin alone group. The decreasing of lipid peroxidation in these tissues actually indicates that EA potently scavenged the free radicals (O_2 and · OH) and suppressed oxidative DNA damage. The antioxidant activity of EA and alleviation of ROS-induced depletion of GSH-Px and CAT activities along with GSH level also illustrate that EA has strong antioxidant activity (Yüce et al., 2007). EA was found to scavenge ROS and RNS such as OH radicals, peroxyl radicals, NO_2 radicals, and peroxynitrite with rate constants equivalent to those of many well-known antioxidants such as vitamin E and vitamin C (Ohan, 2002). Devipriya et al., previous study, has reported that EA, besides acting as an antioxidant, enhances the antioxidant status at both enzymatic and non-enzymatic levels in the tissues and also decreases the lipid peroxidation in tissues during

alcohol-induced toxicity. Thus, an improvement of antioxidant status by EA might decrease the lipid peroxidation in the circulation. They have also shown that EA attenuates the tissue damage by decreasing lipid peroxidation. From the above findings, we could expect that EA scavenges free radicals and regulates endogenous antioxidants status and maintains pro-oxidant/antioxidant balance, which subsequently decreases the attack of free radicals on the membrane lipids and attenuates tissue damage by preventing propagation of lipid peroxidation (Devipriya et al., 2008).

D-GalN is an extensively used hepatotoxicant (Das et al., 2012). Vasanth Raj et al., (2010) proposed that D-GalN mediated hepatotoxicity was chosen as the experimental model. Since the changes associated with D-GalN induced liver damage are similar to that of acute viral hepatitis. D-GalN dose of 700 mg/kg b.wt was finalized after acute toxicity studies and following standard protocol.

D-GalN acts directly or indirectly and alters antioxidant status that makes certain organs, more susceptible to oxidative stress. Several studies have shown that D-GalN causes alteration of hepatic enzymes. D-GalN is known to selectively block the transcription and indirectly hepatic protein synthesis and as a consequence of endotoxin toxicity, it causes fulminant hepatitis (Vasanth Raj et al., 2010). An explanation of the mechanism of D-GalN-induced liver injury was considered as inducing the disruption of liver glucose metabolism, which leads to a decrease in RNA and protein synthesis, resulting in necrotized hepatocytes (Chaung et al., 2003). D-GalN may exhaust several uracil nucleotides including UDP-glucose and UDP-galactose. Accumulation of UDP-sugar nucleotide may contribute to the change in the rough endoplasmic reticulum and to the disturbance of protein metabolism. Severe damage of D-GalN to the membrane structures is thought to be responsible for the loss in the activity of ionic pumps (Raj et al., 2011). The impairment in the calcium pumps, with a consequent increase in the intracellular calcium, is considered to be responsible for cell death. D-GalN has been found to induce extensive liver damage within a period of 24 h following IP administration (Vasanth Raj et al., 2010). Administration of D-GalN disrupts the permeability of the plasma membrane,

causing leakage of enzymes from the cell, leading an increase of the levels of the serum enzymes (Kim et al., 2015). Intracellular enzymes, such as transaminases, ALP, and LDH are useful indicators for liver function; their increased levels are indicators of liver damage. Aminotransferases (AST and ALT) are reliable marker enzymes of the liver and they are the first enzymes to be used in diagnostic enzymology when liver damage has occurred. Because of their intracellular location in the cytosol, toxicity affecting the liver with a subsequent breakdown in membrane architecture of the cells leads to their spillage into serum, and their concentration rises in the latter (Jayakumar et al., 2012). In our study, a considerable elevation of serum ALT, AST, ALP, and LDH was detected after D-GalN injection, and this increase was attenuated by treatment with EA. Normal lobular architecture and cell structure were observed in the control groups, whereas increased hepatocellular necrosis, portal inflammation, and inflammatory cell infiltration were observed in the liver section obtained from D-GalN-treated animals. These pathologic changes were attenuated by pre- and post-treatment with EA, suggesting that EA may have a potential clinical application in the treatment of liver disease.

In this study, the intoxication of rats with D-GalN 700 mg/kg b.wt significantly altered the biochemical parameters when compared with the normal control rats ($p< 0.001$, Table 3.1). A significant increase in the levels of AST, ALT, ALP and LDH levels were observed intoxicant groups demonstrating decreased the functional ability of the liver and sign of poor health. Treatment with EA 20 mg/kg b.wt significantly decreased the level of AST, ALT, ALP and LDH levels towards normal. All these significant changes in the levels of biochemical parameters refer to the effect of EA in protecting the liver by restoring the altered levels in rats. After D-GalN injection the ALT, AST, LDH, and ALP activities were increased to approximately 11 to 15-fold, 7-fold, 1.6-fold, and 1.5-fold, respectively compared to that in control groups. These increases were significantly attenuated by 20 mg/kg of EA. D-GalN caused significant increases in the ALT, AST, LDH, and ALP level of the liver ($p<0.001$) when compared to the control groups. Significant decreases ($p<0.001$) observed in the EA + D-GalN and D-GalN + EA groups compared to the D-GalN alone group with respect to all serum levels. ALT, AST, LDH, and ALP

levels of EA + D-GalN and D-GalN + EA groups slightly increased ($p<0.05$) when compared to the control group. Therefore, it seems that EA suppressed the leakage of AST, ALT, ALP and LDH into the serum, indicating that hepatocyte injury could possibly protect by EA treatment in vivo.

This result was strongly supported by histological observations. Histological changes showed a normal liver lobular architecture and cell structure in the livers of control animals. In control groups (Fig 3.2), cells maintained their original sinusoidal and hepatic morphology. However, livers exposed to D-GalN (Fig 3.3) showed broad hemorrhagic necrosis, extensive areas of portal inflammation complete damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis, congestion of hepatic sinusoids, and a moderate increase in inflammatory cell infiltration. These pathological alterations were attenuated in animals receiving EA treatments (Fig 3.4, 3.5). The disorganization caused by the D-GalN treatment was somewhat (Fig 3.3) to very obviously (Fig 3.4, 3.5) reduced in the sections from the rats given EA. Liver sections of D-GalN +EA and EA + D-GalN groups showed pronounced modulated effects when compared to D-GalN treated groups. EA at a dose of 20 mg/kg b.wt showed good improvement in hepatocytes as compared to that of D-GalN treated animals. There was slight congestion and mild vacuolization observed in EA+ D-GalN and D-GalN + EA treated groups. Furthermore, ultrastructural studies were performed using TEM to confirm the occurrence of morphological changes at the cellular level. The control animals were observed small uniform nuclei, granular cytoplasm, and the normal structure of cells and organelles (Fig 3.6). The hepatocytes of D-GalN treated rats were detected multiple irregulars shaped nuclei close to each other with an irregular cytoplasm and slightly shrunken cell changes, mitochondrial with melting cristae, and damaged endoplasmic reticulum (Fig 3.7). EA administrated groups (EA + DGalN and D-GalN + EA) were examined TEM and the liver cells were observed, the nuclear membrane and chromatin became evident, the damage of mitochondrial cristae and endoplasmic reticulum were restored, regular nuclear membrane structure and accumulation of glycogen inside cells were also observed (Fig 3.8, 3.9). Hence, the results of the biochemical and histological studies undoubtedly confirmed that EA has a potential clinical application for treatment of

liver disorders. In this study EA at 25 mg/kg was selected as the optimal effective dose for evaluating the molecular mechanisms of EA against D-GalN-induced hepatotoxicity.

In conclusion, our findings suggest that EA protect against D-GalN-induced acute liver injury. The mechanism of action of EA seems to involve its antioxidant activities and ability to scavenge free radicals. Results unmistakably found that the significant increase in the levels of serum markers was prevented by EA treatment. It is expected that EA scavenged free radicals and regulates endogenous antioxidants status and maintains pro-oxidant/antioxidant balance, which subsequently decreased the attack of free radicals on the membrane lipids and resulting marker enzymes to impede leaking into the serum. This study provides evidence that EA may be useful as a potential pharmacological alteration for the prevention of hepatic injury. However, further studies are needed to clarify the underlying molecular mechanism for the ameliorating effects of EA treatment on D-GalN liver damage.

REFERENCES

Allen, E. S. (2002). The Liver: Anatomy , Physiology , Disease and Treatment, 3–13.

Ann, D., Putt, D., Kresty, L., Stoner, G. D., Fromm, D., & Hollenberg, P. F. (1996). The effects of dietary ellagic acid on rat hepatic and esophageal mucosal cytochromes P450 and phase II enzymes, *17*(4), 821–828.

Archivio, M. D., Filesi, C., Benedetto, R. Di, Gargiulo, R., Giovannini, C., & Masella, R. (2007). Polyphenols , dietary sources and bioavailability, 348–361.

Câmara, C., Urrea, C., & Schlegel, V. (2013). Pinto Beans (Phaseolus vulgaris L.) as a Functional Food: Implications on Human Health. *Agriculture*, *3*, 90–111. doi:10.3390/agriculture3010090

Celik, G., Semiz, A., Karakurt, S., Arslan, S., Adali, O., & Sen, A. (2013). A comparative study for the evaluation of two doses of ellagic acid on hepatic drug metabolizing and antioxidant enzymes in the rat. *BioMed Research International*, *2013*, 358945. doi:10.1155/2013/358945

Chaung, S. S., Lin, C. C., Lin, J., Yu, K. H., Hsu, Y. F., & Yen, M. H. (2003). The hepatoprotective effects of Limonium sinense against carbon tetrachloride and ??-D-galactosamine intoxication in rats. *Phytotherapy Research*, *17*(May 2002), 784–791. doi:10.1002/ptr.1236

Clifford, M. N., & Scalbert, A. (2000). Review Ellagitannins – nature , occurrence and dietary burden, *1125*(November 1999).

Coen, M., Hong, Y. S., Clayton, T. A., Rohde, C. M., Pearce, J. T., Reily, M. D., … Nicholson, J. K. (2007). The Mechanism of Galactosamine Toxicity Revisited ; A Metabonomic Study research articles, 2711–2719.

Correnti, J. M. (2014). A. in alcoholic liver disease and liver regeneration. T. J. U. (n.d.).

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports*, *26*(8), 1001–43. doi:10.1039/b802662a

Das, J., Ghosh, J., Roy, A., & Sil, P. C. (2012). Mangiferin exerts hepatoprotective activity against D-galactosamine induced acute toxicity and oxidative/nitrosative stress via Nrf2-NF??B pathways. *Toxicology and Applied Pharmacology*, *260*(1), 35–47. doi:10.1016/j.taap.2012.01.015

Devipriya, N., Sudheer, A. R., Vishwanathan, P., & Menon, V. P. (2008). Modulatory potential of ellagic acid, a natural plant polyphenol on altered lipid profile and lipid peroxidation status during alcohol-induced toxicity: a pathohistological study. *Journal of Biochemical and Molecular Toxicology*, *22*(2), 101–12. doi:10.1002/jbt.20226

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology*, *35*, 495–516. doi:10.1080/01926230701320337

Gao, B., Jeong, W. Il, & Tian, Z. (2008). Liver: An organ with predominant innate immunity. *Hepatology*, *47*, 729–736. doi:10.1002/hep.22034

Ghudhaib, K. K., Hanna, E. R., & Jawad, A. H. (2010). EFFECT OF ELLAGIC ACID ON SOME TYPES OF PATHOGENIC BACTERIA, *13*(2), 79–85.

Girish, C., Koner, B. C., Jayanthi, S., Ramachandra Rao, K., Rajesh, B., & Pradhan, S. C. (2009). Hepatoprotective activity of picroliv, curcumin and ellagic acid compared to silymarin on paracetamol induced liver toxicity in mice. *Fundamental & Clinical Pharmacology*, *23*(6), 735–45. doi:10.1111/j.1472-8206.2009.00722.x

Gowda, S., Desai, P. B., Hull, V. V, Math, A. a K., Vernekar, S. N., & Kulkarni, S. S. (2009). A review on laboratory liver function tests. *The Pan African Medical Journal*, *3*(November), 17.

Guicciardi, M. E., & Gores, G. J. (2005). Apoptosis: a mechanism of acute and chronic liver injury. *Gut*, *54*(7), 1024–33. doi:10.1136/gut.2004.053850

Gümüş, M., Yüksel, H., Evliyaoğlu, O., Kapan, M., Böyük, A., Önder, A., & Aldemir, M. (2011). Effects of ellagic acid on copper, zinc, and biochemical values in serum and liver of experimental cholestatic rats. *Biological Trace Element Research*, *143*, 386–393. doi:10.1007/s12011-010-8863-2

Heber, D. (2008). Multitargeted therapy of cancer by ellagitannins. *Cancer Letters*, *269*(2), 262–8. doi:10.1016/j.canlet.2008.03.043

Huetz, P., Mavaddat, N., & Mavri, J. (2005). Reaction between ellagic acid and an ultimate carcinogen. *Journal of Chemical Information and Modeling*, *45*, 1564–1570. doi:10.1021/ci050163c

Hyder, M. (2013). Comparative Levels of ALT, AST, ALP and GGT in Liver associated Diseases. *European Journal of ...*, *3*(2), 280–284. Retrieved from http://www.pelagiaresearchlibrary.com/european-journal-of-experimental-biology/vol3-iss2/EJEB-2013-3-2-280-284.pdf

Ishibashi, H., Nakamura, M., Komori, A., Migita, K., & Shimoda, S. (2009). Liver architecture, cell function, and disease. *Seminars in Immunopathology*, *31*, 399–409. doi:10.1007/s00281-009-0155-6

Jayakumar, S., Madankumar, A., Asokkumar, S., Raghunandhakumar, S., Gokula Dhas, K., Kamaraj, S., … Devaki, T. (2012). Potential preventive effect of carvacrol against diethylnitrosamine-induced hepatocellular carcinoma in rats. *Molecular and Cellular Biochemistry*, *360*, 51–60. doi:10.1007/s11010-011-1043-7

Kew, M. C. (2000). Serum aminotransferase concentration as evidence of hepatocellular damage. *Lancet*, *355*, 591–592. doi:10.1016/S0140-6736(99)00219-6

Khanbabaee, K., & van Ree, T. (2001). Tannins: classification and definition. *Natural Product Reports*, *18*, 641–649. doi:10.1039/b1010611

Kim, S. J., Kim, J. K., Lee, D. U., Kwak, J. H., & Lee, S. M. (2010). Genipin protects lipopolysaccharide-induced apoptotic liver damage in d-galactosamine-sensitized mice. *European Journal of Pharmacology*, *635*(1-3), 188–193. doi:10.1016/j.ejphar.2010.03.007

Kim, S.-J., Choi, H.-S., Cho, H.-I., Jin, Y.-W., Lee, E.-K., Ahn, J. Y., & Lee, S.-M. (2015). Protective effect of wild ginseng cambial meristematic cells on d-galactosamine-induced hepatotoxicity in rats. *Journal of Ginseng Research*, (May), 1–8. doi:10.1016/j.jgr.2015.04.002

Landete, J. M. (2011). Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health. *Food Research International*, *44*(5), 1150–1160. doi:10.1016/j.foodres.2011.04.027

Larrosa, M., García-conesa, M. T., & Juan, C. (2012). FLAVONOIDS AND RELATED COMPOUNDS: Bioavailability and Function, 183–200.

Li, T., Chen, G., Su, C., Lin, J., Yeh, C., Cheng, K., & Chung, J. (2005). Ellagic acid induced p53/p21 expression, G1 arrest and apoptosis in human bladder cancer T24 cells. *Anticancer Research*, *25*(91), 971–980.

Limdi, J. K. (2003). Evaluation of abnormal liver function tests. *Postgraduate Medical Journal*, *79*(6), 307–312. doi:10.1136/pmj.79.932.307

Losso, J. N., Bansode, R. R., Trappey, A., Bawadi, H. a., & Truax, R. (2004). In vitro anti-proliferative activities of ellagic acid. *Journal of Nutritional Biochemistry*, *15*, 672–678. doi:10.1016/j.jnutbio.2004.06.004

Malini, P., Kanchana, G., & Rajadurai, M. (2011). Antibiabetic efficacy of allagic acid instreptozotocin induced diabetes mellitus in albino wistar rats. *Asian Journal of Pharmaceutical and Clinical Research*, *4*(3), 127–128.

Niemetz, R., & Gross, G. G. (2005). Enzymology of gallotannin and ellagitannin biosynthesis. *Phytochemistry*, *66*(2005), 2001–2011. doi:10.1016/j.phytochem.2005.01.009

Ohan, H. A. R. I. M. (2002). Free Radical Studies of Ellagic Acid , a Natural Phenolic Antioxidant, 2200–2206.

Özkaya, A. (2010). The Effects of Ellagic Acid on Some Biochemical Parameters in the Liver of Rats Against Oxidative Stress Induced by Aluminum [1] Alüminyum ile Oksidatif Strese Maruz Kalan Sıçanların Karaciğerindeki Bazı Biyokimyasal Parametreler Üzerine Ellagik Asidin , *16*(2), 2–7.

Raj, P. V., Nitesh, K., Prateek, J., Sankhe, M. N., Rao, J. V., Rao, C. M., & Udupa, N. (2011). Effect of lecithin on d-galactosamine induced hepatotoxicity through mitochondrial pathway involving Bcl-2 and Bax. *Indian Journal of Clinical Biochemistry*, *26*(4), 378–384. doi:10.1007/s12291-011-0155-x

Scalbert, A., Johnson, I. T., & Saltmarsh, M. (2005). Polyphenols: antioxidants and beyond 1–3, *81*, 215–217.

Scalbert, A., & Williamson, G. (2000). Chocolate: Modern Science Investigates an Ancient Medicine. *Journal of Medicinal Food*, *3*, 121–125. doi:10.1089/109662000416311

Seeram, N. P., Lee, R., & Heber, D. (2004). Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (Punica granatum L.) juice. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *348*(1-2), 63–8. doi:10.1016/j.cccn.2004.04.029

Sepúlveda, L., Ascacio, a, Rodríguez-Herrera, R., Aguilera-Carbó, a, & Aguilar, C. N. (2011). Ellagic acid: biological properties and biotechnological development for production processes. *African Journal of Biotechnology*, *10*(22), 4518–4523. doi:10.5897/AJB10.2201

Sohn, E. H., Koo, H. J., Hang, D. T. T., Jang, S. a., Namkoong, S., Lim, J. D., & Kang, S. C. (2013). Protective effects of ellagic acid on ethanol-induced toxicity in hepatic HepG2 cells. *Molecular and Cellular Toxicology*, *9*, 249–256. doi:10.1007/s13273-013-0032-1

Soni, K. B., Lahiri, M., Chackradeo, P., Bhide, S. V., & Kuttan, R. (1997). Protective effect of food additives on aflatoxin-induced mutagenicity and hepatocarcinogenicity. *Cancer Letters*, *115*, 129–133. doi:10.1016/S0304-3835(97)04710-1

Srivastava, T., & Chosdol, K. (2007). Clinical biochemistry.

Stachlewitz, R. F., Seabra, V., Bradford, B., Bradham, C. a, Rusyn, I., Germolec, D., & Thurman, R. G. (1999). Glycine and uridine prevent D-galactosamine hepatotoxicity in the rat: role of Kupffer cells. *Hepatology (Baltimore, Md.)*, *29*, 737–745. doi:10.1002/hep.510290335

Vasanth Raj, P., Nitesh, K., Sagar Gang, S., Hitesh Jagani, V., Raghu Chandrashekhar, H., Venkata Rao, J., … Udupa, N. (2010). Protective role of catechin on D-galactosamine induced hepatotoxicity through a p53 dependent pathway. *Indian Journal of Clinical Biochemistry*, *25*(4), 349–356. doi:10.1007/s12291-010-0073-3

Vattem, D., & Shetty, K. (2005). Biological functionality of ellagic acid: a review. *Journal of Food Biochemistry*, *29*(413), 234–266. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1745-4514.2005.00031.x/full

Verslype, C. (2004). Evaluation of abnormal liver-enzyme results in asymptomatic patients. *Acta Clinica Belgica*, *59*, 285–289. doi:10.1056/NEJM200004273421707

Watanabe, A., Kobayashi, M., Hayashi, S., Kodama, D., Isoda, K., Kondoh, M., … Yagi, K. (2006). Protection against D-galactosamine-induced acute liver injury by oral administration of extracts from Lentinus edodes mycelia. *Biological & Pharmaceutical Bulletin*, *29*(August), 1651–1654. doi:10.1248/bpb.29.1651

Wolf, P. L. (1999). Biochemical diagnosis of liver disease. *Indian Journal of Clinical Biochemistry*, *14*, 59–90. doi:10.1007/BF02869152

Wong, R. S. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research*, *30*(1), 87. doi:10.1186/1756-9966-30-87

Wu, M. (2001). Apoptosis : Molecular Mechanisms.

Yüce, A., Ateşşahin, A., Ceribaşi, A. O., & Aksakal, M. (2007). Ellagic acid prevents cisplatin-induced oxidative stress in liver and heart tissue of rats. *Basic & Clinical Pharmacology & Toxicology*, *101*(5), 345–9. doi:10.1111/j.1742-7843.2007.00129.x

Zhang, H., Guo, Z.-J., Xu, W.-M., You, X.-J., Han, L., Han, Y.-X., & Dai, L.-J. (2014). Antitumor effect and mechanism of an ellagic acid derivative on the HepG2 human hepatocellular carcinoma cell line. *Oncology Letters*, *7*(2), 525–530. doi:10.3892/ol.2013.1740

Zhang, H.-M., Zhao, L., Li, H., Xu, H., Chen, W.-W., & Tao, L. (2014). Research progress on the anticarcinogenic actions and mechanisms of ellagic acid. *Cancer Biology & Medicine*, *11*, 92–100. doi:10.7497/j.issn.2095-3941.2014.02.004