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Research Article
Effect of Ellagic Acid on Tamoxifen-Induced Hepatotoxicity in Rats

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Abstract

Background and Objective: Hepatotoxicity occurs as one of the adverse effects of tamoxifen (TAM), an antiestrogen compound used to treat and prevent all stages of estrogen-dependent breast cancer. It is thought that antioxidant agents could ameliorate TAM's hepatotoxic effect in these patients. In this study, the ellagic acid (EA), an antioxidant compound, was tested for its effect on hepatotoxicity caused by TAM. **Materials and Methods:** Forty-eight female albino Wistar rats were allocated into eight control and treatment groups. After treatment for nine consecutive days, serum samples were collected for serological tests, while liver tissues were collected for histopathological analysis. The body and liver weights were measured to determine their correlation ratio. A one-way analysis of variance was used to compare the data statistically. **Results:** The EA-treated groups showed a significant reduction in the values of Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), Aspartate Transaminase (AST), Lipid Peroxide (LPO) and Hydrogen Peroxide (H_2O_2), with increasing concentrations of Reduced Glutathione (GSH). A statistically significant ($p \leq 0.05$) reduction was found in the EA-treated groups regarding the liver-to-body weight ratio. Moreover, histopathological findings indicated that EA treatments reduced the hepatotoxicity lesions in the liver tissues, such as massive hemorrhage in the subcapsular area/liver parenchyma, severe steatohepatitis, ballooning degeneration of hepatocytes and marked infiltration of polymorphonuclear inflammatory cells in centrilobular/portal area. **Conclusion:** The oral EA treatments at various doses could ameliorate the hepatotoxicity induced through TAM injection in female albino Wistar rats. Thus, supplementation of EA could help alleviate liver injury induced by TAM treatment.

Key words: Biochemical test, hepatotoxicity, herbal product, histopathological analysis, *in vivo* study

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tamoxifen (TAM) is a nonsteroidal antiestrogen medication for treating hormone-dependent breast cancer[1]. It is an estrogen receptor regulator in different cells and tissues[2]. The TAM can also potentially reduce bone and cardiovascular system diseases in women[3]. However, its extensive use has drawn attention to its side effects, especially hepatotoxic effects. The TAM-induced liver toxicity in humans results in cirrhosis, hepatic necrosis, multifocal fatty infiltration, severe steatosis and toxic liver disease. Fatty liver occurs in about one-third of breast cancer patients receiving TAM[4]. Oxidative stress may play a part in TAM-induced toxicity[5], as it tends to cause liver damage by oxidative stress in rodents[6] and its hepatotoxic effect has been associated with decreased fatty acid β-oxidation and the creation of reactive oxygen species (ROS)[7].

When cells are exposed to various endogenous and exogenous agents, reactive oxygen species (ROS) are produced, causing damage to many vital biomolecules related to multiple human diseases[8]. Endogenous antioxidants keep these pro-oxidants in check, but under disease states, the balance shifts in favor of pro-oxidants, resulting in oxidative stress. As a result, dietary antioxidants that can scavenge free radicals may potentially prevent disease[9].

Ellagic acid (EA; $C_{14}H_6O_8$) is a metabolite in medicinal plants, such as pomegranate, raspberries, blackberries, muscadine grape and tropical fruits such as camu camu, walnuts and pecan[10]. The EA and its derivatives are drawing attention to their biomedical applications, including antimicrobial, anti-inflammatory, neuroprotective and hepatoprotective properties[11].

Many of its pharmacological activities are due to its antioxidant properties. Other mechanisms, however, have been concerned with EA's multiple effects, including lowering lipid metabolism and the lipidemic profile, altering pro-inflammatory activators like Tumor Necrosis Factor (TNF), Interleukin (IL)-1 and IL-6 and decreasing nuclear factor (NF)-B activity while enhancing atomic factor erythroid 2-related factor 2 expression. The TAM's antioxidant activities are linked to its ability to scavenge free radicals comparable to essential vitamins[12].

The EA has four hydroxyls and two functional lactone groups, allowing it to clean many ROS and other oxidizing agents[13]. The ability of EA to remove a hydrogen atom from the phenolic constituent to a free radical is the mechanism underlying its scavenging activity. The formal hydrogen removal from the EA has been demonstrated to include complex methods via the transfer of H-atom, single electron followed by proton transfer and sequential proton loss electron transfer[14]. Accordingly, this study aimed to investigate EA's potential for reducing the hepatotoxicity induced by TAM administration.

MATERIALS AND METHODS

Study area: The study was conducted between March and July, 2023 at the College of Veterinary Medicine, University of Sulaimani, Sulaymaniyah, Iraq.

Materials: The TAM was obtained from EBEWE Pharma, Austria and EA was purchased from Sigma-Aldrich, USA. The TAM and EA were prepared in normal saline and corn oil, respectively. All other chemicals were of the highest purity and analytical grade.

Animals: Female Wistar albino rats, 8-12 weeks old, were used in the study. The animal house of the Biology Department at the University of Sulaimani supplied 48 rats weighing 170-200 g. Before starting the study, the rats were acclimatized in plastic cages for ten days under standard conditions with a temperature of 24±2°C and a humidity of 50-70%. Furthermore, the rats were subjected to a 12 hrs light/dark cycle and fed with standard pellets with free admission to drinking water.

Study design: The rats were arbitrarily divided into eight groups of 6 rats each. Group 1 was the negative control and left without treatment; Group 2 was injected with a single intraperitoneal (IP) dose of TAM (90 mg/kg) on the fifth day and considered positive control. Group 3 rats received corn oil (0.7 mL) orally for nine consecutive days and were injected with a single IP dose of normal saline (0.9% NaCl) on the fifth day. Groups 4 and 5 received an oral dose of 10 and 30 mg/kg of EA for nine consecutive days and were injected with a single IP dose (1 mL) of normal saline on the fifth day[15,16]. Rats of groups 6 and 7 were drenched with 10 and 30 mg/kg of EA, respectively, for nine sequential days and injected with a single IP dose of TAM (90 mg/kg) on the fifth day[15]. Lastly, group 8 was injected with a single IP dose of TAM (90 mg/kg) on the fifth day, received corn oil orally for nine successive days and was considered the disease model group.

Collection of blood and liver samples: On the 10th day of the experiment, the rats were subjected to an 8 hrs fast and anesthetized with a 0.2 mL IP mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL). About 5 mL of blood was collected by cardiac puncture into sterile collection tubes with anticoagulants and centrifuged at 5,000 rpm/10 min to collect serum, then stored at -20°C for serological analysis. On the other hand, the liver was excised, blot-dried, cut into

pieces, weighed and put in 10% formalin for histopathological examination. At the same time, 1.0 g of liver tissue was homogenized in phosphate buffer saline (PBS) and stored at -80°C for oxidative stress analysis.

Biochemical analysis: According to the manufacturers' instructions, liver function enzymes, including AST (Diamond Diagnostic, Egypt), ALT (Diamond Diagnostic, Egypt) and ALP (Biodiagnostic, Egypt), were determined in serum samples using an automatic biochemistry analyzer (Cobas, 6000 Module 501, Roche, Germany). The oxidative stress status was assessed by estimating lipid peroxide (LPO), reduced Glutathione (GSH) and H_2O_2 content in liver homogenate using colorimetric assay kits for the GSH/LPO and a fluorometric assay kit for the H_2O_2 (Elabscience® ELISA, USA), based on the manufacturer's protocol.

Histopathological procedure: The protocol used for the histopathological study depended on a procedure in a previous study by Darwish *et al.*[17]. Briefly, after at least 48 hrs of fixation of the liver samples in 10% formalin, samples were dehydrated through ascending concentrations of ethanol (70, 80, 90 and 100%), washed and cleared in xylol, rehydrated and embedded in paraffin. Then, 5 µm thick sections were stained by Hematoxylin and Eosin (H&E), coverslipped and the slides were read under a standard light microscope (Leica, Japan) using various power fields.

Body/liver weight measurements: The rats' body weights were measured using a digital balance before the sacrifice. At the same time, the freshly excised livers of sacrificed animals were also measured after washing in normal saline to determine the liver-to-body weight ratio.

Ethical approval: The Ethical Committee of the College of Veterinary Medicine, University of Sulaimani, Iraq, approved the study protocol (approval No. AUP-2021/19). All methods followed the guidelines of Europe Directive 2010/63/EU. Efforts were taken to reduce pain/discomfort in the studied animals.

Statistical analysis: The data of the results were expressed as Means±Standard Error (SEM). A One-way Analysis of Variance (ANOVA) was used to compare the results, followed by Duncan's *post hoc*. A probability level of 0.05 was considered to determine significant differences.

RESULTS

Ellagic acid alleviated liver toxicity by TAM: The TAM injection caused hepatotoxicity in female Wistar rats at a dose rate of 90 mg/kg, as shown by a significant increase in ALP, ALT and AST compared to the control (Fig. 1a-c). Pretreatment with EA significantly reduced liver injury at dosage rates of 10 and 30 mg/kg compared to the group injected with TAM and the reduction was dose-dependent. However, the ALP level was higher in the groups receiving corn oil and EA without TAM injection. These results indicated the beneficial effects of EA on hepatotoxicity induced by TAM.

EA alleviated TAM-induced oxidative stress: The TAM induced oxidative stress in all groups, indicated by elevated LPO and H_2O_2 levels, with substantial reduction ($p \leq 0.05$) in GSH value in the injected animals compared to the negative control group (Fig. 2a-c). Treatments with EA (10 and 30 mg/kg) ameliorated the oxidative stress as the GSH levels were higher in the treated groups and the LPO and H_2O_2 levels were significantly lower compared to the TAM-treated group. Simultaneously, corn oil-treated animals showed no significant differences from the negative control group. The oxidative stress-alleviating effect of EA was dose-dependent.

A significant ($p \leq 0.05$) increment in the liver-to-body weight ratio was found in the TAM-treated groups in comparison with the negative control group. The liver-to-body weight ratio decreased significantly in the EA-treated groups at 10 and 30 mg/kg doses. Additionally, no significant changes ($p > 0.05$) were found in groups drenched with corn oil compared to either EA-treated or control-negative groups (Table 1).

Table 1: Liver and body weights of the rats

Group	Liver weight (g)	Body weight (g)	Liver-to-body weight ratio (%)
Negative control	13.2±0.2	180.5±2.6^{bc}	7.3±0.1^{ab}
TAM (90 mg/kg)	13.9±0.1	167.0±4.1^{a}	8.3±0.2^{c}
Corn oil+NS	13.4±0.3	188.5±4.5^{c}	7.1±0.2^{a}
EA (10 mg/kg)+NS	13.5±0.2	178.8±2.5^{bc}	7.5±0.1^{ab}
EA (30 mg/kg)+NS	13.2±0.3	179.7±3.1^{bc}	7.3±0.2^{ab}
TAM+EA (10 mg/kg)	13.5±0.2	175.0±4.9^{ab}	7.7±0.3^{b}
TAM+EA (30 mg/kg)	13.3±0.3	178.5±3.6^{bc}	7.5±0.1^{ab}
TAM+corn oil	13.7±0.3	173.5±3.8^{ab}	7.8±0.1^{bc}

Values are shown as Means±SEM, Different superscript letters show significant differences within the column at $p \leq 0.05$, A one-way analysis of variance was used, followed by Duncan's *post hoc* and NS: Normal saline

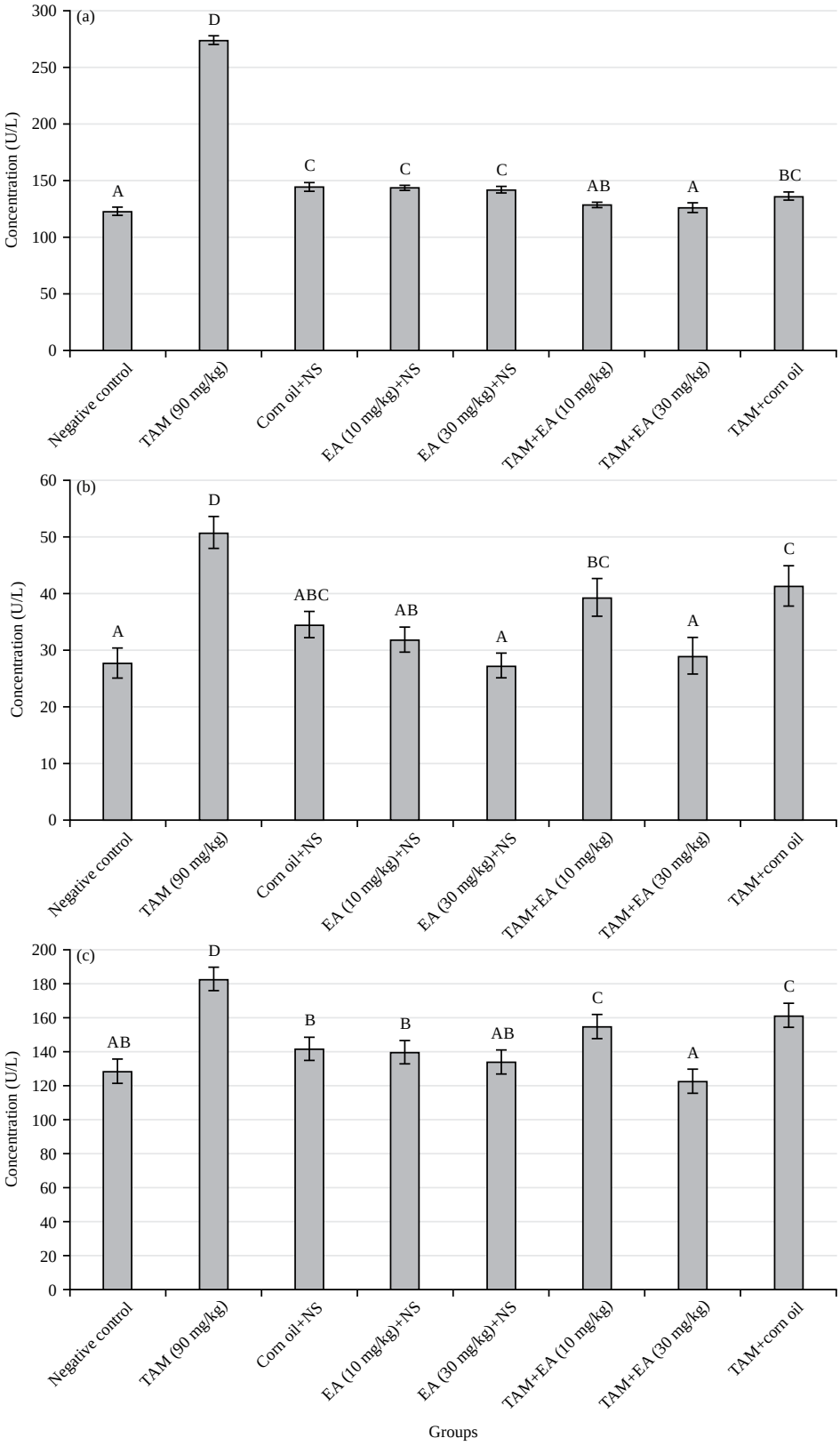

Fig. 1(a-c): EA reduced TAM-induced hepatotoxicity, indicated by a significant reduction in the levels of (a) ALP, (b) ALT and (c) AST five days after TAM injection

Columns represent the mean of six rats per group and the error bars represent the SEM, Different letters (A, B, C and D) indicate significant differences at $p \leq 0.05$, Test: One-way-ANOVA (Duncan's *post hoc* test) and NS: Normal saline

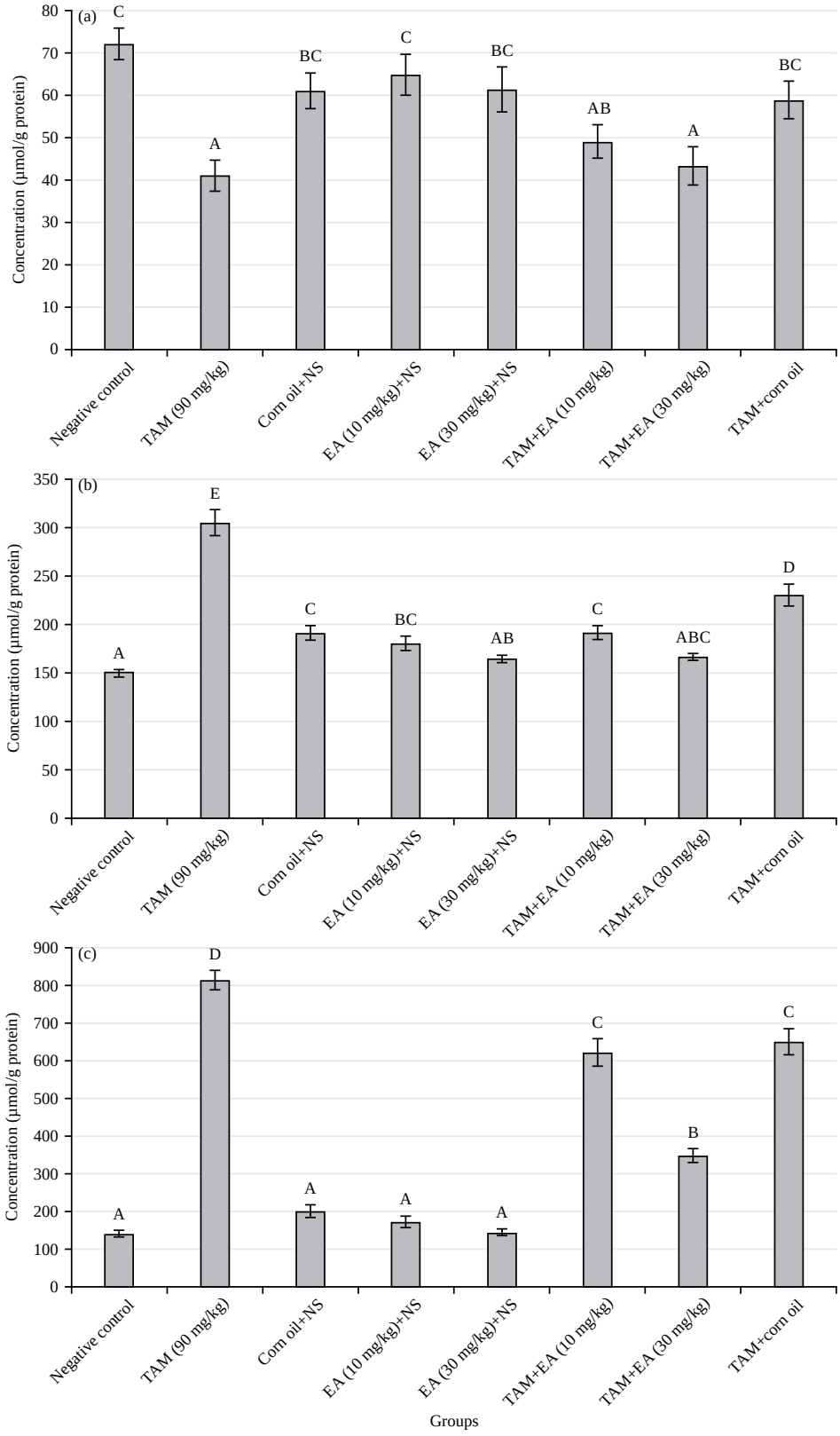

Fig. 2(a-c): EA reduced TAM-induced oxidative stress, rats were given EA for nine consecutive days and injected with TAM on the fifth day, (a) GSH, (b) LPO and (c) H_2O_2

Columns represent the average values of six rats and the error bars represent the SEM, Different letters indicate significant differences at $p \leq 0.05$ and Test: One-way ANOVA followed by Duncan's *post hoc*

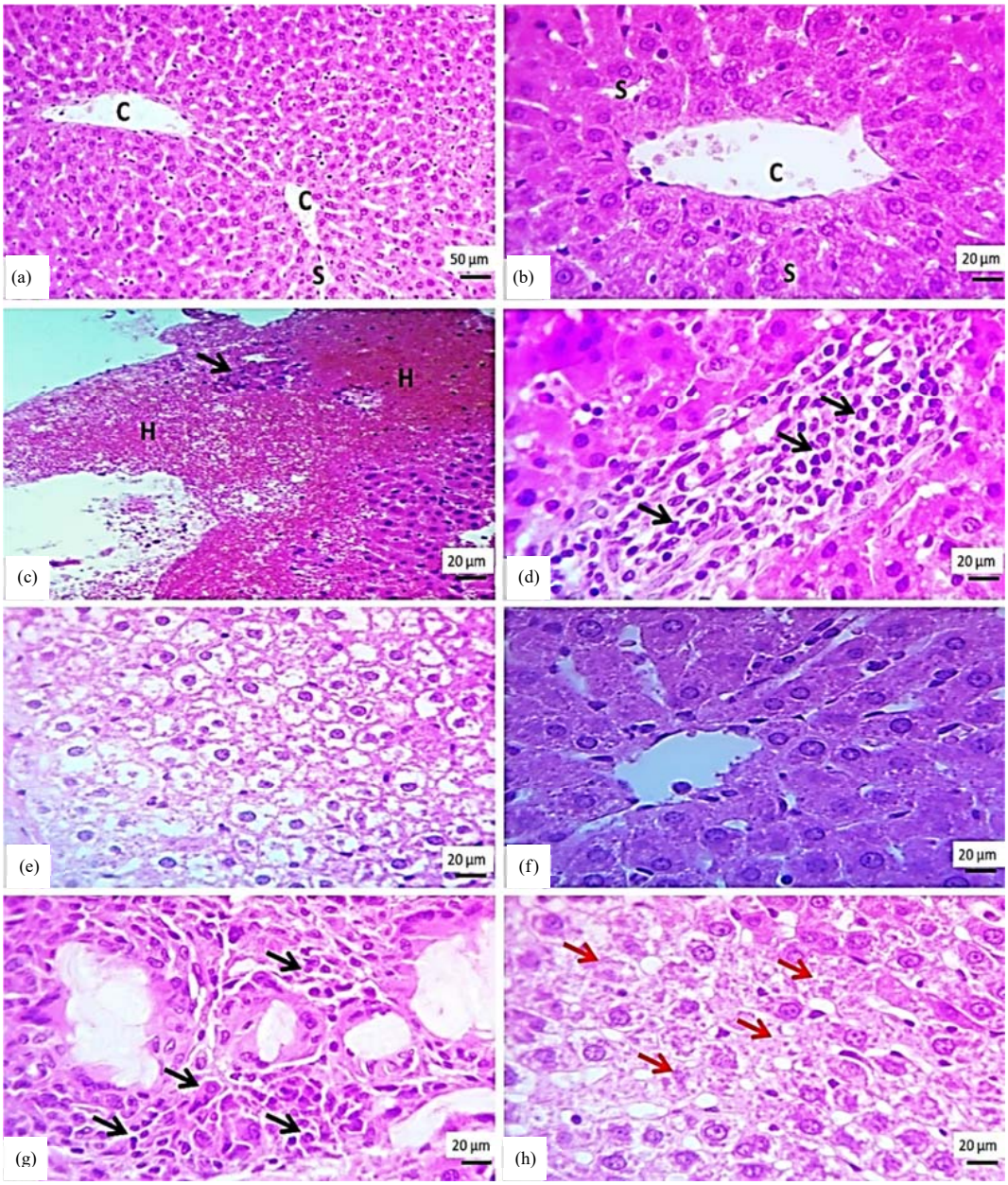

Fig. 3(a-h): Microscopic sections of the liver, (a-b) Normal histological structures of liver parenchyma with the intact central vein (C), sinusoidal capillary (S) in the negative control group, (c) Severe hemorrhage (H) in the subcapsular region and hepatocyte in the positive control group, (d) Marked inflammatory reaction (black arrows) in the centrilobular zone in the positive control, (e) Severe ballooning degeneration with microvesicular steatosis in the positive control, (f) Mild-moderate cell swelling with narrowing of sinusoidal capillary in the corn oil group and (g-h) Moderate-severe inflammatory infiltration in the portal area (black arrows), marked hydropic degeneration with sinusoidal dilation in tamoxifen+corn oil group (red arrows)
H&E stain

Histopathological finding: The histological examination of the liver section in control negative rats showed the standard architecture of hepatic cords separated by sinusoids capillary with normal central vein, intact hepatocytes appeared to be polygonal morphology. They had a large nucleus with apparent mitochondria (Fig. 3a-b). On the contrary, liver tissues that were treated with TAM (90 mg/kg) showed massive bleeding in the subcapsular area and throughout

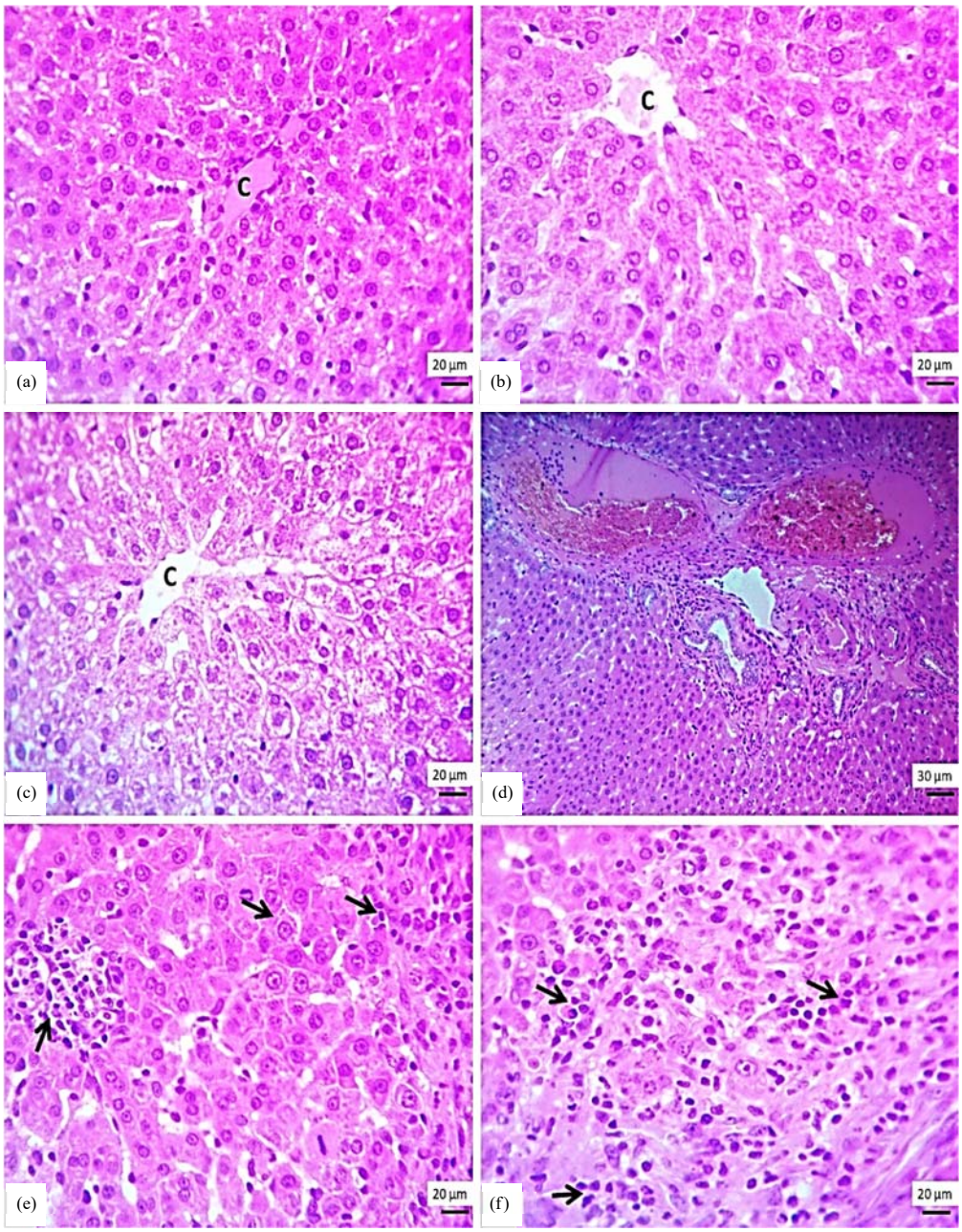

Fig. 4(a-f): Microscopic sections of the liver, (a-b) Congestion of central vein in section a, mild-moderate swelling of hepatocytes in the group that was treated with 10 mg/kg of EA, (c) Marked hydropic degeneration of hepatocytes and (d-f) Moderate inflammatory reaction (black arrows) in the portal area and centrilobular zone and within sinusoidal capillary in the group treated with tamoxifen+10 mg/kg of EA
H&E stain

liver parenchyma, severe steatohepatitis, particularly microvesicular steatosis with severe ballooning degeneration of hepatocytes and marked infiltration of polymorphonuclear inflammatory cells in the centrilobular and portal area in comparison to the control negative group (Fig. 3c-e). However, treatment with corn oil showed an intact central vein, mild-moderate degree of hepatocyte swelling characterized by pale cytoplasm, enlarged cells with centrally located nuclei and narrowing of sinusoid vs control negative and positive group (Fig. 3f).

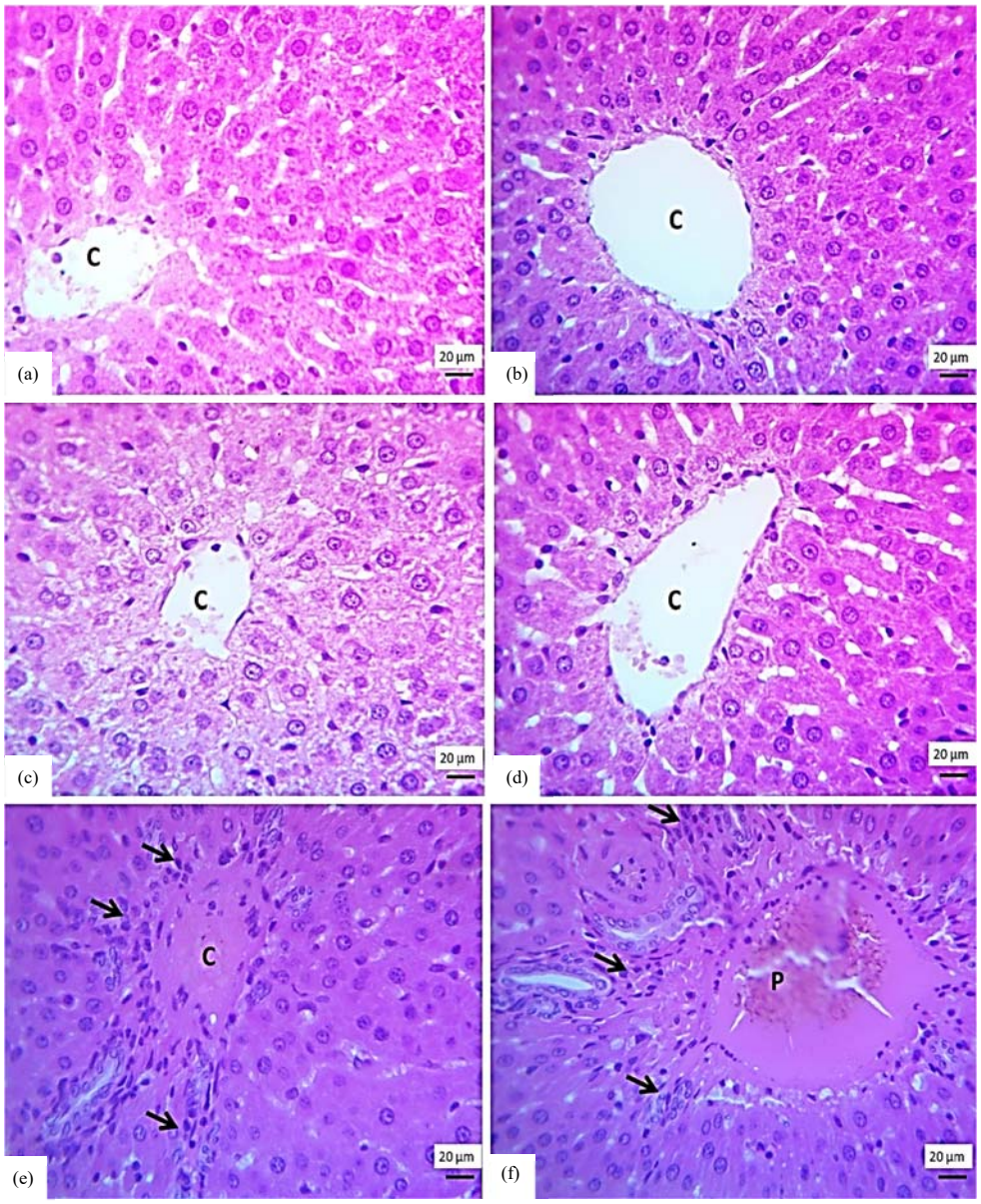

Fig. 5(a-f): Microscopic sections of the liver, (a-b) Congestion of the central vein in section a, mild swelling of hepatocytes with the typical organization of hepatic cord in the group treated with 30 mg/kg of EA, (c-d) Mild-moderate hydropic degeneration of hepatocytes and (e-f) Central vein congestion (C), mild inflammatory reaction (black arrows) in the centrilobular zone and portal area (P) in the group treated with tamoxifen+30 mg/kg of EA
H&E stain

Moreover, liver sections in the TAM+corn oil group exhibited marked ballooning degeneration of hepatocytes with a moderate-severe degree of polymorphonuclear inflammatory cell infiltration in the centrilobular and portal area (Fig. 3g-h). Morphological changes in rats treated with 10 mg/kg of EA (Fig. 4a-b) appeared to have hepatocyte swelling to a moderate degree with congestion of the central vein. The liver section in the group treated with TAM+10 mg of EA revealed marked vacuolar or hydropic degeneration with infiltration of neutrophil and eosinophil in the centrilobular, within the sinusoid and periportal area to a moderate degree (Fig. 4c-f). The liver section after being treated with 30 mg/kg of EA (Fig. 5a-b) represented the typical liver organization with mild swelling of hepatocytes compared to the group that was treated with TAM+30 mg/kg EA, which revealed congestion of central vein, mild-moderate hydropic degeneration of hepatocytes and mild inflammatory reaction in the centrilobular zone and portal area (Fig. 5c-f).

DISCUSSION

The TAM denotes a substantial novelty in treating breast cancer; however, it was confirmed that high doses result in side effects, such as oxidative liver injury, preventing its long-term use. It was considered a hepatocarcinogen based on studies on rats, as it produces five DNA adducts[18]. The TAM is more toxic to the liver than any other organ due to its higher affinity for the liver tissue than others[19]. Various natural plant metabolites have been tried to protect and/or prevent many chemotherapeutics' side effects, including flavonoids, which occur in almost all food categories and the highest rates are present in fruits and vegetables[20]. In this study, pretreatment with EA alleviated TAM-induced increase in the levels of serum AST, ALT and ALP. The aminotransferases and alkaline phosphatase are among the tests that detect hepatocytic injuries and their elevation is an indicator of hepatotoxicity[21]. Based on these outcomes, EA reduced the hepatotoxicity caused by TAM.

Consistent with these results, EA treatment reduced the accumulation of LPO and H_2O_2 and restored GSH levels in the liver. Lipid peroxidation by reactive oxygen species plays a significant role in cell injuries and cell death. Hepatotoxicity by TAM is attributed to oxidative stress by TAM's direct actions on the hepatocytes or during the drug's metabolism in the liver[22]. Hence, the antioxidant effect of EA contributed to reducing the LPO and H_2O_2 levels and, consequently, the hepatotoxic effect of TAM.

The GSH (tripeptide, γ-l-glutamyl-l-cysteinyl-glycine) was another compound tested in this study. It is the most abundant antioxidant compound synthesized in the cells and plays a significant role in ROS removal and protection of cells from oxidative damage[23]. Hence, high GSH levels in tissues contribute to the protection of these tissues from oxidative damage. In this study, EA pretreatment significantly elevated GSH levels, further confirming its hepatoprotective effect from TAM-induced hepatic injury.

These outcomes are explained by the fact that lipid peroxidation may be accredited by hexose monophosphate shunt in rat liver, which is strongly reserved by TAM's high dose so that the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) amounts in cells are diminished. The oxidative stress was detected during TAM management in high doses and was complemented by reduced hepatic GSH levels and increased peroxidation[24]. Similarly, EA was shown in another study to defend against cisplatin-induced hepatotoxicity by reversing the inactivation of GSH and the antioxidant system by cisplatin and up-regulation of GSH-Px and catalase levels in the liver[25]. Thus, the protective role of EA may be due to its antioxidant activity on various free radicals.

Additionally, in the current study, the liver-to-body weight ratio was significantly increased in the TAM-treated groups compared to the negative control, while significantly decreased in the EA-treated groups at both doses of 10 and 30 mg/kg and almost close to that of the negative control group. These outcomes mean that EA protects the average liver/body weight without potential abnormal swelling of hepatocytes and inflammation in the liver tissue and they were concurrent with those obtained by El-Kashef and El-Sheakh[26].

Furthermore, the histopathological analysis of liver tissues in treated animals further established EA's hepatoprotective effects induced by TAM. It was found that TAM induced pathological alterations in liver histology, including lymphocytic infiltration, bile ductular proliferation, edema and hepatocellular degeneration, which agreed with other studies[27-29]. At the same time, EA ameliorated the histopathological alterations and exhibited only mild congestion and infiltration of lymphocytes. These results further confirmed EA's hepatoprotective and antioxidant activities. Albukhari *et al.*[30] observed similar results using caffeic acid phenethyl ester instead of EA and Suddek[31] using thymoquinone.

The results from this study have shown that EA reduces the hepatotoxicity caused by TAM and it can be considered as a food supplement in people using TAM for breast cancer. The hepatotoxicity-ameliorating effect of EA set the ground to test this natural compound in breast cancer patients to determine its effectiveness.

CONCLUSION

The oral EA treatments at different doses ameliorated the TAM-induced hepatotoxicity in female Wistar rats indicated by various parameters, including biochemical and histopathological analysis, despite the live/body weight ratio. Consequently, it is speculated that EA can scavenge free radicals and protect against TAM-induced oxidative stress in a dose-dependent manner. These results show EA's beneficial role in alleviating liver injury caused by TAM treatment.

SIGNIFICANCE STATEMENT

Ellagic acid (EA) is an antioxidant polyphenol found in many fruits and vegetables and it was tested in this study for its potential hepatoprotective effect against oxidative stress by tamoxifen (TAM), an anti-breast cancer medication. Treatment with EA significantly ($p<0.05$) reduced the serum levels of alkaline phosphatase, alanine transaminase and aspartate transaminase. Also, EA treatment reduced hepatic

levels of lipid peroxide and hydrogen peroxide and increased the reduced glutathione levels in the livers of rats that were administered EA. Also, the histopathological analyses indicated that TAM significantly reduced the hepatocellular injury caused by TAM. These results imply that the naturally occurring EA can ameliorate the hepatic injuries caused by the oxidative stress from TAM. Ellagic acid is an antioxidant compound that is potentially helpful in reducing oxidative stress by tamoxifen.

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