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Annona squamosa (custard apple) fruit extract alleviates the oxidative stress and inflammation induced by doxorubicin in the liver of male rats

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Abstract

Background: Doxorubicin (DOX) is a commonly utilized and effective chemotherapeutic agent for treating various cancers, with side effects such as hepatotoxicity. Multiple research studies to alleviate hepatic impairment. *Annona squamosa* Linn is an essential medicinal plant that produces edible fruits known as custard apples. **Aim:** This study aimed to assess the therapeutic effect of *Annona squamosa* fruit extract (ASFE) against liver damage caused by doxorubicin in male rats. **Materials and Methods:** This study utilized thirty-two male adult albino rats. The rats were divided into four groups of eight: the control group, the DOX-treated group (3 mg/kg biweekly via intraperitoneal injection for 4 weeks), the ASFE-supplemented group (350 mg/kg body weight daily for 4 weeks), and the DOX-treated group that also received ASFE, **Results:** Administration of DOX to rats significantly elevated liver function and oxidative stress levels. There was evident hepatic inflammation and histological alterations. ASE effectively reinstated the impaired liver functions caused by DOX to normal levels, as seen by a notable reduction in liver enzymes (ALT and AST) and total bilirubin. ASFE substantially modified the oxidative stress caused by DOX by increasing antioxidants (SOD, CAT, and GSH) and reducing MDA levels. Similarly, the detrimental histological alterations in the liver, such as dilated and congested blood sinusoids, cellular infiltration, and fibrosis induced by DOX, seemingly resolved following treatment with ASFE. Furthermore, ASFE effectively mitigated the inflammatory effects of DOX by downregulating tumor necrosis factor-alpha (TNF-α). **Conclusion:** The extract from *A. squamosa* fruit effectively mitigated hepatic oxidative stress and inflammation induced by doxorubicin in male rats.

Keywords: Doxorubicin, liver dysfunction, fibrosis, oxidative stress, inflammation

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INTRODUCTION

Doxorubicin (DOX) is an antineoplastic agent utilized in the treatment of different human malignancies, particularly solid tumors. Nonetheless, its application is linked to numerous acute and chronic dose-dependent adverse effects, including malfunction of the digestive tract epithelium, degeneration of bone marrow, hepatotoxicity, cardiotoxicity, and nephrotoxicity (1, 2). The primary organ responsible for the degradation and excretion of chemicals is the liver, which is adversely affected by the usage of DOX. Hepatic injury significantly contributes to DOX toxicity, with hepatic toxicity found in 40% of patients (3, 4).

In the liver, cytochrome family enzymes and cytoplasmic reductase convert DOX into Doxorubicinol and other aglycone metabolites, which are hepatotoxic. Numerous research groups have documented that DOX increases the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (5,6). The cell membranes of hepatocytes are susceptible to free radical damage

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generated by DOX. Consequently, peroxidation persists in an autocatalytic manner, resulting in functional and structural defects. DOX-induced oxidative stress significantly disrupts DNA and protein synthesis, hence impairing the regenerative capacity of organelles. This irreversible event results in a significant increase in hepatic enzymes and necrosis or apoptosis of hepatocytes (7). Numerous data indicate increased inflammatory mediators, implying the involvement of inflammation in DOX-induced organ damage (8, 9).

Oxidative stress, a mechanism directly involved in the pathogenesis of several illnesses, can be mitigated by phytochemicals produced by plants (10, 11). Published studies indicate that plant components possess antioxidant and anti-inflammatory properties that may alleviate DOX-induced damage (2, 12, 13). Annona squamosa (custard apple), a member of the Annonaceae family, is prevalent in tropical and subtropical climates and possesses numerous traditional applications suggesting potential medicinal benefits (14). Phytochemical analyses of Annona have revealed many bioactive metabolites, including flavonoids, terpenoids, coumarins, anthraquinones, and phytosterols (15). A. squamosa has been documented to have anticancer, antioxidant, anti-inflammatory, antidiabetic, antihypertensive, hepatoprotective, and antiparasitic properties (16). A. squamosa extracts demonstrate significant hepatoprotective effects against alcohol-induced liver damage in rats due to their antioxidant properties (17). Furthermore, A. squamosa extract has been documented to exhibit anti-hyperglycemic properties and to improve hepato-renal dysfunction linked to diabetes mellitus in rats (18). Leaf extract from another Annonaceae family member, A. muricata, has been investigated for its capacity to protect male rats from testicular injury induced by cadmium (19). A. muricata has been demonstrated to mitigate testicular oxidative damage in rats (20). The purpose of this study was to assess the therapeutic effects of A. squamosa fruit extract against doxorubicin-induced oxidative stress and inflammation in the livers of male rats.

MATERIAL AND METHODS

1. Chemicals

Doxorubicin (DOX) was acquired from BIOVEA (Egypt) and Sigma-Aldrich (USA). The remaining chemicals necessary for the assessment of biochemical parameters were sourced from Biodiagnostic, Cairo, Egypt.

2. Preparation of *Annona squamosa* fruit extract

Fresh Annona squamosa fruits were acquired at the local market in Jazan City, Kingdom of Saudi Arabia. The fruits were washed and peeled to eliminate the seeds. The pulp of Annona squamosa was subjected to oven drying at 45 °C. The desiccated pulp was pulverized using a household electric mill and preserved at 4 °C for subsequent application (21). The pulp powder was measured and immersed in containers with 95% ethanol at a ratio of 1:2 (v/v) (22). The soaking was conducted for three days using 4 liters of 95% ethanol at room temperature (25 °C). This procedure was conducted three times. Following filtration, ethanol was eliminated utilizing a rotary evaporator in a water bath at 40 °C (23).

3. Experimental design

This study employed thirty-two male Wistar albino rats, each weighing between 150 and 160 grams. The animals were accommodated in wire-bottom cages within a room featuring standard illumination, a 12-hour light-dark cycle, a temperature of 25 ± 1°C, and 50% relative humidity. They also have access to potable water and a plentiful, nutritionally balanced diet. The rats were randomly assigned to four groups (n = 8) after a week of acclimation as follows: Control group: The subjects remained untreated and received a saline solution (the solvent of DOX). A. squamosa fruit extract (ASFE) group: The rats were administered ASE orally at a dosage of 350 mg/kg body weight daily (1/10 of LD50) for 4 weeks via gavage. DOX-treated group: The rats were administered DOX dissolved in normal saline at a dosage of 3 mg/kg twice weekly via intraperitoneal

injection for 4 weeks. DOX&ASFE-treated group: The rats received DOX for 4 weeks, followed by treatment with ASFE at the same dosages as in groups 2 and 3.

4. Sample collection and tissue preparation

After the experiment, the rats were anesthetized, and blood was collected in glass tubes. The serum was isolated via centrifugation at 3000 rpm for 10 minutes and preserved at −80°C for liver function analysis. The animals were dissected, and the entire liver was promptly excised, rinsed in normal saline, and sectioned into small fragments. Certain liver segments were preserved in 10% neutral buffered formalin for histological and immunohistochemical analysis, while other segments were homogenized for the assessment of liver oxidative stress and antioxidant markers.

All procedures were performed following the guidelines of the Standing Committee for Scientific Research - Jazan University.

5. Assessment of serum enzyme markers of liver damage

To determine acute liver injury, serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin were measured using enzymatic colorimetric kits (Spectrum Diagnostics, Egypt).

6. Evaluation of liver tissue antioxidants and oxidative stress

The hepatic tissues of each group were homogenized in a KH2PO4 buffer (100 mM) with EDTA (1 mM, pH 7.4). The homogenate was subjected to centrifugation at 12000×g for 30 minutes at 4 °C, and the supernatant was preserved in aliquots at -20 °C for the analysis of antioxidant enzymes and oxidative stress indicators. Catalase (CAT) activity was determined using the previously published method (26). An absorbance change of 0.01 units/ min indicates one unit of catalase activity. The assessment of superoxide dismutase (SOD) activity in hepatic tissue was conducted by Chance and Maehly (27). The results of CAT and SOD were expressed in units per milligram of tissue. The activity of reduced glutathione was assessed according to the methodology outlined by Jollow (28). The GSH activity was quantified in U/mg protein. Hepatic concentrations of malondialdehyde (MDA), an indicator of lipid peroxidation, were assessed using a thiobarbituric acid assay with an MDA kit (Biodiagnostic, Egypt). The colorful thiobarbituric acid reactive product was quantified spectrophotometrically at 534 nm (29). The results of MDA were quantified in nmol/mg protein.

7. Histological examination of liver tissue

Liver specimens were preserved in 10% formalin, dehydrated with escalating concentrations of ethyl alcohol, cleaned with xylene, and embedded in paraffin wax at 58-62ºC. Five-micron slices were prepared, stained with Hematoxylin and Eosin, and assessed for structural alterations using a bright field microscope (30).

8. Immunohistochemistry of TNF-α

Tumor necrosis factor (TNF-α) in liver sections was identified using the methodology of Abd Eldaim et al. (31). The liver sections were deparaffinized, rehydrated in alcohol, and treated in 3% hydrogen peroxide. Tissues were subsequently treated with the primary antibody, rabbit polyclonal anti-TNF-α (ab6671, Abcam), at a concentration of 1 µg/ml in 5% bovine serum albumin overnight. The immune reactivity was observed using diaminobenzidine (DAB; Sigma, USA) and semi-quantitatively assessed in high-power fields (HPF, 40X) according to the percentage of positively stained immune cells.

9. Statistical analysis

Data were represented as mean ± S.E.M. and statistically performed using GraphPad Prism 5.0 Software (USA). Comparison between groups was conducted by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

1. Changes in liver functions

In rats treated with A. squamosa fruit extract (ASFE), the serum levels of aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin exhibited no significant change ($P > 0.05$) in comparison to the control group. In DOX-treated rats, the levels of AST, ALT, and total bilirubin were considerably elevated ($P < 0.05$) compared to the control group. Remarkably, the administration of ASFE to DOX-injected rats for 28 days resulted in AST, ALT, and total bilirubin levels significantly approaching normal values ($P > 0.05$) comparable to the control (Figure 1).

2. Changes in liver tissues and antioxidants

Figure (2) represents the activities of antioxidants and the oxidative stress marker in liver tissues of both the control and experimental groups. The data indicated no significant alterations ($P > 0.05$) in the levels of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and malondialdehyde (MDA) between ASFE-treated rats and the control group. Conversely, in DOX-injected rats treated with ASFE, the levels of antioxidants and MDA were dramatically enhanced, approaching normal values ($P > 0.05$) comparable to the control group.

3. Histological results of the liver

Hematoxylin and eosin-stained section: The liver sections from the control group and rats treated with A. squmosa extract exhibited well-structured strands of hepatocytes surrounding the central vein. The hepatic portal region exhibited well-defined architecture, comprising the hepatic portal vein, bile ductule, and hepatic arteriole (Figure 3A-D). In DOX-injected rats, liver sections exhibited distinct histological characteristics, including dilated and congested central veins with markedly deteriorated endothelium. Furthermore, there were hemorrhage spots, aggregated hepatocytes, prominent cellular infiltrations, and fibrotic accumulations, particularly surrounding the portal region, together with an excess of Kupffer cells (Figure 3E-H). After 28 days of ASFE treatment in the DOX group, the majority of liver histopathological traits significantly disappeared, while certain sections still exhibited mildly dilated sinusoids and an excess of Kupffer cells (Figure 3I-J).

Masson's trichrome stained sections: The liver sections from the control and ASFE extract exhibited low staining of collagenous fibers (blue-stained) surrounding the central and portal regions (Figure 4A & B). Nevertheless, this stain was prominently exhibited throughout the liver sections of DOX-treated animals (Figure 4C). The treatment of DOX-injected rats with ASFE resulted in a significantly reduced expression of Masson's trichrome stain compared to the DOX group (Figure 4D). The quantitative imaging analysis of the positively expressed area of liver sections in control, ASFE, DOX, and DOX+ASFE yielded values of 4.439, 3.6, 608.18, 259, and 9.812, respectively (Figure 4E).

4. Immuunohistochemical changes in TNF-α

The liver sections from the control and ASFE groups (Figure 5A & B) exhibited negative to minimal TNF-α expression; however, this expression was markedly more pronounced in the liver section of DOX-injected rats (Figure 5C). In contrast, post-treatment of DOX animals with ASFE resulted in a significant reduction of TNF-α immunoreactivity compared to the DOX group, although it remained considerably elevated relative to the control (Figure 5D). Image analysis was used to evaluate the quantitative levels of TNF-α positive cells in the liver tissues across the examined groups. The data indicated that the average proportion of TNF-α positive cells in the control, ASFE, DOX, and DOX + ASFE groups were 0.654, 0.484, 12.852, and 2.882, respectively (as illustrated in panel E).

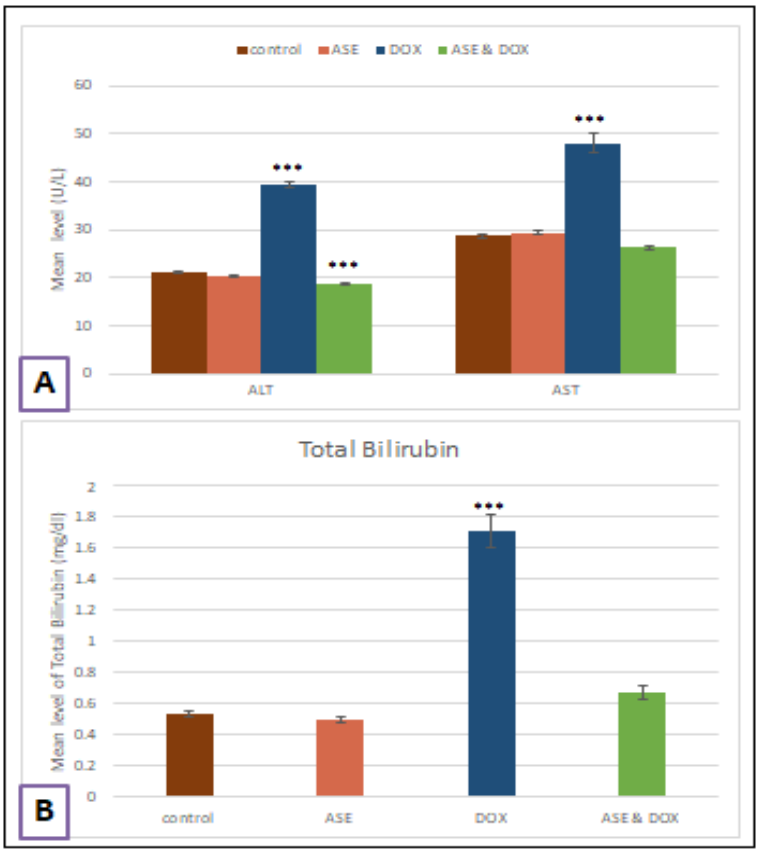

Figure 1: The levels of serum enzymes (ALT & AST) and total bilirubin among the control and experimental groups. The DOXorubicin (DOX) showed a significant increase in the levels of liver enzymes and total bilirubin compared to the control ($P < 0.05$); however, *A. squmaosa* extract (ASE) mitigates this elevation. $P < 0.05$ was considered statistically significant.

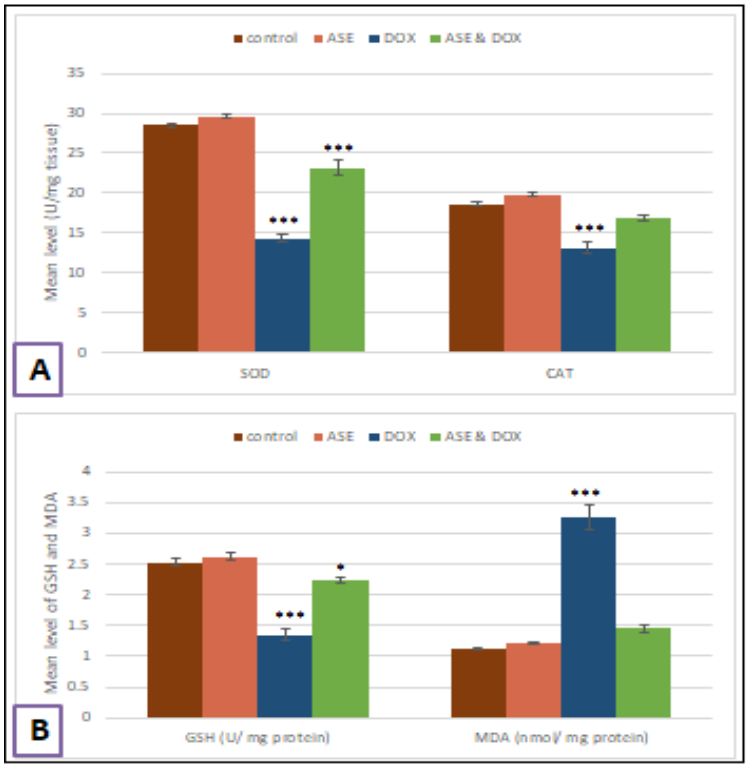

Figure 2: The levels of liver antioxidants (SOD, CAT, and GSH) and oxidative stress marker (MDA) among the control and experimental groups. The DOXorubicin (DOX) group showed a significant decrease in the levels of liver SOD, CAT, and GSH while a significant increase and MDA compared to the control ($P<0.05$). *A. squmaosa* extract (ASE) restored the antioxidants and MDA to the normalcy. $P < 0.05$ was considered statistically significant

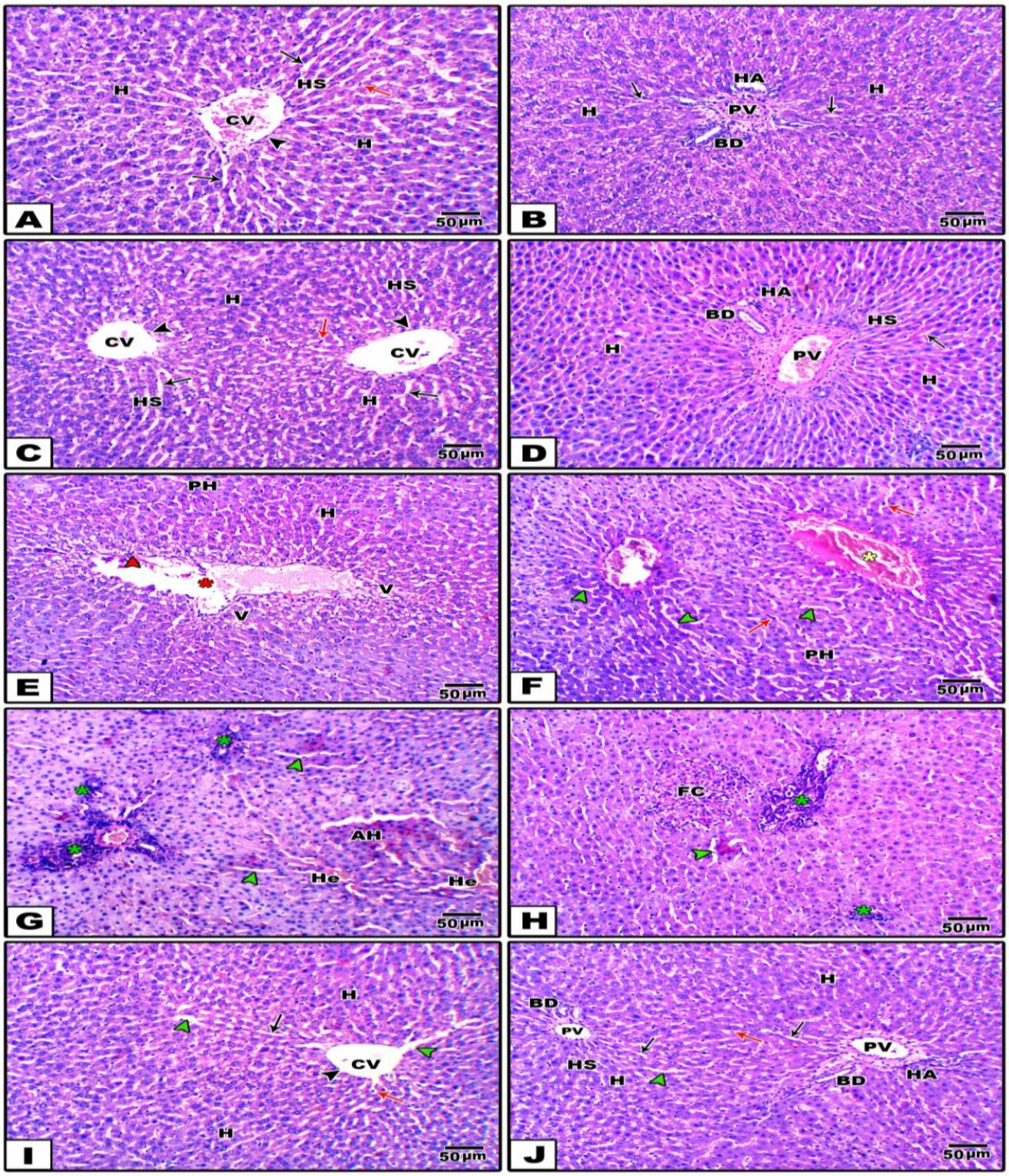

Figure 3: Histological sections through the liver of control (panels A&B), ASE (panels C&D), DOXorubicin (panels E-H), and DOX + ASE (panels I&J) groups of rats. In the control and ASE groups, the liver sections display normal histological traits. The liver sections from DOX-treated rats show dilated central veins (red asterisks), congested central veins (yellow asterisks), and obvious degenerated walls of central veins (red arrowheads). Also, the dilated blood sinusoids (green arrowheads), cellular infiltrations (green asterisks), and excess Kupffer cells (red arrows) were noticed. In the ameliorative group (DOX followed by ASFE), the liver sections appeared more or less similar to the control. (H&E stain, scale bar 50 µm). **Abbreviations:** Hepatocytes (H), Hepatic strands (HS), Central vein (CV), Portal vein (PV), Hepatic arteriole(HA), Bile ductule (BD), Pyknotic hepatocytes (PH), Aggregated hepatic cells (AH), Fibrotic cells (FC), intact central vein endothelium (Black arrow head).

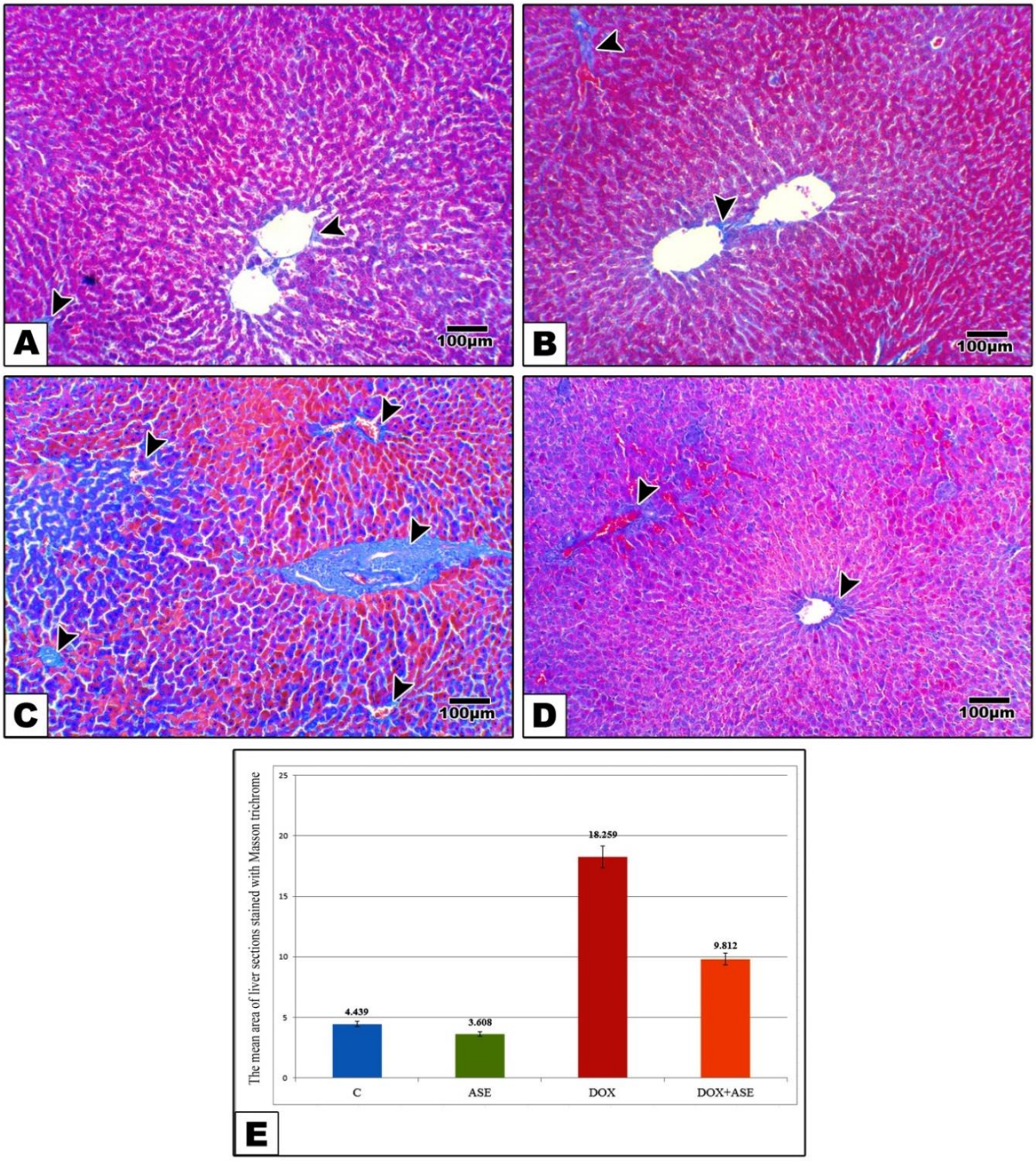

Figure 4: Photomicrograph of histological sections through the liver of the control (panel A), ASE (panel B), DOX-treated group (panel C), and DOX+ASE (Panel D) stained with Masson's trichrome (a specific fibrotic stain). The degree of Masson's trichrome stain (blue color, arrowheads) appears more prominent in the liver section of DOX-injected rats compared to the control and ASE groups. ASE mitigated the degree of liver fibrosis as indicated by alleviation in the Masson's trichrome intensity caused by DOX. Panel (E) illustrates the quantitative analysis of Masson's trichrome-stained tissue in the liver among all groups. (Stain; Masson's trichrome, scale bar: 100µm).

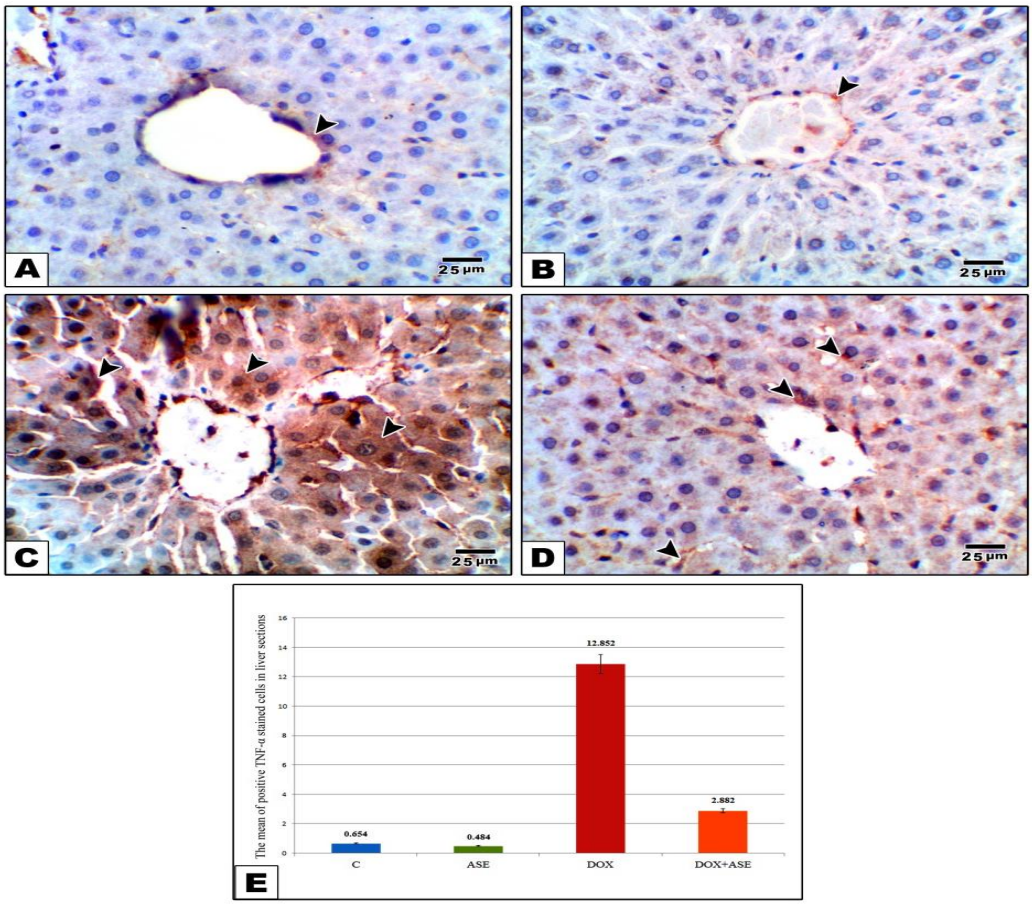

Figure 5: Immunohistochemical staining of TNF-α in the liver tissues among the control (panel A), ASFE (panel B), DOX (panel C), and DOX + ASFE (panel D) groups stained with TNF-α antibody. In the control and ASFE groups, the immunoreactivity of TNF-α protein (brown stain color) appears negative or weakly expressed within the liver cells. Conversely, the immunoreactivity of TNF-α appears strongly expressed in the liver tissues of the DOX-treated group while on post-treatment treatment with ASFE; the TNF-α immunoreactivity markedly declined indicating the powerful anti-inflammatory effect of ASFE. The quantitative analysis of the TNF-α positively expressed cell in the liver of control, ASFE, DOX, and DOX+ ASFE are illustrated on panel (E). (*Arrows point to the immunolocalization of* TNF-α, *Stain:* NF-κB *antibody, scale bar: 25µm).*

DISCUSSION

Doxorubicin (DOX) is a commonly employed and potent antineoplastic drug. Nonetheless, multi-organ toxicity significantly restricts its application (32). The liver is particularly impacted due to its function in the metabolism of DOX. The liver exhibits a strong affinity for DOX, facilitating its preferential accumulation in this organ after systemic treatment (33, 34). The metabolism of DOX in the liver is linked to the generation of reactive oxygen species, inflammation, and mitochondrial dysfunction (35, 36). This diminishes the hepatocytes' capacity to execute energy-dependent mechanisms essential for DOX detoxification and removal, hence promoting cellular death (37). Hepatocellular apoptosis leads to the release of enzymes into the bloodstream, allowing for the assessment of liver damage through the measurement of these enzymes (38). Elevated levels of circulating AST and ALT indicate liver injury (33). The current work demonstrated DOX-

induced hepatic toxicity by elevated blood ALT and AST activity, together with increased total bilirubin levels in DOX-treated rats compared to control rats, indicating significant liver injury, corroborated by histological analyses. These findings align with prior observations on DOX-induced hepatotoxicity and apoptosis in individuals with pre-existing liver injury following DOX administration (9, 39).

DOX has been demonstrated to disrupt redox homeostasis, characterized by an increase in reactive oxygen species (ROS) and a decrease in antioxidant defenses, culminating in the oxidation of DNA and other macromolecules, including lipids, causing liver damage (9,39). Consistent with prior findings, the current investigation demonstrated substantial elevations in liver MDA and notable reductions in liver antioxidants, including SOD, CAT, and GSH levels, in the DOX-injected group. This signified the failure of antioxidant defense systems and the lipid peroxidative deterioration of the biomembrane. The ASFE therapy significantly enhanced the antioxidant/oxidant status in DOX-intoxicated rats by reducing liver MDA levels and increasing SOD, CAT, and GSH concentrations. These findings corroborated the potential antioxidant capacity of ASFE, likely due to its cytoprotective qualities, enabling it to defend liver tissues from oxidative stress generated by DOX in rat liver. A comparable investigation demonstrated the possible protective impact of ASFE against alcohol-induced oxidative stress in the livers of rats, as revealed by modifying the amounts of SOD, CAT, GSH, and MDA toward normal levels (17). The phenolic and flavonoid concentrations indicate that these primary phytochemicals are important for the antioxidant activity of medicinal plants (1). The radical scavenging capacity of numerous Annona species was assessed utilizing diverse antioxidant screening methodologies. Polyphenols were discovered to reestablish the state of balance between endogenous antioxidants and free radicals by directly neutralizing reactive oxygen species (ROS) and inducing endogenous defenses such as Glutathione (GSH) (22, 40).

Besides oxidative stress, inflammation is recognized as a primary factor contributing to hepatotoxicity. DOX therapy may elicit a pro-inflammatory response by elevating pro-inflammatory cytokines such as TNF-α and suppressing anti-inflammatory supportive elements (41). Immunohistochemistry findings revealed heightened hepatic expression of TNF-α, with a greater proportion of hepatic and sinusoidal cells expressing TNF-α in the livers of DOX-treated rats compared to the control group. TNF-α is a pivotal cytokine implicated in hepatic inflammation and injury. It attaches to its receptors on hepatocytes and other hepatic cells, initiating a series of intracellular processes that may lead to cell death via apoptosis or necrosis, TNF-α is recognized for augmenting the generation of reactive oxygen species (ROS), hence intensifying oxidative stress and cellular injury. It also alters the equilibrium of anti-inflammatory and pro-inflammatory cytokines, resulting in fibrosis and cirrhosis (42-44). Furthermore, TNF-α could weaken the integrity of the hepatic blood vessels, resulting in heightened vascular permeability and ensuing tissue injury (45). Treatment with ASFE mitigated hepatic inflammation induced by DOX injection in rats. This was demonstrated by a notable decrease in TNF-α in the liver tissues, indicating the anti-inflammatory effects of ASFE against DOX-induced hepatic inflammation. Previous research (14, 46) demonstrated the anti-inflammatory action of A. squamosa. Reports indicate that custard apple include substances such as alkaloids, phenolic acids, and flavonoids, which possess significant anti-inflammatory properties (47, 48).

Additionally, several histological abnormalities were observed in the livers of DOX-injected rats, including dilated blood sinusoids, inflammatory infiltration, periportal fibrosis, significant disruption of hepatic strands, and considerable damage to

hepatocytes. The area percentage of collagen fibers was significantly elevated in the livers of DOX-injected rats compared to the control group.

Abdelhady et al. (49) documented comparable histopathological findings. Furthermore, DOX has been demonstrated to promote the formation of inflammatory cells (50), correlated with elevated serum aminotransferase activity indicative of liver injury (51). The mechanism via which doxorubicin induces histopathological alterations in the liver remains ambiguous; nevertheless, prior studies have indicated that doxorubicin is converted into a semiquinone free radical by hepatic enzymes, potentially leading to hepatocellular damage and fibrosis (49, 52). Dodda et al. (53) said that lipid peroxidation of liver cell membranes, generated by reactive oxygen species (ROS), is the primary cause of tissue damage resulting from DOX.

The histological defects and liver dysfunctions reported in DOX-treated rats are significantly repaired in the ASFE-treated group, indicating a protective effect against DOX-induced liver injury. Prior research indicated that A. squamosa fruit extract can mitigate hepatic malfunction and cellular damage caused by numerous external causes (17, 54). Zahid et al. demonstrated that ASFE confers a protective effect against alcohol-induced hepatic damage in rats (17,55). The mechanism through which ASFE mitigates the histopathological characteristics induced by DOX remains ambiguous; however, prior studies suggest that the phytochemical constituents of A. squamosa, such as acetogenins, alkaloids, diterpenes, and cyclopeptides, play a crucial role in resisting oxidative stress by scavenging free radicals, thereby preventing cellular damage.

Conclusion

A. squamosa fruit extract (ASFE) has a powerful ameliorative role against oxidative stress, inflammation, and histopathological changes induced by doxorubicin in the liver of rats. The restoration of liver functions and the normalcy of antioxidants such as superoxide dismutase, catalase, and GSH, along with MDA, served as evidence of this. In addition, ASFE effectively reduced the activity of the pro-inflammatory cytokine tumor necrosis factor-alpha and the fibrosis that DOX caused in the liver.

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