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In vivo, effects of combined treatment of female mice with *Phoenix dactylifera* extract and doxorubicin on reducing neighboring cell death during hepatic cancer therapy

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Abstract:

The extract of date fruits (*Phoenix dactylifera)* was orally administered or combined with 0.026 mg/ml water of doxorubicin (Dox) once every 7 days for 30 days to female mice to determine their abilities as anti-cancer effects using real-time PCR technique and histopathological examination to evaluate the apoptosis in the liver of treated mice. Mice treated with Agwa date extract (ADE) in combination with doxorubicin (Dox) showed high cancer cell apoptosis with healthy normal cells, while mice treated only with ADE showed a mild apoptosis process, indicating that ADE can protect the healthy tissue from the Dox chemotherapy side effects while revealing the apoptotic process of the hepatic cancer tissue. Also, mice treated with ADE and then Dox exhibited normal histology of the hepatic cords, portal triads, and central veins. Therefore, date fruits can be used as safe, edible natural resources and nutritional supplements during Dox chemotherapy to alleviate the side effects of Dox.

Keywords: Cancer, date fruits; doxorubicin chemotherapy, PCR, histopathology; apoptosis.

Introduction:

One of the most fatal and prevalent tumors around the world is hepatocellular carcinoma (HCC) (1,2). Generally, the cell cycle regulatory system ensures that the cellular mitotic division runs correctly to yield two genetically identical cells; however, when the Cell cycle checkpoints prevent genetic mistakes from spreading and accumulating during mitotic cell division according to the DNA surveillance mechanism, the genetically damaged cells turn to the apoptosis process, and the continuous division of the genetically damaged cells is enabled by cancer-associated mutations resulting in altering the capability of cells to exit the cell cycle and disrupt cell cycle regulation (3). Several trials have been approached and are up to date for the treatment of HCC, such as chemo-immobilization, and liver transplantation, and the most common is systematic chemotherapy (4).

Doxorubicin (Dox) is one of the chemotherapeutic drugs that is approved by the FDA (Food and Drug Administration) as a kind of chemotherapy treatment in which the naphthacenequinone nucleus is linked to an amino sugar, daunosamine, by a glycosidic bond (5,6); however, dose-related organ damage limits its clinical application (7). It is used in clinical

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trials and frequently used to treat a variety of human malignancies, including postmenopausal women's breast cancer (8-10). Dox has a highly effective anti-tumor effect for various tumors; however, severe cytotoxic effects, drug resistance, and oxidative damage to the cellular lipid membrane and cellular components were accompanied by its treatment (11,12), resulting in delaying the relief and outcome of the carcinoma (11). Therefore, the inhibiting accumulation of Dox in target organs is a method that shows promise to attenuate the toxicity of Dox (13).

Date palm fruits (*Phoenix dactilyfera)* are growing in many countries, especially the Arabian Gulf, and have a high content of flavonoids, which have antioxidant benefits (14). Furthermore, Ajwa dates are date palm fruits that have Soft, delightful berries with black exocarp and basal white lines are primarily grown in Al-Madina Al-Munawaroh (15) and are claimed to have many health advantages, including hepatoprotective, antioxidant, anticholesteremic, and anticancer properties, in both traditional and alternative medicine (16) because they contain a lot of phytochemicals such as carotenoids (lutein and beta-carotene) and polyphenols (luteolin, quercetin, iso-quercetin, rutin, apigenin, and anthocyanins) more than other varieties of fruits (17). Many studies demonstrated that extracts derived from date palm fruit are great sources of biologically active components and support their potential application in novel natural resource-based anticancer treatments (18).

Folklore Medicine has recognized Date fruits, especially species Ajwa dates to have many health advantages due to having pharmacological importance and being considered anti-cancer because of their contents that inhibit the up-normal cell cycle progress by inducing apoptosis throughout regulating signaling pathways involving apoptotic factors such as Bcl-2 and Bax and intracellular reactive oxygen species (ROS) (17). Additionally, Ajwa dates have anti-inflammatory, antiestrogenic, antidiabetic, anxiolytic, antiviral, anti-hypertensive, and antimicrobial effects (19,20), anti-cancer effects, and can reduce the side effects of Dox (21). Because of the high mortality and the related adverse effects of chemotherapy, there is an increasing demand to get an effective and safe treatment with earlier cancer diagnosis (22). To decrease the side effects of chemotherapy, patients often look for alternative treatments such as natural products (23); therefore, this work aimed to simultaneously compare cancer treatment with ADE alone and in combination with Dox chemotherapy to mitigate and minimize the side effects of Dox used in cancer treatment. Real-time PCR technique and histopathological examination were conducted to confirm the apoptosis in the liver of treated mice.

Materials and Methods:

1. Chemicals

Doxorubicin hydrochloride (5,12-Naphthacenedione, 10-[(3-amino-2,3,6-trideoxy-L-lyxohexopyranosyl) oxy]-7,8,9,10-tetrahydro-6,8, 11-trihydroxy-8-(hydroxylacetyl)-1-methoxy-hydrochloride (8S-cis) was obtained from Khandelwal Laboratories Pvt. Ltd Factory Thane, India (lyophilized Doxorubicin 50 mg for intravenous injection).

2. Collection of Date fruits and Preparation of Extract

Semi-dry Ajwa date fruits were bought from El-Madina Al-Munawwara City, Saudi Arabia's local market. and Agwa date extract (ADE) was prepared as described elsewhere with some modifications (24). In brief, seeds were removed manually and discarded, then the flesh was washed with double-distilled water, dried, and ground into fine particles. The dried powder was homogenized in 95% ethyl alcohol (Sigma-Aldrich) at a ratio of 1:3 (W/V), mixed vigorously, and then left at room temperature for one week. The mixture was filtrated through Wattman filter paper grade 520, then centrifuged at 4000 ×g for 15 min. A rotary evaporator was used to evaporate the supernatant at 30 °C (Buchi Rotavapor R-205, Switzerland). In a water bath (Lab1st-GX),

the obtained extract was heated at 37 °C for 72 hours until it became a semisolid paste and then stored at -20 °C until used. The semisolid paste was suspended in distilled water to prepare 10, 20, and 40 mg/0.5 ml (mouse) of date fruit extract.

3. Treatment of Animals and Tissue Sampling:

The experiments and handling of animals were carried out in compliance with the guidelines set forth by the Research Ethics Committee at Zagazig University, Egypt (ZU-IACUC/1/F/111/2023). Female adult mice (*Mus musculus*) weighing 20–25 g (10 weeks old) were obtained from the Animal House of the Research Center, Cairo, Egypt, and housed in well-ventilated plastic cages, fed on a commercial diet, and given water ad libitum. Animals were randomly divided into 9 groups (10 mice each) maintained at 25 ± 2 °C temperature, 70% humidity, and 12:12 light to dark, and given a week to acclimatize before beginning the study. Animals of the first group received distilled water and were kept as negative controls, while animals of the second group were intraperitoneally injected with a single dose of Ehrlich Ascites Carcinoma, EAC (10% in saline solution) and kept as positive controls (25). EAC is one of the most prevalent malignancies and can induce undifferentiated carcinoma (25). The third group was injected intraperitoneally with 0.026 mg/ml of Dox chemotherapy once every 7 days for 30 days, as described by Paget and Barnes (26), while groups 4, 5, and 6 were orally given 10, 20, and 40 mg/mouse. of ADE once daily for 30 days. The 7, 8, and 9 groups were orally administered 10, 20, and 40 mg/mouse of ADE once daily for 30 days and then treated intraperitoneally with 0.026 mg/ml of Dox chemotherapy once every 7 days for 30 days, respectively. The weight of the mice was recorded after 7 days after the EAC injection until the 30th. At the end of the experiment, a simple capillary tube was used to take blood from a peripheral vein into sterile, heparinized tubes twenty-four hours after the last dosage. The cells were counted by the hemocytometer after the cells were suspended in 0.02 ml of sterile isotonic saline cells (27). Mice were put under anesthesia by intraperitoneal injection of 50 mg/kg of body weight of thiopentone sodium (EMC, Panpharma UK Ltd) (28), sacrificed, and dissected to get livers at the end of the experiment. Aliquots of livers were quickly submerged in liquid nitrogen and kept at -80 °C for RNA extraction and qRT-PCR (quantitative real-time PCR). For further histopathological examinations, livers were submerged in 10% neutral buffered formalin.

4. Trypan Blue Viability Test:

Trypan blue is a stain usually used for detecting living cells, where cells without interactions with the dye are viable (29). In brief, after thoroughly staining and mixing 0.5 ml of the cell suspension with 0.4% Trypan blue stain, it was allowed to stand at room temperature for five minutes and then subjected to a light microscope to be counted using the hemocytometer. The stained non-viable cells were counted under the microscope for not more than 30 minutes because it was possible to observe an increase in dead cells due to trypan toxicity.

5. RNA extraction and qRT- PCR:

Total RNA was extracted from livers using a HiSenScript^{TM} RH-ccDNA Synthesis Kit (iNtRON Biotechnology Co., South Korea) following the instructions from the manufacturer. In brief, 20 µl reaction volume (10 µl master mix and 10 µl RNA sample containing 1µg RNA), 1µg of extracted RNA was reversely transcripted into cDNA using the High-Capacity cDNA Reverse Transcription Kit cDNA Kit complying with the instructions from the manufacturer (Applied Biosystems TM USA). The purity and concentration of RNA were performed at 260 nm and 260 nm/280 nm ratios using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, United States). Additionally, complementary DNA (cDNA) was synthesized from 1 to 2 µg of extracted RNA using the HiSenScriptTM RH [-] RT PreMix

Kit (iNtRON Biotechnology), and the reaction was conducted for 60 minutes at 37 °C. The synthesized cDNA was used as a template for PCR amplification. RT-PCR was performed using Topreal Cyber-Green qPCR Mix Plus (Enzynomics, Korea) in a Rotor-Gene Q PCR System (Qiagen, Germany). Following the instructions of the manufacturer, the PCR assay was conducted under the following conditions: Initial denaturation at 95 °C for 12 min, followed by 40 cycles of denaturing at 95 °C for 20 sec, annealing at 60 °C for 30 sec, extending at 72 °C for 30 sec, and then cooling at 40 °C for 30 sec (30). Further, Rotor-Gene Q series software (Qiagen) was used to evaluate the CT values and melting curves of each sample. The PCR products were analyzed using 1% agarose gel electrophoresis. Gel images were analyzed using real-time PCR (SYBR Green qRT-PCR), and the relative level of gene expression in the liver was calculated by using the $2^{-\Delta\Delta CT}$ method to determine relative fold changes in target genes (BAX, BCL2, and caspase 3), and the gene expression level was normalized using GAPDH, which is the internal control. The amount of target gene expression levels was quantified (31) using the formula:

$$\Delta\Delta CT = (CT\ target - CT\ GAPDH)\ treated - (CT\ target - T\ GAPDH)\ untreated.$$

The RT-qPCR primers were produced by Sangon Biotech (Beijing, China) and are listed in Table 1.

Table 1: Forward and reverse sequences for the internal control Gapdh and the apoptotic factors BAX, Bcl2, and Caspase 3 genes.

Gene	Forward primer	Reverse primer
BAX	5′ CTGGATCCAAGACCAGGGTG3′	5′ GTGAGGACTCCAGCCACAAA3′
Bcl2	5′ GAACTGGGGGAGGATTGTGG3′	5′ GCATGCTGGGGCCATATAGT3′
CASPASE3	5′ GGGGAGCTTGGAACGCTAAG3′	5′ GAGTCCACTGACTTGCTCCC3′
Gapdh	5′AAATGAGAGAGGCCCAGCTAC3`	5′ GAGGGCTGCAGTCCGTATTTA3′

6. Histopathological studies

Liver tissue was carefully removed from the mice, fixed in 10% neutral formalin (Sigma-Aldrich), dehydrated in increasing grades of ethyl alcohol, cleared in xylene, and then embedded in melted paraffin wax at 58 °C (32). An automatic rotary microtome M-240 (Myr, S.L.) was used to section paraffin blocks at 3–4 mm thickness. An Olympus BX 21 light microscope connected to a digital DSC-W 800 super steady camera (Sony, Japan) was used to examine stained sections.

7. Statistical analysis

Using the statistical package of SPSS version 28.0 (SPSS Inc., Chicago, Illinois, USA), data were statistically analyzed using one-way analysis of variance (ANOVA) at a probability of 0.05. Analyses were carried out at least in 10 replicates. All values were calculated as the mean ± standard deviation (SD).

Results:

1. Cell Viability

EAC showed a high count of 2.5 million obtained from the peritoneal cavity aspiration, crowded cells with rounded morphology in the isotonic suspension, and high viable cells detected by trypan blue stain (Figure 1).

2. Effect of treatments on the body gain

Figure 2 illustrates that there was no statistically significant difference ($p > 0.05$) regarding the body gain among the treated mice and the control groups on the 8th day ($p > 0.708$) after treatment. However, the body gain revealed a statistically significant decrease in mice treated only with ADE or Dox on the 10th day ($p \leq 0.05$) in comparison with control groups. Furthermore, significant decreases were recorded in mice treated only with Dox and when combined with different doses of ADE on the 20th day. On the 30th day of treatment, the body gain significantly decreased following Dox treatment, whereas the ADE at all the tested doses significantly attenuated the effect of Dox.

3. Detection of the concentration and purity of extracted RNA

The isolated RNA was validated before cDNA synthesis, and the absorbency 260\280 ratio was calculated to assess the purity and quality of the RNA extract. The yield of RNA extract ranged in liver samples from 4.016 to 7.672 µg/ml, while the isolated RNA had an absorbency 260/280 value ranging between 1.8 and 2.0 indicating high purity.

4. Apoptotic genes of Caspase3, BAX, Bcl2, and BAX/Bcl-2 expressions detected by qRT-PCR

The apoptotic gene expressions from qRT-PCR for caspase3, BAX, Bcl2, and the BAX/Bcl-2 ratio are shown in Figure **3.** The results revealed significant up-regulation ($P<0.05$) in caspase3, BAX, and the BAX/Bcl-2 ratio expressions compared to the positive control, while Bcl2 values showed significant up-regulated ($P<0.05$) compared to the positive control. Mice treated with ADE at all the tested doses combined with Dox showed significant up-regulation for caspase3, BAX, and the BAX/Bcl-2 ratios compared to other groups; however, the obtained Bcl2 values were found to be significantly down-regulated in mice treated with ADE and Dox in comparison with the positive control.

5. Effect of different treatments on the architecture of livers

The histological results help describe how treatment stress affects the integrity of cells and tissue. Figures 4-6 demonstrate the ability of different treatments to cause histopathological alterations in the livers of mice during 30 days of treatment. Livers of mice treated with EAC as positive control showed a perihepatic solid neoplastic mass with necrotic areas, atrophy on the adjacent hepatic parenchyma, and several anaplastic cells and giant tumor cells seen within the hepatic parenchyma (Figures 4A–6A). On the 10th day after the treatments, hyperplastic hepatocytes (arrowheads) and interstitial round cells infiltrated the mice's livers treated with Dox and then with 20 mg/mouse of ADE (Figures B and C), while mice treated with Dox and then with 20 mg/mouse of ADE showed moderate focal round cell infiltration and degenerated cells were observed. Few numbers of apoptotic and degenerated cells with round cell aggregates, degenerative changes with a moderate number of hepatocytes, and apoptosis with interstitial lymphocytic infiltrates were observed in the mice's livers treated first with Dox and then with 40 mg/mouse of ADE (F, G, and H). On the 30th day (Figure 6), after the treatments with Dox and then either 10, 20, or 40 mg/mouse of ADE, animals showed fewer numbers of hyperplastic hepatocytes, degenerative changes in a large number of hepatocytes, or interstitial round cell infiltrates in the livers of mice than those treated for the 20th day (Figure 5) than the 10th day (Figure 4) in a dose-dependent manner.

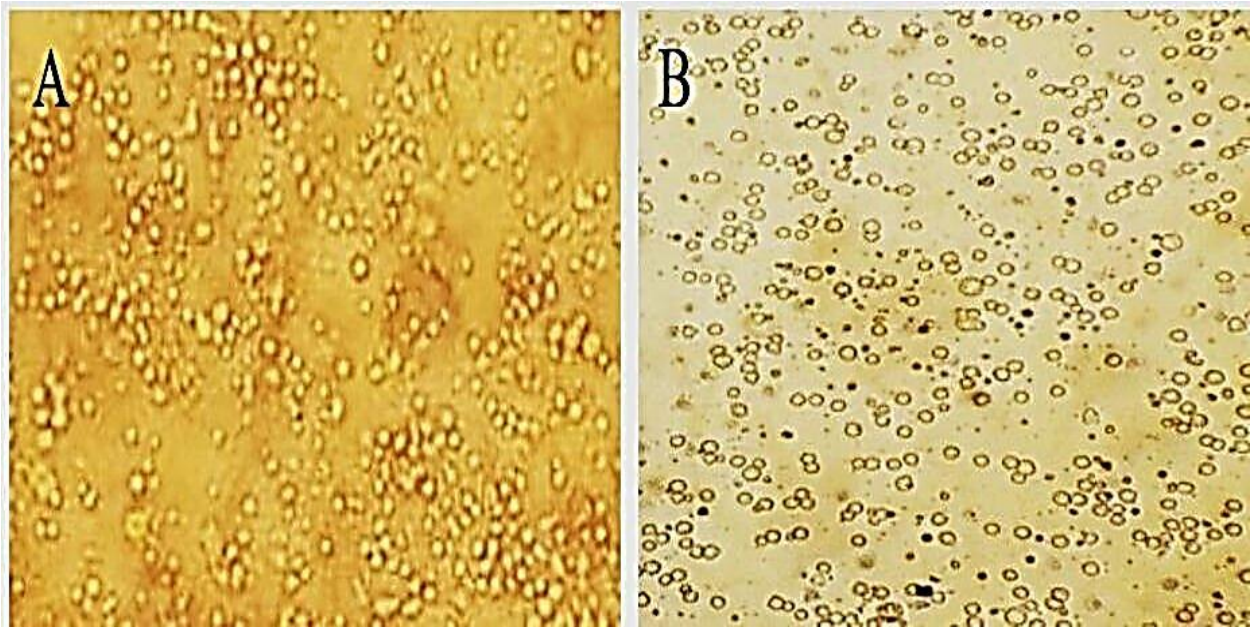

Figure 1: Effect of EAC on the viability of cells. Non-stained viable cells (A) and (B) dead stained cells with trypan blue take the dark color stained. (Trypan blue, x 40).

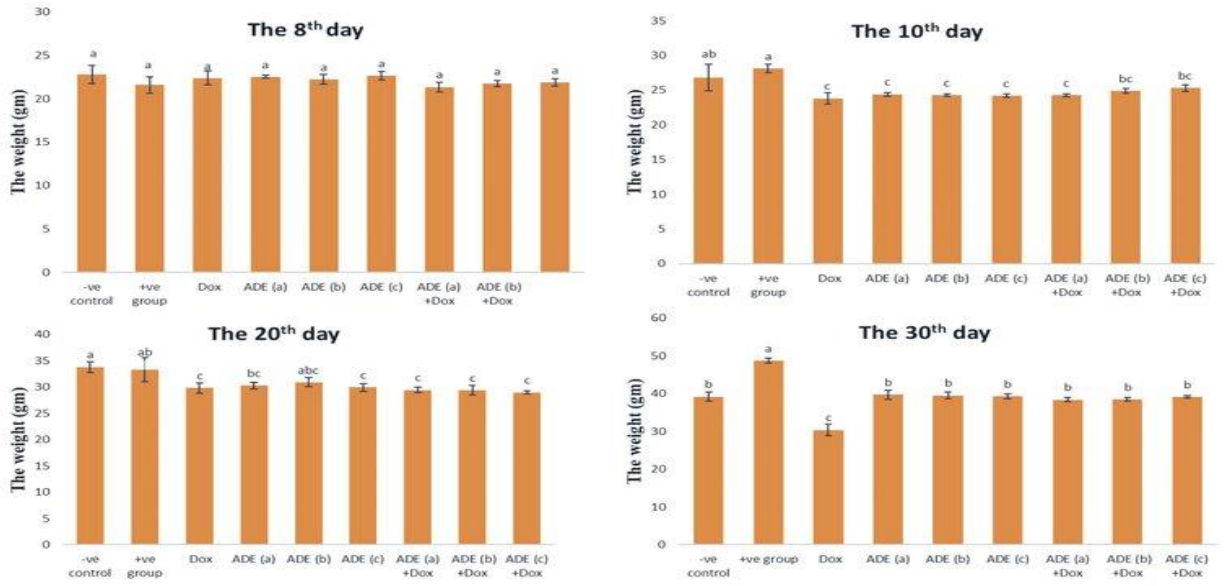

Figure 2. Impact of different treatments on the body weight of mice treated for 30 days. Means share the same letters and are not significantly different from one another.

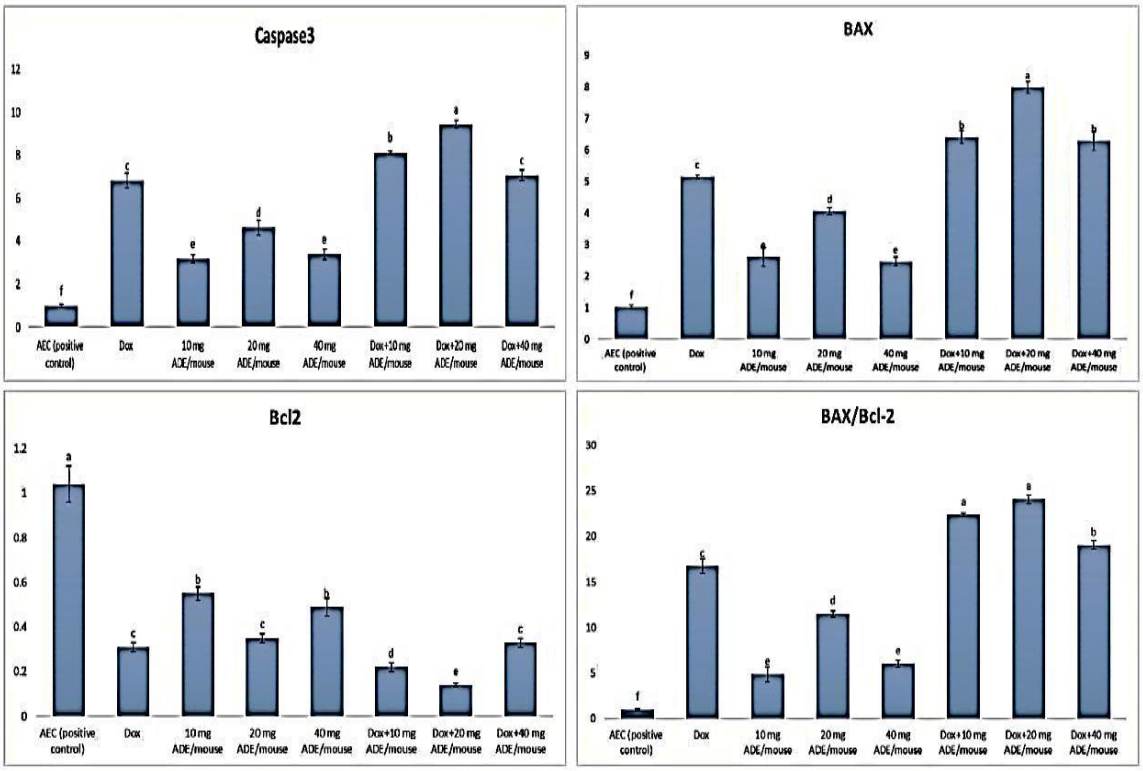

Figure 3. Effect of various treatments on the gene expressions of caspase3, BAX, Bcl2, and BAX/Bcl-2 ratio. This means share the same letters is not significantly different from one another.

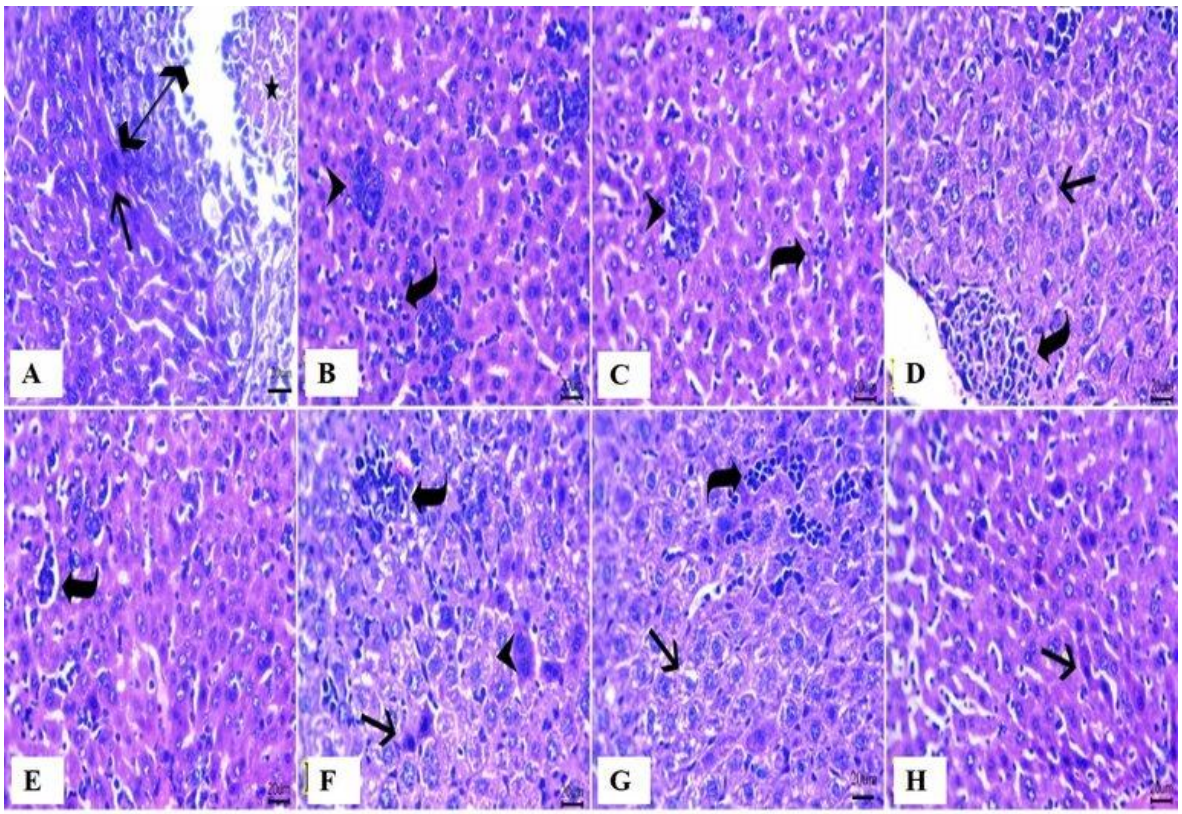

Figure 4. Histopathological lesions in livers of mice treated groups after 10th days; nominal treatments were negative control, Dox+10 mg/mouse, Dox+10 mg/mouse, and Dox+10 mg/mouse of ADE for A, (B & C), (D & E), and (F, G, & H) slides, respectively. (A): perihepatic solid neoplastic mass (double-headed arrow) with necrotic areas (star) beside pressure atrophy on the adjacent hepatic parenchyma (arrow). (B & C): Hyperplastic hepatocytes (arrowhead) and interstitial round cells infiltrate (curved arrow). (D, E): Focal round cells infiltrate (curved arrow), and hydropic degenerated cells (arrow), with a moderate number of hepatocytes (arrowhead) and apoptosis (arrow) with interstitial lymphocytic infiltrates (curved arrow). (F, G, & H): few numbers of apoptotic and degenerated cells (arrow) with round cell aggregates. H&E ×100

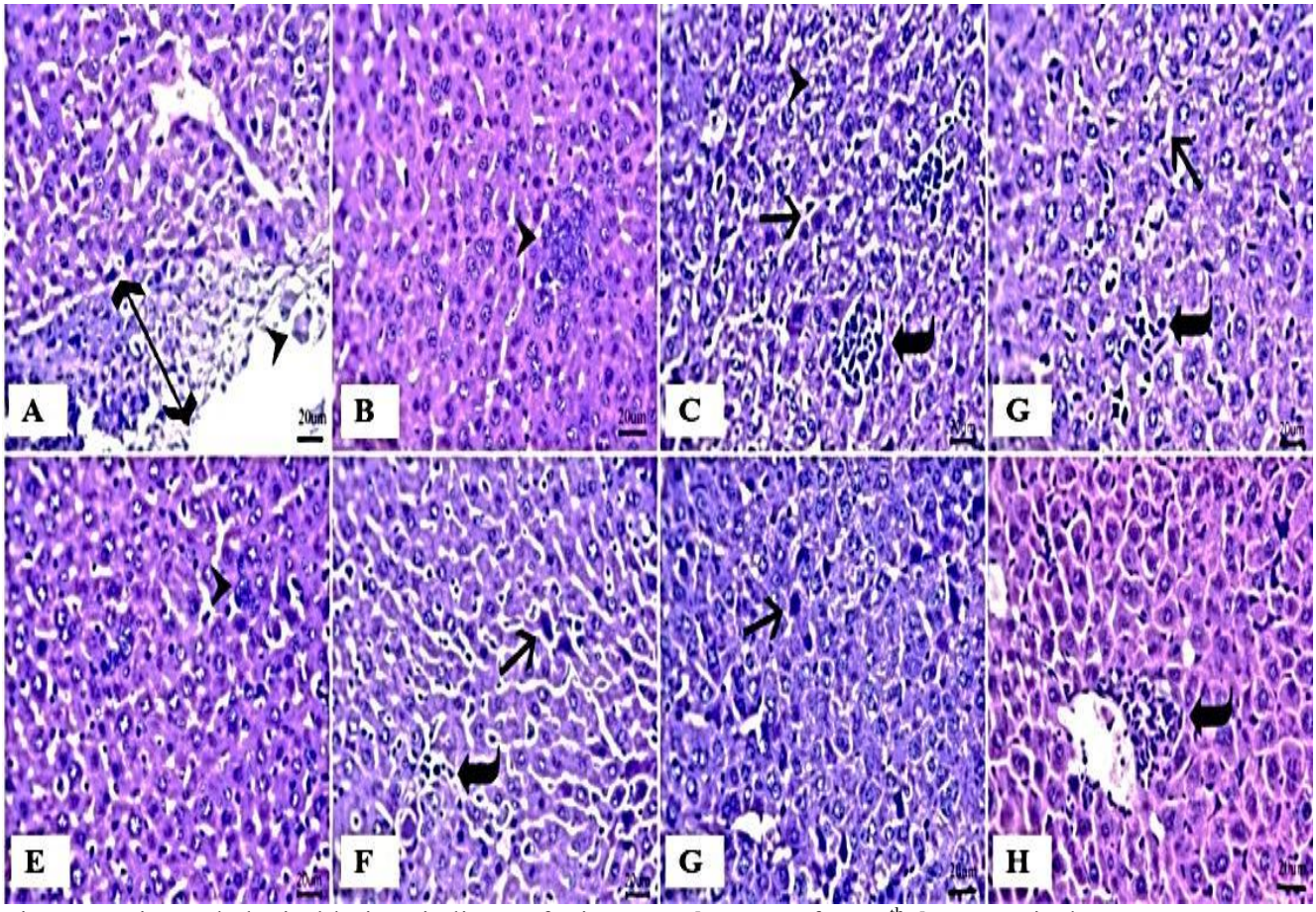

Figure 5. Histopathological lesions in livers of mice treated groups after 20th days; nominal treatments were positive control, Dox+10 mg/mouse, Dox+10 mg/mouse, and Dox+10 mg/mouse of ADE for A, (B & C), (D & E), and (F, G, & H) slides, respectively. (A): perihepatic solid neoplastic mass (double-headed arrow) with necrotic areas beside the presence of tumor giant cells. (B & C): Combination of randomly distributed apoptotic hepatocytes (arrows), degenerated cells (arrowhead), and round cells aggregates between hepatocytes (curved arrow). Few degenerated hepatocytes (arrow) and focal lymphocytic aggregates (curved arrow). (D, E): Few degenerated hepatocytes (arrow) and focal lymphocytic aggregates (curved arrow) in the ADE 20 mg/mouse group (D). (F, G, & H): Apparent normal hepatic parenchyma with perivascular inflammatory cells infiltrates (curved arrow). H&E ×100.

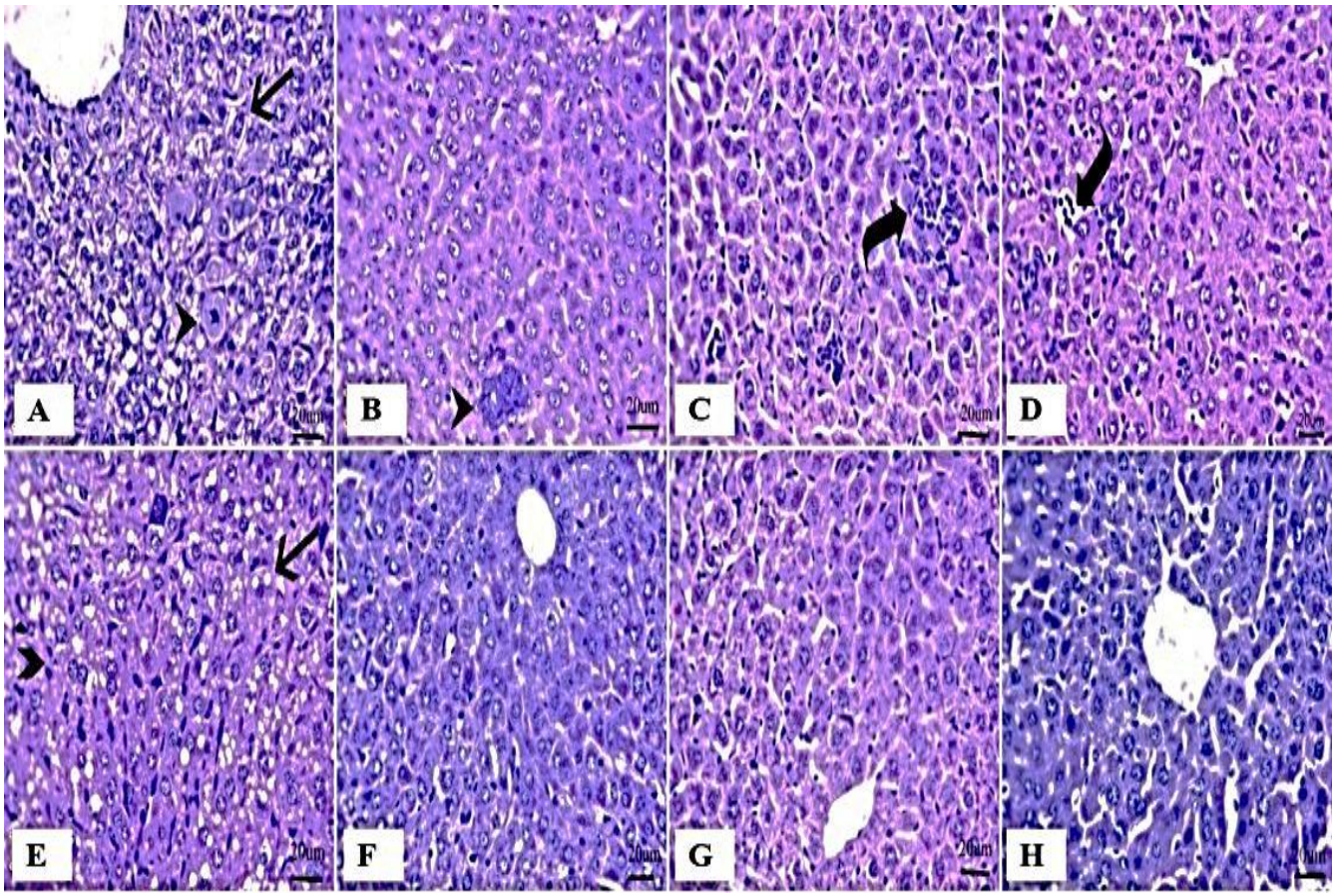

Figure 6. Histopathological lesions in livers of mice treated groups after 30th days; the nominal treatments were positive control, Dox+10 mg/mouse, Dox+10 mg/mouse, and Dox+10 mg/mouse of ADE for A, (B & C), (D & E), and (F, G, & H) slides, respectively. (A): Few numbers of hyperplastic hepatocytes (arrowhead), anaplastic cells (arrowhead), and hydropic degenerated cells (arrow). (B & C): Tiny aggregates of lymphocytes with normal histological structures of hepatic cords and central veins. (D, E): Minute aggregates of lymphocytes with normal histological structures of hepatic cords and central veins. (F, G, & H): Fatty degenerations within most hepatic cells (arrow) with the presence of hypertrophied Kupffer cells (arrowhead), normal histological structures of hepatic cords, and central veins. H&E ×100.

Discussion:

The present results revealed the body weight of mice treated with Dox was significantly decreased during all the tested times of the experiment and the most significant decrease in body weight was noted following 30 days of treatment, whereas when mice treated with ADE at doses of 10, 20, and 40 mg/mouse after Dox treatment showed improved in their body gains. The more cancer volume increased, the more body weight increased in case of stable feed intake and absence of diarrhea (33). In the present study, the ADE + Dox groups showed less weight loss, as Dox decreased the cancer volume in the mice, which agreed with that found in albino rats (33) who illustrated that Dox detected a loss in tumor size by Dox resulted in increasing body weight. However, decrease in body weight loss in the Dox + ADE groups more than in the Dox groups. The body loss in Dox chemotherapy is due to the reduction of skeletal muscle weight and adipose tissue mass (8). In agreement with our results, it was found that when exposed to the chemotherapy drug doxorubicin, wild-type mice show a considerable loss in body weight, with both lean mass and total body fat markedly declining. This is a phenomenon occurring in clinical settings (8,34). The genetically

expressed results revealed that Ajwa date is considered an elegant anti-cancer agent without any side effects in the healthy cells surrounding the cancer tissue (16). Date fruit extract contains specific constituents that act against the apoptosis pathway that arrests the cancer cell cycle up the normal mitotic division by changing in cancer cell's membrane blebbing. The apoptotic gene expressions found in the present results showed a significant difference among the groups. The caspase3 and Bax apoptotic genes were found to be more significantly induced in mice treated with Dox combined with 20 mg/mouse of ADE than in other treated groups. Date fruit extract was found to arrest the hepatic cancer cells during the mitotic division mechanism by decreasing cancer growth (16,35). On the contrary, the expression of the anti-apoptosis gene Bcl-2 was consequently reduced due to the significant influence of the combined effect of ADE + Dox on the downregulation of Bcl-2, hence ADE+Dox resists the hepatic cancer division by reduction of anti-apoptotic gene expression (16). Dox alone showed a lower effect on the Bcl-2, therefore, in this study, the Bax\Bcl-2 transcription ratio increased because Bax apoptosis expression was more than Bcl-2 indicating the death of hepatic cancer cells increased after administration of ADE + Dox together (16). As documented, ADE contains a quercetin which contributes to the expression of the Bax gene. Flow cytometric analyses of human breast adenocarcinoma cells with ADE (15 and 20 mg/ml) for 24 hrs showed 'S' phase cell cycle arrest; elevated expression of the p53 and Bax proteins; activation of caspase3; and a decrease in the potential of the mitochondrial membrane (16,35).

The activation of caspase expression throughout the change in the integrity of the mitochondrial membrane leads to enhancing the apoptosis mechanism in hepatic carcinoma (16,35). Therefore, following treatment of mice, ADE can trigger cell cycle arrest and prevent cancer cells from proliferating (16). Also, the anti-apoptotic Bcl-2 was found to decrease, while the ratio of Bax/Bcl-2 transcription increased, indicating that p53-mediated signaling triggered apoptosis and carcinoma cell death after ADE therapy (16).

The histopathological evaluation of the treated groups reported that the ADE + Dox groups showed normal histology of hepatic tissue with the absence of cancer lesions at the end of the experiment. Cancer lesions were still found in the other groups due to the combined anti-cancer agents of both Dox and ADE and the specific characteristics of the ADE in protecting the healthy cells from the destructive effect of Dox chemotherapy (5,16,36). After the 10 and 20th days of treatment, mice treated only with Dox showed lesions with degenerative and apoptotic changes similar to those found in mice receiving EAC for hepatic cancer induction (37). In the present study, mice receiving only EAC as a positive control showed severe lesions such as perihepatic solid neoplastic mass with necrotic areas, several anaplastic cells, and giant tumor cells within the hepatic parenchyma. These observations were related to the mice's high mitotic division rate and the aggressive mobility of the transplanted EAC (38).

Interestingly, in the present study, ADE showed ameliorative changes in the architecture of the liver that were indicative of apoptosis.

Conclusion:

This work dealt with the combined effect of Dox chemotherapy and ADE as a natural product, as well as the impact of such a combination on protecting healthy cells from the destructive side effects of chemotherapy. Natural products such as Ajwa date fruits, which contain flavonoids, aglycones, and terpenoids, have been proven to modulate apoptotic pathways and/or trigger apoptosis. and have cancer-inhibitory properties **(**39**)**. The purification and identification of date fruit extract components, which are the reason behind its anti-cancer properties, are needed. In conclusion, this study recommends using Agwa date fruits as safe, edible

natural resources and nutritional supplements during doxorubicin chemotherapy.

Conflict of interest: NIL
Funding: NIL

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