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Egyptian Propolis Extract Attenuates Hepatotoxicity Induced by Doxorubicin via Increasing Antioxidant Defense and Decreasing Inflammatory and Apoptotic Markers: Targeting Nrf2 and Bcl-2

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Abstract

Doxorubicin (DOX) is a chemotherapy medication that is used to treat different types of cancers. Propolis is commonly used as a hepatoprotective agent against oxidative stress. Therefore, the present study was designed to investigate the possible protective role of the Egyptian propolis extract (EPE) against DOX-induced hepatic toxicity in rats. The study was carried out on forty male adult albino rats divided into four groups (control group): received normal saline by oral gavage daily for 28, (EPE group): received EPE (200 mg /kg) daily by oral gavage for 28 days. (DOX group): rats were injected once with DOX (20 mg/kg) intraperitoneally on the 24th day (EPE treated DOX group): received EPE (200 mg /kg) daily by oral gavage for 28 days and injected with DOX 20 mg/kg intraperitoneally on the 24th day. Our results revealed that liver enzymes, MDA, TNFα, interleukin -1β (IL-1β) and IL-6 and caspase-3 were significantly increased in DOX group compared with control, while EPE treated DOX group showed significant decrease. Catalase and superoxide dismutase were significantly decreased in DOX group compared with control while EPE treated DOX group showed significant increase. Moreover, gene expression of TNF α, nuclear factor erythroid 2–related factor 2 (NRF-2), heme oxygenase -1 (HO-1) have been elevated significantly in DOX group when compared with control and their mRNA levels have been downregulated significantly by EPE treatment while EPE treatment has upregulated gene expression of BCL-2. Conclusion: our results raised the idea that EPE protecting the liver from DOX-related oxidative and apoptotic effects.

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Introduction

Doxorubicin (DOX) is a chemotherapy medicationthat is used to treat different types of cancers like bladder cancer, breast cancer, lymphoma, Kaposi's sarcoma and acute lymphocytic leukemia. It is originally extracted from the bacterium Streptomyces peucetius**(1).**It successfully works not only in combination with other antitumor drugs but also in combination with both radiation and surgery**(2).**The liver is considered the main metabolic organ of DOX. It was recorded that40% of the patients who use DOX as a chemotherapeutic drug experienced varying degrees of hepatic damage**(3)**. DOX has been attributed to causinga significant increase of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) indicating hepatic tissue damage which could be due to oxidative stress caused by DOX**(4).**It was reported that hepatic damage originated due to activation of genes responsible for different vital biological processes including response to oxidative stress, damage and repair of DNA, cell cycle progression, mitochondrial dysfunction and apoptosis**(5).**

Injacet al(6)reported the hepatotoxicity in the rats with mammary carcinomas that received DOX. They explained that this is because the occurrence ofoxidative stress, inflammation and lipid peroxidation induced by reactive oxygen species (ROS) production. This hepatotoxicity may also include other features like cell cycle obstruction and respiratory chain disturbance. Imbalance redox potential induced by ROS production during the metabolism of DOX was also recorded **(7)**.

Propolis, is a natural resinous mixture produced by honeybees and other bees, from resinous and gummy substances that they collect from leaves, buds and other plant structures **(8).** It is well known as the “bee glue”.The constituents of the propolis are greatly varied depending not only on its flora and geographic region but also the local climate **(9, 10).** It has anti-inflammatory, antimicrobial, antioxidant immunomodulatory, and anticancer activities **(11, 12).**Propolis has a high content ofphenolic compounds. Therefore, it has the ability to reduce oxidative stress and inhibit the formation of free radicals**(13)**.**Kumazawaet al (14)**stated that propolis has several antioxidative components like caffeic acid, ferulic acid and caffeic acid phenethyl ester. It was found that Egyptian propoliscould provoke antioxidant mechanisms in the liver and also reduce apoptosis in aflatoxin-treated mice**(15).** Propolis is commonly used as ahepatoprotective agentagainst oxidative stress**(16).**Therefore, the present study was designed to investigate the possible protective role of a kind of the Egyptian propolis extract (EPE)against DOX-induced hepatic toxicity in rats.

1. Material and methods

1.1 Chemicals

EPE : Propolis was purchased from the commercial market at Elgharbia governorate then extracted as reported before**(17, 18).**DOX was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) .

1.2 Animals:

The present work was performed at the animal house of Faculty of MedicineTanta University. The study was carried out on forty male adult albino rats weighing 150 to 200 grams were purchased from Faculty of Science. The rats were housed at room temperature, in animal cages (5/cage), under controlled environmental

conditions with free access to food and water.All procedures were done according to the guidelines of the Local Ethical Committee.

2.3 Ethics statement

All the experiments were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85–23, revised 1996). The animal study was reviewed and approved by the Ethical Animal Research Committee of Tanta University approval number (36122/11/22)

Animal handling was followed according to Helsinki declaration of animal ethics. Animals in this study were not used in other experiments. All the remnants of sacrificed animals were discarded by safe disposal measures in the general incinerator of Faculty of Medicine, Tanta University according to the research and safe disposal rules.

2.4 Experimental design:

After one week of acclimatization, rats were randomly classified into 4 equal groups:

Group I; (control group):rats received normal saline by oral gavage daily for 28 days.

Group II; (EPE group):rats received EPE in the dose of (200 mg /kg) **(19)**daily by oral gavage for 28 days.

Group III; (DOX group):ratswere injected once with DOX in the dose of 20 mg/kg intraperitoneally on the 24th day**(20)**

Group IV; (EPE treated DOX group):rats received EPE in the dose of (200 mg /kg) daily by oral gavage for 28 days and injected with DOX in the dose of 20 mg/kg intraperitoneally on the 24th day.

2.5 Blood Sampling

At the end of the study, all rats which were fasting overnight were weighed then sacrificed by decapitation; fasting blood samples were obtained and taken into dry sterile centrifuge tubes, allowed to clot at room temperature for 30 minutes, then centrifuged for 10 minutes at 5000 rpm. Sera were separated and divided into small aliquots and frozen at -20 ºC until analysis

2.6Preparation of tissue homogenate:

The livers were cut into pieces. Onepiece was fixed in 10% neutral buffered formaldehyde for further histopathological study. The remaining pieces were wrapped in aluminum foil and stored at -80 º C until analysis.

2.7 Biochemical measurements:

2.7.1 Analyses of liver enzymes in serum:

Serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined using using commercial ELISA kits, provided by MyBioSource (ALT cat#MBS269614, AST ca#MBS264975) according to the manufacturers' instructions.

2.7.2 Analyses of lipid peroxidation:

Malondialdehyde (MDA) was determined in hepatic tissueusing commercial ELISA kits, provided by MyBioSource (cat#MBS268427) according to the manufacturers' instructions.

2.7.3 Assays of catalase (CAT) and superoxide dismutase (SOD)

Catalase activityandSuperoxide dismutase (SOD) were determined in hepatic tissue using commercial ELISA kits, provided by MyBioSource (catalase cat#MBS726781, SOD cat# MBS266897(according to the manufacturers' instructions.

2.7.4 Analysis of inflammatory markers:

Tissue TNFα, IL-1β and IL-6 were determined using commercial ELISA kits, provided by MyBioSource(TNFα cat# MBS2507393, IL-1 β cat#MBS825017, IL-6 cat#MBS269892)according to the manufacturers' instructions.

2.7.5 Analysis of caspase-3:

Caspase-3was determined using commercial ELISA kits, provided by MyBioSource(cat#MBS018987) according to the manufacturers' instructions.

2.8 Assay of TNF α ,superoxide dismutase (SOD),heme oxygenase -1 (HO-1) , BCL-2 and The nuclear factor erythroid 2–related factor 2 (Nrf2) by real time PCR:.

The TRIzol™ reagent (Thermo Scientific, Waltham, MA, USA) was utilized to extractthe total RNA from the liver tissue according to the manufacturer's guidelines. The spectrophotometer was used to investigate the purity and concentration of total RNA. The integrity of RNA was assessed on agarose gel electrophoresis. cDNA was synthesized using Revert Aid H Minus Reverse Transcriptase which is a genetically modified M-MuLV RT **(21)**. The isolated cDNA was amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol (Thermo Scientific, USA). Primers were selected from PrimerBank (https://pga.mgh.harvard.edu/primerbank/) as in **Table (1).** An internal control GAPDH was used as a standard for the RT-PCR analysis. Data were analyzed using the 2-△△C t method.

2.9 Histopathological study:

Light microscopic study

Specimens were fixed for 24 hours in 10% neutral buffered formalin, then subjected to the following stains to examine the normal histological structure and pathological changes in rat's liver in the different experimental groups:

i. Hematoxylin and eosin (H&E) stain

H&E stain were used to study the normal histological structure of rat's liver tissue and the pathological changes among experimental groups according to**(22)**

The first step is dewaxing Then sections need rehydration by putting the slides in descending series of alcohol from 90%,70% to 50%. Then the slides were immersed in distilled water. The sections were stained in hematoxylin for 15 minutes and washed in tape water for 10 minutes. Then, they were stained in eosin for 1minute.

ii. **Bcl-2 immunostain:**

Serial tissue sections of 4 μm were deparaffinized in xylene and rehydrated in graded ethanol. Then sections were pre-treated with 10 mm citric acid buffer (pH = 6) for antigen retrieval. Then they were pre-incubated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min. The specimens were then incubated with anti-Bcl-2 polyclonal antibody overnight within the refrigerator at 4 °C. Following incubation, two washes in PBS for 10 min were done **(23, 24).**

2.10 Statistical analysis

Statistical significance was determined by ANOVA with a Tukey post-hoc test for multiple comparisons. Results were considered statistically significant with P value <0.05. (GraphPad Prism, San Diego, CA).

3.Results:

3.1 Effect of EPE and DOX on the liver functions:

Our study examined the effect of DOX on serum biomarkers as indicators of hepatic function. So,

ALT and AST levels were measured. It showed a non-significant ($p \geq 0.05$) change in the values of ALT and AST in the EPE group compared to the control group. The DOX group showed a significant increase ($p \leq 0.05$) in both ALT and AST as compared to control group. **(Table.2).**WhileEPE treated DOX group showed a significant decrease($p \leq 0.05$) as compared to the DOX group.

Table 1: Primer sequences used in PCR

Gene	Forward	Reverse
TNF-α	CTTCTGTCTACTGAACTTCGGG	CAGGCTTGTCACTCGAATTTTG
SOD	TGTGTCCATTGAAGATCGTGTG	TCCCAGCATTTCCAGTCTTTG
Nrf2	AGTGGCAAGATCTCATGTCC	TTGATGGGAGTGTTGGGTG
HO-1	ACAGAGGAACACAAAGACCAG	GTGTCTGGGATGAGCTAGTG
BCL-2	GGACTTGAAGTGCCATTGGTA	GTTATCATACCCTGTTCTCCCG
GAPDH	TTCACCACCATGGAGAAGGC	TGATGGCATGGACTGTGGTC

Primer sequences of tumor necrosis factor alpha (TNF-α),superoxide dismutase (SOD),nuclear factor erythroid 2–related factor 2 (NRF-2), heme oxygenase -1 (HO-1),Bcl-2

Table 2: Effect of EPE treatmenton liver enzymes and oxidative stress markers

	Control	EPE	DOX	DOX+EPE
ALT (IU/L)	21.72 ± 7.22	18.61 ± 5.49	87.51 ± 9.81^{a,b}	52.07 ± 5.86^{a,b,c}
AST (IU/L)	29.38 ± 6.34	29.13 ± 8.41	93.35 ± 17.79^{a,b}	56.52 ± 9.95^{a,b,c}
MDA (nmol/mg)	6.6 ± 0.66	6.978 ± 0.48	14.99 ± 1.71^{a,b}	11.53 ± 0.94^{a,b,c}
SOD (U/mg)	6.604 ± 0.66	6.620 ± 0.68	1.898 ± 0.26^{a,b}	3.875 ± 0.57^{a,b,c}
CAT (U/mg)	0.22 ± 0.07	0.19 ± 0.08	0.048 ± 0.015^{a,b}	0.19 ± 0.04^{c}

Values are represented as mean ± SD (n=10)
a denote a statistically significant difference at ($P < 0.05$) compared to control group
b denote a statistically significant difference at ($P < 0.05$) compared to EPE group
c denote a statistically significant difference at ($P < 0.05$) compared to DOX group

3.2 Effect of EPE and DOX on the oxidative stress markers of liver:

Analysis of SOD, HO-1 and NRF-2 gene expression showed that EPE group showed a non-significant ($p \geq 0.05$) change in the expression of NRF-2, HO-1 and SOD genes compared to the control group. While in DOX group, rats showed a significant ($p \leq 0.05$) decrease in all genes compared to the control group. On the other hand, EPE treated DOX group showed significant ($p \leq 0.05$) increase in the level of these genes **(Fig.1A-C)**.

Also our results showed that EPE group showed a non-significant ($p \geq 0.05$) change in the values of MDA, SOD and CAT compared to the control group. Remarkably, DOX caused a significant ($p \leq 0.05$) increase in MDA and a significant ($p \leq 0.05$) decrease in SOD and CAT as compared to the control group **(Table 2).** While EPE treatment reversed these changes. All these findings reveal the potential for the antioxidant action of EPE.

3.3 Effect of EPE and DOX on inflammatory markers:

EPE group showed a non-significant ($p \geq 0.05$) change in the values of IL-1β, TNFα and IL-6 compared to the control group. Injection of DOX showed a significant increase ($p \leq 0.05$) in the values of IL-1β, TNFα and IL-6 compared to the control group. While in EPE treated DOX group, rats showed a significant ($p \leq 0.05$) decrease as compared with DOX group. **(Fig. 2A, B, C).**

Profiling of gene expression for TNF-α showed non-significant ($p \geq 0.05$) change in both control group and EPE group. While DOX group showed a significant increase ($p \leq 0.05$) in TNF-α gene expression compared to the control group. While EPE treated DOX group caused a significant ($p \leq 0.05$) decrease compared with DOX group **(Fig. 2D).**

3.4 Effect of EPE on apoptosis

DOX group showed significant increase ($p \leq 0.05$) of caspae-3 level as compared to control group. While EPE treated DOX groupshowed significant decrease ($p \leq 0.05$) of caspase-3 levels as compared DOX treatment. On the other hand, DOX group showed significant decrease ($p \leq 0.05$) in BCL-2 gene expression as compared to control group. While EPE treated DOX group showed an opposite effect as it caused significant increase ($p \leq 0.05$) in BCL-2 expression as compared to DOX group**(Fig. 3A-B).** These findings suggest that EPE may reduce DOX induced apoptosis in the liver.

3.5 Histopathological evaluation:

3.5.1 Hematoxylin & Eosin (H&E):

H&E stainedsections of rat's liver tissue of the control group showed normal liver cells with apparent nuclei, liver sinusoids were seen between liver cell cords and normal central vein was seen **(Figs.4 a&b).** In group II (EPE group): H&E stainedsections of rat's liver tissueshowed a picture the same as the control group **(Fig.4 c&d).** In group III (**DOX group)**: H&E stainedsections of rat's liver tissue demonstrated extensive swelling and haemorrhage in the central vein with prominent cellular infiltration (CI) and hepatocytes show apoptotic changes in the form of vacuolations and apoptotic nuclei (**Fig.4 e&f).**H&E stained-sections of **Group IV (EPE+DOX group)** illustrated return of normal liver architecture with a normal appearance of central vein, liver cells and sinusoids (**Fig.4g&h).**

3.5.2 Results of expression of Bcl-2 in liver tissue evaluated by immunohistochemical technique:

Light microscopic examination of sections in liver cells of the control group stained with Bcl- 2 immunostain showed positive cytoplasmic Bcl-2 immunoreactivity which appeared as cytoplasmic brown colouration**(Fig. 5a).** Group II (EPE group): showed positive cytoplasmic Bcl2 immunoreactivity similar to control group **(Fig.5b).** Group III (**DOX group)**: showed decreased cytoplasmic Bcl2 immunoreactivity than control group **(Fig. 5c),** while group IV (**EPE+DOX group)** showed increased cytoplasmic Bcl2 immunoreactivity than group III **(Fig.5d).**

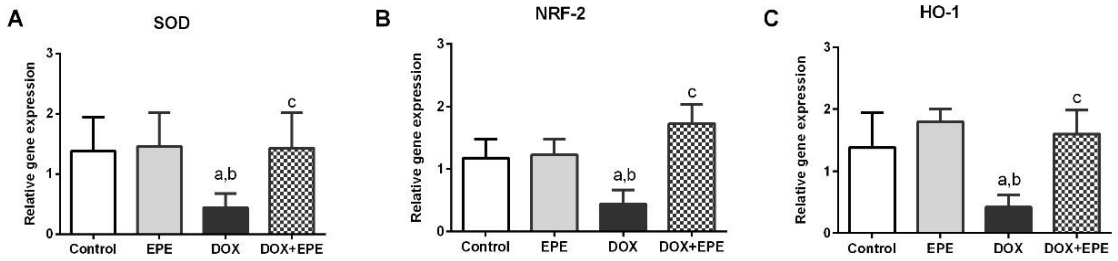

Figure 1: Effect of EPE treatmenton the oxidative stress markers

A) SOD relative gene expression in hepatic tissue B)NRF-2 relative gene expression in hepatic tissue C)HO-1 relative gene expression in hepatic tissue

Values are represented as mean ± SD (n=10).P was considered significant at < 0.05
a denote a statistically significant difference at (P < 0.05) compared to control group
b denote a statistically significant difference at (P < 0.05) compared to EPE group
c denote a statistically significant difference at (P < 0.05) compared to DOX group

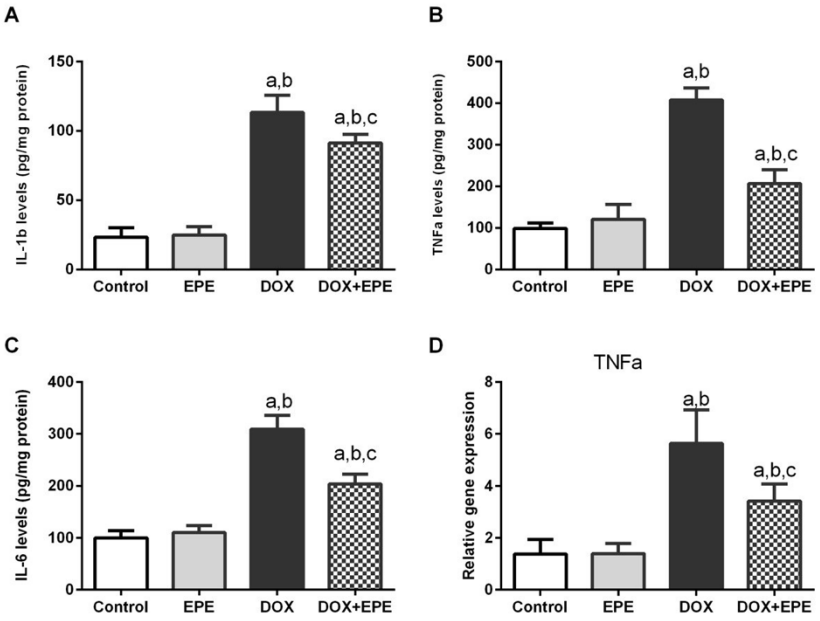

Fig 2. Effect of EPE treatmenton inflammatory markers

A)IL-1b level in hepatic tissue B)TNF-α level in hepatic tissue C)IL-6 levelin hepatic tissue D)TNF-αrelative gene expression

Values are represented as mean ± SD (n=10)
a denote a statistically significant difference at (P < 0.05) compared to control group
b denote a statistically significant difference at (P < 0.05) compared to EPE group
c denote a statistically significant difference at (P < 0.05) compared to DOX group

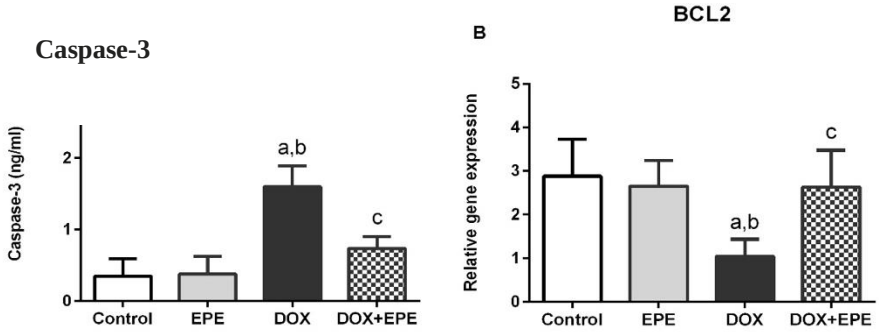

Fig 3. Effect of EPE treatmenton apoptotic markers

A) Caspase-3 level in hepatic tissue B)Bcl-2relative gene expression

Values are represented as mean ± SD (n=10)
a denote a statistically significant difference at (P < 0.05) compared to control group
b denote a statistically significant difference at (P < 0.05) compared to EPE group
c denote a statistically significant difference at (P < 0.05) compared to DOX group

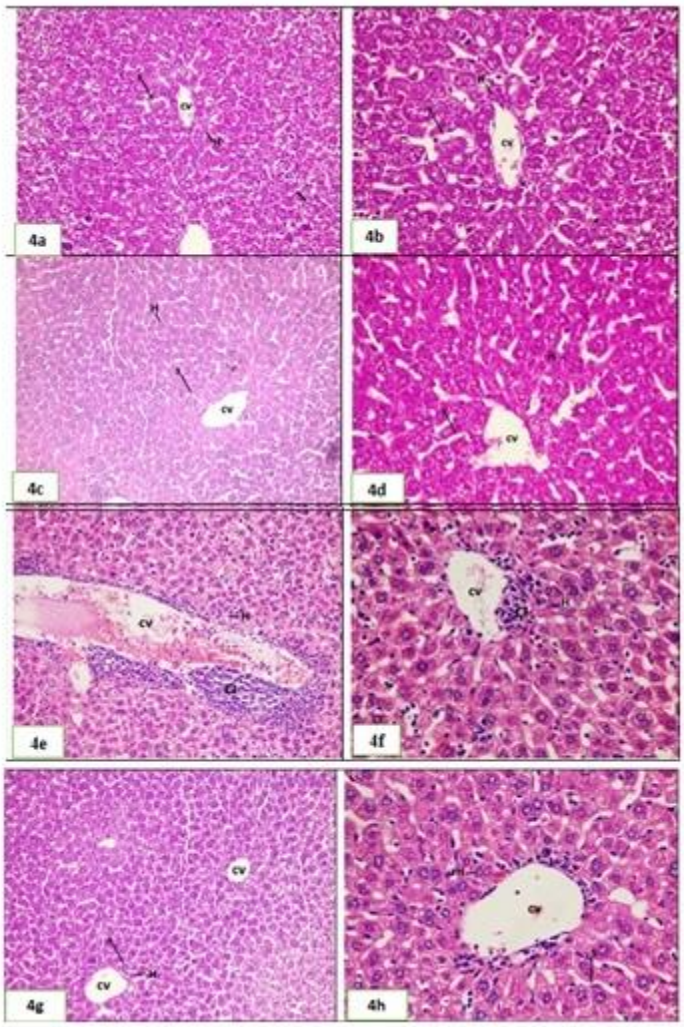

Fig. 4: figures 4a,c,e and g: magnification X200. Figures 4b,d,f and h: magnification x400. (a&b) are photomicrographs of the **control group** show normal histological picture of the liver with a normal central vein (cv). Hepatocytes (H) appear normal with prominent nuclei and sinusoids (s) can be seen between hepatocyte cords. **(c&d)** (**EPE group):**show pictures similar to the control group. **(e&f) (DOX group)**: demonstrate extensive swelling and haemorrhage in the central vein with prominent cellular infiltration (CI) and hepatocytes show apoptotic changes in the form of vacuolations and apoptotic nuclei. **(g&h) (EPE+DOX group)**: illustrate the return of normal liver architecture with normal appearance of central vein, liver cells and sinusoids.

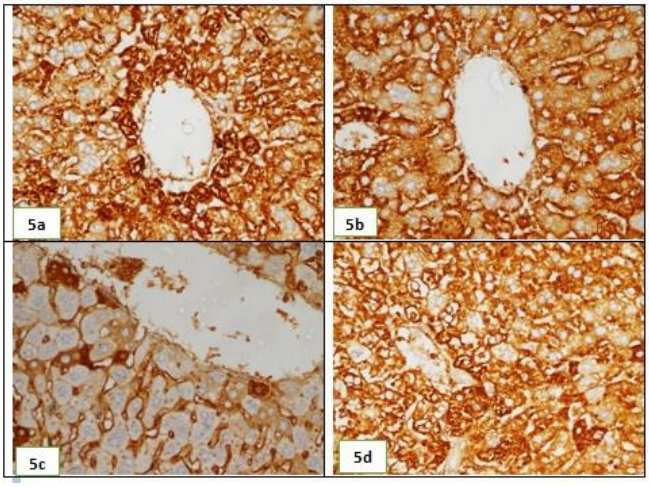

Fig.5: magnification X400:(a) is a photomicrograph showing positive Bcl2 expression (brown colouration) in the control group. **(b)** (**EPE group):** shows Bcl2 expression similar to the control group. **(c) (DOX group):** demonstrates less Bcl2 expression than the control group. **(d) (EPE+DOX group)** Bcl2 expression is increased in comparison with the **DOX group**.

4. Discussion

DOX is one of the most commonly used drugsin clinical practice for the treatment of a wide range of tumors **(25)**. Although it has been shown to cause many side effects in normal organs. Many studies have reported multi-organ involvement with the use of DOX **(26).**

The harmful effects of DOX on the liver were demonstrated in the current study both histologically and biochemically. Much work has gone into understanding the mechanisms underlying DOX toxicity and developing treatments, including antioxidants, that lessen these negative reactions. The purpose of this study was to evaluate the potential hepatoprotective effects of EPE in the face of DOX toxic damage. In addition to histological and immunohistochemical results in the rat model, these effects were validated based on a number of biochemical markers exploring their mechanisms.

Serum transaminases are used as an indicator of hepatic injury **(27)**. Hepatic impairment changes cell membrane transporting mechanisms leading to the release of such enzymes in the serum causing their elevation **(7).** Our results have revealed the hepatotoxic effect induced by DOX proved by a significant increase in serum ALT, and AST. This has been previously reported in animal models after DOX treatment **(3).** Since that hepatocytes are the primary target of reactive oxygen species damage, an increase in apoptotic processes in liver tissue may be the cause of the rise in these liver function indicators **(28).**Administration of EPE has caused significant reduction of ALT and AST indicating it may have considerable hepatoprotective effect**.** This may be due to the antioxidant activity of phenolics in EPE as stated before**(15).**

The present data showed the rats treated with DOX had increased levels of MDA and decreased levels of SOD and CAT as compared to the control group. Interestingly all these parameters were improved with administration of EPE showing its protective effect and antioxidant effect. This suggests that DOX can form free radicals following oxidative damage to biological molecules and lipid peroxidation in the membrane **(29).** Additionally, research has shown that administering DOX causes an increase in MDA content **(30, 31).**

Nrf2, transcription factor, is a target player in regulating the endogenous antioxidant defence. Activation of Nrf2 leads to its entrance to the nucleus, followed by binding with a specific antioxidant element, and this results in the transcription of a specific cytoprotective protein that restores the redox balance. Pharmacological agents can protect animals from oxidative damage by restoring the Nrf2 activity **(32).** It was stated that Nrf2 can regulate many antioxidant proteins such as SOD, HO-1, and CAT **(33)**. HO-1 is a key heme-degrading enzyme that shares in keeping homeostasis.

Previous research revealed that administration of DOX cause a reduction of NRF-2, HO-1 and SOD and this coincides with our results which show significant decrease in these antioxidative parameters **(34)**. Remarkably, the administration of EPE restored those parameters.

According to our findings, rats given DOX had higher levels of the inflammatory markers TNFα, IL-6, and IL-1β than the control group. This highlights a crucial inflammatory rule in the

pathophysiology of DOX-induced hepatotoxicity. Inflammation and oxidative stress play a crucial part in the pathophysiology of DOX-induced multi-organ toxicities. It has been demonstrated that DOX-induced oxidative stress may increase the production of pro-inflammatory cytokines and the release of additional inflammatory mediators (NF-κB), leading to a variety of pathological changes **(35).**

Administration of EPE was shown to cause a reduction of levels of inflammatory markers as compared to the DOX group showing that EPE potentially has anti-inflammatory properties**.** In harmony with the present results, a preceding work showed that EPE anti-inflammatory effects were observed via reducing IL-1β expression along with COX-2, and IL-6 expression ROS and NO production **(36).** It has been shown that propolis could push macrophages towards the M2 anti-inflammatory type **(37).**

The activation of the cysteine protease caspase family results in the production of a deleterious signal for cell death. The last executor of apoptosis is thought to be caspase-3 **(38).**Conversely, BCL-2 is a necessary cell survival factor for all cells. It stops caspase activation by preserving mitochondrial integrity and blocking apoptotic pathways**(39).**

In the current study, we found that DOX has caused significant elevation of caspase-3. On the other hand, it has caused a significant decrease of antiapoptotic Bcl-2 but EPE was shown to cause the opposite effect on these parameters.

Previous researches proved that DOX could affect the apoptotic pathways. These observations are in accordance with previous findings **(40).** It is believed that oxidative stress could be the reason for changes in apoptotic cascades **(41).** Damage to the mitochondrial membrane has been critically linked to episodes of organ toxicity. These changes in the apoptotic pathways could be due to ROS excess and how it could stimulate intrinsic pathways for apoptosis triggering the release of caspases **(42).** Similar protective effects against DOX-induced hepatoxicity were further confirmed by histopathological and immunohistochemical results.

5. Conclusions

These results raised the idea that EPE might function as an adjuvant therapy, protecting the liver from DOX-related oxidative and apoptotic effects and averting their negative effects. More research is required to confirm EPE 's efficaciousness in treating DOX-induced multiple toxicities or to develop innovative drug delivery techniques.

6. Declarations and statements

Ethics approval and consent to participate:

We conducted the study protocol according to The Local Committee of Research and Medical Ethics of the Faculty of Medicine, Tanta University.

Availability of data and material:

The corresponding author can provide the datasets used and/or analyzed during the current work upon request.

Competing interests:

The authors declare to have no conflicts of interest.

Funding:

The authors received no specific funding for this work.

Authors' contributions:

All authors contributed to the data analysis and interpretation of the data, drafted, and revised the

manuscript, and approved the final version of the manuscript.

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