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Evaluating the potential protective effect of virgin coconut oil against doxorubicin-mediated hepatotoxicity in rats

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ABSTRACT

Doxorubicin (DOX) is a chemotherapeutic drug with a broad spectrum of antineoplastic effects against several malignancies. Nonetheless, its clinical utility is limited due to its dose-dependent chronic cardiotoxicity, myelosuppression, and hepatotoxicity. Virgin coconut oil (VCO) consumption is associated with numerous biological and pharmaceutical impacts. The goal of the current study was to assess the protective effects of VCO against doxorubicin-induced hepatotoxicity. Four groups of rats were assigned: control, DOX (15 mg/kg, single dose intraperitoneally), VCO (10 ml/kg, orally for 7 days), and VCO+DOX. Rats given DOX experienced significant rises in ALT and AST serum levels as well as a decrease in albumin levels. Additionally, a significant elevation in MDA level was accompanied by a significant reduction in GSH, CAT, and SOD in the liver tissue following DOX exposure. Moreover, the levels of pro-inflammatory (NF-κB, TNF-α, and IL-6) and pro-apoptotic (caspase-3) markers were significantly increased, while the anti-apoptotic marker (Bcl-2) was significantly decreased. On the other hand, the pretreatment with VCO significantly reduced the level of MDA, caspase-3, and liver function markers and enhanced the antioxidative molecule in response to DOX injection. However, VCO supplementation failed to inhibit the inflammatory response in the liver tissue following DOX exposure which may be due to its high saturated fatty acids content.

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1. Introduction

Doxorubicin has a broad spectrum of actions and is a powerful anticancer agent. It is used to treat a variety of cancers, including solid and hematological cancers [1]. Its application is associated with hepatotoxicity [2], nephrotoxicity [3], cardiotoxicity [4], and testicular impairments [5]. DOX is mainly metabolized in the liver. Numerous studies recorded different grades of hepatic injuries [6]. It is still unclear how DOX causes cell damage. Nonetheless, it is believed that free radical generation, lipid disorders, and mitochondrial dysfunction may all be important contributors [7]. Oxidative stress plays a critical role in the development of DOX-induced hepatic damage [8]. DOX with anthraquinone structure is converted into an unstable semiquinone intermediate metabolite which can enhance the formation of reactive oxygen species (ROS) through deactivating endogenous antioxidants such as reduced glutathione (GSH) and superoxide dismutase (SOD), resulting in oxidative insults [9-10]. In addition, DOX-derived ROS can directly or indirectly stimulate apoptotic events (extrinsic or intrinsic pathways), both of which can promote the release of cytochrome C and stimulate caspase-3 activity that enhances cell loss [11-12]. Moreover, ROS activates immune cells and the overproduction of pro-inflammatory mediators leading to hepatic damage following DOX exposure [3].

Natural resources and traditional remedies that are accessible and affordable across cultures are becoming more popular. Organ toxicity caused by DOX is prevented by plant components and natural compounds with antioxidant and anti-inflammatory properties [3,13]. Virgin coconut oil (VCO) is a medicinal and nutritional food in conventional coconut growing areas. It is an unrefined kernel oil extracted from mature and fresh coconut (Cocos nucifera) by natural or mechanical means [14]. Because saturated fatty acids make up more than 90% of VCO, it is considered as a saturated fat [15]. Nowadays, VCO has gained popularity because of its advantageous benefits. Analgesic, anti-inflammatory, and antipyretic effects of VCO have been demonstrated [16]. VCO consumption encourages antithrombotic effects linked to low cholesterol and platelet coagulation inhibition [17]. VCO has been shown to have more antioxidant activity than coconut oil that has been refined [18]. It has also been proven that VCO increases antioxidant activity and inhibits lipid peroxidation [19]. Phytochemical analysis revealed that p-coumaric and ferulic acids are the major powerful phenolics in the VCO [20]. Due to its high flavonoids and phenolic constituents, several biological and pharmaceutical benefits are associated with VCO supplementation such as neuroprotective, cardioprotective, hepatoprotective, and renoprotective [21].

Based on the data presented above, the current investigation aims to study the potential protective impact of VCO against DOX-mediated hepatic injury in rats by examining liver function markers, oxidative stress, and inflammatory and apoptotic indices in liver tissue.

2. Materials and Methods

2.1 Drugs and Chemicals

Doxorubicin (Doxorubicin hydrochloride 2 mg/ml) was purchased from EBEWE pharma, Austria. Whereas, virgin coconut oil was purchased from National Research Centre (NRC, Egypt). All other used chemicals were of analytical grade.

2.2 Determination of fatty acid methyl esters of the virgin coconut oil using Gas-chromatography coupled with mass spectrometry (GC-MS)

Gas-chromatography coupled with mass spectrometry (GC-MS) was used to identify and measure the composition of fatty acids present in virgin coconut oil according to the previously reported method [22]. Table 1 showed the Fatty acid compositions of virgin coconut oil using GS-MS method.

2.3 Laboratory animals and experimental approval

Male Wistar albino rats weighing between 170-200 g at the age of 2 months were purchased from VACSERA (Cairo, Egypt). Rats were housed in clean, tightly regulated laboratory conditions (23°C temperature, 55–58% humidity, and 12-hour light/12-hour dark cycles). Rats were provided with free access to water and standard diet pellets. The protocol for this experiment was approved by the Helwan University Faculty of Science's Institutional Ethics Committee for Laboratory Animal Care (approval number: HU2018/Z/NAA0518-01). This procedure adhered to the National Institute of Health's standards for the use of laboratory animals, 8th edition.

Table 1: Fatty acid compositions of virgin coconut oil obtained by GS-MS method

Fatty acid	**Fatty acid**	**Virgin coconut oil (%)**
Hexanoic acid, methyl ester	**(**Caproic acid)	0.1223
Octanoic acid, methyl ester	(Caprylic acid)	1.6643
Decanoic acid, methyl ester	Capric acid	2.9162
Dodecanoic acid, methyl ester	Lauric acid	56.2903
Tetradecanoic acid	Myristic acid	28.6154
Hexadecanoic acid, methyl ester	Palmitic acid	7.2905
Methyl stearate	Stearic acid	3.1011

2.4 Experimental design

Twenty-four male Wistar albino rats were allowed to acclimatize for two weeks, then, four groups of six rats each were divided randomly:

Control group: Rats were given a normal saline orally for seven days.

DOX group: Rats were intraperitoneally injected with a single dose of DOX (15 mg/kg) on the 5th day [23].

VCO group: Rats administered virgin coconut oil (10 mL/kg, orally) for seven days [24].

VCO+DOX group: Rats administered with VCO and DOX, respectively with the same doses foe seven days.

Animals of all groups were sacrificed in 8th day.

2.5 Samples collection

Cervical decapitation was used to sacrifice animals of all species. In order to assess liver function markers, blood was collected, allowed to clot in clean, dry test tubes, and then centrifuged at 3000 x g for 10 mins. Yet, the liver tissues were quickly separated into two pieces. For histological analysis, the first portion was maintained in a neutral formalin buffer (10%). The second part was homogenized in a cooled phosphate buffer for biochemical examination (0.1 M, pH 7.4). In a cooling centrifuge set at 4°C, the homogenate was spun at 3000 x g for 10 minutes. After that, the supernatant was stored at -80°C. The Lowry et al. [25] technique was used to measure the total protein level.

2.6 Estimation of liver function markers

Serum alanine transferase (ALT) and aspartate transferase (AST) activities, and albumin levels were quantified colorimetrically using commercial kits obtained by Biodiagnostic (Giza, Egypt) following the protocols described by Reitman and Frankel [26] and Doumas [27], respectively.

2.7 Hepatic oxidant/antioxidant status

To estimate the oxidative stress markers, lipid peroxidation expressed as malondialdehyde (MDA) was measured in liver tissues homogenates following the method reported by Ohkawa et al. [28]. At 405 nm, reduced glutathione (GSH) levels were determined using Ellman's method [29] which detects the yellow-colored 5-thionitrobenzoic acid that is proportional to the GSH level. The activities of superoxide dismutase (SOD) and catalase (CAT) were measured spectrophotometrically in tissue homogenates using the methods described by Nishikimi et al. [30] and Aebi [31], respectively. Bio diagnostic, Giza, Egypt, provided commercial kits.

2.8 Inflammatory parameters in hepatic tissue

According to the manufacturer's instructions (Novus Biologicals, Centennial, CO, USA), ELISA kits used to measure the protein levels of interleukin-6 (IL-6), nuclear factor kappa B (NF-κB), and tumor necrosis factor-alpha (TNF-α).

2.9 Apoptosis parameters in hepatic tissue

The apoptotic proteins including B cell lymphoma2 (Bcl-2), and caspase-3 were determined using ELISA kits purchased from Novus Biologicals (Centennial, CO, USA) based on the manufacturer's guidelines.

2.10 Histopathological architecture assessment

Before being dehydrated and paraffinized, the liver samples were fixed for 24 hours in a 10% neutral formalin buffer. After that, 5-micron-thick blocks were cut and stained with hematoxylin and eosin (H&E). Nikon microscope (E200 LED, Japan) was employed for the inspection. Cellular deterioration, inflammatory cell infiltration, and apoptosis events were investigated in liver sections using a 400x magnification.

2.11 Statistical analysis

The SPSS (version 21) software was used to statistically analyze all the data (Version X, IBM, Armonk, NY, USA). The analysis's findings were presented as means standard deviations (SD). One-way ANOVA with Tukey's multiple comparison tests was used to compare various groups. Statistics indicated a significant difference when the P-value was less than 0.05.

3. Results

3.1 Liver functions after exposure of rats to DOX and/or VCO

As presented in Figure 1, DOX increased significantly ($p<0.05$) the serum levels of ALT and AST activities while the level of albumin was significantly decreased compared to the control group. Interestingly, the pretreated with VCO restored significantly the levels of ALT, and albumin levels near the control value; suggesting its protective role against DOX-induced hepatocellular impairments.

Average values are presented as means ± SD of 6 rats /group. Data were analyzed by one-way ANOVA. a and b point to the significant differences ($p<0.05$) compared to the control and DOX-exposed rats, respectively. c point to the significant differences ($p<0.05$) versus VCO-treated group.

3.2 Oxidative stress in the liver after exposure of rats to DOX and/or VCO

It was evident from this investigation that the liver tissue experienced oxidative stress induced by DOX. The levels of GSH, CAT, and SOD were significantly ($p<0.05$) lower than the control levels, as shown in Figure 2. In addition, after the injection of DOX, the level of MDA in the liver was substantially higher than in the control group. Notably, with VCO pre-administered rats showed improvement in the oxidative status compared to the DOX-treated rats.

The values are presented of means ± SD. One-way ANOVA was applied to analyze the obtained data. ^{a} and ^{b} point to the significant variation ($p<0.05$) compared to the

control and DOX-exposed groups, respectively. c point to the significant variation ($p<0.05$) versus VCO-administered rats.

3.3 Hepatic inflammatory response after exposure of rats to DOX and/or VCO

This investigation exhibited that DOX-induced inflammation in the liver tissue as presented by the elevations ($p<0.05$) in levels of the inflammatory indices (NF-κB, TNF-α, and IL-6) than the control group, as presented in Figure 3. remained unchanged versus DOX-injected animals. Surprisingly, the VCO group showed a significant elevation in the level of NF-κB versus the control group.

ANOVA was applied to analyze the obtained data. a and b point to the significant variation ($p<0.05$) compared to the control and DOX-exposed groups, respectively. c point to the significant variation ($p<0.05$) versus VCO-administered rats.

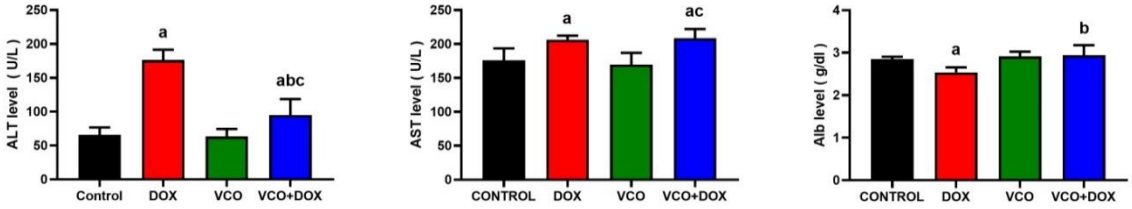

Fig. 1: Blood levels of ALT, AST, and Alb after exposure of rats to DOX and/or VCO.

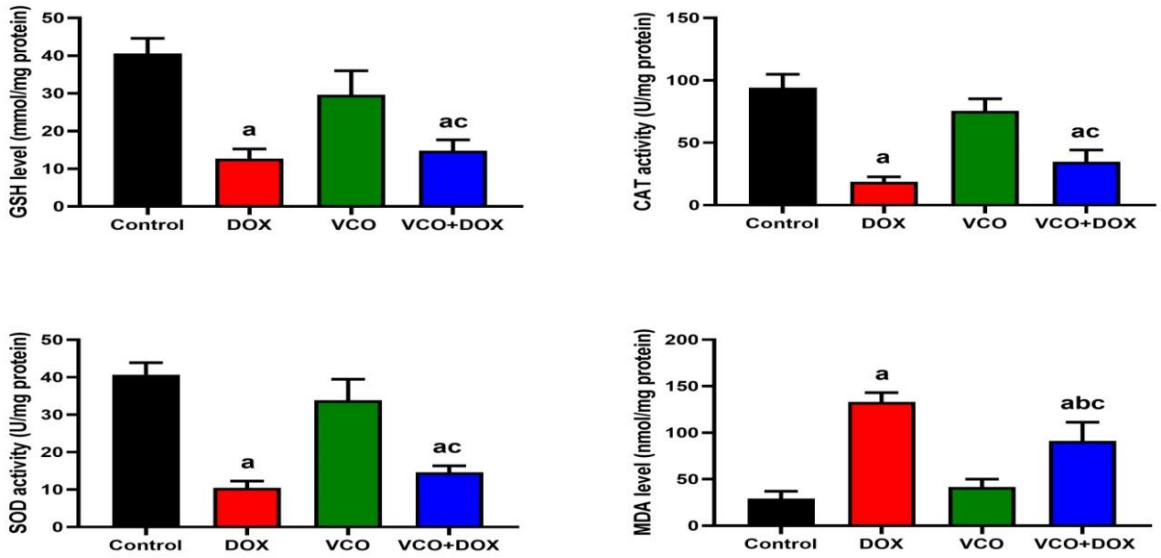

Fig. 2: GSH, CAT, SOD and MDA levels in hepatic tissue after exposure of rats to DOX and/or VCO.

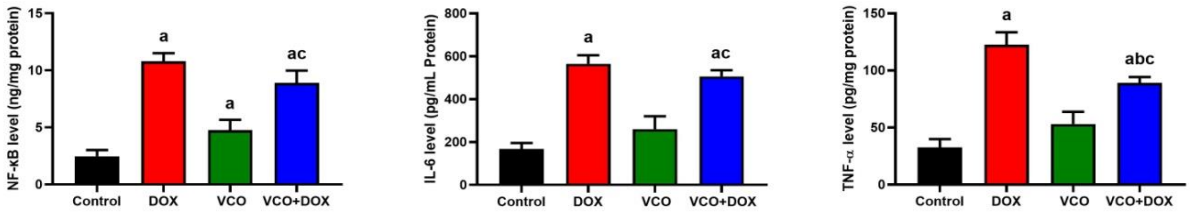

Fig. 3: Inflammatory markers (NF-κB, TNF-α, and IL-6) levels in hepatic tissue after exposure of rats to DOX and/or VCO. The values are presented of means ± SD.

Additionally, the pretreatment with VCO dramatically lowered the level of TNF-α, however, IL-6 and NF-κB

3.4 Hepatic apoptotic response after exposure of rats to DOX and/or VCO

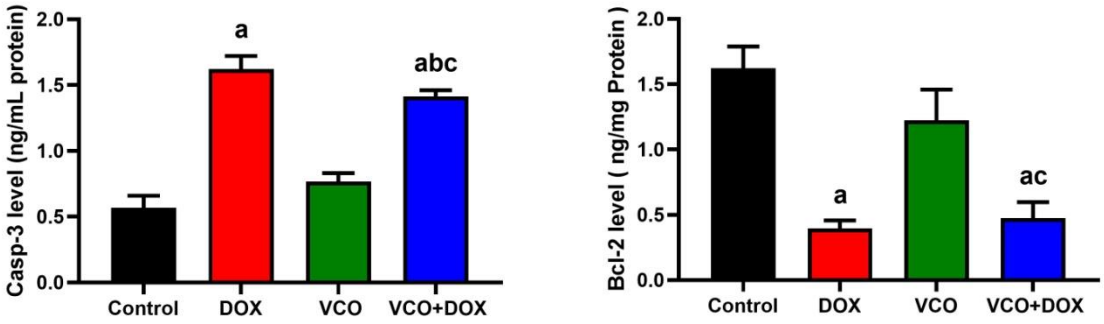

Fig. 4: Figure 4: Bcl-2 and caspase-3 levels in hepatic tissue after exposure of rats to DOX and/or VCO.

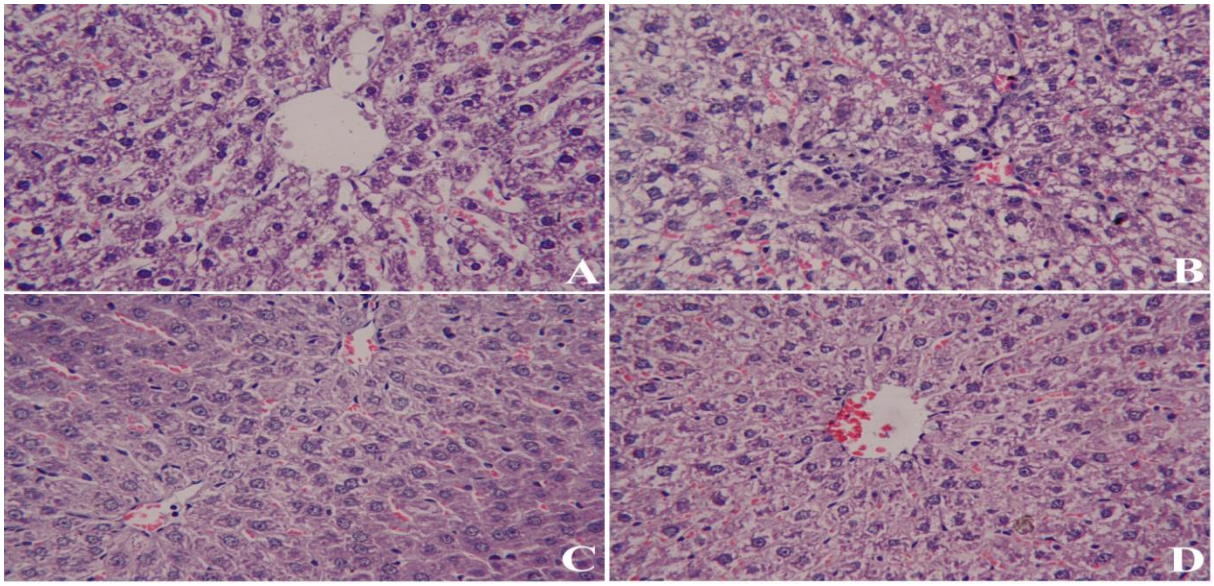

Fig. 5: Histopathological changes in the liver in response to DOX exposure in rats (15 mg/kg) and/or virgin coconut oil (VCO, 10 ml/kg). A: Control, B: Dox, C: VCO, and D: DOX+VCO. Magnification = x400

DOX injection was found to enhance hepatocellular loss as demonstrated by the significant decreases in the Bcl-2 level, and the significant increases in the caspase-3 level against to the control rats, as presented in Figure 4. However, the pretreatment with VCO significantly reduced the DOX-induced elevation in the pro-apoptotic protein, caspase-3 but couldn't significantly increase the DOX impact on the level of Bcl-2.

Average values are presented of means ± SD of 6 rats/group. The values are presented of means ± SD. One-way ANOVA was applied to analyze the obtained data. a and b point to the significant variation ($p<0.05$) compared to the control and DOX-exposed groups, respectively. c point to the significant variation ($p<0.05$) versus VCO-administered rats.

3.5 Histopathological changes in the liver after exposure of rats to DOX and/or VCO

Hepatic tissue from control and VCO-treated rats was histopathologically screened, and the results revealed typical hepatic histoarchitecture with a central vein and neatly organized hepatocytes (Figure 5A and 5C, respectively). Additionally, earlier biochemical findings are confirmed by a histopathological examination of liver sections from rats that had been DOX-intoxicated. Hepatic capsule thickening, hepatocyte cytoplasmic vacuolization, inflammatory cell infiltration, and hepatocyte apoptosis were all visible in liver slices (Figure 5B). It's interesting to note that the hepatic lesions in the DOX-treated rats with VCO were only partly improved, indicating the protective effect of VCO against DOX-mediated injury (Figure 5D).

4. Discussion

Doxorubicin is a broad-spectrum anthracycline antibiotic commonly used with many drugs to treat a variety of tumors, including solid tumors, leukemias, and lymphomas [32]. Due to the DOX's severe organ toxicity, including the nervous system, liver, kidney, heart, testis and lung, its therapeutic application is rather constrained. [33-34]. DOX is converted

in the liver into doxorubicinol metabolite by cytochrome enzymes and cytoplasmic reductase which can cause liver damage [35]. The mechanism responsible for DOX-induced hepatotoxicity is due to the formation of ROS which eventually causes cell death [36-37]. DOX has been demonstrated to disrupt redox homeostasis, which is characterized by elevated ROS, depleted antioxidant defenses, oxidation of DNA and other macromolecules, including lipids, results in liver damage [38-39]. According to recent studies, DOX's hepatotoxicity may be reduced by its combination with a potent natural antioxidant [40-41]. The current investigation aimed to estimate the potential protective role of VCO on hepatotoxicity induced by DOX in adult male albino rats. VCO is known for health benefits related to its high flavonoid and phenolic acids content [21].

Serum ALT and AST levels are used to detect hepatotoxicity, the disturbed hepatocytes membrane causes the leak out of intracellular enzymes into the serum. Therefore, the elevated serum ALT and AST levels indicate liver membrane integrity loss [42]. DOX is an anthracycline antibiotic that acts by altering membrane function, forming free radicals, and intercalating DNA. [43]. The obtained results revealed that DOX (15 mg/kg) increased the serum levels of ALT and AST, and decreased the albumin level. These findings are in line with Wali et al. [34] and Ahmed et al. [44] who explained the increased in serum enzyme activities following DOX exposure to their excess leakage from degenerated hepatocytes membranes as a result of toxicity. According to EL-Maraghy et al. [45].

Serum albumin levels decreases as a result of changes in protein and free amino acid metabolism and synthesis in the injured hepatocytes, as well as increased protein degradation. Meanwhile, the pretreatment with VCO improved ALT and albumin levels, which may be due to improvement of antioxidant levels and suppression of MDA level, which may be attributed to the high flavonoid and phenolic acids content in VCO [21]. AST is not only a hepatic marker but is also used as a myocardial function marker [46]. The Severe cardiotoxicity induced by DOX [46] may be the reason why VCO couldn't improve AST serum level.

Oxidative stress is the primary cause and initiating factor in DOX-induced hepatotic injury [47]. Many studies have shown that DOX can lower levels of naturally occurring antioxidants and raise levels of ROS including superoxide anion and hydrogen peroxide [48]. These overabundant and difficult-to-scavenge free radicals can enhance lipid peroxidation and cell damage [9]. MDA, which is the primary byproduct of lipid peroxidation and is represented as a particular indicator of oxidative damage, while GSH, CAT, and SOD are the essential endogenous antioxidants that protect cells from oxidative damage [38,49-50]. The current study's findings shown that DOX could dramatically raise liver MDA levels while significantly lowering GSH, CAT, and SOD levels in liver tissues. These findings are consistent with those attained by several authors. Sirwi et al. [41] and Ahmed et al. [44] who reported that one of the most convincing explanations for the hepatic damage mediated by DOX administration is that the drug has the capacity to produce excessive amounts of free radicals and lipid peroxides while inhibiting antioxidant defense mechanisms and free radical scavenging ability. Moreover, the pretreatment with VCO led to a considerable decrease in the hepatic concentration of MDA while increasing the hepatic concentration of the antioxidants (GSH, CAT, and SOD). So, it is likely that the phytochemical ingredients found in VCO is responsible for its antioxidant effects [51].

Inflammation, oxidative stress and activation of cell loss are the significant features of DOX-induced hepatotoxicity [52]. Inflammatory response is a physiological response that occurs after an injury or infection, with the goal of boosting tissue regeneration and eliminating irritating agents [53]. Several inflammatory mediators are released in this process, such as cell adhesion molecules, chemokines, and cytokines [54]. To maintain homeostatic balance, a controlled inflammatory response is required. Therefore, excessive or inappropriate inflammation causes a pathological inflammatory status [53]. The obtained results showed the development of inflammatory response in the hepatic tissue following the exposure to DOX as represented by the significantly elevation in levels of IL-6, NF-κB and TNF-α. These findings were consistent with previous research reported by Wali et al. [34] and Sirwi et al. [41] who demonstrated that DOX increased the levels of NF-κB, TNF-α, and IL-6 in the liver tissue, resulting in undesired inflammatory response. In the current study, the pretreatment with VCO significantly decreased the level of TNF-α and couldn't affect the levels of NF-κB, and IL-6. Fail of VCO to inhibit the inflammatory marker IL-6, and NF-κB in the liver tissue may be related to the high content of saturated fatty acids in this oil especially lauric acid. Previous studies have revealed that lauric acid activates pro-inflammatory response by TLR2 and TLR4, thereby mediating cyclooxygenase-2 and NF-κB activation and expression [55]. de Moura e Dias et al. [56] revealed that VCO has been shown to raise the concentration of the pro-inflammatory cytokines IL-1β and IL-12 in the intra-abdominal adipose tissue of rats. Additionally, it promotes the incorporation of saturated fatty acids into the hepatic and adipose tissues. This may explain the elevation of level of NF-κB in rats treated with VCO group compared to the control group.

A hypothesis has been proposed that oxidative stress is a key factor in triggering apoptotic events [57]. The alteration in mitochondrial membrane was shown to be linked with the overproduction of ROS that activates intrinsic events in the apoptotic machinery [58]. As a result of the disturbed mitochondrial membrane, cytochrome c is released into the cytosol and activating caspase-9 leading to cell death [59]. Other enzymes like caspase-3 may be stimulated as a result of this process. Instead, Bax, a pro-apoptotic protein thought to maintain the membrane's porosity, causes the release of cytochrome c, which results in an intrinsic apoptosis-

mediated activity in cells and/or tissues [59]. Bcl-2, on the other hand, is an anti-apoptotic protein that is mostly present in mitochondria's outer membrane. It has the ability to maintain mitochondrial integrity and prevent the release of cytochrome C into the cytoplasm. Consequently, the Bax/Bcl-2 ratio is largely maintained by cellular survival [60]. In the current investigation, rats given DOX had significantly higher levels of the apoptotic marker, caspase-3 and significantly lower levels of the anti-apoptotic marker Bcl-2 in the liver tissue compared to the control group. These findings are supported by previous reports, AlAsmari et al. [38] and Tan et al. [40] who explained the increased apoptosis in DOX-exposed rats to the development of oxidative challenge. In turn, the current experiment exhibited that the pretreatment with VCO could significantly inhibit the activity of caspase-3 in the liver tissue and this may be result of the blocked oxidative stress and the enhancement of antioxidant defense system. The present study showed that the pretreatment with VCO couldn't significantly improve the level of the anti-apoptotic marker Bcl-2 in liver tissue and this may be due to that the inflammatory response following the exposure to DOX affected the Bcl-2 level as it has anti-inflammatory capacity [61].

Conclusions

The obtained findings showed that a single dose of DOX disrupted the level of liver function markers namely, ALT, AST, and albumin. Additionally, DOX impaired the balance between the oxidant (MDA) and antioxidants (GSH, SOD, and CAT) in the hepatic tissue. This was accompanied by the development of inflammatory response by elevating the levels of NF-κB, TNF-α, and IL-6 and stimulated hepatocyte loss by increasing the activity of caspase-3 and decreasing the level of Bcl2, which was confirmed by the histopathological changes. Unexpectedly, VCO supplementation improved partially the biochemical alterations and couldn't restore the hemostasis in the liver tissue, which may be due to its high content of saturated fatty acids. Therefore, recommendations regarding using VCO against DOX-induced hepatotoxicity should be made with caution to avoid its potential side effects. However, further research is mandatory to find out the appropriate safe dose of VCO which can successfully inhibit the hepatotoxicity induced by DOX.

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