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The Protective Effects of the Aqueous Extract of Saliva Against Biochemical and Histopathological Changes in Kidney and Liver of Male Rats Treated with the Anticancer Drug Doxorubicin.

Khalid Al-Syaad[1] and Essam H. Ibrahim*[1,2]

[1]Biology Department, Faculty of Science, King Khalid University, Abha, P.O Box 9004, Saudi Arabia.
[2]Blood Products Quality Control and Research Department, National Organization for Research and Control of Biologicals, Cairo, Egypt.

ABSTRACT

Doxorubicin drug is an anti-cancer substance, but unwanted side effects result from its use. The objective of this research was to study the possibility of whether the Sage plant extract has the ability to reduce the harmful effects of the drug on vital organs like liver and kidneys. Sexually mature male albino rats were divided into four groups (8 each); the control group injected intraperitoneally (i.p.) with a saline solution, DOX group injected i.p. weekly with doxorubicin (Dox) drug (4 mg/kg for 7 weeks); DOX and Salvia group were injected i.p. weekly with Dox (mg/kg for 7 weeks) and swallow daily oral Sage leaf extract (85 mg/kg) for the same period and Salvia group swallow daily Sage leaf extract (85 mg/kg) for 7 weeks. After completion of the experiment animals were dissected and samples of kidneys and livers to prepare textile sections for routine histopathological examination were taken. The histopathological examination in doxorubicin-treated rats revealed some deterioration in both liver and kidneys functions and histopathology. These deteriorations were partially healed by treatments with Salvia extract. In conclusion, the use of Salvia extract with toxic anticancer agent DOX may decrease the bad side effects of that drug.

Key words: Doxurobicin; Salvia; plant extract; liver; Kidneys; histopathology.

Corresponding author

INTRODUCTION

Since the introduction of doxorubicin (DOX) for the treatment of cancer in 1969, this compound has demonstrated high antitumor efficacy. DOX's cytotoxic effect on malignant cells, as well as its toxic effects on various organs is thought to be related to its DNA intercalation and cell membrane lipid binding activities [1]. It has been suggested, that DOX-induced apoptosis may be an integral component of the cellular mechanism of action responsible for its therapeutic effects, toxicities, or both [2, 3]. DOX's use in chemotherapy has been limited largely due to its diverse toxicities, including cardiac, renal, pulmonary, hematological and testicular toxicity. DOX-induced changes in the kidneys of rats include increased glomerular capillary permeability and glomerular atrophy. Although the exact mechanism of DOX-induced nephrotoxicity remains unknown, it is believed that the toxicity is mediated through free radical formation, iron-dependent oxidative damage of biological macromolecules, and membrane lipid peroxidation [4]. In animal trials, DOX demonstrated nephrotoxic activity and produces chronic progressive glomerular disease. In rats with DOX-induced nephropathy, heavy proteinuria associated with swelling and vacuolation of epithelial cells were reported in short-term experiments. DOX-induced nephrosis provides a well characterized model of progressive renal damage, induced by a uniform challenge at a single point in time. This results in proteinuria and subsequent structural renal damage with a relatively large variability among individual animals. Severe renal damage, extensive glomerular lesions, tubular dilatation, vacuolization of renal glomeruli, protein deposits in tubular lumen, and stromal fibrosis have been observed in long-term studies. These experiments indicated that DOX-induced nephropathy has chronic and self-perpetuating continual effects leading to terminal renal failure. The dose and the duration of DOX required to induce renal diseases vary among investigations. It was demonstrated that a 3 mg/kg dosage of DOX induced renal damage after 6 weeks. On the other hand, nephrotoxicity can be induced by 25 mg/kg of DOX after only 2 days [4-11].

Hepatotoxicity is associated with impaired liver function, caused by exposure to a drug or other factors severely impairing its function [12]. The liver is responsible not only for many crucial functions within the body, but also for biotransformation of drugs, their detoxification and conversion into the forms that can be readily eliminated from the body. Interaction of anthracyclines, e.g. doxorubicin with DNA is considered as the main mechanism of their toxicity, both in cancer and normal cells. Anthracycline drugs are effective inhibitors of the activity of topoisomerase II or I. Doxorubicin is widely applied in chemotherapy, but its use is limited by the high risk of cardiomyopathy and congestive heart failure development [13]. Cytotoxicity of anthracyclines is also associated with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [14]. *In vivo* doxorubicin undergoes reduction to a semiquinone-free radical by microsomal and nuclear enzymes. Molecule of O2 can accept an electron from semiquinone, which results in generation of superoxide anion radical. Doxorubicin can also bind ionic iron (Fe3+). This complex is highly toxic to membrane lipids, proteins and DNA. The drug can also bind to the DNA and uncoil the double-stranded helix with generation of free radicals and DNA damage [15]. Cytosolic fraction of doxorubicin may be converted to doxorubicinol by NADPH-dependent aldo-/keto- or carbonyl reductases. This metabolite inhibits several membrane ATP-ases and isometric contraction of heart muscle [16].

MATERIALS AND METHODS

Animals

All of the procedures involving animals in this study were approved by the institution's animal welfare regulatory committee. Male Albino rats were maintained at King Khalid University animal house on a 14:10-hour light:dark cycle. Control and treated rats were provided with food and water *ad libitum*; there were no differences in food intake. One week after arrival rats were randomly divided into 4 treatment groups, each composed of 8 rats.

Preparation of *Salvia officinalis* extract

Water extract of *Salvia officinalis* was done by soaking 10 g of leaves and stems of the plant in pure boiling water for 30 minutes with continuous stirring. The mixture was filtrated and the clear filtrate was kept in sterile dark containers at 4 °C till use.

Treatment of animals

Adult Albino rat (*Rattus norvegicus*) were divided into four groups, containing 8 rats in each group, first group negative control received normal saline intraperitoneally (i.p.). While the second group were i.p. treated weekly with doxorubicin (Dox; 4 mg/kg) for seven weeks. The third group were weekly i.p. treated with doxorubicin (4 mg/kg) and orally extract of salvia (85 mg/kg) for seven weeks. The fourth group received Salvia extract (85 mg/kg) orally for seven weeks.

Blood collection

Blood samples were collected in plastic syringes and transferred to plastic tubes to get sera and kept at -80°C till use.

Histological studies

The livers and kidneys were collected from all the groups, fixed in 10% formalin in saline, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and mounted in molten paraplast at 58-62 ºC. Five micron sections were obtained, stained with Harris Hematoxylin & Eosin and evaluated for any structural changes under a bright field microscope.

Liver and kidney function tests

Colorimetric determination of ALT, AST, Urea and creatinine activities were determined by kinetic method using commercially available kits according to Talke and Schubert (1965) [17], , Larsen (1972) [18], Bergmeyer *et al*. (1978) [19] following the manufacturer instructions.

Statistical Analysis

The biochemical and weight data recorded were expressed as mean±SD and statistical and correlation analyses were undertaken using the One-way ANOVA followed by a post-hoc LSD (Least Significant Difference) test. A p value < 0.05 was statistically significant. A Statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (SPSS, version 15.0, Chicago, IL, USA).

RESULTS

Preparation of *Salvia officinalis* extract

Preparation of water extract of *Salvia officinalis* was done and the clear filtrate was kept in sterile dark containers at 4 ^{o}C till use.

Effects of Sylivia extract and Dox in animal weights

No animal deaths were observed in the course of the experiments. All animals used in this experiment were weighed before and after treatment as shown in table (1). Animals in control untreated group and Salvia group showed normal growth rate while growth rate was diminished in Dox alone and Dox and Salvia treated groups.

Organ collection

Kidneys and livers of all animals used in this experiment were collected and studied. The weight of these organs in different groups were recorded as shown in table (2). From the table it was shown that there were no significant differences among groups after treatment.

BIOCHEMICAL TESTS

Liver enzymes

The liver enzymes AST and ALT were measured in sera of all animals used in this experiment as shown in table (3). It was shown that the level of ALT was significantly (<0.05) increased in groups treated with Salvia

alone or in combination with Dox while there were no change in AST levels in all groups. There were no significant increase in AST in different treated groups.

Kidney function markers

The kidney function markers, urea and creatinine, were measured in sera of all animals used in this experiment as shown in table (4). It was shown that the level of creatinine in sera of animals treated with Dox was significantly increased. This increase was diminished when the animals were treated with Salvia in addition to Dox. Also treatment of the animal with Dox increased the level of urea, but this increase was not diminished when treating animal with Salvia in addition to Dox.

Histological studies

The livers and kidneys were collected from all the groups, fixed in 10% formalin in saline, stained with Harris Hematoxylin & Eosin and evaluated for any structural changes under a bright field microscope.

Histological studies of the kidney
Control Groups

Histopathological examination of the kidneys revealed that control and Salvia groups had normal histological features. The section indicated a detailed cortical parenchyma and the renal corpuscles appeared as dense rounded structures with the glomerulus surrounded by a narrow Bowman's spaces. (**Fig. 1**).

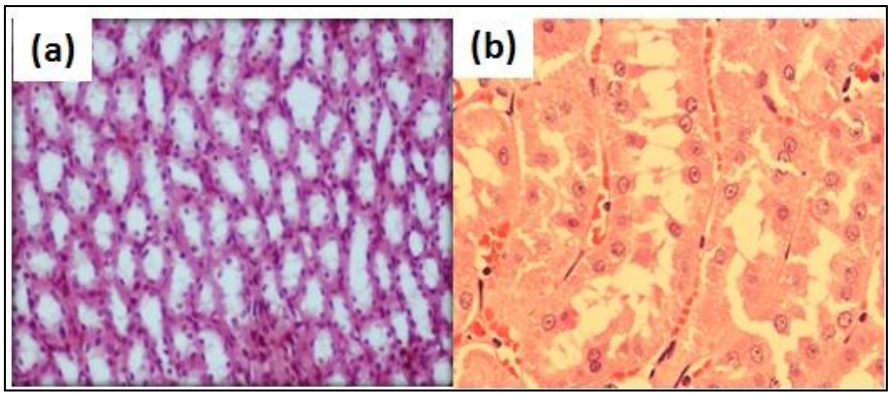

Figure 1: a: Kidney of control group showing normal glomeruli, cortical and medullary tubules. [H&E] Mag. x 400. b: Kidney of Salvia group showing normal glomeruli, cortical and medullary tubules. [H&E] Mag. x 400.

Experimental Groups

The kidney sections of animals in Dox group revealed marked distortion of cyto-architecture of the renal cortical structures, and degenerative and atrophic changes. There were tubular necrosis, interstitial haemorrhage, moderate-severe chronic inflammatory cell infiltrates, vascular hypertrophy, protein casts, cystic dilatation and vacuolations appearing in the stroma. (**Fig. 2**).

The kidneys of the animals in Dox and Salvia group revealed some level of cyto-architectural distortion of the cortical structures, vascular hypertrophy, interstitial oedema, mild chronic inflammatory infiltrates and haemorrhage as compared to the control (**Fig. 3**). Concomitant administration of Salvia with Dox resulted in mild reversal of histopathological damage induced by Dox, with mild regeneration of renal epithelial cells lining of cortical tubules and mild restoration of normal morphology to renal cortex.

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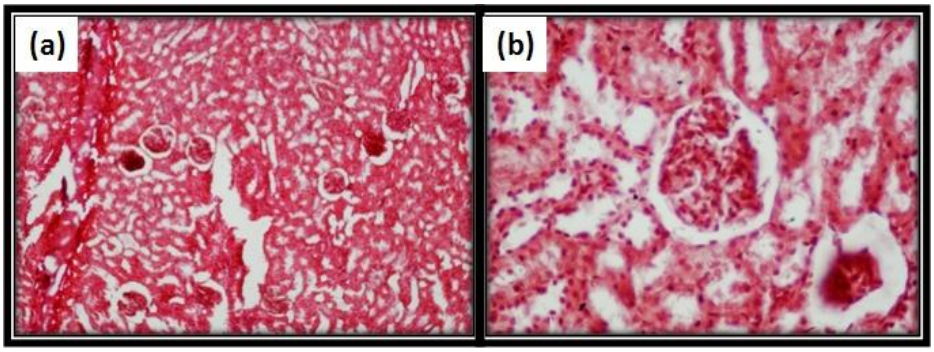

Figure 2: a: Dox Group treated kidney showing tubular necrosis, interstitial haemorrhage and moderate-severe chronic inflammatory cell infiltrates, and vacuolation in the stroma [H&E x200]. b: Dox Group treated kidney showing tubular necrosis, moderate-sever chronic inflammatory cell infiltrates, vacuolations and interstitial fibrosis [H&E x400].

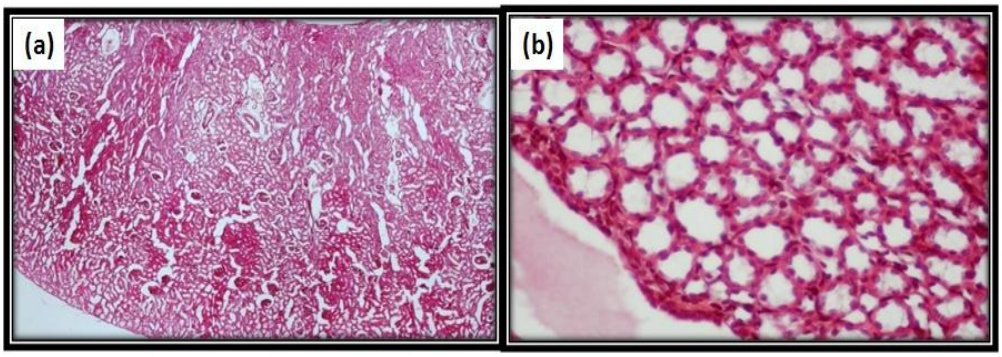

Figure 3: a: Dox and Salvia Group treated kidney showing vascular hypertrophy, interstitial oedema, chronic inflammatory infiltrates and haemorrhage [H&E x 200].
b: Dox and Salvia Group treated kidney showing Tubular necrosis (A), interstitial haemorrhage(B) Mild chronic inflammatory cell infiltrates(C) and Vascular hypertrophy (D) [H&E x 400]

Histological studies of the liver

Control Group

The control sections of the liver showed normal histological features (**Fig. 4**). It is composed of hexagonadal or pentagonadal lobules with central veins and peripheral hepatic triads or tetrads embedded in connective tissue. Hepatocytes are arranged in trabecules running radiantly from the central vein and are separated by sinusoids containing Kupffer cells. They are regular and contain a large spheroidal nucleus with a distinctly marked nucleolus and peripheral chromatin distribution. Some cells have two nuclei each.

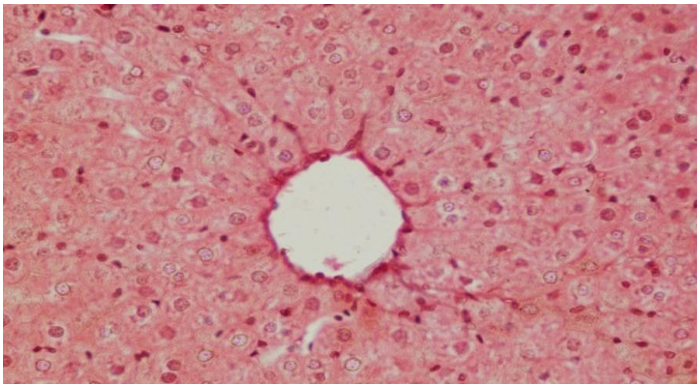

Figure 4: Normal liver showing hexagonadal or pentagonadal lobules with central veins and peripheral hepatic triads or tetrads embedded in connective tissue. [H&E] Mag. x 400.

Experimental Groups

The liver of the animals in Dox and Salvia group revealed some level blurring. Following exposure to Salvia alone, the trabecular structure of the lobules was slightly or distinctly blurred. The cytoplasm of hepatocytes, contained empty vacuole-like spaces, and were enlarged. Some sinusoids were overfilled with erythrocytes and the walls of most sinusoids showed numerous Kupffer cells. Locally, mononuclear cell infiltrates were observed, most frequently in the hepatocytes. In a few animals of this group, an increased density of nuclear chromatin and a very compact nuclear structure were noted. Sporadically, single necrotic cells were evident. After exposure to DOX alone, the trabecular liver structure was more seriously affected than after Salvia administration (**Fig. 5**). DOX-induced degenerative changes were evident in numerous hepatocytes the cells were enlarged and had light and foamy cytoplasm filled with vacuoles. The walls of the sinusoids in both zones showed numerous Kupffer cells. In a few hepatocytes, necrotic changes were evident; a small, pycnotic cellular nucleus with condensed chromatin, lack of nucleolus and strongly acidophilic cytoplasm were observed. Mononuclear cell infiltrates were also noted hepatocytes. In rats co-exposed to DOX and Salvia, the trabecular structure of the lobules was blurred (**Fig. 6**). The cytoplasm of some hepatocytes was light, enlarged and contained vacuoles (less numerous than after DOX alone). Numerous Kupffer cells were found in the sinusoid walls. These changes were observed mainly in the hepatocytes. Mononuclear cell infiltrates were evident.

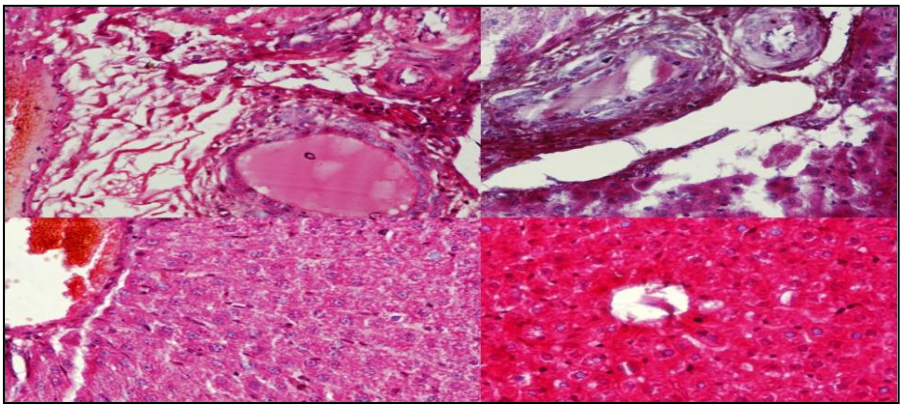

Figure 5: Dox Group treated liver showing enlarged hepatocytes and had light and foamy cytoplasm filled with vacuoles. The walls of the sinusoids showed numerous Kupffer cells. In a few hepatocytes, necrotic changes were evident; a small, pycnotic cellular nucleus with condensed chromatin, lack of nucleolus and strongly acidophilic cytoplasm were observed. Infiltration of mononuclear cell is evident. [H&E x400].

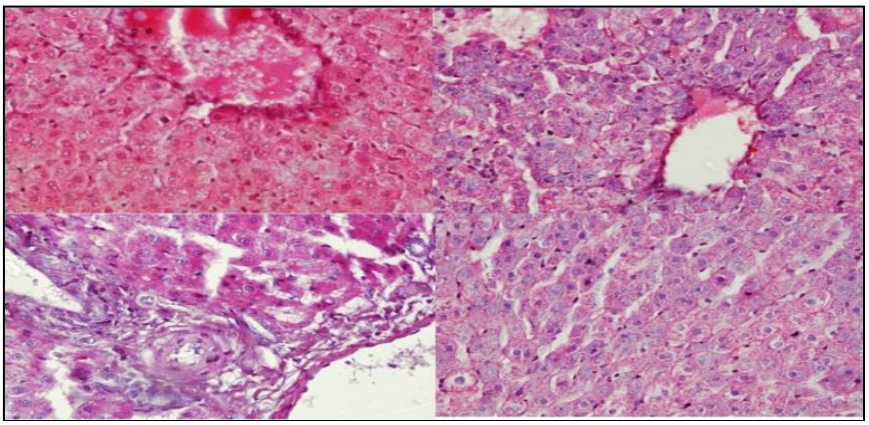

Figure 6: Dox and Salvia treated liver showing the cytoplasm of some hepatocytes as light, enlarged and contained vacuoles (less numerous than after DOX alone). The presence of numerous Kupffer cells in the sinusoid walls and mononuclear cell infiltrates are evident. An increased density of nuclear chromatin and a very compact nuclear structure were noted. [H&E x400].

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Table 1: Animal weights before and after Salvia and Dox treatment.

Group (N=8)	Weight before treatment (g) (Mean ± StDev)	Weight after treatment (g) (Mean ± StDev)
Control	**375.66** ± 28.79153	**382.57** ± 19.33563
DOX	**369.28** ± 33.79842	**335.5**0 ± 41.20968
DOX + Salvia	**349**.23 ± 49.68702	**333.6**0 ± 42.60561
Salvia	**377.71** ± 19.8637	**3**85.44 ± 25.54548

Table 2: Weights of livers and kidneys of animal of different groups after treatment.

Group (N=8)	Weight of liver (g) (Mean ± StDev)	Weight of kidneys (g) (Mean ± StDev)
Control	15.22 ±1.43	4.02±1.28
DOX	12.83±1.36	3.95±0.93
DOX + Salvia	14.74±4.56	4.82±1.03
Salvia	15.95 ± 0.97	5.13 ± 0.28

Table 3: Liver enzyme levels in different animal groups after treat with Salvia and Dox.

Group (N=8)	ALT (U/L) (Mean ± StDev)	AST (U/L) (Mean ± StDev)
Control	69.87 ± 5.44	43.98 ± 14.65
DOX	69.87 ± 5.44	34.98 ± 6.38
DOX + Salvia	102.13 ± 46.61	39.97± 12.68
Salvia	104.08 ± 18.37	37.28 ± 4.98

Table 4: Levels of urea and creatinine in sera of animals after treat with Salvia and Dox.

Group (N=7)	Creatinine (mg/dL) (Mean ± StDev)	Urea (mg/dL) (Mean ± StDev)
Control	0.697±0.23	54.44 ± 8.76
DOX	0.92±0.476	76.20 ± 13.10
DOX + Salvia	0.63 ±0.24	82.99 ± 20.04
Salvia	0.68 ±0.06	52.87± 5.83

DISCUSSION

DOX is a very important agent in the treatment of cancer patients although its use may be complicated by the presence of acute and chronic side effects. Despite the wide use of DOX in the treatment of cancer, its mechanism of action is still not well known and has often been the subject of controversy [3]. Anticancer therapy usually demolishes the physiological homoeostasis and affects multiple organs during treatment process. Effective anticancer therapy with anthracyclines is limited because of its toxicity to various organs including kidneys and liver [20, 21]. The toxicity has been attributed to radical formation and oxidant injury. Nephrotoxic action of DOX is also considered to be via drug-induced free radical generation [22, 23]. One of the mechanisms suggested is free radical formation and oxidative stress [24]. The level of the endogenous antioxidant CoQ10 seems to increase in human plasma after Dox therapy [25]. This is probably through upregulation of CoQ10 gene expression as a cellular defense mechanism against chemotherapy to promote cell survival [26].

The dose of Dox used in this study corresponds to the dose that is currently being used in clinical practice [27]. In the present study, this dose produced acute renal and liver function deteriorations in the animal group receiving it. In the present study treatment of animals with DOX lead to the histopathological and functional bad effects to both liver and kidneys. The formation of free radicals as well as an increase in response to DOX treatment has already been documented. The disturbance in oxidant-antioxidant systems results in tissue injury that is demonstrated with protein oxidation in tissue and protein oxidation in renal tissue, is recognized as one of the possible biochemical mechanisms of DOX-induced nephrotoxicity [28]. DOX has widely been used in many countries for hematological malignancies. However, the toxic effect on the kidneys and consequently acute renal failure producing effect of DOX is a limiting factor of its usefulness.

Therefore, novel therapeutic agents with improved efficacy seem to be considerable for clinical approach. Many antioxidants have been assayed with very different results. Among these molecules, metal ion chelators, like transferrins, metallothionein, desferrioxamine or proteins that oxidize ferrous ions, such as ceruloplasmin, have been widely investigated in relation to DOX.

Salvia has been shown to possess anti-inflammatory, anticancer, and antioxidant properties [29-31]. In recent years dietary plants with antioxidative property have been the center of focus. It is believe that these plants can prevent or protect tissues against damaging effect of free radicals [31, 32]. In addition, it has been shown that dietary supplementation with natural antioxidants such as, vitamins E and flavonoids attenuated the oxidative stress induced by oxidative agents [31, 33-36]. Polyphenolic compounds are widely distributed in plants and known to be excellent antioxidants *in vitro*. They have the capacity to reduce free-radical formation by scavenging free radicals and protecting antioxidant defenses. In the present study Salvia diminished the side effects of DOX both histopathologically and functionally of both liver and kidneys. Previous studies have demonstrated that Salvia exhibits antioxidant properties against oxidant conditions that cause tissue injury and may prevent induced kidney failure and liver fibrosis in rat via antioxidant mechanism [37, 38]. Improvement of Dox-induced nephrotoxicity was previously tried by compounds that partially succeeded in preserving normal renal function and structure probably through their antioxidant effects, as caffeic acid phenethyl ester [10, 39-41], Zingiber officinale Roscoe [42], and Solanum torvum [43]. Effect of Salvia on liver was not so good but in the same time it lowered the bad effects of DOX on liver. Indeed, some antioxidants were reported to possess prooxidant effects at higher doses, as the flavonoids: quercetin, myricetin, kaempferol [44], and curcumin [45] that were found to mediate induction of reactive oxygen species at high concentration.

CONCLUSIONS

In conclusion, the present study demonstrates that injection of DOX at dose of 4 mg/kg for 7 weeks to the male albino rats (Rattus norvegicus) caused renal and liver injuries. Furthermore, this study revealed that treatment with Salvia protected renal and liver tissues against DOX-induced nephrotoxicity hepatotoxicity. Preventive effects of Salvia on these renal lesions may be via its antioxidant and anti-inflammatory action. Although the exact mechanisms remain to be clarified, Salvia could be an effective course of therapy to enhance therapeutic efficacy and to lessen DOX toxicity in clinical chemotherapy.

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