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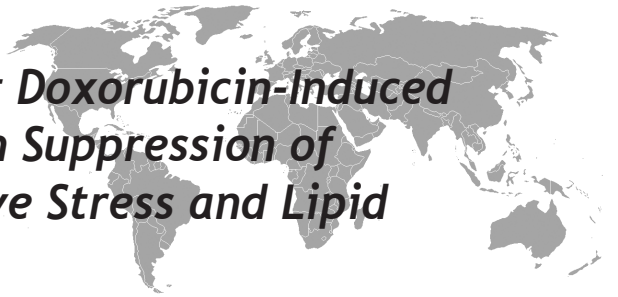
*Original Article*



# *Effect of Urtica Dioica against Doxorubicin-Induced Cardiotoxicity in Rats through Suppression of Histological Damage, Oxidative Stress and Lipid Peroxidation*

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*ABSTRACT*

***Objective:*** *Doxorubicin (DOX) is a highly effective anti-cancer drug with limited clinical use due to its serious cardiotoxicity. Urtica dioica L. seeds (UD), have been widely used in folk medicine, particularly in the therapy for advanced cancer patients, possesses a potent anti-oxidant properties. The goal of present study was to investigate the cardioprotective effects of UD on DOX-induced cardiotoxicity.* ***Method:*** *The rats in the UD treated group were given intraperitoneally 2 ml/kg UD. To induce cardiotoxicity, 30 mg/kg DOX was injected intraperitoneally by a single dose and the rats were sacrificed after 48 h.* ***Results:*** *The present study revealed for the first time a protective role of UD against DOX-induced cardiotoxicity. UD therapy significantly protected against DOX-induced myocardial damage which was characterized by conduction abnormalities, vacuolization, inflammatory cell infiltration, hemorrhages, and myofibrillar disarrangement. As indicators of oxidative stress, DOX caused significantly increase lipid peroxidation and reduction in activities of antioxidant enzymes; superoxide dismutase, glutathione peroxidase, and catalase. UD treatment significantly attenuated DOX-induced oxidative injury.* ***Conclusion:*** *The present study showed that UD might be a suitable cardioprotector against toxic effects of DOX.*

***Key words:*** *Doxorubicin, urtica dioica, cardiotoxicity, oxidative stress, rat*

***Urtica Dioica'nın Sıçanlarda Doksorubisine Bağlı Kardiyotosisitede Histolojik Hasar, Oksidatif Stress ve Lipid Peroksidasyon Bakılayıcı Etkileri***

*ÖZET*

***Amaç:*** *Doksorubisin (DOX) son derece etkili bir anti-kanser ilacı olmasına karşın, klinik kullanımı ciddi kardiyotoksik etkiler nedeniyle sınırlı kalmaktadır. Urtica dioica L. tohumları (UD) geleneksel tıpta özellikle ileri evreli kanser hastalarının tedavisinde yaygın biçimde kullanılagelmiştir ve güçlü anti-oksidan özelliklere sahiptir. Bu çalışma UD'nin DOX'a bağlı gelişen kardiyotoksisite üzerindeki kardiyoprotektif etkilerini araştırmak amacıyla düzenlenmiştir.* ***Yöntem:*** *UD-tedavi grubundaki sıçanlara intraperitoneal yolla 2 ml/kg UD verilmiştir. Kardiyotoksisiteye yol açmak amacıyla, intraperitoneal yolla tek doz olarak 30 mg/kg DOX enjeksiyonu yapılmış ve sıçanlar 48 saat sonra sakrifiye edilmişlerdir.* ***Bulgular:*** *Bu çalışmada UD'nin DOX'a bağlı gelişen kardiyotoksisiteye karşı koruyucu rolü olduğu ilk kez gösterilmiştir. DOX kullanımından kaynaklanan ve ileti bozuklukları, vakuolizasyon, enflamatuvar hücre infiltrasyonu, hemoraji ve miyofibril düzeninde bozukluğa yol açan kardiyotoksisiteye karşı UD tedavisi önemli koruma sağlamıştır. Oksidatif stres göstergelerine bakıldığında, DOX tedavisinin anlamlı düzeyde lipid peroksidasyonu artışına ve antioksidan enzimlerin (süperoksid dismutaz, glutatyon peroksidaz ve kataloz) aktivitelerinde azalmaya neden olduğu görülmüştür. UD tedavisi DOX'a bağlı oksidatif hasarı da anlamlı ölçüde azaltmıştır.* ***Sonuç:*** *Bu çalışma UD'nin DOX'un toksik etkilerine karşı kalbi koruyucu etkilerde bulunabileceğini göstermiştir.*

***Anahtar kelimeler:*** *Doksorubisin, urtica dioica, kardiyotoksite, oksidatif stres, sıçan*

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## INTRODUCTION

Doxorubicin (DOX) is an antibiotic derived from anthracycline and represents the first anti-cancer drug in its class. It is commonly used in the treatment of a number of tumors including leukaemia, lymphoma, soft tissue and bone sarcomas, Wilm's tumor, neuroblastoma, and hepatoblastoma thanks to its wide-spectrum antineoplastic efficacy. The major factor limiting the use of DOX and other anthracyclines in the treatment of cancer is the cardiotoxic adverse effects (1,2).

Although the exact mechanisms responsible for the cardiotoxic effects associated with the use of DOX are unknown, the diversity observed in histopathological effects suggests that different factors may play a role in their development simultaneously. Also, in previous studies oxidative stress emerged as a major factor in the pathogenesis of DOX-related cardiotoxicity, with a particular emphasis on the effect of radical oxygen species (ROS) generated through redox reactions. DOX-related ROS production results in lipid peroxidation, ultimately leading to increased malondialdehyde (MDA) levels. In addition to increased ROS generation, reduced levels of anti-oxidative enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase by DOX have also been implicated in the development of cardiotoxic effects (3,4).

In early stages, DOX is associated with a number of different structural alterations in myocytes that can be identified by electron microscopy only (5). Initial sign of myocyte injury involves the dilation of the sarcoplasmic reticulum, which progresses to vacuolization, followed by membrane thinning, intense formation of inclusion bodies, as well as mitochondrial swelling. Finally, the cellular necrosis is accompanied by nuclear changes. Under light microscopy, vacuolization and degeneration of some myocytes occur in addition to areas of myocardial tissue. Subsequently, fibrosis takes place. The severity of histological changes is proportional to the dose administered (6,7).

Antioxidants serve to neutralize the effects of molecules generated through the processes of oxidative stress. Free radical formation may occur during normal cellular metabolism as well, and the maintenance of the balance between antioxidants and free radicals sustains the viability of the organism, which utilizes a number of enzymes such as SOD, catalase (CAT), and GSH-Px to suppress these reactions (8,9). Although the role of numerous antioxidant molecules in the prevention of DOX-associated cardiotoxicity have been tested previously (10,11), no studies examined the histopathological and biochemical effects of Urtica dioca L. (UD) within the context of DOX-induced cardiac toxicity.

Urtica dioica is a perennial plant with stinging hairs belonging to the plant family Urticaceae growing to a height of 30–100 cm. It is endemic in many parts of Turkey, and the seeds have been widely used in folk medicine, particularly in the therapy for advanced cancer patients, for a long time (12). It has several pharmacological properties such as anti-oxidant, anti-inflammatory, anti-apoptotic, and anti-fibrotic activities (13-15).

In view of that, our study aimed to investigate the protective effect of UD against DOX-induced cardiotoxicity using histological and biochemical parameters.

## MATERIAL AND METHODS

### Animals

Eighteen female Sprague-Dawley rats, weighing 200-220 gr and averaging 8 weeks old were used in our study. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by Namik Kemal University, Local Animal Ethics Committee, and ethical rules were observed during the study (Permission number: 2015/06-09, 07.05.2015).

### Experimental design

A total of 18 female Sprague-Dawley rats were divided into three groups: control, DOX, DOX+UD; each group consisted of six animals. The rats in the UD treated group were given intraperitoneally (i.p.) 2 ml/kg UD. The control group was given the same volume of saline. This application continued daily for a total of 14 days. To induce cardiotoxicity, 30 mg/kg DOX (Carlo Erba, Milan, Italy), was injected i.p. in a single dose and the rats were sacrificed after 48 h. At the end of the experiment, all rats were sacrificed by decapitation under i.p. ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia. The anesthetized rats were sacrificed and the cardiac tissues were removed for histopathological and biochemical investigation. The dose of DOX and UD was selected by previous studies respectively (15,16).

***Table 1**. Cardiomyopathy scores in control, DOX-treated, and DOX-treated with UD groups. (n: 6 for each gruop)*

| | Control | DOX | DOX+UD |
|---|---|---|---|
| *Myofibrillar loss* | *(-)* | *(++) to (+++)* | *(+) to (++)* |
| *Cytoplasmic vacuolization* | *(-)* | *(+) to (++)* | *(-) to (+)* |
| *Myocardial disorganization* | *(-)* | *(+++)* | *(+) to (++)* |
| *Inflammatory cell infiltration* | *(-)* | *(+++)* | *(-) to (+)* |
| *Hemorrhages* | *(-)* | *(+++)* | *(+) to (++)* |

*The scoring system was as follows: (-) no damage, (+) mild, (++) moderate, and (+++) severe damage.*

Histopathological Examination

The cardiac tissues were individually immersed in Bouin's solution, dehydrated in alcohol and embedded in paraffin. Sections of 5 µm were obtained, deparaffinized and stained with hematoxylin and eosin (H and E). The cardiac tissue was examined, evaluated and photographed in random order under blind conditions with standard light microscopy (Olympus CX41 microscope (Olympus, Japan)). The severity of changes was quantitative as none (-) to severe (+++) based on the degree of myofibrillar loss, cytoplasmic vacuolization, myocardial disorganization, inflammatory cell infiltration, and hemorrhages as according to modified Alpsoy et al.'s method (17). The scoring system was as follows: (-) no damage, (+) mild, (++) moderate, and (+++) severe damage.

Biochemical Analysis

Heart tissue samples were cut into small pieces and homogenized in 2 mL Tris-HCl buffer (pH 7.4) for 3 min at 16,000 rpm by a homogenizer (yellow line DI25 digital; IKA, Burladingen, Germany). Protein measurements were made at all stages according to the Lowry's method (18). The homogenates were used to measure levels of MDA level (nmol/mg protein) (19). The homogenates were then centrifuged at 5000×g for 60 min to remove debris. Clear upper supernatant fluid was taken and assayed for SOD (U/mg protein) (20), CAT (U/mg protein) (21), and GSH-Px (U/g protein) (22) activities.

***Statistical Analysis***

All statistical analyses were carried out using SPSS statistical software (S0064 Minitab Release 13, License number: WCP1331.00197). All data were presented in mean ± SD. Differences in the measured parameters among the four groups were analyzed with a nonparametric test (Kruskal–Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney U-test. These differences were considered significant when probability was less than 0.05.

## RESULTS

Histopathological findings

Cardiotoxicity induced by DOX was further evaluated using the H&E staining followed by light microscopy. Cardiac tissues from control rats showed normal myocardium architecture (Figure 1a). Degenerative changes were apparent for rats in the DOX group including myofibrillar loss, cytoplasmic vacuolization, inflammatory cell infiltration, and hemorrhages (Figure 1b). On the other hand, UD treatment significantly reduced the DOX-induced histopathological changes in the cardiac tissue and maintained the normal histological appearance of myocardium (Figure 1c and Table 1).

Biochemical findings

The MDA levels of cardiac tissue significantly increased in the DOX groups rats that were administered DOX. However, this elevation was significantly suppressed when UD therapy followed DOX administration. Additionally, treatment with UD significantly attenuated the depletion of reduced SOD, GSH-Px, and CAT activity in cardiac tissue resulted from DOX administration (Table 2).

## DISCUSSION

Doxorubicin is an anti-tumoral agent with non-specific effects on the cell cycle. Although it is effective in a wide range of neoplasias, cardiotoxic side effects represent a major limitation against its use. A cumulative DOX dose of 550 mg/m2 has been shown to be associated with acute cardiotoxicity in humans. These toxic side effects are largely accounted for by the free oxygen radicals.

***Table 2.*** *Tissue MDA (nmol/mg protein), SOD (U/mg protein), CAT (U/mg protein), and GSH-Px (U/g protein) levels in all groups (n=6) (n: 6 for each gruop)*

| | Control | DOX | DOX+UD |
|---|---|---|---|
| MDA | 62.21±6.13 | 93.71±8.89[a] | 75.13±7.22[b] |
| SOD | 5.13±0.72 | 3.29±0.29[a] | 4.48±0.56[b] |
| CAT | 7.42±1.46 | 4.23±1.01[a] | 6.55±1.77[b] |
| GSH-Px | 14.42±2.61 | 4.32±1.06[a] | 9.67±1.95[b] |

*Kruskal-Wallis test was used for statistical analysis. Values are expressed as means ± SD, n=6 for each group. [a]P<0.001 compared with control group. [b]P<0.01 compared with DOX group.*

Although the exact pathogenesis of DOX-induced cardiotoxic effects remains unknown, accumulation of ROS has been demonstrated to lead to cellular injury in the absence of scavenger activity (23-25). Free radicals implicated in the pathogenesis include superoxide, hydroxyl radicals, and nitric oxide. DOX not only leads to increased generation of free oxygen radicals, but also reduces the levels of antioxidant enzymes such as GSH-Px, GSH, and CAD, leading to toxicity (26). A primary characteristic of these enzymes is their protective effect on the membrane lipids against peroxidation, through the inhibition of peroxidation chain reactions and scavenging activity on ROS (27). Most studies on the prevention of DOX-induced cardiotoxicity are experimental in nature, using a number of mice, rat, rabbit, swine, or dog models with clinical and morphological characteristics of DOX-induced cardiotoxicity that are similar to those observed in humans (28). Thus, in our study we studied the cardioprotective effects of UD with known anti-oxidant and anti-apoptotic properties on cardiac histological injury, lipid peroxidation, and the anti-oxidant system after a single 30 mg/kg dose of DOX in rats.

In studies examining the toxic effects of DOX on the cardiac muscle, the morphological findings detected by the light microscopy include hypertrophy of the myocardial fibrils, edema, vacuolization, interstitial edema, and hemorrhage (17). In many previous studies, DOX was associated with injury and loss of myofibrils. The anti-tumoral activity of DOX is known to be associated with the inhibition of protein and nucleic acid synthesis. Examination of the effects occurring after treatment with DOX demonstrated atrophy, degeneration, cytoplasmic vacuolization, and irregularity of the myofibrils in cardiomyocytes (29). In the study by Zhang et al. severe hyperemia and hemorrhage in the cardiac tissue as well as pyknotic nuclei and eosinophilic cytoplasm have been found after a single dose DOX injection (30). Again in another study, varying doses of DOX (30 mg/kg, 15 mg/kg, 10 mg/kg) were found to induce a number of pathological changes in the cardiac tissue (31,32). Consistent with the literature, similar types of changes have been observed after DOX injection. On the other hand, we have histologically shown that UD administered at a dose of 2 ml/kg can effectively prevent the DOX-induced injury.

In previous studies, no correlation between plasma antioxidant enzyme activity and tissue enzyme activity was observed. This discrepancy was found to arise from the variation between organs in terms of the antioxidant capacity. Thus, tissue enzyme activity was proposed to provide a more reliable assay for studies measuring antioxidant enzyme activity (33). Using the same approach, some authors studied the tissue anti-oxidant and lipid peroxidation capacity (34,35). By planning almost the same experimental setup, we decided to examine the cardiac tissue levels (but not plasma levels) of antioxidant enzymes, i.e. SOD, GSH-Px, and CAT, in addition to MDA, a marker of lipid peroxidation.

As in the pathogenesis of many other conditions, ROS has been implicated in the development of DOX-induced cardiotoxicty (36). Under physiological conditions, the effect of ROS generated through a number of processes is counterbalanced by the effect of antioxidant defense mechanisms. A change in this balance in favour of ROS results in oxidative stress and tissue injury, which is initiated by the formation of lipid radicals in the cellular membrane and followed by the formation of lipid hydroperoxides, and finally by the formation of toxic products such as aldehyde, alkenes, and MDA (37). In other words, MDA is an end-product associated with tissue injury. Many previous studies have implicated elevated cardiac tissue MDA levels in the pathophysiology of DOX-associated cardiotoxicity (17,31,32). Also, several antioxidant molecules have been shown to prevent DOX cardiotoxicity by reducing tissue MDA levels (38,39). In our study, elevated MDA lev-

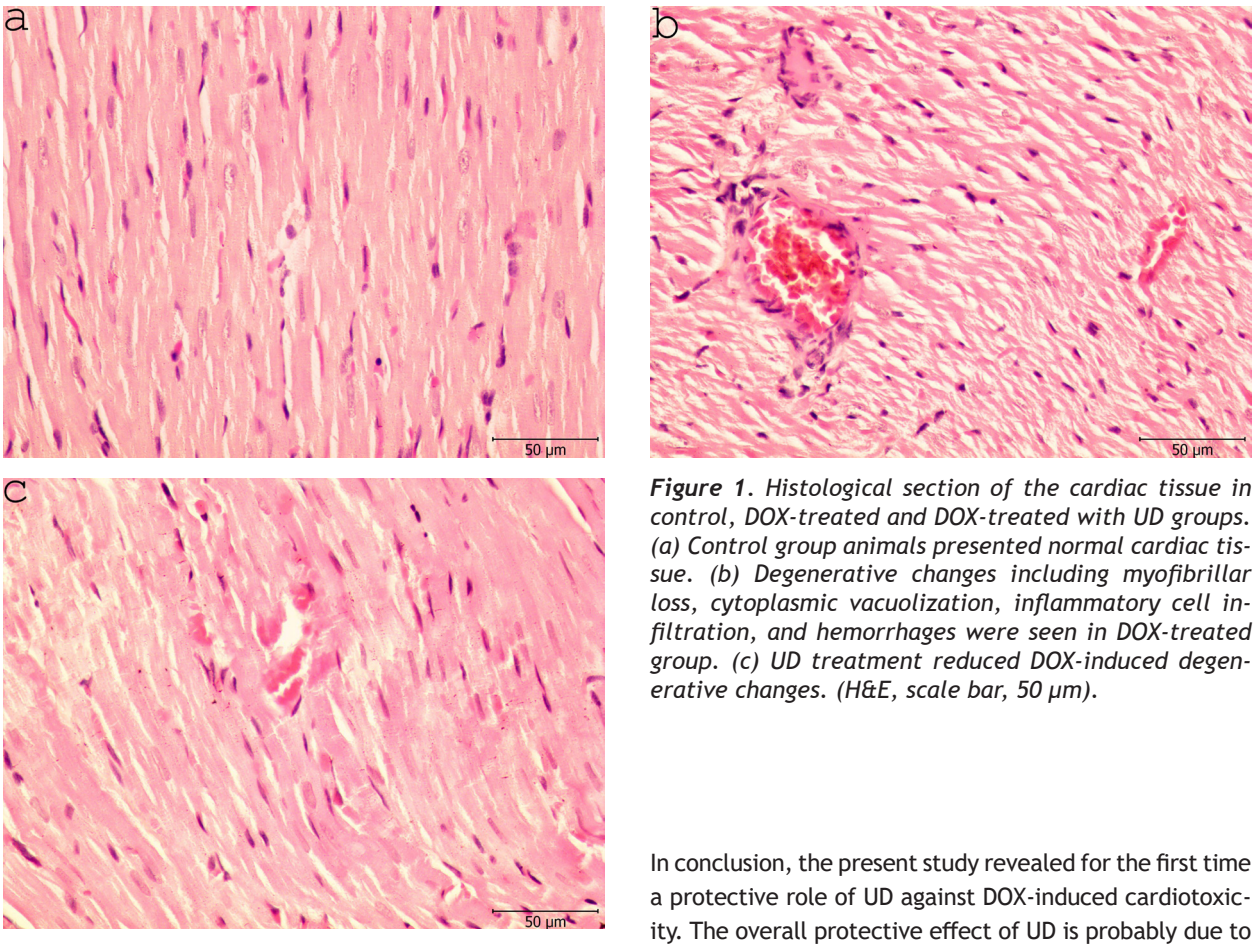


***Figure 1**. Histological section of the cardiac tissue in control, DOX-treated and DOX-treated with UD groups. (a) Control group animals presented normal cardiac tissue. (b) Degenerative changes including myofibrillar loss, cytoplasmic vacuolization, inflammatory cell infiltration, and hemorrhages were seen in DOX-treated group. (c) UD treatment reduced DOX-induced degenerative changes. (H&E, scale bar, 50 µm).*

els after DOX treatment have been found to decline following UD treatment. These results suggest an increased tissue lipid peroxidation with DOX causing tissue injury, while UD has protective effects at a biochemical level.

Previous studies showed that DOX treatment was associated with reduced antioxidant enzyme levels, i.e. SOD, GSH-Px and CAT. In the study by Subburaman et al. (39) examining the protective effect of naringen against DOX-induced cardiotoxicity, DOX was found to result in reduced SOD, CAT, and GSH-Px. Alpsoy et al. (17) in their study where cardiotoxicity was induced by DOX administered at a dose of 30 mg/kg, a similar reduction in SOD, CAT, and GSH-Px antioxidant enzyme levels was observed. In a 2013 study, DOX was found to lead to reduced SOD and GSH levels, while no change in CAT was found (40). In line with previous reports, DOX was associated with reduced levels of SOD, CAT, and GSH-Px, while UD was associated increased levels of these enzymes.

In conclusion, the present study revealed for the first time a protective role of UD against DOX-induced cardiotoxicity. The overall protective effect of UD is probably due to counter action of free radicals by its antioxidant nature hence its ability to restore normalcy in tissue under oxidative stress. However, the precise molecular mechanism by which UD exerts in protective action against oxidative damage remains to be investigated. If this protective function is confirmed in cancer patients, UD may have an important clinical significance as an adjuvant therapy with DOX.

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