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DOSE DEPENDENT CARDIAC EFFECTS OF DOXORUBICIN IN WISTAR RATS: A BIOCHEMICAL AND HISTOPATHOLOGICAL ANALYSIS

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Doxorubicin, Cardiotoxicity, Dose dependence, Oxidative stress, Histopathology, Wistar rats

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ABSTRACT: Although doxorubicin is used widely in animal models to induce cardiotoxicity, serial dose-dependent cardiac effects have not been investigated in experimental animal models to date. Therefore, the objective of this study was to find out the dose-dependent changes of doxorubicin-induced acute cardiotoxicity, both biochemically and histopathologically in Wistar rats. Wistar rats were divided into nine groups. Group 1: normal control; Groups 2-9: eight doses of doxorubicin (13, 14, 15, 16, 17, 18, 19 & 20 mg/kg, intraperitoneal injection, after 16 h fast). Animals were sacrificed on the 4th day, and blood was collected for the estimation of cardiac troponin I (cTnI), aspartate aminotransferase (AST) and superoxide dismutase (SOD) activities and heart tissues were collected for histopathological assessment. Mean cTnI concentrations of groups 1-9 were 0, 39.46 ± 1.8, 60.92 ± 2.7, 87.79 ± 1.8, 116.96 ± 2.7, 147.79 ± 2.3, 163.96 ± 1.5, 197.38 ± 2.3 and 221.54 ± 1.8 pg/mL respectively. In groups 1-9, mean AST activities were 38.90 ± 0.98, 42.23 ± 0.99, 44.07 ± 1.20, 50.77 ± 1.70, 57.93 ± 3.40, 58.54 ± 0.65, 63.57 ± 0.69, 68.94 ± 2.20 and 79.83 ± 1.20 U/L and SOD activities were 77.73 ± 0.86, 72.92 ± 1.3, 66.49 ± 0.91, 54.06 ± 0.92, 28.01 ± 0.45, 21.12 ± 0.56, 16.12 ± 0.46, 14.34 ± 0.70 and 8.75 ± 0.94% respectively. Significant differences ($P<0.05$) between group 1 and groups 2-9 were evident in all three diagnostic parameters. Degree of histopathological damage due to doxorubicin increased with increasing dosage of doxorubicin.

INTRODUCTION: Over the last 20 years, the survival rate of cancer patients has significantly increased due to the progress of modern cancer therapy [1]. The U.S National Cancer Institute estimates that at least 15.5 million cancer survivors were alive in the United States in 2016, and this number will approach 20 million by 2026. [2]

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67% of adults diagnosed with cancer today will be alive in 5 years, and 75% of children with cancer today will be alive in 10 years.

According to Ozdogru, the introduction of the anthracycline group of chemotherapeutics into the cancer treatment regimens increased the survival rate from 30% to 70% [3]. Doxorubicin, the highly effective chemotherapeutic agent which belongs to the anthracycline family, is frequently used for various malignancies including leukemia, lymphoma, breast cancer, lung cancer, multiple myeloma, and sarcoma mainly as a first line treatment [4, 5]. However, the clinical use of doxorubicin is hampered by its serious

cardiotoxicity, which often leads to irreversible degenerative cardiomyopathy and heart failure [6]. Doxorubicin-induced cardiotoxicity can be acute, occur during and within 2-3 days of its administration and chronic, usually evident within 30 days of administration of its last dose, but it may occur even after 6-10 years after its administration [7]. Incidence of acute cardiotoxicity is approximately 11%, while the incidence of chronic doxorubicin cardiotoxicity is much lower, with an estimated incidence of about 1.7%. The incidence of doxorubicin cardiomyopathy is primarily related to its dose. The incidence is about 4-5% when the cumulative dose of doxorubicin is 450-500 mg/m^2 and 18% when the dose is 550-600 mg/m^2. [8] According to previous studies, dose-dependent changes have been observed in left ventricular ejection fraction following anthracycline chemotherapy, particularly at cumulative doses of doxorubicin >350 mg/m^2. [9] According to the literature, some studies reported that histopathologic changes in the endomyocardial biopsy of patients who have received as little as a cumulative dose of 240 mg/m^2 of doxorubicin.

Also, to the cumulative dose, age of the patient also influences to develop doxorubicin cardiomyopathy [8]. Very young and very old individuals have greater risk. Other risk factors include female gender, prior mediastinal radiation therapy, hypertension, concomitant treatment with cyclophosphamide, trastuzumab or paclitaxel, and the presence of cardiac disease [9]. Chemotherapeutic cardiotoxicity can be characterized as type 1 or type 2 cardiotoxicity [10]. Type I cardiotoxicity is caused by cardiomyocyte death, due to necrosis or apoptosis, and it is not reversible. Type II cardiotoxicity is caused by cardiomyocyte dysfunction, and therefore, it may be reversible. The long-term cardiotoxicity caused by the anthracycline represents type I toxicity. A widely accepted mechanism for anthracycline-induced anti-tumor activity relates to DNA damage. In this mechanism, doxorubicin intercalates into DNA and disrupt topoisomerase II-α mediated DNA repair by inhibiting its relegation reaction and cause the accumulation of protein-linked double and single-stranded DNA breaks (cleavable complexes) and induction of apoptosis that ultimately lead to cytotoxic DNA damage and cell death [11, 12]. Redox injury and interference with protein synthesis play an important role in doxorubicin cardiotoxicity [13]. Conditions that exacerbate free-radical formation may enhance doxorubicin-induced cardiotoxicity. In the mechanism of free radical formation caused by doxorubicin metabolism, doxorubicin is converted to the semiquinone radical by several mitochondrial enzymes such as NADH dehydrogenase and cytochrome P-450 reductase in mitochondrial respiratory complex I that can react with molecular oxygen to form the superoxide radical [9, 13] - subsequently, redox cycling results in the production of hydrogen peroxide and the hydroxyl radical.

Another reported mechanism of doxorubicin-induced cardiotoxicity is the formation of doxorubicin-iron complexes which catalyze a Fenton reaction (Fe^{2+} -catalyzed conversion of hydrogen peroxide to hydroxyl radical) resulting in the generation of reactive oxygen species [14, 15]. Cardiomyocytes are more prone to stress caused by doxorubicin due to several reasons [16]. Some of them are having a high content of mitochondria and a relatively low amount of anti-oxidants. Mitochondria have been identified as the main target of doxorubicin-induced toxicity as a result of the dose-dependent increase in the mitochondrial accumulation of doxorubicin. This is because doxorubicin has high affinity to cardiolipin, a phospholipid that is uniquely expressed on the inner mitochondrial membrane [17].

The pathophysiological background of the cardiotoxic effect of doxorubicin is multifactorial and not completely explained [18, 19]. The damage produced by doxorubicin is dose-related and may lead to cardiomyopathy. Although doxorubicin is used widely in animal models to induce cardiotoxicity, dose-dependent cardiac effects have not been investigated as a systematic study both biochemically and histopathologically in an experimental animal model to date. Therefore, the objective of this study was to find out dose-dependent changes of doxorubicin-induced acute cardiotoxicity, both biochemically and histo-pathologically in healthy Wistar rats.

MATERIALS AND METHODS:

Experimental animals: Healthy male and female Wistar albino rats, 6-8 weeks old and weighing 150-200 g were purchased from the Medical

Research Institute, Colombo, Sri Lanka. The animals were housed in a well-ventilated hygienic experimental animal house at the Faculty of Medicine, University of Ruhuna, Sri Lanka. All rats were kept in the cages with softwood shavings as bedding materials.

They were maintained on a standard laboratory diet of rat pellets and water *ad libitum*. Rats were acclimatized to the environment (temperature, 23 ± 2 °C; humidity, 50 ± 5%; and 12-h light-dark cycle) for one week before experimental use. All animals fasted for 16 h before administration of doxorubicin. All protocols used in this study were approved by the ethics committee of the Faculty of Medicine, University of Ruhuna, Sri Lanka (23.10.2014:3.10), guided by the CIOMS international guiding principles of biomedical research involving animals.

Chemicals: Diagnostic kit for serum cTnI was purchased from Elabscience Biotechnology Co., Ltd, China, and SOD (EC 1.15.1.1) inhibition assay kit was purchased from Sigma Aldrich, Co, USA. AST (EC 2.6.1.1) activity assay kit was purchased from Biorex Diagnostics Limited, United Kingdom and doxorubicin was purchased from United Biotech (P) Limited, India. Chemicals used for histopathological analysis included, formalin, hematoxylin, eosin, absolute ethanol, xylene, and DPX. Alcohol used was of analytical grade.

Treatment of Animals:

Control Group: Ten healthy Wistar albino rats with an equal number of male and female animals served as the normal control group, and a single intraperitoneal injection of normal saline (10 mL/kg) was administered after a 16 h fast. All animals were sacrificed by cervical dislocation three days after the respective intraperitoneal injection.

Doxorubicin-Induced Cardiotoxicity: Rats were randomly divided into eight groups of ten animals in each. A series of a single intraperitoneal dose of doxorubicin (13, 14, 15, 16, 17, 18, 19 and 20 mg/kg) was injected to each animal after a 16 h fast. All animals were sacrificed by cervical dislocation three days after the administration of doxorubicin. Blood was collected by cardiac puncture, and heart tissue was collected and fixed in 10% buffered formalin for histopathological assessment of cardiac damage.

Assessment of Cardiotoxicity: Serum cTnI, AST, and SOD inhibition activity were measured using commercially available assay kits. Myocardial tissue was sampled for histopathological assessment from the ventricle, 5mm above the apex of the heart of all animals and fixed in 10% formal saline. They were processed, cut the sections in 3µm thickness, and stained with hematoxylin and eosin. The sections were examined under the light microscope, and pathological changes were scored. Scoring of pathological changes was performed according to a grading system developed by the authors as follows: 0, no cells with necrotic changes; 1, up to 10 cells with necrotic changes; 2, 10-50 cells with necrotic changes; 3, 50-100 cells with necrotic changes; >100 cells with necrotic changes **Table 2**. Myocytes with nuclear pyknosis or karrheorhexis or karyolysis with hypereosinophilic cytoplasm and no striation were identified as necrotic myocytes. Necrotic myocyte density was assessed separately in peripheral and subendocardial regions of the myocardium.

Statistical Analysis: The results were evaluated by one-way analysis of variance. A probability (P) value less than 0.05 was considered significant.

RESULTS: A gradual, significant increase in serum cTnI concentration was observed in rats treated with doxorubicin, from the lowest dose of 13 mg/kg to the highest dose of 20 mg/kg, compared to the normal control group **Table 1**. Consistently, Wistar rats treated with doxorubicin also showed a significant ($P<0.05$) sequential increase in serum AST activity compared to the control with the highest value observed in the animal group treated with 20 mg/kg of doxorubicin **Table 1**. Wistar rats treated with doxorubicin showed a significant sequential depletion ($P<0.05$) of SOD activity in serum compared to the control group showing the lowest value in the group of rats treated with 20 mg/kg of doxorubicin **Table 1**.

Cross sections of the cardiac tissues from the control group showed the normal myocardial architecture, **Fig. 1A**. Early cellular changes of necrosis including pyknotic nuclei, hypereosinophilic cytoplasm were seen in all

groups of rats who received doxorubicin, and the effect was dose-dependent since highest score for the necrotic changes was found in the group exposed to 20 mg/kg of doxorubicin **Table 2** (**Fig. 1** and **2**).

TABLE 1: EFFECT OF DIFFERENT DOSES OF DOXORUBICIN ON SERUM cTnI, AST AND SOD ACTIVITY

Treatment	cTnI (pg/mL)	AST (U/L)	SOD (%)
Control	0.00	38.90 ± 0.98	77.73 ± 0.86
13 mg/kg	39.46 ± 1.8***	42.23 ± 0.99*	72.92 ± 1.3*
14 mg/kg	60.92 ± 2.7***	44.07 ± 1.20**	66.49 ± 0.91***
15 mg/kg	87.79 ± 1.8***	50.77 ± 1.70**	54.06 ± 0.92***
16 mg/kg	116.96 ± 2.7***	57.93 ± 3.40**	28.01 ± 0.45***
17 mg/kg	147.79 ± 2.3***	58.54 ± 0.65***	21.12 ± 0.56***
18 mg/kg	163.96 ± 1.5***	63.57 ± 0.69***	16.12 ± 0.46***
19 mg/kg	197.38 ± 2.3***	68.94 ± 2.20***	14.34 ± 0.70***
20 mg/kg	221.54 ± 1.8***	79.83 ± 1.20***	8.75 ± 0.94***

All values are expressed as mean ± SE (n=10). P values: * < 0.05, **< 0.01, *** <0.001

TABLE 2: AVERAGE GRADING OF CELLS WITH NECROTIC CHANGES IN ANIMALS EXPOSED TO DIFFERENT DOSES OF DOXORUBICIN

Animal group	Sub-endocardial region of heart tissues (score out of 4)	The peripheral region of heart tissues (score out of 4)	Total score (out of 8)
Control	0	0	0
13 mg/kg	3.0	0	3.0
14 mg/kg	3.0	0.33	3.33
15 mg/kg	3.2	1.05	4.25
16 mg/kg	3.0	1.67	4.67
17 mg/kg	3.33	1.67	5.0
18 mg/kg	4.0	3.6	7.6
19 mg/kg	4.0	3.6	7.6
20 mg/kg	4.0	3.7	7.7

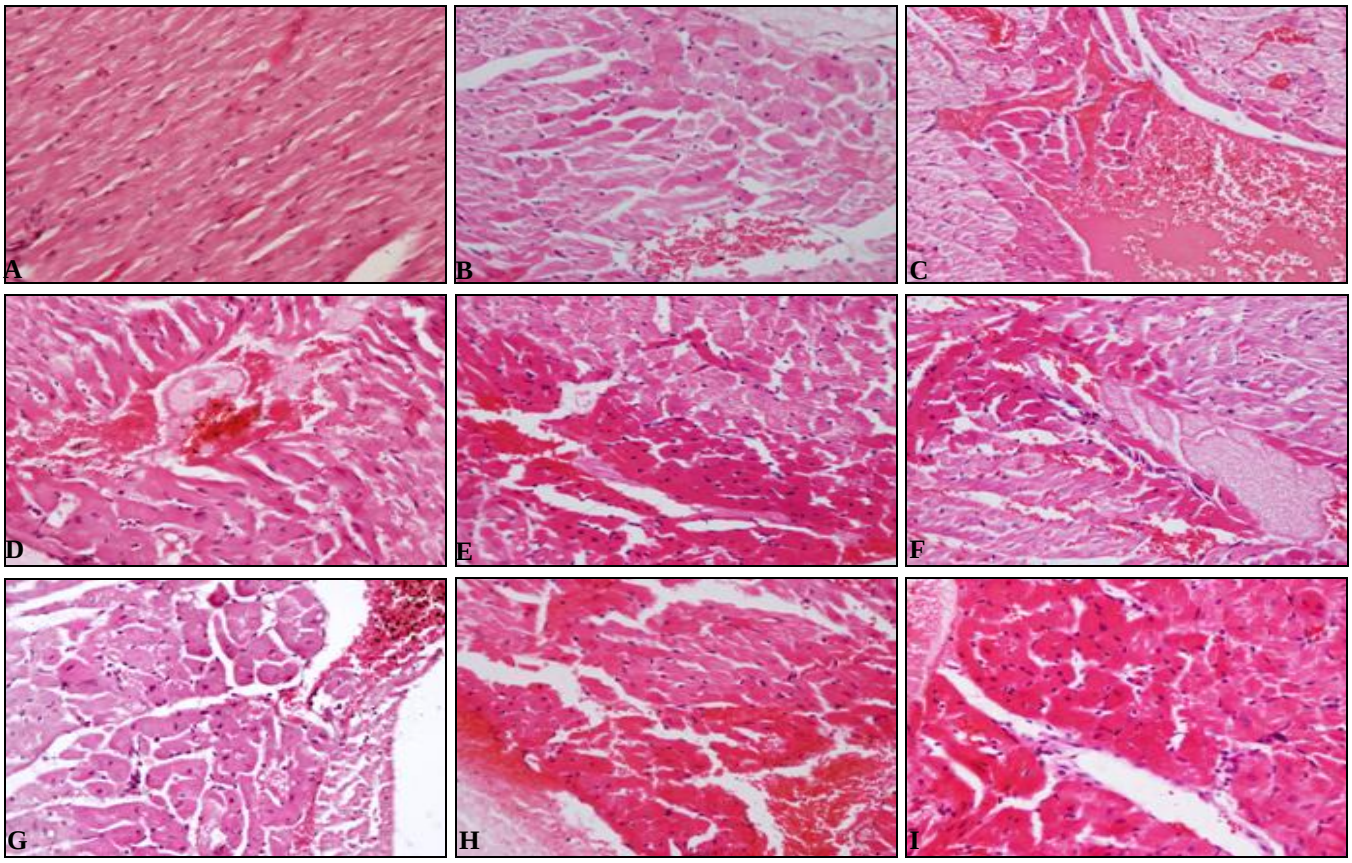

FIG. 1: CELLS WITH EARLY NECROTIC CHANGES IN SUBENDOCARDIAL REGION AFTER ADMINISTRATION OF DIFFERENT DOSES OF DOXORUBICIN. A - Normal control showing normal morphology, rat groups treated with; B – 13 mg/kg of doxorubicin (grade – 3.0), C – 14 mg/kg of doxorubicin (grade – 3.0), D – 15 mg/kg of doxorubicin (grade – 3.2), E – 16 mg/kg of doxorubicin (grade – 3.0), F – 17 mg/kg of doxorubicin (grade – 3.33), G – 18 mg/kg of doxorubicin (grade – 4.0), H - 19 mg/kg of doxorubicin (grade – 4.0), I - 20 mg/kg of doxorubicin (grade – 4.0) (Light micrograph of rat heart, H & E, ×40).

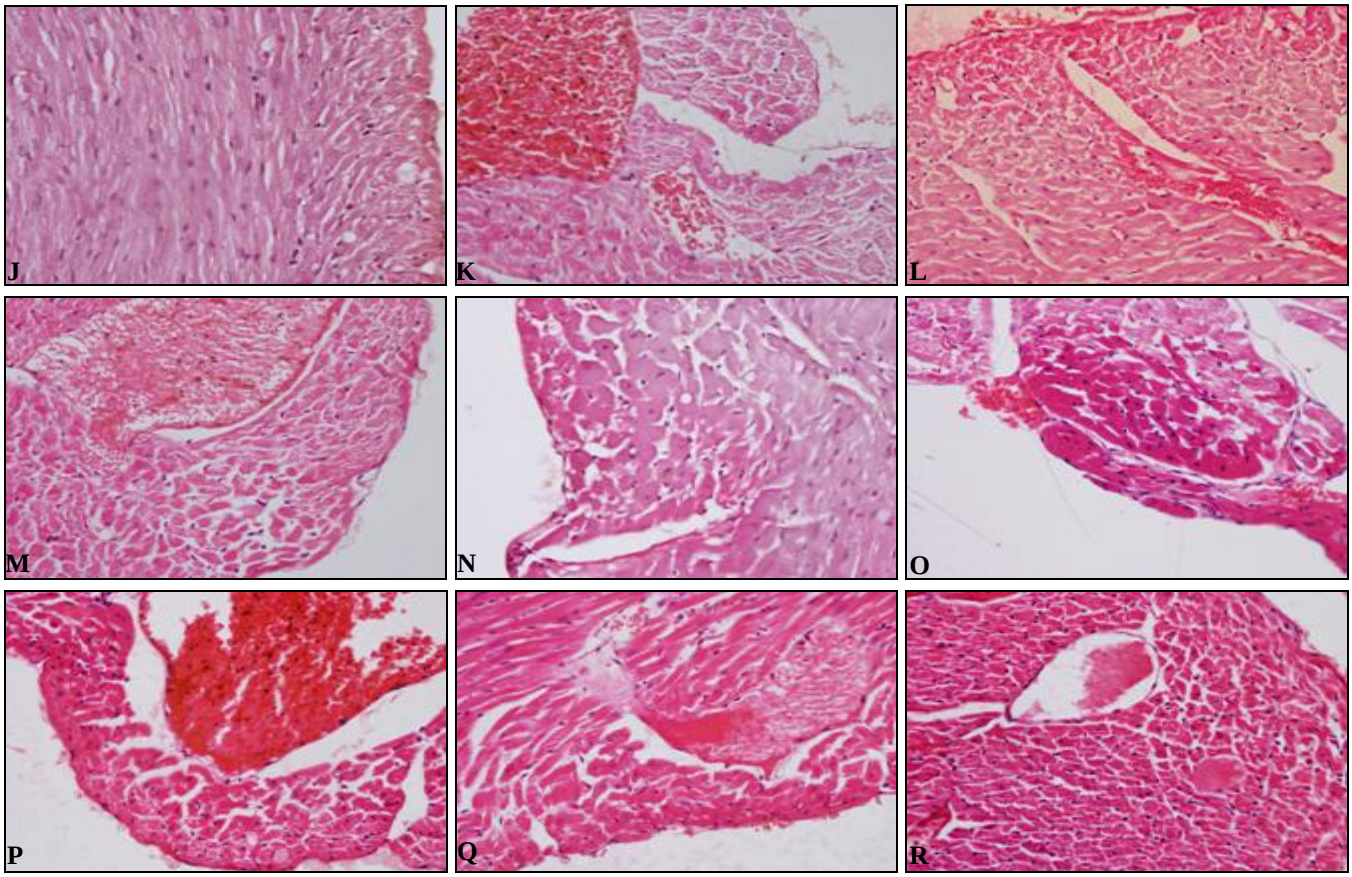

FIG. 2: CELLS WITH EARLY NECROTIC CHANGES IN PERIPHERAL REGION AFTER ADMINISTRATION OF DIFFERENT DOSES OF DOXORUBICIN. J - Normal control showing normal architecture, rat groups treated with; K - 13 mg/kg of doxorubicin (grade – 0.0), L – 14 mg/kg of doxorubicin (grade – 0.33), M – 15 mg/kg of doxorubicin (grade – 1.05), N – 16 mg/kg of doxorubicin (grade – 1.67), O – 17 mg/kg of doxorubicin (grade – 1.67), P – 18 mg/kg of doxorubicin (grade – 3.6), Q – 19 mg/kg of doxorubicin (grade – 3.6), R – 20 mg/kg of doxorubicin (grade - 3.7) (Light micrograph of rat heart, H & E, ×40).

Early reversible changes of cell injury were apparent in all groups of rats that received doxorubicin including hemorrhages, interstitial edema, congestion, wavy fibers, and vacuoles **Table 3** (**Fig. 3**). Inflammatory cell infiltrations were observed only in groups that received 18 mg/kg - 20 mg/kg of doxorubicin **Fig. 3**.

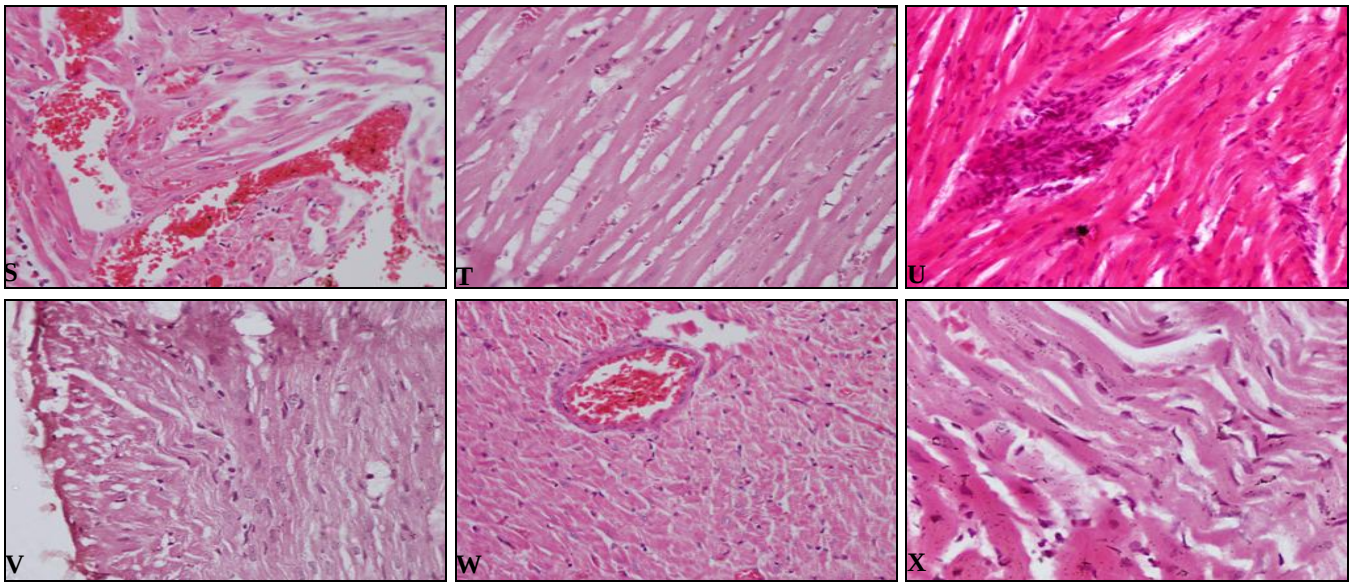

FIG. 3: HISTOPATHOLOGICAL CHANGES OBSERVED IN THE CARDIAC TISSUES OF RATS TREATED WITH DOXORUBICIN. S – Haemorrhages, T – Interstitial edema, U – Inflammatory infiltrations, V – Vacuoles, W – Congested capillaries X – Wavy fibers (Light micrograph of rat heart, H & E, × 40).

TABLE 3: DOSE-RESPONSE EFFECT OF HISTOPATHOLOGICAL CHANGES OF CARDIAC TISSUE OF WISTAR RATS EXPOSED TO DOXORUBICIN

Animal group	Haemorrhages	Interstitial edema	Inflammatory infiltrations	Vacuoles	Congested capillaries	Wavy fibers
Control	Absent	Absent	Absent	Absent	Absent	Absent
13 mg/kg	Absent	Absent	Absent	Present	Present	Present
14 mg/kg	Absent	Absent	Absent	Present	Present	Present
15 mg/kg	Absent	Absent	Absent	Present	Present	Present
16 mg/kg	Present	Present	Absent	Present	Present	Present
17 mg/kg	Present	Present	Present	Present	Present	Present
18 mg/kg	Present	Present	Present	Present	Present	Present
19 mg/kg	Present	Present	Present	Present	Present	Present
20 mg/kg	Present	Present	Present	Present	Present	Present

Hemorrhages and interstitial edema were apparent in rats treated with more than 16 mg/kg of doxorubicin. Wavy fibers were easily seen with higher doses of doxorubicin. Necrotic areas were visible more in the subendocardial regions of the heart tissue compared to the peripheral region.

DISCUSSION: Doxorubicin belongs to the group of anthracyclines which were introduced for the use of cancer treatment in the late 1960s and remained one of the most commonly prescribed anticancer drugs [7, 20]. However, its dose-dependent cardio-toxicity limits the efficacy as an antitumor treatment. Several hypotheses have been suggested to explain the cardiotoxicity induced by anthracycline. The most accepted hypothesis is the doxorubicin-induced free radical formation, which causes lipid peroxidation and oxidative damage in the cardiac muscle [21]. Myofibrillar deterioration and intracellular calcium dysregulation are also important mechanisms commonly associated with doxorubicin-induced cardiac toxicity.

According to the mechanism put forward by Goormaghtigh *et al*., mitochondria are the most extensively injured sub-cellular organelles in doxorubicin-induced cardiotoxicity [22]. Since doxorubicin is a cationic drug, it is believed that it retains in the inner mitochondrial membrane and forms an almost irreversible complex with cardiolipin [23]. Due to the formation of this complex, it is near the electron transport chain. Previous researches suggest that this enables redox cycling of doxorubicin to be mediated through its interaction with NADH dehydrogenase of the electron transport chain. Oxidation of the doxorubicin-semiquinone radical to the doxorubicin-quinone form leads to the generation of the highly reactive superoxide [24]. Other membrane proteins which are responsible for the transfer of carnitine can also be adversely affected by doxorubicin, which can contribute to the decrease in mitochondrial function. Since mitochondria produce more than 90% of the ATP utilized by cardiomyocytes; the above events disrupt the cellular metabolism of mitochondria [25].

This functional disruption leads to ultrastructural pathologic changes such as mitochondrial swelling and myelin figures within the mitochondria [26]. In 1980 May *et al*., demonstrated that doxorubicin had a strong affinity for iron, and it suggests that cellular damage induced by doxorubicin may be mediated by iron - doxorubicin complex [27]. Subsequent studies followed on this hypothesis revealed that this iron complex could cause lipid peroxidation through its interactions with the negatively-charged membranes [28]. Also, to this doxorubicin reduction in the presence of free iron could undergo redox recycling which is responsible for generating free radicals and doxorubicinol, the metabolite of the doxorubicin interacts with thiol groups on proteins, compounding the damages to the cell [29]. Anthracycline-induced cardiotoxicity can be divided into acute, subacute, and chronic forms. Acute cardiotoxicity may start during and within 24 h of the infusion and following ECG abnormalities could be seen [30].

They are atypical ST-T changes, reduced QRS voltages, sinus tachycardia, premature supra-ventricular, and ventricular complexes, QT interval prolongation, and rarely, acute myocardial ischemia. These electrocardiographic changes may be completely asymptomatic, but sometimes may be associated with few symptoms. In most patients, these symptoms usually resolve naturally within several hours or weeks after the completion of

chemotherapy. Sub-acute cardiotoxicity is usually rare, it may appear several weeks or months (as late as 30 months) after the last dose of anthracycline and myocarditis, or pericarditis is the most frequent manifestation. The chronic cardiotoxicity may not appear until as many as 4 to 20 years after the last administration of doxorubicin and is associated with progressive myocardial dysfunction [7]. It has been revealed that the mortality rate in these patients is 50% after five years.

In this study, 13-20 mg/kg of doxorubicin was used to determine the dose-dependent cardiac effects of doxorubicin. This range was selected according to the cardiotoxic doses used in previous studies. In Kang *et al*., they have used 10, 12, 14 and 16 mg/kg of cumulative dose of doxorubicin [31]. According to the results of that study, systolic functions were significantly reduced in the rat group that received 16 mg/kg doxorubicin compared to the control. cTnI levels were significantly increased with the rise of total cumulative doses of doxorubicin. The severity of the pathological changes in the myocardium was significantly higher in the groups that received 14 and 16 mg/kg doxorubicin, respectively. Another study conducted by Sayed-Ahmed *et al*., have used 10, 15, 20 and 25 mg/kg cumulative dose of doxorubicin to detect the effect of doxorubicin-induced cardiomyopathy using increased plasma endothelin-1 and cardiac nitric oxide [32].

The results showed that doxorubicin caused a significant increase of plasma endothelin-1 at a cumulative dose of 10, 15, and 20 mg/kg of doxorubicin. They also showed a dose-dependent increase of serum LDH and creatine phosphokinase activity. Although a few doses were tested in some of the studies reported before, both biochemical, as well as histopathological investigations, have never been reported. Therefore, a range of doses from 13-20 mg/kg was selected with 1mg/kg interval as even a smaller difference in dosage as 1 mg/kg exerted a significant biochemical and histopathological change on the cardiac tissue.

Therefore, the objective of this study was to provide a comprehensive report on the biochemical and histopathological changes observed after the administration of a range of doses of doxorubicin to Wistar rats.

Previous studies have also shown that cardio-myopathy is more severe when higher doses are administered within a short period of time than when the same cumulative dose is administered as a repeated dose over a long period [33]. Therefore, this study was conducted with a single dose of doxorubicin. Sublethal dose used in the study was 20 mg/kg as the doses above 20 mg/kg led to higher mortality in Wistar rats.

The present study was conducted to determine the dose-dependent cardiotoxic changes observed in doxorubicin-induced acute cardiotoxicity because the acute cardiotoxicity has a higher prevalence than the chronic cardiotoxicity [13]. Although, the chronic cardiotoxicity is dose-dependent, and the extent of damage depends on the cumulative dosage, the acute cardiotoxicity can be evident even with the administration of one dose of doxorubicin. In the present study, the extent of damage in acute cardiotoxicity was determined by the measurement of serum biomarkers of cardiac damage, oxidative stress as well as by the assessment of histopathological changes in cardiac tissue. As biomarkers of cardiac damage, serum cTnI concentration and AST activity were measured while oxidative damage was measured by the depletion of SOD level in serum.

In the present study, for the estimation of cardiotoxicity, blood samples and tissue samples were collected after three days of doxorubicin administration. The three-day time-point was selected based on data from pharmacokinetic studies showing that the average terminal half-life of doxorubicin lies between 12 and 48 h and because acute cardiotoxicity appears between 48 and 72 h [20]. In cardiomyocyte damage, usually, cTnI concentration increases in blood 3-7 h after cell damage reaches to maximum level 10-20 h and returns to normal value within 10 days [34]. AST level increases in blood 3-4 h after cell damage reach the maximum level in 15 -28 h and returns to normal values within 5 days. Increased level of cTnI in the bloodstream is a well-established biomarker with high sensitivity and specificity for myocardial infarction or necrosis in man and animals [35, 36]. The cTnI elevation is common in patients with congestive heart failure [37] and it has also been reported as an independent predictor of cardiac mortality in heart failure [38].

Studies done on animal models have shown that the release of cTnI from myocardium is proportional to the size and extent of tissue injury in several animal models of cardiotoxicity [36]. The current study showed an increase in cTnI levels with each dose of doxorubicin, and the maximum level was observed with the highest dose of doxorubicin (20 mg/kg). The probable cause of the increase of cTnI level is damage to myocardium [39]. A dose-dependent increase in cardiac lesions was previously reported when Fischer rats were treated with 1-2 mg/kg of doxorubicin for up to 10-14 weeks [40,] and Wister-Kyoto rats were treated with a cumulative dose of 12 mg/kg of doxorubicin [41]. Previous studies also reported that myocardial injury increased with increase in dose, as observed in Sprague Dawley rats treated with 10, 13.5 and 18 mg/kg of doxorubicin [40] and similar results were observed in the present study.

According to literature, transaminases such as alanine aminotransferase and AST are liberated into the serum after extensive tissue injury [42]. The heart muscle is rich in AST; it suggests that their increased level is an indicator of myocardial damage. Therefore, higher the activity of AST, the size of the injury to the myocardium is large [42, 43] and the current study also revealed that AST level increases with the increase of doxorubicin dosage concluding that the myocardial injury increases in a dose-dependent manner concerning doxorubicin. According to Octavia *et al*., the sequence of events initiated by free radical formation is believed to be the major mechanism by which doxorubicin injures the myocardium [23]. To confirm the induction of oxidative stress by doxorubicin, SOD activity in serum was measured in the present study. It is commonly accepted that SOD protects against the free radical injury by converting superoxide radical to hydrogen peroxide and prevent the formation of hydroxyl radicals through O_2^- driven Fenton reaction [44]. In this study, doxorubicin treated animals showed a decrease in SOD activity in a dose-dependent manner with the lowest level of SOD in the rat group that received the highest dose of doxorubicin which confirms an increase of the oxidative stress and cardiac damage in a concomitant dose of doxorubicin.

Histopathological assessment of cardiac damage is considered as the gold standard to detect doxorubicin-induced cardiotoxicity [23]. Cardio-toxicity caused by chemotherapies can be indicated by a variety of pathological changes such as arrhythmia, changes in blood pressure, myocardial ischemia or necrosis [45]. The study was done by Sawyer *et al*. has shown the ultrastructural changes of endomyocardial biopsies of anthracycline-treated patients and rodents such as loss of myofibrils, the disarray of myofibrils of the sarcomere, dilation of the sarcoplasmic reticulum, swelling of the mitochondria and cytoplasmic vacuolization [46]. The main reason for the breakdown of sarcomeres after anthracycline exposure is believed to be the degradation of titin, a giant myofilament protein, and an integral part of the sarcomere [47]. The integrity of titin is important for the dynamic regulation of the contractile function of the heart. Loss of integrity of titin has been demonstrated in ischemic human hearts indicating its disruption in the pathogenesis of progressive ventricular dysfunction in this condition [48].

Therefore, it can be suggested that histopathological changes observed in doxorubicin-induced cardiotoxicity may be similar to ischaemic changes. Molecular basis of the doxorubicin-induced cardiotoxicity has also shown similarities between ischemia and doxorubicin-induced necrosis. A study was done by Matsui *et al*., has shown that the PI-3K/Akt pathway is responsible for regulating the survival of cardiac myocytes from insults such as transient ischemia [49]. Similar to this result, a study was done by Fukazawa *et al*., has shown that activation of PI-3k/Akt signaling suppresses anthracycline-induced apoptosis, and inhibition of PI-3K / Akt increases anthracycline-induced apoptosis [50]. Not only this, myopathic hearts from anthracycline-treatment have presented with increased Bax/Bcl-2 ratio similar to what has been seen after ischemia-reperfusion [51].

Therefore, histopathological changes seen in doxorubicin cardiotoxicity may be similar to ischemic changes. The present study showed necrotic cells with pyknotic nuclei and hypereosinophilic cytoplasm, hemorrhages, interstitial edema, inflammatory cell infiltrations, vacuoles, congested capillaries, and wavy myocardial fibers. These changes are mostly similar to acute ischaemic changes.

In acute ischaemic change wavy myocardial fibers are observed within 4 h, especially at the periphery of the ischaemic zone [52]. With time, interstitial edema increases and the fibers appear thinner, wavy and elongated. Within 12 h of ischemia, initiation of coagulative necrosis, interstitial edema, focal hemorrhages and infiltration of neutrophils could be seen [53]. Within 12-18 h, myocytes become pallor due to nuclear shrinkage and eosinophilic cytoplasm. Due to the continuation of the coagulative necrosis, focal and larger areas of myocyte contraction banding can be observed. Within 18-24 h, continuation of coagulative necrosis, nuclear pyknosis, and marginal contraction bands may be seen. The present study also showed most of these changes similar to acute ischemia which leads to myocardial infarction.

One of the studies conducted by Razmaraii *et al*., showed that cumulative dose of 12 mg/kg of doxorubicin caused histological alterations in cardiac tissues such as cytoplasmic vacuolization, interstitial edema, hyaline degeneration, and Zenker's necrosis [54]. Another study conducted in 2012 showed that pathological changes of cardiac tissues were dose dependent since massive necrosis was found in animals exposed to 14 mg/kg cumulative dose of doxorubicin [55]. Dose dependence was also confirmed in terms of eosinophilic degeneration and interstitial edema.

The present study also revealed the dose-dependent histological changes in cardiac tissues. All groups of doxorubicin-treated animals in this study showed cells with early changes of necrosis, including pyknotic nuclei and hyper-eosinophilic cytoplasm while giving the highest score for the 20 mg/kg dosage. Although cytoplasmic vacuolation and congested capillaries were observed in almost all doxorubicin treated groups of animals, histological changes such as hemorrhages, interstitial edema and inflammatory infiltration were dose-dependent and were observed only in groups who were treated with more than 16 mg/kg of doxorubicin. Since doxorubicin is widely used as an animal model to induce cardiotoxicity, dose-dependent biochemical and histopathological changes observed after the administration of doxorubicin in Wistar rats are useful in the ethnopharmacological evaluation of the activity of medicinal plant extracts.

CONCLUSION: In conclusion, doxorubicin induced oxidative stress, cardiomyocyte damage and changes in cardiac morphology were dependent on the dose of doxorubicin. The incidence of cardiac necrosis and other histological features of doxorubicin-induced cardiotoxicity were more pronounced in rats treated with a higher dose of doxorubicin. According to the results obtained in this study, the maximum damage was observed with 20 mg/kg of doxorubicin in Wistar rats.

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CONFLICT OF INTEREST: The authors declared that they have no competing interests.

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