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ASSESSMENT OF COMPOUNDED DOXORUBICIN IN CARDIAC TISSUE OF EXPERIMENTAL ANIMALS

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ABSTRACT

Doxorubicin is one of the most important anticancer agents, but its clinical uses are limited due to cardiotoxicity, the present study evaluated the effects of new derivatives of doxorubicin on cardiac tissue by the measurement of serum cardiac troponin I (cTnI) and CK-BM assays as biomarkers as well as heart tissue after 3 weeks of treatment in albino mice. The results indicated that TnI was significantly elevated ($P \leq 0.05$) in all studied groups when compared with the control, also serum CK-MB was significantly elevated ($P \leq 0.05$) in all studied groups except that treated with a new compound when compared with the control group. The histopathological study of heart tissue revealed that the treatment with this compound showed less changes in heart tissue.

Key words: Doxorubicin, troponin I, CK-BM, histopathology

INTRODUCTION

The severe toxicity of most anti-carcinogenic drugs encourages to search for new drug and efforts for the development of newer agents, that can prevent or slow-down cancer growth with less toxicity and more safety. Efficiency, low outcome of cure rate and health care costs make cancer the real challenge for medical therapy and prevention (Kroschinsky *et al*., 2017).

Doxorubicin (DOX), also known as Adriamycin, is an anti-cancer chemotherapeutic drug classified as an anthracycline antibiotic; this drug, is widely used as an antineoplastic agent in the treatment of a variety of solid malignancies, such as leukemia, bladder, lung, breast and ovarian cancers but it´s cardiotoxicity has long been recognized as a complicating factor (Hosseini and Mahdian, 2016, Ejam, 2016). Methotrexate (MTX) another effective and extensively used chemotherapeutic agent to treat range of malignancies, but its therapeutic use is limited because of dose dependent hepatotoxicity influence and it can induced acute liver injury (Al-Fatlawi and Al-Shammari, 2017).

One novel way of attempting to improve anticancer efficacy is to design a compounded DNA replication inhibitor, and that was achieved by Fischer-Speier substitution of DOX with MTX. The proposed approach, modifies the toxicity of each one of the anticancer drugs and specifies their efficacy differentially to the transformed neoplastic cells since the expression of higher lytic enzymes lead to liberate the two drugs in adjacent or inside the tumor cells.

MATERIALS AND METHODS

Chemicals and reagents: Methotrexate, doxorubicin hydrochloride, and penicillin/streptomycin, were purchased from Sigma Aldrich (Gillingham, UK). DMEM media (GIBCO, USA), supplemented with 10% heat inactivated fetal bovine serum (Capricorn Scientific, Germany). MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) purchased from Bioworld, (USA. All other reagents were of analytical grade and used as received.

Synthesis of target compound: The target compound was prepared by Fischer-Speier esterification (Otera and Nishikido, 2009) by mixing 0.5g of doxorubicin (2mmol) with methotrex at 25ml then 0.412g of N, N-dicyclohexylcarbodiimide (DCC) (2mmol) were added with continuous stirring on a magnetic stirrer hot-plate at 70ºC for 6 hours.

Chemical analysis of drug

HPLC analysis: The analysis depended on the union method in the detection of drugs DOX, MTX and DMT using C18 column, injection volume (20 µl), Detector (UV-visible).

DMT analysis by Fourier transform infrared spectrophotometer (FTIR): DOX, MTX, and the synthesized compound (DMT) are milled with potassium bromide (KBr) to form a fine powder, this powder is then compressed into a thin pallet and analyzed directly by FTIR spectrophotometer (Shimadzu, FT-IR-8400S, Japan) to determine the functional group present in the drugs. Each sample was run at infrared region between 400 and 4000 nm.

Preparation of experimental animals and ethical approval: Male Swiss albino mice *Mus musculus*, weigh between 20–30 g, and aged between 8 to 12 weeks, were used in the present study. The mice were obtained from the animal house, Department of Biology, University of Babylon. All animals were kept for 15 days before experiment in well ventilated cages, at 25±2ºC, 12:12 h light: dark

cycle, balanced rodent food pellet and water. All experimental protocols using live animals were first reviewed, approved and accepted according to Guidelines for Care and Use of Laboratory Animals in Biomedical Research.

Groups of Experimental Animals: A total number of 40 Swiss albino mice were used in the present study. Animals were divided into five groups, (n= 8) as follows:

Group1: Control group. Group2: inoculated in the back between the thighs with RD cells. Each mouse was injected subcutaneously with 2 x 10^6 cell/ml. Group 3, 4, and 5: mice of these groups were inoculated with 2 x 10^6 cell/ml in the back between the thighs and injected intraperitoneally (i.p.), depending on the body weight, with doxorubicin (5mg/kg), MTX (15 mg/kg), and DMT (5mg/kg).

Biochemical Analysis: Biochemical estimations of serum Troponin-I (Tn-I) and creatine kinase (CK-MB) were performed using commercially available kit using an autoanalyzer (Mindray chemistry analyzer, BS-230, China).

Preparation of tissue sample: The heart was exteriorized and excised. Specimens were immediately fixed in 10% formaldehyde solution. After fixation they were processed in the usual manner, and embedded in paraffin for subsequent histopathological examination for toxicity analysis.

Statistical Analysis: Mean, SE and one-way Anova were performed using IBM SPSS statistics 21.0. In all tests, P value of less than 0.05 was considered statistically significant.

Results

Chemical formula: C16H15NO4; Physical appearance: red powder; Mp: 200-201ºC; The HPLC for DOX, MTX, and DMT by (C18) column using a mobile-phase showed that retention time for DOX, MTX, and DMT as (1.916, 0.806 and 6.644) respectively and their areas were (425.51, 1261.72 and 2237.48) respectively (Table 1 and Figure 1). The spectra revealed stretch bands at 3441 cm^{-1} and 3417 cm^{-1} which correspond to O-H stretching of carboxylic acid, while asymmetrical and symmetrical stretching of aldehyde occurred at 2933 cm^{-1} and 2976 cm^{-1} respectively. The absorption band at 1726 cm^{-1} was attributed to C=O stretching from esters. At 1581 cm^{-1}, a N-H bend is found, the bands at 1449 cm^{-1} and 1411 cm^{-1} corresponded to the C-H (CH_3). The band at 1510 cm^{-1} corresponded to the C=O in the aromatic plane binding. The lower range of the fingerprint region below 1300 cm^{-1} represents different kinds of C-O of ethers, alcohol, carboxylic acid, and C-N vibrations, which cannot be allocated more specifically

Table 1: Area and retention time of compounds

No.	Drug	Retention time	Area.
1.	DOX	1.916	425.51
2.	MTX	0.806	1261.72
3.	DMT	6.644	2237.48

In this experiment serum was TnI significantly elevated (P≤ 0.05) in all groups RD xenograft, (RD+ MTX), (RD+ DOX), and (RD+ DMT) (0.267±0.011, 0.324±0.007, 0.296±0.003, 0.408± 0.004) respectively as compared with the control group (0.21±0.008). While CK-MB was signifycantly elevated (P≤ 0.05) in all groups RD xenograft, (RD+ MTX), (RD+ MTX) (41.3±0.1.53, 24.8±0.83, 44.2± 0.07) respectively except (RD+ DMT) group (27.2±1.19) as compared with the control group (28.25 ± 1.93) (Table 2).

Histological examinations of heart tissues from normal rats revealed that the cardiac muscle fibers appeared as short branching and anastomosing cylinders with moderately stained eosinophilic sarcoplasm and centrally located oval nuclei (Figure 2); though, histological sections of cardiac tissue of (RD + DMT) group also revealed intact myocardium as illustrated in figure (3). Conversely focal cellular injury, disoriented nuclei, massive degenerative changes, myofibrillar fragmentation of cardiac myocytes, inflammatory cell infiltration, and intermuscular hemorrhage in mice of (RD+DOX) group as briefed in figures (4, 5, 6 and 7). In group (RD+MTX), mild intermuscular hemorrhage and degenerated cardiac muscle fibers were observed (Figure 8 and 9).

Table 2: TnI and CK-BM levels in the serum of different experimental groups.

No.	Group	Tn-I (pg/mL)	CK-MB (ng/mL)
1	Control	0.21± 0.008[a]	28.25± 1.93[a]
2	RD xenograft	0.267±0.011[b]	41.3 ± 1.53[b]
3	RD+DOX	0.324±0.007[c]	33.25 ± 0.83[c]
4	RD+MTX	0.296±0.003[d]	44.2 ± 0.70[b]
5	RD+DMT	0.408±0.004[e]	27.2 ± 1.19[a]

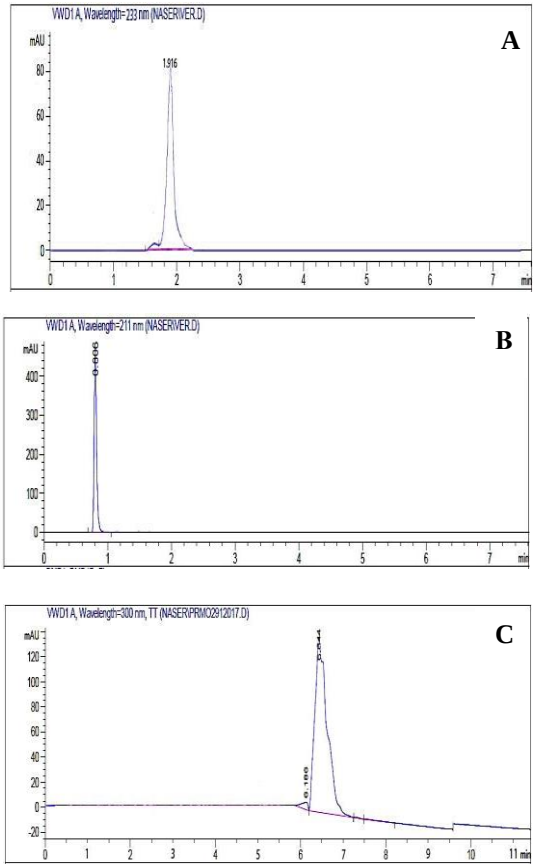

Figure 1: HPLC chromatogram of A: DOX, B: MTX and C: DMT solutions.

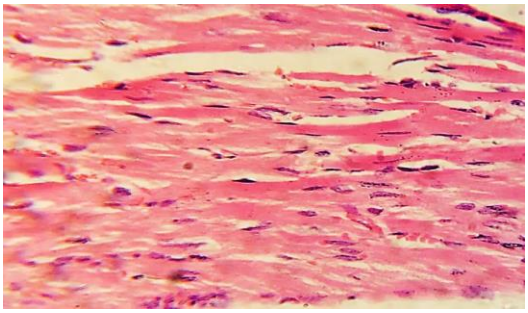

Figure 2: Histological section of cardiac tissue of control group showing normal cardiac myocytes, branching, anastomosing cardiac muscle fibers with A: central vesicular nucleus and B: interstitial connective tissue spaces. 400X (H & E stain).

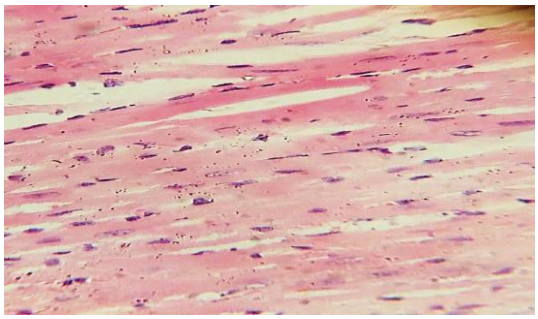

Figure 3: Histological section of cardiac tissue of (RD+DMT) group showing normal cardiac myocytes, branching, anastomosing cardiac muscle fibers with A: central vesicular nucleus and B: interstitial connective tissue spaces. 400X (H & E stain).

(RD+ DOX: RD xenografted and treated with DOX, RD+ MTX: RD xenografted and treated with MTX; RD+ DMT: RD xenografted and treated with DMT. Similar letters indicate non-significant, while the different letters indicate significant compared with the control group; n=8 for each group, ($p \leq 0.05$)**).**

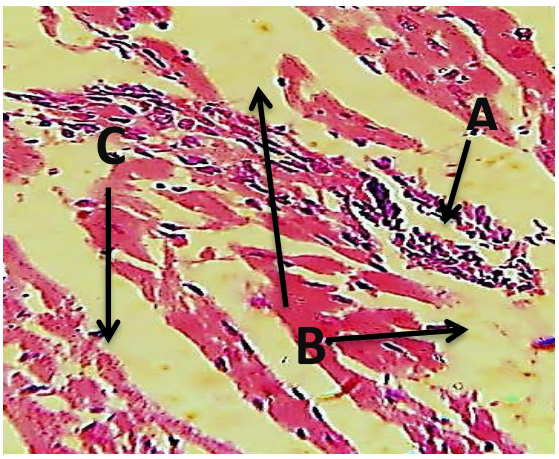

Figure 4: Histological section of cardiac tissue of (RD+DOX) group showing A: inflammatory cell infiltration, B: widened connective tissue spaces between cardiomyocytes, C: myofibrillar fragmentation of cardiac myocytes. 400X (H & E stain).

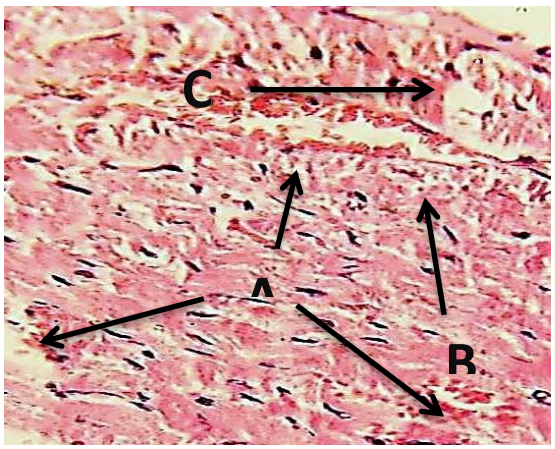

Figure 5: Histological section of cardiac tissue of (RD+DOX) group showing A: Hemorrhage B: myofibrillar fragmentation of cardiac myocytes. C: disorganization of nuclei. 400X (H & E stain).

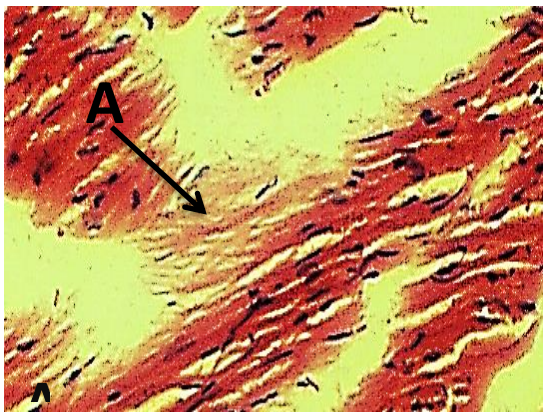

Figure 6: Histological section of cardiac tissue of (RD+DOX) group showing, A: degeneration of cardiomyocytes. 400X (H & E stain).

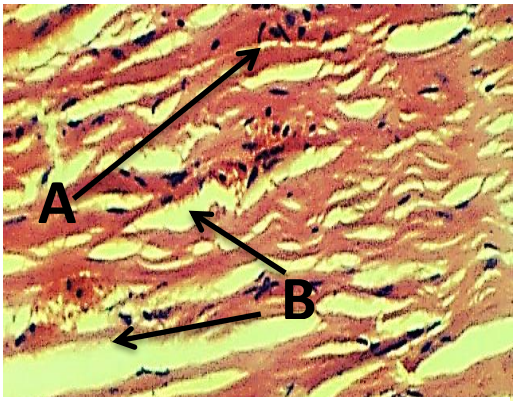

Figure 7: Histological section of cardiac tissue of (RD+DOX) group showing, A: disorginazation of nuclei, B: widened conective tissue spaces between cardiomyocytes. 400X (H & E stain).

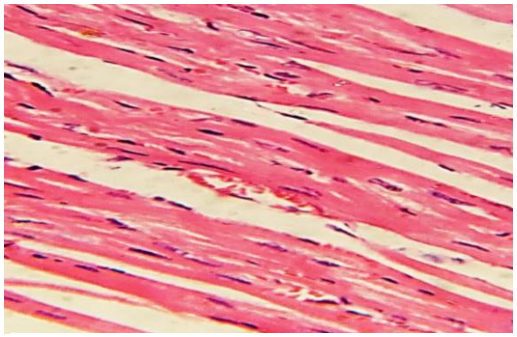

Figure 8: Histological section of cardiac tissue of (RD+MTX) group showing A: hemorrhages. 400X (H & E stain).

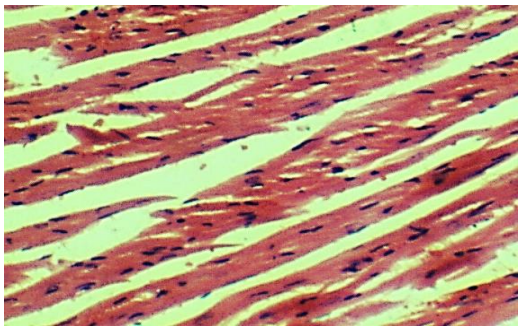

Figure 9: Histological section of cardiac tissue of (RD+MTX) group showing normal cardiomyocyte. 400X (H & E stain).

DISCUSSION

Cardiac toxicity is a major dose-limiting factor for application of some medication like DOX which well known as cardiac toxic compound (Beak *et al*., 2017). So, the present study evaluated the effects of DOX and other drugs on cardiac function by measuring of serum cardiac troponin I (cTnI) and CK-BM as biomarkers after completion of the treatment schedule (3 weeks) in the mice.

Cardiac troponins are sensitive clinical biomarkers for all heart muscle related diseases (Hessel *et al*., 2008, Westermann *et al*., 2017) especially troponin I (TnI) which, represents the best-characterized biomarker for evaluating anthracycline-induced cardiac toxicity (Henri *et al*., 2016). The results of table 1 showed that TnI was significantly elevated in all groups and this suggests the release might be caused by a combination of a disruption of the cardiomyocyte membrane and a dissociation of the thin-filament troponin complex in the cell (Hessel *et al*., 2008, Gaze and Collinson, 2008).

Elevation of this marker in RD xenograft group may be related to the ability of human tumors of various tissue origins to show an intriguing over-expression of genes not considered as oncogenes, such as encoding TnI (Casas-Tintó *et al*., 2016). Therefore, the presence of RD cells in animals may be the cause of elevated levels of this protein in this group and other groups in addition to the effects of injected drugs on cardiac tissue.

The elevation effect of DOX on serum TnI in (RD+DOX) group supported the hypothesis of DOX cardiotoxicity. Although the precise mechanisms whereby DOX induces myocardial injury are multiple and remain incompletely understood. It is widely accepted that the DOX induces cardiac injury via several mechanisms, including activation of ubiquitin-proteasome system, sarcomere reorganization, induction of pro-inflammatory cytokines, free radical generation and apoptotic cell death that are the typical changes in DOX-induced heart failure (Octavia *et al*., 2012).

Ahmad *et al*., (2017) used DOX to induce Cardiomyopathy in male Wistar rats with six intraperitoneal injections of 2.5 mg/kg over a period of two weeks, this dose produced delayed, progressive, and chronic cardiotoxicity with myocardial lesions that are similar to those reported in human.

Although in the present study where a slight elevation of circulating level was detected in (RD +MTX) group, as far as we know, there are no studies about the toxicological effects of MTX on the cardiac troponin. This elevation may be caused by a disruption of the cardiomyocyte membrane that resulted from oxidative stress generated by MTX (Doostan *et al*., 2017).

The high level of TnI with DMT treatment, may be related to its activity which can be resulted from its lysis inside the cell after entrance of cell membrane or may be from its conversation to another substitution of DOX that it might be lead to a similar effect; or due to its high dose as Compared to DOX, or maybe it contains residues of DOX and MTX or other impurities, which resulted incidentally during the reaction.

The present study was further evaluated the effects DOX, MTX and DMT on serum CK-MB. Generally, the enzyme Creatine kinase, consist of two cytosolic subunits either from brain (B) or muscle (M), appeared as three isoforms including CK-BB, CK-MB and CK-MM. Of these, CK-MB was known as one of the specific indicators for evaluating myocardial tissue injury (Chang *et al*., 2015).

In this study, serum CK-MB was significantly elevated in all groups except (RD+ DMT) and control groups. The high activity and concentration of serum CK-MB of RD xenograft group may be originated from the tumor cells. These results are in agreement with Moss *et al*., (1989) and Sawabe *et al*., (1999) who stated that the concentrations and activity of non-cardiac origin CK- MB in the patient's serum of rhabdomyosarcoma (RMS) were at high levels. Also, Sawabe, *et al*., (1999) suggested that CK-MB, both concentration and activity, are sensitive markers of disease states of RMS than neuron-specific enolase (NSE) and lactate dehydrogenase (LD) to follow up the patients with RMS.

Additionally, the present results showed an increase of serum CK-MB in DOX treatment group (RD+DOX), in addition to (TnI). It might be another evidence for the damage that can be manifested by DOX on the heart tissue by destruction of the cell membrane, increasing permeability of them, and so leakage of these enzymes to the blood stream. These results are in agreement with the findings of Ahmad *et al*., (2017), QuanJun *et al*., (2017) and with other numerous former studies that confirmed similar augmentation in the activity of cardiac enzymes in animals subsequent to a challenge by DOX single and/or cumulative doses (Al Mukhtar *et al*., 2015)

The reduction of serum CK-BM in (RD+ DMT) group may indicate its safety and its nontoxicity on heart cells unlike DOX and MTX and this may be resulted from its different structure and/or activity.

Histological sections of heart tissue of (RD+ DOX) treated group showed focal cellular injury, vaculation of cardiomyocytes and disoriented nuclei, massive degenerative changes, myofibrillar fragmentation of cardiac myocytes, inflammatory cell infiltration between cardiomyocytes, moderate congestion, breakdown of cardiac and secondary fibers and intermuscular hemorrhage, these histopathological changes seem to be early onset chronic, according to Alkuraishy *et al*., (2017) who classified-DOX cardiotoxicity- typically into three types: acute, early onset chronic (within days or weeks) and chronic progressive cardiotoxicity (weeks to months after drug administration). DOX is known to induce NFKB activation and COX-2 expression in cardiomyocytes (Jindal *et al*., 2006). It was reported that DOX can induce changes in the properties of membrane bound ATPases of cardiac cells affecting cardiac function and lead to lethal myocardial cellular injury (Ragavendran *et al*., 2012). Also, DOX therapy augments oxidative stress and disturbs cytosolic calcium homeostasis, increases intracellular calcium levels from the sarcoplasmic reticulum through activation of the ryanodine receptor and by blighting calcium clearance systems in cardiomyocytes (Alkuraishy *et al*., 2017). In addition to that, DOX mediated vascular congestion may contribute to this pathogenesis (Singal and Iliskovic,1999). The present results are in agreement with other studies which found a strong relationship between oxidative stress and the inflammatory response of the cardiac tissue includ-ing release of cytokine release following DOX administration (Bien *et al*., 2007; Rasha *et al*., 2010).

Al Mukhtar *et al*., (2015) concluded that the administration of single dose of (15mg/kg) of DOX caused cardiomyopathy which was manifested by cardiac histopathological sections that showed moderate cytoplasm vacuolization and inflammatory cells infiltrate with vascular congestion, also previous study by Francis, and Nayak, (2017) stated that single dose of 20 mg/kg of DOX caused wide spread of myofibrillar disorganization and extensive myocardial fiber necrosis as evident for two weeks.

Histopathological analysis in group (RD+ MTX) showed mild intermuscular hemorrhage and degenerated cardiac muscle fibers, the administration of MTX may induce oxidative and nutritive stress on heart tissues (Doostan *et al*., 2017). These results are in agreement with what was reported by Abdel-Daim *et al*., 2017 that MTX induces marked histopathological changes in cardiac tissue of mice.

Examination of cardiac muscle sections of (RD +DMT) group showed some changes that indica-

ted its cardiotoxicity and has less significant effect than DOX and MTX with a moderate influence.

CONCLUSIONS

DMT has less cardiotoxicity than DOX and it is highly recommended. Further confirmatory studies need to be conducted using different concentrations and durations of DMT on heart tissue and other tissues with measuring of tissue oxidative stress.

REFERENCE

Kroschinsky, F., Stölzel, F., von Bonin, S., Beutel, G., Kochanek, M., Kiehl, M. and P. Schellongowski, New drugs, new toxicities: severe side effects of modern targeted and immunotherapy of cancer and their management. *Critical Care 21*(1): 89 (2017).

Hosseini, A., and D. Mahdian, Protective effect of lactuca serriola on doxorubicin-induced toxicity in h9c2 cells. *Acta Poloniae Pharmaceutica 73*(3): 659-666 (2016).

Ejam, S.S., Doxorubicin Induced Nephrotoxicity: Protective Effect of Rosmary leaves Journal of Babylon University/Pure and Applied Sciences 7(24): 1898-1908 (2016).

Atheer Abbas Yaseen Al-Fatlawi and Majida Malik Meteab Al-Shammari. Rice bran phytic acid protects against methotrexate -induced oxidative stress and acute liver injury in rats. Kufa Journal For Veterinary Medical Sciences 8 (1): 246-260 (2017).

Beak, J.Y., Huang, W., Parker, J.S., Hicks, S.T., Patterson, C., Simpson, P.C., Ma, A., Jin, J. and B.C. Jensen, An oral selective alpha-1A adrenergic receptor agonist prevents doxorubicin cardiotoxicity. *JACC: Basic to Translational Science 2*(1): 39-53 (2017).

Henri, C., Heinonen, T. and J.C. Tardif, The role of biomarkers in decreasing risk of cardiac toxicity after cancer therapy. *Biomarkers in cancer 8*(Suppl 2): 39 (2016).

Westermann, D., Neumann, J.T., Sörensen, N.A. and S. Blankenberg, High-sensitivity assays for troponin in patients with cardiac disease. Nature Reviews Cardiology (2017).

Gaze DC, Collinson PO (2008) Multiple molecular forms of circulating cardiac troponin: analytical and clinical significance. Ann Clin Biochem 45:349-355 (2008).

Casas-Tintó, S., Maraver, A., Serrano, M. and A. Ferrús, Troponin-I enhances and is required for oncogenic overgrowth. Oncotarget 7(33): 52631 (2016).

Octavia, Y. *et al.* Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. *Journal of molecular and cellular cardiology* 52: 1213–1225 (2012).

Shafique Ahmad, Bibhu Prasad Panda, Kanchan Kohli, Mohammad Fahim and Kiran Dubey, Folic acid ameliorates celecoxib cardiotoxicity in a doxorubicin heart failure rat model, Pharmaceutical Biology, 55:1, 1295-1303 (2017).

Doostan, F., Vafafar, R., Zakeri-Milani, P., Pouri, A., Afshar, R.A. and M.M.Abbasi, Effects of Pomegranate (Punica Granatum L.) Seed and Peel Methanolic Extracts on Oxidative Stress and Lipid Profile Changes Induced by Methotrexate in Rats. *Advanced pharmaceutical bulletin 7*(2): 269 (2017).

Chang,C.C., Liou,C.B., Su1,M.J., Lee,Y.C., Liang, C.T., Ho, J.L., Tsai, H.W., Yen, T.H. and F.Y. Chu, Creatine Kinase (CK)-MB-to-Total-CK Ratio: a Laboratory Indicator for Primary Cancer Screening. *Asian Pacific Jour-nal of Cancer Prevention 16*(15): 6599-6603 (2015).

Moss, D.W., Whitaker, K.B., Munro, A.J. Creatine kinase MB isoenzyme in rhabdomyosarcoma. *Clin.Chem.* 1989, *35*, 896–897.

Sawabe, S., Hara, K. and M. Maekawa, High creatine kinase MB concentration and activity in patients with rhabdomyosarcoma. Rinsho byori. The Japanese journal of clinical pathology 47(11): 1079-1082 (1999).

QuanJun, Y., GenJin, Y., LiLi, W., YongLong, H., Yan, H., Jie, L., JinLu, H., Jin, L., Run, G. and G. Cheng, Protective Effects of Dexrazoxane against Doxorubicin-Induced Cardiotoxicity: A Metabolomic Study. *PloS one 12*(1): e01695 67 (2017).

Al Mukhtar, E.J., Sahib, Z.H., Jawad, A.M. and H. Naji, Rosemary Leaves Aqueous Extract for Protection against Acute Doxorubicin-Induced Cardiotoxicity in Mice. *proteins 7*: 19 (2025).

Alkuraishy, H.M., Al-Gareeb, A.I. and H.A. Alhussaniy, Doxorubicin Induced Cardiotoxicity: Molecular Mechanism and Protection by Conventional Drugs and Natural Products. International Journal of Clinical Oncology and Cancer Research 2(2): 31-44 (2017).

Hamza, A.A., Ahmed, M.M., Elwey, H.M. and A. Amin, Melissa officinalis Protects against Doxorubicin-Induced Cardiotoxicity in Rats and Potentiates Its Anticancer Activity on MCF-7 Cells. PloS one 11(11):1-25 (2016).

Abdel-Daim, M.M., Khalifa, H.A., Abushouk, A.I., Dkhil, M.A. and S.A. Al-Quraishy, Diosmin Attenuates Methotrexate-Induced Hepatic, Renal, and Cardiac Injury in Mice: A Biochemical and Histopathological Study. Oxidative Medicine and Cellular Longevity (2017).