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GENOTOXIC AND CARDIO-PROTECTIVE EFFECTS OF *KALANCHOE INTEGRA* IN DOXORUBICIN-INDUCED CARDIOTOXICITY IN A RAT MODEL

BY

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DECLARATION

DECLARATION BY THE CANDIDATE

I hereby declare that this is the product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

Signature…….. Date: 22/06/2022

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DECLARATION BY SUPERVISORS

We hereby declare that the principal work and presentation of the thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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ABSTRACT

Background: Advancement in cancer therapy has improved survival among patients. However, the use of anticancer drugs like the anthracyclines (e.g., doxorubicin) are not without untoward or side effects. Notable among these is its cardiotoxic effect which ranges from mild transient blood pressure changes to potentially serious heart failure. *Kalanchoe integra* (KI) is used in folklore medicine in the management of several clinical conditions. The components of *KI* predict its cardioprotective potential which could be beneficial among its numerous uses. The aim of the study was to determine the cardio-protective effect of KI against doxorubicin-induced cardiotoxicity using a rat model.

Methods: The leaves of *Kalanchoe integra (KI)* were collected, air-dried, pulverized and extracted using 70% ethanol. HPLC fingerprinting analyses of *KI* was carried out using an Agilent 1100 system (Santa Clara, CA, USA), composed of quaternary pump, autosampler, diode array detector (DAD), and HP ChemStation Software. Chromatographic separation was carried out on a Tskgel ODS C18 (250 x 4.6 mm i.d., 5 µm particle size) analytical column maintained at 40 ºC.

The single cell gel electrophoresis assay (SCGE, also known as comet assay) was also employed to ascertain the genotoxic effects of the *KI* extract. The comet assay was used to study the potential genotoxic effect of *KI* on the liver, kidney, epithelium of the rectum and bone marrow.

A total number of 42 Sprague-Dawley rats (150-200g) were put into 7 groups (n=6). Group I: Vehicle control, received normal saline (1 ml/kg p.o) for 30 days. Group II: Toxic control, received doxorubicin (DOX) (20 mg/kg i.p.) once on the 29th day. Group III: KI control, received KI (300 mg/kg p.o) for 30 days. Group IV: Vitamin E control, received Vitamin E (100 mg/kg

p.o) for 30 days. Group V: KI treated-1, received KI (300 mg/kg p.o) for 30 days and DOX (20 mg/kg i.p) on the 29th day. Group VI: KI treated-2, received KI (600 mg/kg p.o) for 30 days and DOX (20mg/kg i.p) on the 29th day. Group VII: Vitamin E treated, received Vitamin E (100 mg/kg p.o) for 30 days and DOX (20mg/kg i.p) on the 29th day. Aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), enzymatic antioxidants such as glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) were assayed from blood taken via cardiac puncture 36 hours after last treatment administration. Excised hearts from rats from each group were taken through histopathological examination.

Results: In the HPLC fingerprint analysis, 13 peaks were identified. The first peak with retention time of 3.4min had a peak area of 1.1754 x10 ^{4} mAU and overall percentage peak area of 19.2%. Also peak with retention time of 24.0 min had the highest peak area of 3.223 x10^{4} mAU and overall percentage peak area of 52.63% which is directly proportional to high concentration of that compound in the plant extract.

The criterion for toxicity was a statistically significant increase in the number of deoxyribonucleic acid (DNA) comets in the organs and tissues studied compared with controls. Results showed that the *KI* extract was non-genotoxic.

Pre-treatment with *KI* protected rats against doxorubicin- induced cardiotoxicity as evidenced by the low levels of AST, ALT, ALP, CK and LDH compared with the controls ($p < 0.05$). SOD, CAT and GPX levels were also high for *KI* extracts; further showing that *KI* protected rats against doxorubicin induced cardiotoxicity. Histological examinations revealed that in the *KI* pretreated groups there were no signs of abnormal myocardial fibers. The myocardia were of

normal shape, size and configuration. It was also observed that there was no evidence of vacuolation, neither was there any sign of necrosis or inflammation. The observations contrasted with what was observed in the doxorubicin only group (without treatment). Rats in the doxorubicin only group showed signs of abnormal hypertrophic myocardial fibers, and the myocardia also had small and large vacuoles.

Conclusion: The current study showed that the ethanolic (70%) leaf extract of KI offered cardioprotection in rats administered with doxorubicin. This is evident in AST, ALT, ALP, CK, LDH, GPX, SOD and CAT levels of assayed blood samples. KI also protected the heart of the rats against histopathological changes such as necrosis, abnormal myocardial fibers, and edema. The study also showed that *KI* is non-genotoxic.

DEDICATION

This work is dedicated to God Almighty and to the Department of Pharmacology and Toxicology, School of Pharmacy, University of Ghana.

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ACE	Angiotensin-converting enzyme
ALP	Alanine phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AREs	Antioxidant response elements
ASK1	Apoptosis signal-regulating kinase 1
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
Bcl 2	b-cell lymphoma 2
CBR3	type 3 carbonyl reductase
CAT	Catalase
CARP	Cardiac ankyrin repeat protein
CHF	Congestive heart failure
CK	Creatine kinase
DAD	Diode array detector
DAU	Daunorubicin
DMSO	Dimethyl sulfoxide

DOX	Doxorubicin
DPPH	2,2-diphenyl-1-picrylhydrazyl
DR	death receptors
ECG	Electrocardiographic
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial NOS
ERNA	Equilibrium radionuclide angiography
GSH	Glutathione
GPX	Glutathione peroxidase
iNOS	Inducible NO synthase
IREs	Iron-responsive elements
IRP	Iron regulatory protein 1
H&E	hematoxylin–eosin
HPLC	High performance liquid chromatography
JNK	c-Jun NH2-terminal kinase
KI	*Kalanchoe integra*
LDH	Lactate dehydrogenase
LV	Left ventricular

LVEF	Left Ventricular Ejection Fraction
MDA	Malondialdehyde
miRNA	MicroRNAs
MnSOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenosine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthases
NOX	NADPH oxidase
Nrf2	Nuclear factor-erythroid 2 related factor 2
PBS	Phosphate buffered saline
PPAR	Peroxisome proliferator-activated receptor
RNS	Reactive nitrogen species
ROX	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Scientist
TNFR1	tumor necrosis factor receptor 1
TnI	Troponin I

TnT	Troponin T
Top 2	Topoisomerase 2
VDAC	Voltage-dependent anion channels
VE/VIT E	Vitamin E
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background

Pharmacotherapeutic advancement has improved cancer management. Although these pharmacological agents are effective in cancer management, they often possess some cardiotoxic effect (Smith et al. 2010). Anti-cancer drugs known to be cardiotoxic include: anthracyclines (eg. doxorubicin), taxoids (eg. paclitaxel), 5-fluorouracil, cyclophosphamide and trastuzumab (monoclonal antibody). Cardiotoxic potential of these drugs could range from mild transient blood pressure changes to potentially serious heart failure (Chen et al., 2010).

Doxorubicin (DOX) belongs to the family of anthracycline antibiotics. DOX, a highly proven antineoplastic agent is effective in the treatment of both adult and pediatric leukemia, lymphoma, breast cancer and solid tumors (Allen, 1992; Saeed et. al, 2015). DOX is known to possess cardiotoxic effects like most anti-cancer drugs (Kojima et. al, 1995; Smith et. al, 2010). The cardiotoxic effect of DOX can be acute, which can result in atrial and ventricular dysrhythmias (Friess et. al, 1985; Steinberg et. al, 1987). Chronic cardiotoxic effect of DOX can result in cumulative dose-dependent cardiomyopathy, which may eventually lead to congestive heart failure (Lipshultz, et. al, 1991; Nysom et. al, 1998).

The principle mechanism of doxorubicin-induced cardiotoxicity is oxidative stress, which can cause increased levels of reactive oxygen species (ROS). An increase in ROS causes lipid peroxidation (Xu. P et al. 2002; Li. R, et al. 2012) which leads to the activation of apoptotic signals (Nicobe et al. 2003). Another mechanism of doxorubicin-induced cardiotoxicity is intracellular calcium dysregulation (Kalivendi et. al, 2005) which could lead to the activation of

calcium-dependent proteases (calpains) (Jang et al. 2004) and caspase-12 cleavage. These can further cause apoptotic pathway activation (Nagakawa et al. 2000). It has been suggested that the use of agents with strong antioxidant potential could help avert doxorubicin-induced cardiotoxicity (Deres et al. 2005).

Kalanchoe is a genus of succulent perennial plants that thrive at temperate areas of the world. Over 200 *Kalanchoe* species have been identified in Africa which have been used medicinally, especially *Kalanchoe pinnata* and *Kalanchoe integra*. It has been reported that *Kalanchoe* contain appreciable amounts of both phenolic compounds and flavonoid (Adenike and Eretan, 2004; Asiedu-Gyekye et al. 2012). *K. integra* is reported to possesses strong antioxidant property in a study where 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used in the evaluation of antioxidant activity of aqueous and methanolic extracts of *K. integra and K. pinnata* (Asiedu-Gyekye et al. 2012). The cogent antioxidant activity exhibited by these two species of *Kalanchoe* shows that they could be free radical scavengers. It is thus postulated that these plant species could possibly decrease the free radical chain of reactions associated with doxorubicin-induced cardiotoxicity.

1.2 Problem Statement

Although DOX has become one of the most effective chemotherapeutic agents, its use is complicated by cardiotoxicity (Singal et al. 2000). In a retrospective study with over 4000 patients receiving DOX, 2.2% of the patients developed clinical signs and symptoms of congestive heart failure (Von Hoff et al. 1979).

Subsequent analysis of three trials of DOX treatment of breast cancer or small cell lung cancer (Multi-center trials 088001, 088006 and 088002) in which left ventricular ejection fraction

(LVEF) was measured by equilibrium radionuclide angiography (ERNA) demonstrated that 5.1% of patients had evidence of congestive heart failure or a significant decline in left ventricular function, thus, confirming the cumulative dose-dependence of doxorubicin cardiotoxicity (Swain et al. 2003).

Despite several research, formal estimates of the worldwide prevalence of anthracycline-induced cardiotoxicity is lacking (Gianni et al. 2008). This is due to differences between the presentation of anthracycline-induced cardiotoxicity among pediatric, adult and elderly patients. Furthermore, there is a lack of uniformity in detecting and reporting cardiac events which make such estimates even more difficult to ascertain (Gianni et al. 2008).

There have been many attempts to mitigate anthracycline-induced cardiotoxicity but with little success (Ewer and Ewer, 2015). Some of these include the use of angiotensin-converting enzyme inhibitors (Colombo et al. 2013), beta blockers (Colombo et al. 2013), liposomal and nanoparticle anthracycline delivery systems (Zhu et al. 2015) and cardio-protective agents such as dexrazoxane (Hale JP and Lewis IJ, 1994). Although there has been technological improvement in the treatment of anthracycline-induced cardiotoxicity, these adverse effects still exist and often decrease the quality of life of patients (Gibson et al. 2014).

1.3 Justification

Doxorubicin (anthracycline antibiotic) is among the most effective and commonly used anticancer drugs. However, its clinical use is limited by its dose-dependent cardiotoxicity. There is, therefore, the need to identify effective cardio-protective agents to mitigate anthracycline-induced cardiotoxicity. About 80% of the world's population, primarily those of developing countries rely on traditional medicine for their primary health care needs (WHO, 2003). This is

because it is cheaper and more accessible than orthodox medicine (Sofowora, 1982). *Kalanchoe species* has been found to possess hypotensive and central nervous system depressant effect (Kukuia et. al 2015). *Kalanchoe integra* is known to have substantial amounts of flavonoids and phenols and has a relatively high antioxidant potential (Asiedu-Gyekye et al, 2012). There is, however, no known report on the cardio-protective profile of Kalanchoe integra. This study, therefore, sought to ascertain the cardio-protective property of *Kalanchoe integra*, a plant known to have good antioxidant property.

Additionally, agents used for their therapeutic properties have to be assessed for safety. There are several assays and animals' models of assessment. Amongst these include assessment of genotoxicity so as to avoid the potential DNA damage that can be caused by *Kalanchoe integra* extract. The comet assay technique was used in determining whether ethanolic leaves extract of *Kalanchoe integra* exhibited any form of DNA damage to the animals. Also, HPLC fingerprinting analysis was performed to assess the number of phytochemical compounds present in the *Kalanchoe integra* extract.

1.4 Hypothesis

Kalanchoe integra extract could protect rats from doxorubicin-induced cardiotoxicity.

1.4 Aim

This research sought to determine genotoxic and cardio-protective potential of *Kalanchoe integra* against doxorubicin-induced cardiotoxicity in a rat model.

1.5 Specific Objectives

1. To examine the possibility of genotoxicity of *KI* in selected organs.

2. To determine levels of selected biomarkers as an index of cardiotoxicity in serum obtained from different rat treatment groups.
3. To determine histological changes in the heart tissue of rats following doxorubicin-induced toxicity and administration of the agent.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cardiotoxicity associated with cancer chemotherapy

Cancer mortality rate has fallen due to the improvement in screening techniques, diagnostic testing and more productive therapies. Among these novel therapies are cardiac imaging for early detection of cardiotoxicity. The goal of cardiac imaging is to assess cardiac structure and function to identify early cardiac injury. This includes echocardiography, nuclear imaging, and magnetic resonance imaging (MRI). Although survival rates of cancer patients have improved, there has been greater recognition of the late effects associated with cancer treatment including cardiotoxicity. The US National Cancer Institute defines cardiotoxicity as one that usually affect the heart (NCI, 2014). In the case of cancer chemotherapy the definition encompasses defects such as left ventricular (LV) dysfunction, cardiac dysfunction and heart failure, myocardial ischemia or infarction, valvular abnormalities, coronary heart disease, pericardial disease, hypertension, and arrhythmias (Yeh and Bickford, 2009). A wide range of research has been conducted into Chemotherapy-induced cardiotoxicity especially anthracycline-induced cardiotoxicity where cardiotoxicity generally refers to cardiac dysfunction and failure (Scherrer-Crosbie, 2017). New chemotherapeutic agents, in particular the vascular endothelial growth factor (VEGF) inhibitors such as bevacizumab, sunitinib, and sorafenib, are able to increase the incidence of myocardial ischemia (Choueiri *et al*., 2010; Ranpura *et al*., 2010) and exhibit indirect cardiotoxicity masked as hypertension. Even radiotherapy which is the treatment of choice for many has even broader cardiotoxic effects, including significant increases in cardiac ischemia (Heidenreich *et al*., 2007; Nilsson *et al*., 2012), valvular abnormalities (Heidenreich *et al*., 2003) and pericardial disease. However, the largest body of research has been dedicated to

anthracycline-induced cardiotoxicity owing to improved outcomes as a result of targeted therapy with anthracyclines and its attendant cardiotoxicity (Scherrer-Crosbie, 2017; McGowan *et al*., 2017).

2.1.1 Anthracycline-induced cardiotoxicity

Anthracyclines are potent antineoplastic agents with proven efficacy in the treatment of many pediatric and adult hematologic and solid organ cancers (Groarke and Nohria, 2015). The coincidental discovery of doxorubicin from *Streptomyces peucetius* and its precursor daunorubicin was a turning point in chemotherapy (Rimal *et al*., 2015). Other anthracyclines include epirubicin and idarubicin. Dose-dependent anthracycline-induced cardiomyocyte injury and death resulting in left ventricular (LV) dysfunction and heart failure, is the most notorious and well-studied chemotherapy-induced cardiovascular toxicity that was first described for different tumors (Groarke and Nohria, 2015). The problem of anthracycline-induced cardiotoxicity is growing with increasing cancer survivorship and increasing use of anthracyclines in people predisposed to adverse cardiac effects such as the elderly, those with cardiovascular comorbidities as well as those receiving additional chemotherapies (Henriksen, 2018). Disease spectrum for anthracycline-induced cardiotoxicity ranges from development of heart failure with symptoms and clinical signs to asymptomatic decline in systolic function determined through measurement of left ventricular ejection fraction (LVEF) (Henriksen, 2018). There is a dearth of information regarding the relationship between asymptomatic decline in LVEF following anthracycline treatment and subsequent development of heart failure but asymptomatic LV dysfunction occurs as a result of increased risk of future congestive cardiac failure and death (Wang *et al*., 2003; Henriksen, 2018).

2.1.1.1 Risk factors of anthracycline-induced cardiotoxicity

The most common risk factors associated with anthracycline-induced cardiotoxicity include cumulative dose, gender, African-American ancestry, age (>65 years or <18 years), renal failure and concurrent exposure of the heart to radiation therapy (Henrikson, 2017). Medical conditions such as cardiac diseases that cause myocardial strain including hypertension and valvular disease also increase the risk of heart failure (Zamorano *et al*., 2016; Henriksen, 2017). Individual susceptibility to anthracycline-induced cardiotoxicity could also be linked to genetic predisposition. Increased tissue iron concentration orchestrated by diseased conditions intensify cardiotoxicity through the formation of anthracycline–iron complexes. A typical example is the case of Hemochromatosis whereby tissues are overloaded with iron and that carriers of the haemochromatosis C282Y HFE genetic mutation are highly susceptible to cardiotoxicity (Lipshultz et al., 2013; Henriksen, 2018). Differences in rates of metabolism of anthracyclines and the production of secondary metabolites influence cardiotoxicity. Overexpression of type 3 carbonyl reductase (CBR3) has been associated with increased production of secondary metabolites in response to doxorubicin with triggering a rapid onset of cardiomyopathy (Henriksen, 2018; Forrest *et al*., 2000).

A study conducted by Vejpongsa *et al.* (2014) proposed that levels of Top 2β in peripheral blood leucocytes could be used as a substitute of cardiac expression and a marker of susceptibility to anthracycline cardiotoxicity. The findings from this study demonstrated that resistance to anthracycline cardiotoxicity in patients could be linked to low Top 2β concentrations (Vejpongsa *et al*., 2013).

2.1.1.2 Classification of anthracycline-induced cardiotoxicity

Anthracycline-induced toxicity of the cardiovascular system can be categorized into acute, chronic and late-onset (delayed) (Balindiwe, 2015) even though studies have reported subclinical cardiotoxicity, which expresses as congestive heart failure (CHF).

2.1.1.2.1 Acute cardiotoxicity

Acute and sub-acute cardiotoxicity is a dose-independent cardiotoxicity classified as asymptomatic electrocardiographic (ECG) changes, transient arrhythmias, tachycardia, hypotension, and myocarditis (Marechal *et al*., 2011). These abnormalities following anthracycline administration are generally not considered a cause for major concern because they are reversible, they resolve unexpectedly, and/or are clinically manageable or are even usually undetected (Marechal *et al*., 2011). Assay of plasma concentrations of cardiac troponin I (TnI), a regulatory protein responsible for initiating contractile activity in the myocardium, is a sensitive tool used to identify acute myocardial injury, left ventricular damage and inadequate cardiac output (Barrett-Lee *et al*., 2009) caused by TnI elevation immediately after a high dose of anthracyclines. TnI elevation can also be used as a predictor of the development of ventricular dysfunction. Incidentally, troponin T (TnT) has also been implicated in the diagnosis and prognosis of damage to cardiomyocytes (Sparano *et al*., 2002).

2.1.1.2.2 Chronic Cardiotoxicity

Chronic anthracycline-induced cardiotoxicity has the tendency to manifest several months or even years after treatment and is classified clinically as the most injurious type of toxicity as it is dose-dependent (Von Hoff *et al*. (1979). Chronic cardiotoxicity ultimately leads to irreversible

cardiomyopathy in affected patients. Typical clinical manifestations of chronic anthracycline-induced cardiotoxicity include an excessive decline in both blood pressure and ejection fraction, a distinctly increased heart rate, and ventricular dilatation preceding heart failure (Lefrak *et al*., 1973). Additionally, cardiomyopathy induced by chemotherapeutic agents can be further classified following specific pathological changes in order to determine the severity of heart damage using the Billingham scale (Billingham *et al*., 1978). Cardiac biopsies from affected patients feature atrophic cells with smaller diameters as well as cytoplasmic vacuolization caused by dilatation. The actual prevalence of this type of cardiotoxicity is, however, difficult to predict or even to determine accurately since it can take years to manifest and clinical trial follow-up time has been inadequate (Swain *et al*., 2003).

2.1.1.2.3 Late-onset (delayed) cardiotoxicity

Late-onset or delayed cardiotoxicity is a dose-dependent cardiotoxicity which occurs several years or even decades after exposure to anthracyclines including doxorubicin. This adverse form of toxicity has been detected in patients who have previously been exposed to anthracyclines during childhood (Šimùnek *et al*., 2009). Cardiovascular stressors such as surgery, pregnancy, weight-lifting, and acute viral infection are possible triggers for late-onset anthracycline-induced cardiotoxicity (Sereno *et al*., 2008).

2.2 Doxorubicin

Doxorubicin (also called Adriamycin) together with daunorubicin (DAU, also known as Daunomycin and Rubidomycin) were obtained from the bacterium *Streptomyces peucetius var.*

caesius, which secretes pigments in the early 1960s and is a member of the family of drugs known as anthracyclines (Arcamone *et al*., 1969; Dimarco et al., 1964; Takemura et al., 2007; dos Santos and dos Santos Goldenberg, 2018). Doxorubicin is one of the most prescribed antineoplastic agents. It is effective for the management of different types of cancers in both the old and young (including children) (Takemura *et al*., 2007; Octavia *et al*., 2012; Simůnek *et al*., 2009). Doxorubicin is used in the treatment of breast cancer, soft tissue sarcomas, childhood tumors such as Wilms' tumor, leukemias, Hodgkin's and non-Hodgkin's lymphoma and several other cancer types (Octavia *et al*., 2012; Simůnek *et al*., 2009). In spite of its strong anticancer properties, usage of doxorubicin has been impeded by typical toxicological events such as hematogenic inhibition, nausea, vomiting, burping, loss of hair, formation of impervious tumor cells or damage to healthy tissues, mostly with severe cardiac toxicity exhibited by congestive cardiomyopathy (Octavia *et al*., 2012; Simůnek *et al*., 2009; Minotti *et al*., 2004; Outomuro *et al*., 2007). Several analogues of the parent doxorubicin compound have emerged with the aim of lowering untoward events of doxorubicin but only a few analogues have progressed to the clinical development and approval phase, one such analogue is epirubicin (EPI) which has a spectrum of activity similar to doxorubicin. (Minotti *et al*., 2004). Despite the development of new alternative drugs to replace doxorubicin, the risk of developing cardiotoxicity still persist (Minotti *et al*., 2004; Outomuro *et al*., 2007; dos Santos and dos Santos Goldenberg, 2018) since doxorubicin continues to be considered as a first line antineoplastic drug (Outomuro *et al*., 2007).

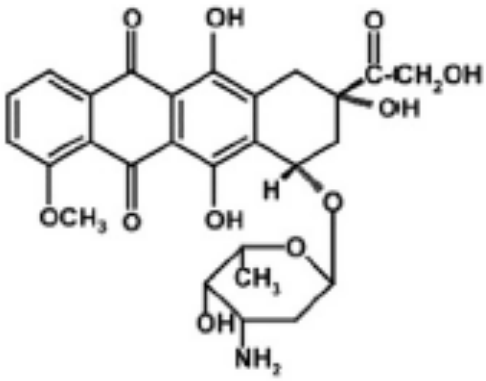


Fig. 2.1 Chemical structure of doxorubicin

2.2.1 Doxorubicin-induced cardiotoxicity

DOX-evoked cardiotoxicity has long been identified as a drawback of cancer therapy (Simůnek *et al*., 2009). The first reported event of DOX related cardiotoxicity in literature was of a 23-year-old patient with evidence of osteosarcoma, he received DOX treatment for 9 months but a month post treatment he died. The post -mortem results showed that the patient died because he developed congestive heart failure as a result of the treatment (Lefrak *et al*., 1973). Long exposure cardiotoxic activity were later recognised in children with acute lymphoid leukaemia (Lipshultz *et al*., 1991; dos Santos and dos Santos Goldenberg, 2018). Children diagnosed with cancer who are on doxorubicin therapy stand a higher chance of showing symptomatic cardiac toxicity at early stages of treatment. The risk stays high even 30 years after treatment. It is believed that for every 8 patients treated with doxorubicin one of them will show symptoms of extreme cardiac illness. (Raj *et al*., 2014; dos Santos and dos Santos Goldenberg, 2018). There are several manifestations of doxorubicin-induced cardiotoxicity, extending from asymptomatic electrocardiography (ECG)-modifications to deteriorated cardiomyopathy marked by declining left ventricular ejection fraction (Octavia *et al*., 2012; Outomuro *et al*., 2007).

2.2.2 Mechanisms of Doxorubicin-induced Cardiotoxicity

Despite its well-documented cardiotoxic effects, doxorubicin is an important antineoplastic agent because of its high antitumor efficacy in various cancer types. Even though the mechanisms involved in Doxorubicin-induced cardiotoxicity are not completely understood, research suggest that it is a multi-facet process, with varying possible pathways involved which eventually result in death of cardiomyocytes (Raj *et al*., 2014; Salazar-Mendiguchía *et al*., 2014; Ghigo *et al*., 2016). Different studies have suggested involvement of oxidative stress, iron metabolism, Ca^{2+} homeostatic imbalance, structural modifications of the sarcomere, issues with regulation of gene expression, and apoptotic dysregulation (Geiger et al., 2010; Octavia *et al*., 2012; Salazar-Mendiguchía *et al*., 2014; Ghigo *et al*., 2016).

2.2.2.1 Oxidative Stress Mechanism in Dox-induced Cardiotoxicity

The most frequently proposed mechanism for the pathophysiology of DOX-induced cardiotoxicity has been attributed to Oxidative Stress (Takemura et al., 2007; Simůnek *et al*., 2009; Salazar Mendiguchía *et al*., 2014). This is defined as the disparity between the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and intrinsic antioxidant system (Renu *et al*., 2018). The heart's unique vulnerability to oxidative stress (Doroshow *et al*., 1980) has given this aspect of doxorubicin-induced cardiomyopathy an overwhelming prominence in literature. The reactive oxygen species, reactive nitrogen species generated in the cardiomyocytes are not successfully offset by the intrinsic antioxidant systems of the cell (Tham *et al*., 2015). Oxidative stress increases when the collective dose of doxorubicin goes beyond 500mg/m^2 body surface area (Pereira *et al*., 2011). The Doxorubicin-induced oxidative stress mechanism is mediated via several molecular mechanisms.

2.2.2.1.1 Mitochondrial dependent reactive oxygen species

Subcellular organelle modifications occur frequently in DOX-induced cardiotoxicity and one of such organelle modifications is as a result of changes in mitochondria of cardiac muscles. A huge percentage of ATP (90%) available to the cardiomyocytes is generated by the mitochondria. High reactive oxygen species generation in doxorubicin chemotherapy is mediated via the lowering of the redox cycling in complex I of electron transport chain (ETC) (Alexieva *et al*., 2014; Davies and Doroshow, 1986; Davies *et al*., 1983; Doroshow and Davies, 1986; Renu *et al*., 2018). Structural changes in the ultrastructure of mitochondria leads to interference of ATP synthesis. In contrast with other tissues cardiac muscles are supplied with 35–40% more mitochondrial number (Goffart *et al*., 2004). One of the most important components of inner mitochondrial membrane which is implicated in the occurrence of doxorubicin-regulated disease-causing situation is cardiolipin. The anion charge on the cardiolipin attracts the cationic charge on doxorubicin resulting in the formation of an irreversible complex (Parker *et al*., 2001). When this happens, doxorubicin remains in the mitochondria. High amount of doxorubicin within the mitochondria exceeding 50–100 μM implies a high level of reactive oxygen species (Sarvazyan, 1996) leading to oxidation of signaling molecules (Kuznetsov *et al*., 2011). The vital role of cardiolipin is the actuation of enzymes such as cytochrome C oxidase, NADH cytochrome C oxidoreductase among others within the electron transport chain. The bonding between cardiolipin and doxorubicin makes it unavailable for triggering the enzymes which are important constituents of complex II and complex IV of the electron transport chain. This impacts on oxidative phosphorylation which ultimately leads to cardiotoxicity (Goormaghtigh *et al*., 1980). The formation of free radicals from mitochondria is mitigated by the increase in manganese superoxide dismutase (MnSOD) (Pani *et al*., 2000). MnSOD is mostly located within the matrix

of mitochondria and thus serves as a superoxide scavenger in mitochondria (Fridovich, 1995). Expulsion of MnSOD decreases mitochondrial survival and activity as well as its stability thus leading to apoptosis and subsequent myocardial dysfunction (Li et al., 1995).

DOX -evoked programmed cell death is mediated via high superoxide dismutase (SOD) and haem oxygenase and maintaining mitochondrial membrane integrity which can be protected by calceolarioside (Kim et al., 2006). Doxorubicin thus halts the mitochondrial metabolism and generation of the end product, which results in cell death. This mechanism in mice can be repaired permitting the mice to breathe in carbon monoxide. The carbon monoxide controls the coding of the gene essential for mitochondrial process and also increasing the expression of gene needed for the heme oxygenase (Piantadosi et al., 2008; Suliman et al., 2007). High quantities of reactive oxygen species cause pathological modifications in lipid, protein, nucleic acids and signaling molecules (Berthiaume and Wallace, 2007).

2.2.2.1.2 *Role of NADPH in the production of reactive oxygen species*

The principal mechanism by which doxorubicin exhibits its toxicological effects is mostly associated with the production of free radicals which ultimately leads to the activation of free radical species. Doxorubicin redox cycle produces these free radicals catalyzed by enzymes such as nicotinamide adenosine dinucleotide phosphate (NADPH), mitochondrial NADH dehydrogenase (Bahadır *et al*., 2014). The level of NADPH oxidase is activated by Angiotensin II (Ang II) which controls reactive oxygen species generation when 1µM dose of doxorubicin treatment is administered (Gilleron *et al*., 2009). Doxorubicin cardiotoxicity is suppressed by blocking angiotensin-converting enzyme (ACE) (Sacco *et al*., 2009). When doxorubicin is used in the treatment of cardiomyoblast, it increases the concentration of NADPH oxidase (NOX) and

enzymes like P450 reductase, nitric oxide synthase resulting in elevated concentrations of ROS and eventually leading to oxidative stress (Gilleron *et al*., 2009).

2.2.2.1.3 *Role of nitric oxide in oxidative stress*

Nitric oxide (NO), a vasodilator which mediates contraction of the heart and that it is present in larger quantities in a diseased heart. Nitric oxide synthases (NOS) have been implicated in doxorubicin-induced ROS generation in the muscle tissue of the heart. NOS regulates nitric oxide (NO) synthesis from L-arginine and oxygen (Moody et al., 2011; dos Santos and dos Santos Goldenberg, 2018). cardiac NO production is accomplished by endothelial NOS (eNOS and inducible NO synthase (iNOS). During doxorubicin treatment, the level of iNOS and NO increases and superoxide anions generated by the actuated NADPH oxidase (NOX), reacts with NO resulting in the generation of peroxynitrite through lipid peroxidation (Renu *et al*., 2018). The peroxynitrite oxides causes oxidative stress in the mitochondria, programmed cell death and necrosis. Using amino guanidine to block iNOS shields against doxorubicin-induced cardiotoxicity (Bahadır *et al*., 2014; Renu *et al*., 2018). NO production can also be impeded by directly binding doxorubicin to the endothelial NOS (eNOS) reductase domain. This causes a decrease in the doxorubicin semiquinone radical, which reacts with oxygen and generates superoxides (dos Santos and dos Santos Goldenberg, 2018).

2.2.2.1.4 Role of nrf2 in oxidative stress

Nuclear factor-erythroid 2 related factor 2 (Nrf2) is a common master transcription factor that upregulates antioxidant response elements (AREs)-mediated expression of antioxidant enzyme

and cytoprotective proteins (Zhao *et al*., 2017). Nrf2 is also implicated in doxorubicin-evoked cardiotoxicity. Low levels of Nrf2 intensifies cardiotoxicity which affects cardiac activity (Renu *et al*., 2018). Compounds that have the ability to increase the expression of Nrf2 exhibit protection against doxorubicin-induced cardiomyopathies (Renu *et al*., 2018; Zhao *et al*., 2017). Doxorubicin impairs autophagy which leads to cardiotoxicity but Nrf2 inhibits oxidative stress via self-instigating autophagy and controlling the parity between oxidative stress and autophagy although the specific pathway has not been elucidated (Li *et al*., 2014).

2.2.2.2 Apoptosis

Pathological conditions such as left ventricular dysfunction and hypertrophy, infracted and reperfused myocardium, diabetes and others could induce apoptosis directly (Freude et al., 2000; Guerra et al., 1999; Sam et al., 2000; Tea et al., 1999; Zhou et al., 2000). Programmed cell death occurs because of high levels of oxidative stress, calcium deficits, opening of the mitochondrial pore, compromising on mitochondrial integrity and function (Tokarska-Schlattner et al., 2006). Doxorubicin-induced apoptosis however occurs via different mechanisms such as oxidative stress in both acute and chronic cardiotoxicity. Increase in oxidative stress has been long implicated in apoptosis with several antioxidants proven to inhibit the process (dos Santos and dos Santos Gute, 2018). Oxidative stress causes increased release of cytochrome c via voltage-dependent anion channels (VDAC) and triggering of caspase 3 in mitochondria which results in apoptosis within the myocardium (Renu *et al*., 2018). The effect of cell death is elevated by b-cell lymphoma 2 – (Bcl 2): Bcl-2-like protein 4 (bax) ratio. The effectiveness of mitochondria is elevated (P/O ratio) and a high action of SOD (Childs et al., 2002). Generation of ROS also elevates apoptotic extent by triggering the apoptosis signal-regulating kinase 1(ASK1) signaling

(Gilleron et al., 2009) that triggers the c-Jun NH2-terminal kinase (JNK) and p38 MAPK mechanisms to evoke cell death (Kim et al., 2007).

Doxorubicin mediates apoptosis by enhancing p53, decreasing the amount of GATA-4 expression and p300 breakdown (Aries *et al*., 2004; Kawamura et al., 2004; Kim *et al*., 2003; Liu *et al*., 2008, 2004; Park *et al*., 2010; Poizat et al., 2005). In doxorubicin cardiotoxicity, there is a triggering of oxidative stress-dependent heat shock factor such as HSF-1 that in turn triggers HSP25 and eventually, p53 is equalized resulting in high generation of pro-apoptotic proteins (Vedam *et al*., 2010). During doxorubicin treated conditions, there is a collection of ceramide (Andrieu-abadie *et al*., 1999; D'Anglemont De Tassigny *et al*., 2004) which alters the signaling mechanisms and increases apoptosis in cardiomyocytes by either mitochondrial L-carnitine and via the channel volume-sensitive chloride ion. Doxorubicin also triggers MAPK, p38 and JNK (c-Jun-NH(2)-terminal kinase) (Xie et al., 2009; Xu et al., 2005) resulting in cell death by inhibiting the Bcl2, Bax, cleaved caspase-9 and cleaved caspase-3 (Chatterjee et al., 2010; Liu et al., 2004). It increases the expression of the death receptors (DR) like tumour necrosis factor receptor 1 (TNFR1), fas cell surface death receptor (Fas), DR4, and DR5 in cardiomyocyte. The increased expression accounts for activation of caspase cascade which leads to apoptosis (Zhao and Zhang, 2017).

2.2.2.3 Iron Metabolism

Doxorubicin alters iron synthesis because of its robust affinity for iron, as a result forming iron-doxorubicin complexes which consequently react with oxygen and trigger ROS production (Gutteridge, 1984). In functional situations, there is not sufficient available iron to react with doxorubicin to cause cardiomyopathy (Minnotti *et al*., 2004), but iron-doxorubicin induced

cardiotoxicity results from the disruption of doxorubicin in the function of proteins which conveys and bind iron within the cell (dos Santos and dos Santos Goldenberg, 2018). Doxorubicinol a metabolite is involved in the mechanism which removes iron from the catalytic Fe-S cluster of the cytoplasmic aconitase (also called iron regulatory protein 1; IRP-1), changing the enzyme to a null protein. Eventually there is an elevation in the integrity of transferrin mRNA and inhibiting translation of iron sequestration proteins. This leads to a decline in IRP-1 which brings about high amount of free iron, which can cause free radical formation (Minotti 1998; Minotti, 2001). Other researchers report that doxorubicin can react with iron-responsive elements (IREs) of the ferritin heavy and light chains which works as an iron transporter, decreasing the amount of available iron intracellularly. The eventual disturbance of this protein causes in an upsurge of free iron, which evokes damages to the myocardium (Canzoneri et al., 2008). Accumulation of free iron inside the myocardium after doxorubicin therapy maybe the primary causal factor of DIC (Ichikawa et al., 2014). It is therefore probable that the decrease in iron numbers is an efficient approach to inhibiting doxorubicin-evoked cardiomyopathy.

2.2.2.4 Sarcomeric structure alterations

Doxorubicin-induced cardiotoxicity causes a depletion of myofilaments of the sarcomere. A giant protein known as titin which happens to be an essential constituent of the cardiac sarcomeres, extends from the M-line to the Z-disk has both structural and regulatory functions (Gautel, 2011). It is believed that compromising on the stability activity of titin is directly linked to the occurrence of dilated cardiomyopathy (Herman *et al*., 2012; McNally, 2012). Doxorubicin causes fast breakdown of titin by activating proteolytic pathways, causing a disparity in the strength of the heart muscle tissue. There is evidence to show that breakdown of titin also takes

place by triggering proteases that are reliant on calcium (calpains) whose inhibition is responsible for preserving cardiac function following doxorubicin treatment (Nakagawa et al., 2000). Reduction in the cardiac ankyrin repeat protein (CARP) can cause marked sarcomeric disarray (Lim *et al*., 2004). CARPs are involved in negative control of cardiac gene expression. It is essential to acknowledge that other proteins are vital for sarcomeric cytoskeleton like myomesin, α-actinin and nebulin (dos Santos and dos Santos Goldenberg, 2018). In essence the sarcomeric structure stability is central in preventing doxorubicin-induced cardiotoxicity.

2.2.2.5 Calcium homeostasis dysregulation

Evidence from studies points to the fact that uncontrolled homeostasis of calcium is implicated in the pathophysiology of doxorubicin-induced cardiotoxicity. The regulation of calcium quantity during the contraction-relaxation cycle in cardiomyocytes is very essential for typical heartbeat contractile action (Zarain-Herzberg *et al*., 2012). Increase in intracellular calcium concentrations has been attributed to several mechanisms (Octavia *et al*., 2012; Salazar-Mendiguchía *et al*., 2014) one of which is linked to doxorubicin synthesis, which produces a dangerous metabolite (doxorubicinol), via the decrease of its carbonyl group. This metabolite has the potential to impair the sodium-calcium exchanger medium (Fu et al., 1990). The activity of the sodium/potassium pump of the sarcolemma is also affected by the doxorubicinol, which disrupts the sodium gradient needed for calcium to flow into the sarcolemma of a cardiomyocyte (Zhou *et al*., 2001). This causes a disparity in the strength of the myocardium and diminishes systolic activity (Fu et al., 1990).

The adverse effects of doxorubicinol is due to accumulation of this metabolite which accounts for notable misregulation of calcium homeostasis, causing myocardial injury. Typical calcium homeostasis is modified by reactive oxygen species and hydrogen peroxide through interference of normal sarcoplasmic reticulum activity. This is achieved by blocking the Ca^{2+}-ATPase pumps, through reduction in the expression of SERCA2a mRNA levels and/or the direct activation of the ryanodine calcium-release channels themselves (Arai et al., 2000). Another research proposes that DOX evokes calcium release from the sarcoplasmic reticulum by elevating the number of times the channels are opened (Holmberg, 1990) and concurrently evoking the impeding of sodium-calcium channels in the plasma membrane while elevating L-type calcium channel activation (Caroni et al., 1981; Keung et al., 1991). Doxorubicin has also been shown to cause a decrease in the calcium storage capacity of mitochondria by specifically activating the selective CsA-sensitive calcium channel, amplifying the calcium-overload (Zhou et al., 2001). This leads to high calcium cytoplasmic concentrations, resulting in mitochondrial abnormality and apoptosis (Mitry and Edwards, 2016). Hence, the maintaining of calcium homeostasis is vital in inhibiting DOX-evoked cardiomyopathy.

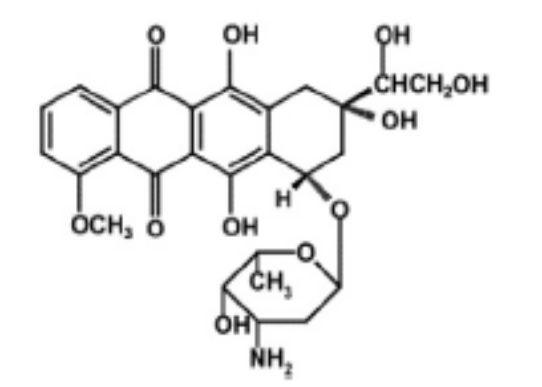


Fig. 2.2 Chemical structure of doxorubicinol a metabolite of doxorubicin. From (Wang *et al.,* 2001; Mitry and Edwards, 2016)

2.2.2.6 The Role of Topoisomerase 2β

DNA topoisomerases can induce temporary single or double-strand breaks to regulate the topological changes during DNA replication, transcription, recombination and chromatin remodeling (Champoux, 2001; McGowan *et al*., 2017). In humans, Top2 is expressed in Top2α and Top2β (Wang, 2002; McGowan *et al*., 2017). Top2α is the most prevalent and is highly expressed in proliferating malignant and non-malignant cells. This is necessary for segregation of chromosomes and its expression changes during the cell cycle, peaking during G2/M phases (Carpenter and Porter, 2004; McGowan *et al*., 2017). Alternatively, Top2β is abundant in quiescent cells, such as adult mammalian cardiomyocytes, and the expression remains the same throughout the cell cycle. Doxorubicin exerts its cytotoxic effect by intercalating DNA through binding with DNA and topoisomerase 2 (Top2α and Top2β) isoenzymes forming a tertiary Top2-doxorubicin-DNA complex, which triggers double-strand DNA breaks. When the complex is bound to Top2α, it inhibits DNA replication; stops the cell cycle in G1/G2; and induces apoptosis (Tewey et al., 1984; McGowan *et al*., 2017) as intended in proliferating malignant cells. Alternatively, when bound to Top2β, mitochondrial dysfunction occurs by the decreasing peroxisome proliferator-activated receptor (PPAR), which regulates oxidative metabolism (Finck and Kelly, 2007; McGowan *et al*., 2017). In cardiomyocytes of adults, this leads to an activation of altered P53 tumor suppressor pathway, β-adrenergic signaling, impairment of calcium signaling, mitochondrial dysfunction and increased apoptosis. Without Top2β, doxorubicin cannot bind directly to DNA (Tewey *et al*., 1984; McGowan *et al*., 2017).

2.2.2.7 Role of microRNAs (miRNA)

Various pathological conditions of the heart results in the dysregulation of miRNAs. MicroRNAs are involved in all cardiac activities, which includes conductance of electrical signals, heart muscle contraction, and growth (Ruggeri *et al*., 2018). The primary enzyme responsible for the generation of mature miRNAs is dicer and that altering the expression of this enzyme in cardiomyopathy causes to an aberrant profile of miRNAs (Asrih and Steffens, 2013). Apoptosis induced by treatment with doxorubicin is caused by the increase of miR-146a, miR-367, miR-215, miR-216b, miR-208b and miR-34c leading to cardiomyopathy (Roca-Alonso et al., 2012; Vacchi-Suzzi *et al*., 2012).

2.3 Biomarkers of Cardiotoxicity

On endomyocardial biopsies, evidence of cardiomyocytes injury and death including myofibrillar loss and vacuolization has been observed. In early myocardial injury, markers such as circulating cardiac troponin and B-type natriuretic peptide have been investigated. B-type natriuretic peptide is established as a reliable marker of decompensated heart failure and may have a role in the later surveillance of cancer survivors particularly where dyspnoea is a presenting symptom. Cardiac troponin I (cTnI) and cardiac troponin T have shown promise as indicators of muscle injury prior to the onset of LV dysfunction (Henriksen, 2018).

2.4 Prevention and treatment of cardiotoxicity

Doxorubicin is an effective chemotherapeutic agent and treatments that would protect patients from acute and chronic cardiotoxicity without altering cytotoxicity of abnormal cells would be ideal for the management of cancer patients. Several cardioprotective agents have been explored

in the treatment and prevention of doxorubicin-induced cardiomyopathy, including drugs that directly interfere with the cellular mechanisms of doxorubicin or are used in the traditional management of heart failure (Mitry and Edwards, 2016). Common strategies for preventing cardiotoxic effects consist in modifying the chemical structure and dosages of ANT and in using cardioprotective agents (Lipshultz et al., 2014). For example, N-benzyladriamycin-14-valerate (AD 198) is a less cardiotoxic anthracycline with a modified chemical structure, but with the same antitumor efficacy as doxorubicin (Cai et al., 2010). Other preventive and treatment strategies have been elaborated below.

2.4.1 Antioxidant agents

Free radicals have been implicated in the pathophysiology of doxorubicin-induced cardiotoxic effects as such antioxidant agents such as dexrazoxane is approved as a cardioprotective agent, being considered the treatment of first choice for doxorubicin induced cardiotoxicity (Goey et al., 2010). Dexrazoxane known as ICRF-187, is an adjunctive agent derivative of ethylenediaminetetraacetic acid (EDTA), which serves as a free radical scavenger by interfering with iron-moderated oxygen free radical production and, subsequently, lipid peroxidation (Seifert et al., 1994; Hochster, 1998). Dexrazoxane also directly competes with topoisomerase II and may stimulate expression of mitochondrial antioxidant enzymes (Mitry and Edwards, 2016). It is taken up rapidly by the myocardium following infusion and competes with ATP-binding sites on Top 2β and producing a configuration change which prevents complex formation with doxorubicin and in so doing reduces cardiotoxicity (Bures et al., 2017; Henrikson, 2017; Menna and Salvatorelli, 2017). Even though vitamin E has also been tested against DOX-induced

cardiomyopathy. Research has proven that vitamin E only inhibits the acute action of DOX cardiotoxic events in mice (El-Demerdash et al., 2003).

2.4.2 Angiotensin inhibition

Angiotensin-converting enzyme (ACE) inhibitors such as enalapril, zofenopril, and lisinopril are frequently administered to patients with heart failure as afterload-reducing agents following doxorubicin-induced heart failure (dos Santos and dos Santos Goldenberg, 2018). ACE inhibitors inhibit the neurohormonal conversion of angiotensin I to angiotensin II, a potent vasoconstrictor. In general, they reduce afterload on the heart and potentially alter cardiac remodeling and fibrosis (Geisberg and Sawyer, 2010) caused by doxorubicin-induced cardiotoxicity.

2.4.3 Beta blockers

Doxorubicin-induced cardiovascular complications have been treated with β-blockers, and growth hormone replacement therapy (Lipshultz, 2014). Carvedilol, which is a nonspecific, adrenergic blocker may be useful in the inhibition of doxorubicin-evoked cardiotoxicity because of its added antioxidant and anti-apoptotic effects (Elitok *et al*., 2014). Carvedilol also protects against calcium dysregulation by blocking the doxorubicin-induced down-regulation of sarcoplasmic reticular calcium pumps (Kalay *et al*., 2006). Randomized trials with carvedilol, (Kalay *et al*., 2006), nebivolol (Kaya et al., 2013) and carvedilol and enalapril in combination (Bosch *et al*., 2013) have demonstrated similar protection from LVEF decline following doxorubicin treatment compared with untreated patients.

2.4.4 Other therapies

Due to the high efficacy of DOX as a chemotherapeutic drug, research efforts are directed towards prevention and treatment of its associated cardiotoxicity using medicinal plants since it continues to be a remedy for many childhood and adult cancers. Medicinal plants or herbal preparations have played an essential role in disease management since prehistoric times (Goldman, 2004) and about 80% of the population in developing countries depend on herbal remedies for primary health care (Kukuia et al., 2014). Plants with proven antioxidant, anti-inflammatory, and anti-apoptotic activities are a good alternative to orthodox medicines for the management of cardiotoxicity (Abdel Daim et al., 2017).

2.5 The Plant *Kalanchoe integra* var. Crenata

Botanical name: ***Kalanchoe integra* var. *Crenata***

Family: *Crassulaceae*

2.5.1 Description and medicinal properties

Kalanchoe integra var. Crenata is commonly referred to as flame kalanchoe; life-plant; never-die. It is a perennial, glabrous, succulent herb with a stem between 0.2-3 m high, which is branched, erect and stout with simple, opposite, succulent leaves (Flora of Pakistan, 2016). The plant is widely distributed but is originally a native to the tropical Himalaya region, India, China, Bhutan and Java (Sharma et al., 2011; Flora of Pakistan, 2016). It can also be found in the Americas and the Caribbean, and Africa (Oviedo-Prieto et al., 2012) since it grows naturally in temperate areas. It is also found growing in tropical regions and is known as "life-plant" (Bailey, 1954; Boyle, 1995). The plant has several common names some of which are ‘flame kalanchoe’, ‘Mother of millions’, ‘Never die’, ‘Dog's liver’, ‘Orange forest kalanchoe’ etc. In

Ghana the plant is called by various local names (for example, the Ewes call it 'aflatoga', Fantes -'eporow', Twi and Ga 'egoro' and 'tamiwu' respectively) and grows widely along footpaths and in forests (Asiedu-Gyekye et al., 2012). It is an ornamental herbaceous perennial succulent shrub forming one to several erect stems 0.3-2 m in height. It yields inflorescences during late autumn and winter. The flowers have rounded heads, long and orange in colour. They have long taproots. The stem is erect, cylindrical, glabrous towards the base and glandular pubescent above; the hairs are short and usually not more than 0.5 mm long. The leaves are irregularly shaped with blunt teeth, sometimes have reddish edges (Wickens, 1987).

All the plants in the kalanchoe genus is made of succulent perennial plants belonging to the class Bryophyllum and over 200 species have been identified, many of which have medicinal properties especially *Kalanchoe integra*. The plant has been used traditionally for the treatment of many disease conditions like peptic ulcer, upper respiratory tract infections, coughs and as anti-infective in Ghana (Dokosi, 1998 and Torres-Santos *et al*., 2003; Asiedu-Gyekye et al., 2012). The plant was first used as an antipsychotic agent way back in 1921 (Pattewar, 2012). The plant is also used traditionally in treating stroke (Dokosi, 1998). The hepatoprotective effect in rat models of toxic hepatitis induced by carbon tetrachloride have also been reported on as is seen in the work by Asiedu-Gyekye and others (Asiedu-Gyekye *et al.*, 2002). It is also used for prophylactic and treatment of adenoma of the prostate gland have been stated (Asiedu-Gyekye *et al.*, 2002). The antidepressant-like effects have been assessed by Kukuia and other (Kukuia et al., 2015). Kalanchoe is reported to contain considerable amounts of flavonoid and phenolic compounds (Gaind and Gupta, 1971; Adenike and Eretan, 2004) with antioxidant properties (Asiedu-Gyekye et al., 2012) against free radicals. This makes the plant a good candidate for the management of doxorubicin induced cardiotoxicity.

Fig 2.3. A picture of *Kalanchoe integra*

2.6 Review on Methods

2.6.1 Genotoxicity assay (Comet)

In 1984 Ostling and Johanson described the method of microgel electrophoresis which measures single DNA strand breaks (Ostling & Johanson, 1984). In 1988 Singh and teammates came up with a modified form of measuring DNA strand breaks (Singh *et al.*, 1988). The aim was to combine DNA gel electrophoresis with fluorescence microscopy to make it easier to visualize the movement of DNA strands from agarose-embedded cells. When DNA breaks are present it favours migration towards to the anode. The amount of DNA moving to the anode makes it easier to quantify the number of DNA breaks present (Olive & Banáth, 2006). In 1990 the comet assay was developed as a modification of the microgel electrophoresis. The comet head had high molecular weight DNA and the comet tail had the migrating fragments of the DNA breaks and

these are measured in real time using a software for digital images (Olive *et al.*, 1990). The amount of DNA in the tail, the distribution of the DNA in the tail, tail length and percentage of DNA in the tail are all measured with the comet assay (Olive & Banáth, 2006).

The comet assay has the following features:

- It is sensitive and reliable detecting different types of DNA damage in different cells (Fairbairn *et al.*, 1995; Moller, 2005).
- It enhances sample handling abilities
- It allows for determination of double DNA strand breaks separate from the single DNA breaks (Olive & Banáth, 2006).
- It has the ability to detect interstrand cross linkages (Merk & Speit, 1999)
- It detects large DNA fragments in apoptotic cells (Olive *et al.*, 1993)
- It is used in detecting heterogeneity in response to DNA destroying agents such as cancer chemotherapeutic drugs (Ostling & Johanson, 1987)
- It can be used to predict tumor response to some treatments which destroys DNA (Almeida *et al.*, 2006).

2.6.2 High performance liquid chromatography (HPLC) fingerprinting

Fingerprint has become an important tool for quality control analysis of herbal samples. This is so because of the growing interest in medicines of plant origin. Fingerprint analysis is applied to identify closely related plant species, to detect adulterations, and also to identify the quality of a finished product. Herbal sample or plant extract fingerprint may be a set of characteristic chromatographic or spectroscopic data that aids unambiguous sample recognition. Several

chromatographic methods have been applied for fingerprinting. These include high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), gas chromatography (GC), or high-speed counter current chromatography (HSCCC)

HPLC is a technique used in separating substances. It identifies, quantifies and purifies compounds (Kumar & Kumar, 2012). It works by distributing the sample or analyte between a mobile phase and a stationary phase. The molecules of the sample interact with the packing material which determines their time- 'on column'. This process is specific indicating that different components of the sample migrate at different times. A UV detector identifies the analyte or sample after it has left the column. The signals from the UV detector are then converted, recorded by a software and displayed as a chromatogram (Böttcher *et al.*, 2020). The HPLC system is made of a solvent reservoir, pump, an injection valve, a column, a detector unit and a data processing unit. The solvent is pumped at high speed and constant speed and by means of an injection valve the sample is supplied to the solvent. An effective separation depends on the inner diameter, length of the column, the type and particle size of the packing material (Meyer, 2010). The role of the detector is to record time and amount of the sample which is eluted or migrated from the column (Meyer, 2010). The detector senses a change in the composition of the sample and proceeds to convert the information into a signal which is estimated by a computer. Examples of detectors used are fluorescence, refractometric, UV and electrochemical (Meyer, 2010). Each peak of the chromatogram gives information on both the qualitative and quantitative nature of the sample. The shape, intensity of the signal, time of appearance and area of the peak provides relevant information on the type of sample being separated (Kromidas & Kuss, 2008).

The HPLC technique is highly sensitive, it does not destroy the sample, it is simple to operate and reliable (Kumar & Kumar, 2012). The technique is applied in biochemical purification, purifying of platelet derived growth factors, it is used in the discovery, development and manufacturing of pharmaceutical products (Kumar & Kumar, 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

The study design was experimental.

3.2 Drugs and Chemicals

All drugs and chemicals used were of analytical grade. Doxorubicin injection (Doxil, U.S.A, batch number NDA 50-718/S-50-FDA) was procured from Ernest Chemist Ghana Limited. Normal saline and Vitamin E were purchased from Gilan Pharmaceuticals Limited, Accra, Ghana. All kits used were obtained from Medilab Diagnostics, Accra, Ghana.

3.3 Collection and identification of Plant Material

Fresh leaves of *Kalanchoe integra* (*KI*) were collected from the Botanical Gardens of the University of Ghana, Legon. Collection was done in mid-July because the leaves of the plant are known to possess high concentrations of its active compounds during this season (Asiedu-Gyekye *et al.*, 2012). The plant was identified at the Centre for Plant Medicine Research, Mampong-Akuapem, given a voucher specimen number (IAGSP-004) and stored at the herbarium.

3.4 Preparation of Ethanolic Extract

The leaves of *KI* were shade-dried for 2 weeks and then pulverized into fine powder using a hammer mill. A mass of 3 kg of the powder of *KI* leaves was weighed and placed in a flat bottom

flask and extraction was done by cold maceration using 70% ethanol. Supernatant obtained was filtered using Whatman No. 1 filter paper and the remaining residue was re-extracted till exhaustion. The extract was freeze dried and powdered sample was stored at 4°C until use.

3.5 Place and Time of Experimentation

The research was carried out at the Animal laboratory of the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, University of Ghana, Korle Bu. All drug administrations were performed during the day: between 7:00 am and 3:00 pm.

3.6 Animal Handling and Ethics

Forty-two (42) female Sprague Dawley rats 6 - 8 weeks of age (weighing 150 - 200 g) were obtained from the Centre for Plant Medicine Research, Akuapem, Mampong, in the Eastern Region of Ghana. The animals were kept at the Animal Experimentation Unit of the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, University of Ghana. The animals were housed in stainless steel cages (34 cm x 47 cm x 18 cm) (n = 6 per cage) with wood shavings as bedding and fed with Growers Mash feed obtained from the Poultry Farmers Association of Ghana, Sakaman, Accra. The animals were given water, fed twice daily, and bedding changed once daily. The temperature of at the Animal House was room temperature (25˚C) and with relative humidity of 45 - 65%. The animals used in the study were handled according to the guide for the care and use of laboratory animals (NRC, 1996). During experimental period, animals were observed: physical characteristics (weight), behavioral changes, and death.

The study protocol was approved by the Ethics and Protocol Review Committee for College of Health Sciences, University of Ghana, with a Protocol Identification Number: CHS-Et/M.4-P6.9/2018-2019.

3.7 Phytochemical Analysis (HPLC Fingerprinting) of *KI*

The HPLC fingerprinting analyses of *KI* was done as described elsewhere (Ameyaw *et al.*, 2014). HPLC analyses was carried out using an Agilent 1100 system (Santa Clara, CA, USA), composed of quaternary pump, autosampler, diode array detector (DAD), and HP ChemStation Software. Chromatographic separation was carried out on a Tskgel ODS C18 (250 x 4.6 mm i.d., 5 µm particle size) analytical column maintained at 40 ^{o}C. The eluents, Water in 0.1 % Phosphoric acid (A) and methanol (B) as mobile phase at a flow rate of 1 mL/min. The gradient program used was set as follows: 0–10 min, 5–15 %B; 10–20 min, 15–45 %B; 20–30 min, 45–60 %B; 30–40 min, 60–90 %B; 40–50 min, 90–90 %B; 50–55min, 90–10 %B , 55-60, 10-10%B. The injection volume 20 µL (1 mg/mL of ethanolic extract was dissolved in 50% methanol, vortexed and filtered).

3.7 Induction of Cardiotoxicity

Cardiotoxicity was induced as described earlier by Khan *et al.* (2014b). A single dose of doxorubicin (20 mg/kg body weight i.p) was administered on the 29th day of the experiment.

3.8 Animal grouping for experimentation on cardioprotective potential of *KI*

After 1 week of acclimatization, the animals were randomly divided into 7 groups of 6 animals in each. Group I received normal saline (1 ml/kg p.o) for 30 days. Group II: Toxic control received normal saline and DOX (20 mg/kg i.p.) once on the 29th day. Group III: KI control received KI (300 mg/kg p.o) for 30 days. Group IV: Vitamin E control received Vitamin E (100 mg/kg body weight p.o) for 30 days. Group V: KI treated-1 received KI (300 mg/kg p.o) for 30 days and DOX (20 mg/kg i.p) on the 29th day. GROUP VI: KI treated-2 received KI (600 mg/kg p.o) for 30 days and DOX (20mg/kg i.p) on the 29th day. GROUP VII: Vitamin E treated received Vitamin E (100mg/kg p.o) for 30 days and DOX (20 mg/kg i.p) on the 29th day.

3.9 Animal sacrifice and sample collection

The animals were sacrificed 36 h after the last experimental treatment under ether anesthesia. The anterior wall of the thoracic region was dissected and reflected superiorly to expose the thoracic viscera. The blood samples were taken via cardiac puncture into serum tubes. The heart was then harvested, washed in ice-cold saline, dried on filter paper and weighed with a chemical balance. A representative portion of each heart was taken from all the groups, and a 30% w/v homogenate made in 0.9% buffered KCl (pH 7.4) for the estimation of glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT). The remaining portion of the heart was stored in 10% buffered formalin.

3. 10 Oxidative Marker Enzyme Assay

The blood samples obtained were analyzed for biomarkers indicative of cardiotoxicity using rapid and immunoassay test kits, according to manufacturers' protocols. The biomarkers included creatine kinase (CK), lactate dehydrogenase (LDH), alanine phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

3.11 Histopathological Study

The remaining heart sample was dehydrated in graded alcohol, embedded in paraffin wax and stained with hematoxylin–eosin (H&E). Pathological examination was conducted under light microscope (40x) for determination of structural abnormality. The histopathological examination was done by a Medical Pathologist at the Department of Medical Laboratory Science, School of Biomedical and Allied Health Sciences, University of Ghana.

3.12 DNA Comet Assay Test

The alkaline single cell gel electrophoresis analysis (comet assay) was used to study the genotoxic potential of *Kalanchoe integra (KI)* on selected tissues/organs such as the liver, kidney, epithelium of the rectum and bone marrow.

In this study, male C57Bl/6 mice were randomly divided into 4 groups of 5 animals each and administered single doses of KI, and methyl methanesulfonate orally as follows:

The High dose group - *6000 mg/kg KI*

The Therapeutic dose group - *600 mg/kg KI*

The Negative control group - *Solvent, 2% starch*

The Positive control group - *methyl methanesulfonate (40 mg/kg)*

After treatment, male C57Bl/6 mice were euthanized by dislocation of the cervical vertebrae 3 hours after the single oral administration of test agents. The intestine, liver, bone marrow and kidney were isolated.

The epiphyses of the femurs were cut off and bone marrow cells were washed from the diaphysis using 2 ml of phosphate buffered saline (PBS) pre-cooled to 4°C and containing 20 mM EDTA-Na2 and 10% DMSO (pH 7.5). The liver, kidney and intestine were homogenized in 3 ml of the same buffer. The tubes were held for 5 minutes at room temperature to precipitate large fragments, after which 1.5 ml of the top layer was transferred to a new tube.

Cell suspensions in volumes of 60 µl were introduced into a test tube with 240 µl of 0.9% low melting point agarose solution (<42°C) in PBS heated to 42°C (micro thermostat, 'TERMIT') and resuspended. Then, 60 µl of the agarose solution with the cells was applied to pre-coated 1% versatile agarose slides that were covered with a cover slip and placed on ice. All subsequent operations were carried out in a dark room with yellow light. After hardening of the agarose (about 10 minutes), the coverslips were carefully removed, micro-preparations placed in a glass cuvette (Schiffendecker type) and poured with preliminarily cooling. After 4°C lysis buffer (10 mM Tris-HCl [pH 10], 2.5M NaCl, 100 mM EDTA- Na2, 1% TritonX-100, 10% DMSO) was added and incubated for at least 1 hour. After lysis, the micro-preparations were transferred to the electrophoresis chamber (Sub Cell GT, "Bio-Rad"). The chamber was filled with an electrophoresis buffer (300 mM NaOH, 1 mM EDTA-Na2, pH>13). The prepared micro-preparations were then incubated for 20 minutes to produce alkaline labile sites and alkaline

DNA denaturation. Then, electrophoresis was performed for 20 minutes at a field strength of 1V/cm and a current strength of ~300 mA. At the end of electrophoresis, the micro-preparations were transferred to a glass cuvette and fixed in a 70% solution of ethyl alcohol (fixation time 15 min). After fixing, the micro preparations were dried and then microscopy performed. Just before microscopy, they were stained with SYBR Green I fluorescent dye (Sigma-Aldrich, USA) (1: 10,000 in TE buffer with 50% glycerol) for 20 minutes in the dark. The analysis was carried out with a fluorescence microscope (Micromed 3 Lum, Russia) combined with a high-resolution digital camera (x 200). The images of the DNA comets obtained from micro-preparations were photographed and analyzed using Comet D software. As a measure of DNA damage, the percentage of tail DNA (tail % DNA) was used. From each micro-preparation, 100 cells were analyzed. As a negative control, 2% starch (solvent) was used. As a positive control, an alkylating agent and a carcinogen - methyl methanesulfonate (methyl mesylate) 40 mg/kg was used.

The criteria for toxicity was a statistically significant increase in the number of DNA comets in the organs/tissues studied compared with negative control.

3.14 Statistical Analysis

GraphPad Prism for windows version 5.0 (GraphPad Software, San Diego, CA, USA) and Statistical Package for Social Scientist (SPSS) version 20.0 were used for data and statistical analysis. Results were expressed as mean ± standard error of the mean (SEM). Comparison among groups was done using One-way analysis of variance (ANOVA) followed by Dunnett's

multiple comparison tests. Data for DNA asset comet were analyzed with Wilcoxon-Mann-Whitney test. P values < 0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 Percent Yield

The percentage yield of the ethanolic extract of *Kalanchoe integra* was 3.46 %. This was obtained from formula below.

$$\text{Percentage yield} = \left[\frac{\text{weight of final extract(g)}}{\text{Soaked sample material}}\right] \boldsymbol{x}\ \mathbf{100\%}$$

$$\text{Percentage yield} = \left[\frac{100.38\ g}{3000 g}\right] \boldsymbol{x}\ \mathbf{100\%}\ = 3.46\%$$

4.2 Phytochemical Analysis (HPLC Fingerprinting)

In the HPLC fingerprint analysis, 13 peaks were identified. The first peak with retention time of 3.4min had a peak area of 1.1754 x10 ^{4} mAU and overall percentage peak area of 19.2%. Also peak with retention time of 24.0 min had the highest peak area of 3.223 x10^{4} mAU) and overall percentage peak area of 52.63% which is directly proportional to high concentration of that compound in the plant extract. Thirteen smaller peaks were also identified with their corresponding smaller peak area and peak height which indicates low compounds present.

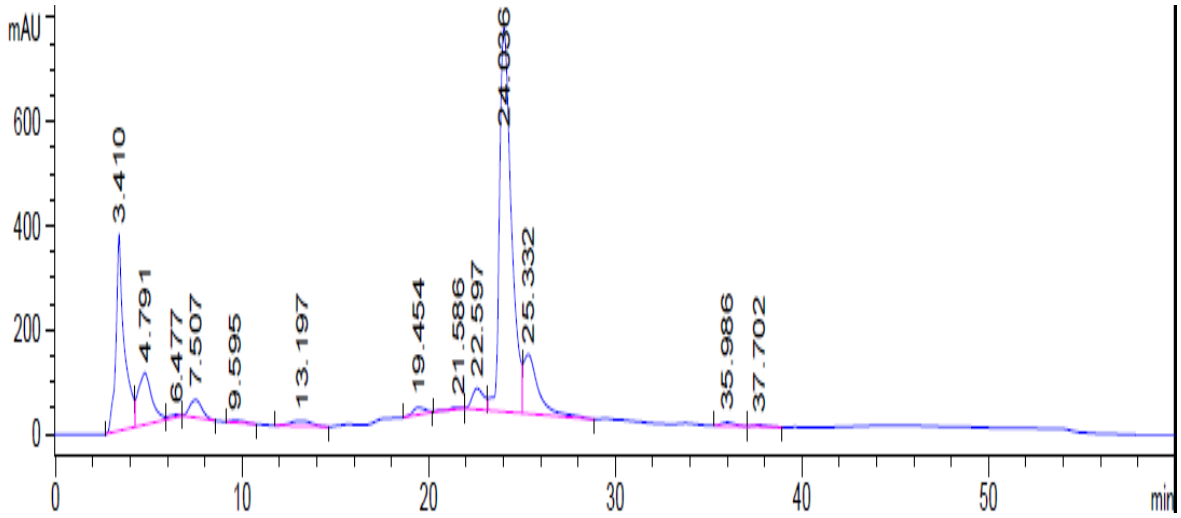


Fig. 4.1 HPLC fingerprinting analysis of *Kalanchoe integra (KI)* showing the various peaks.

Note* The higher the peak the higher the compounds present in the mixture *(KI)* and vice-versa.

4.3 Behavioral Observations of Animals

The behaviors of the rats were observed during period of experimentation. There were no physical observable signs of toxicity in movement, salivation, sleep, lethargy and piloerection among all treatment groups. No mortality was also recorded during the study period.

4.4 Body Weight Assessment

The weekly mean body weights for the rats are represented in Table 4.1. There were slight variations of the weights of the rats within their respective groups, however, these variations were not statistically significant. For example, the mean weight of animals in the saline group on day 1 was 167.7 g but rose to 174.4 g on day 7 and by day 28 it was 180.1 g. The situation was not any different within the various treatment groups. One-way analysis of variance showed that

there was no statistically significant difference between the mean weights of rats throughout the study.

Table 4.1: Weekly mean weights in grams for rats during the experiment

Weeks	Saline	Dox only	KI 300	VITE 100	DOX+KI300	DOX+KI600	DOX+VITE100	P-value
Week 0	167.7(3.6)	199.4(11.4)	192.7(11.5)	187.7(5.78)	158.2(7.2)	178.5 (11.0)	188.9(10.2)	0.218
Week 1	174.4(5.6)	199.4(10.6)	188.4(12.0)	186.3(6.2)	158.7(6.3)	177.1(12.1)	191.2(10.2)	0.451
Week 2	178.9(5.2)	200.1(10.6)	186.1(14.1)	187.0(5.9)	160.0(6.6)	180.1(11.4)	192.7(10.3)	0.141
Week 3	183.5(4.8)	199.8(10.6)	189.1(13.8)	189.4(5.7)	162.5(6.8)	181.2(12.1)	193.6(10.3)	0.202
Week 4	180.1(7.2)	193.6(11.0)	187.7(10.7)	188.9(10.2)	167.7(5.6)	178.9(6.7)	190.8(11.0)	0.235

Values are expressed as mean (SEM). P-values represent significance level after one-way ANOVA between group comparisons

4.5 Organ Weights

One-way analysis of variance (ANOVA) revealed that there was no significant difference in the weight of the heart and liver excised from the various treatment groups of the rats (Figures 4.2).

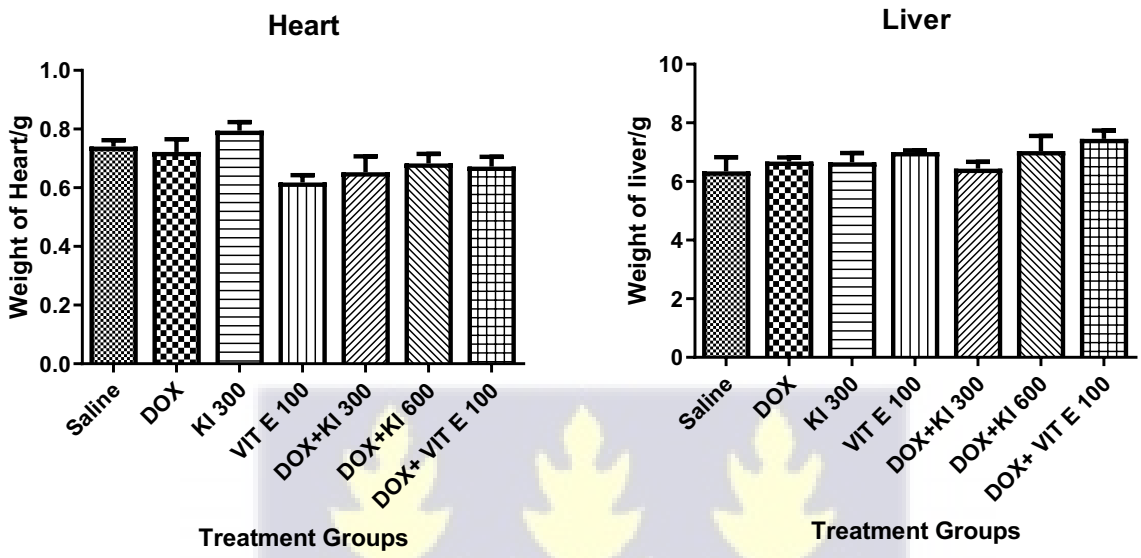


Figure 4.2: Weight of heart and liver of the various treatment groups

4.6 Biochemical Analysis

The serum biochemistry analysis showed statistically significant differences in ALT ($P < 0.0001$) when the various treatment groups was compared to saline. A significant increase in ALT level was found in the Doxorubicin only group when compared with saline whereas a significant decrease was seen in KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg groups when compared to the doxorubicin only group. The analysis showed statistically significant differences in AST ($P=0.0017$) when doxorubicin was compared to saline and when doxorubicin only was compared to KI 300 mg/kg, VIT E 100

mg/kg and DOX + KI 600 mg/kg. A significant increase in the AST level was observed in the doxorubicin only group with a significant decrease observed in the KI 300 mg/kg, VIT E 100 mg/kg and DOX+ KI 600 mg/kg groups when compared to the saline and doxorubicin only groups respectively. Table 4.2 showed that there are statistically significant differences in ALP ($P < 0.0001$) when doxorubicin was compared to saline and when doxorubicin was compared to KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg. A significant increase in ALP level was seen in the Doxorubicin only group whereas a significant decrease was observed in KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg groups when compared to the saline group and doxorubicin only group respectively. Table 4.2 showed that there is statistically significant differences in CK ($P < 0.0001$) when saline was compared to doxorubicin and when doxorubicin was compared to KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg. A significant increase in CK level was seen in the Doxorubicin only group when compared to the saline group and a significant decrease in KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg groups when compared to the doxorubicin only group. The analysis showed that there is statistically significant differences in LDH ($P = 0.0088$) when saline was compared to doxorubicin and when doxorubicin was compared to KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg. A significant increase in LDH level was seen in the Doxorubicin only group when compared to the saline group and a significant decrease in KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg groups when compared to the doxorubicin only group.

The analysis showed that there is statistically significant differences in CAT (P <0.0001) when saline was compared to doxorubicin, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg and when doxorubicin was compared to KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg. A significant decrease in CAT level was seen in the Doxorubicin only group when compared to the saline group and a significant increase in KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg groups when compared to the doxorubicin only group. The analysis showed that there are statistically significant differences in GPX (P =0.0017) when saline was compared to doxorubicin and when doxorubicin was compared to KI 300 mg/kg, Dox + KI 600 mg/kg and VIT E 100 mg/kg. A significant decrease in GPX level was seen in the Doxorubicin only group when compared to the saline group and a significant increase in KI 300 mg/kg, Dox + KI 600 mg/kg and VIT E 100 mg/kg groups when compared to the doxorubicin only group. The analysis showed that there is statistically significant differences in SOD (P =0.0001) when saline was compared to doxorubicin, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg and when doxorubicin was compared to KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg. A significant decrease in SOD level was seen in the Doxorubicin only group when compared to the saline group and a significant increase in KI 300 mg/kg, Dox + KI 300 mg/kg, Dox + KI 600 mg/kg, VIT E 100 mg/kg and Dox + VIT E 100 mg/kg groups when compared to the doxorubicin only group.

Table 4.2: Serum biochemical analysis among various treatment groups

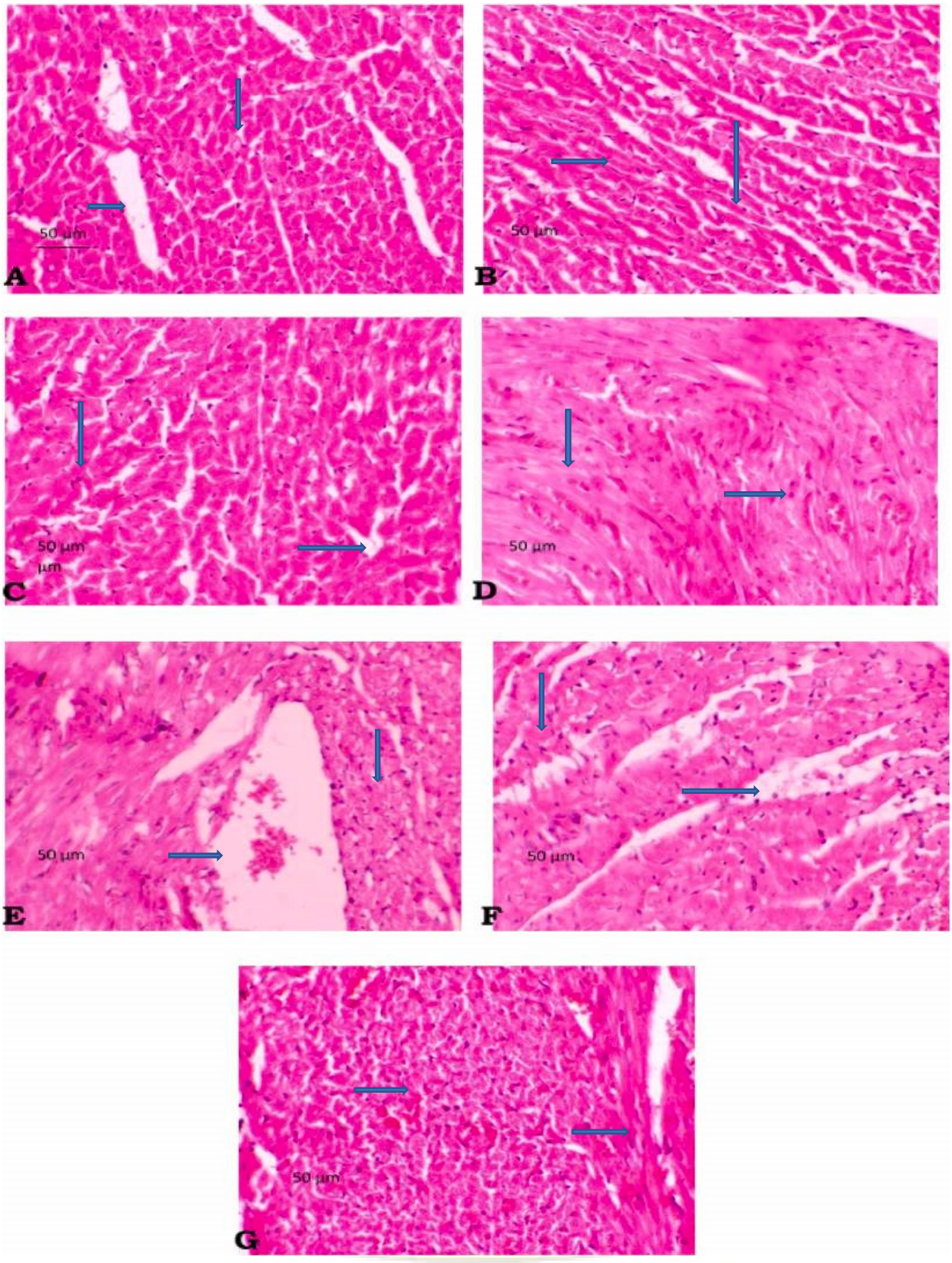
Parameters	Saline	DOX	KI300	VITE100	DOX+KI300	DOX+KI600	DOX+VITE100	P-value
ALT (U/L)	78.7± 11.2	241.7± 50.7***	97.0± 12.9$^{###}$	61.8± 13.9$^{###}$	127.0± 11.4$^{###}$	108.3± 4.0$^{###}$	65.8±11.1$^{###}$	<0.0001
AST (U/L)	162.5±13.9	323.0± 58.2*	145.8± 11.2$^{##}$	168.6± 13.3$^{#}$	244.2± 49.9	173.3± 13.8$^{##}$	266.7± 16.7	0.0017
ALP (U/L)	175.2± 9.0	256.5±27.5***	117.4±10.2$^{###}$	125.5±21.4$^{###}$	126.4±4.6$^{###}$	115.7±13.9$^{###}$	124.8± 6.3$^{###}$	<0.0001
CK (IU/L)	326.0±60.9	2488±378.3***	357.6±181.6$^{###}$	600.4±126.7$^{###}$	925.8±373.8$^{###}$	138.3±10.6$^{###}$	557.8±217.3$^{###}$	<0.0001
LDH(IU/L)	1002±239.8	3366±428.3***	870.8±115.7$^{###}$	466.4±80.62$^{###}$	1943±338.0$^{###}$	327.4±167.4$^{###}$	1726±170.3$^{###}$	0.0088
CAT (IU/L)	23.66±0.04	8.37±0.13***	23.87± 0.14$^{###}$	30.87±0.05$^{###***}$	26.8±0.05$^{###***}$	26.4± 0.0$^{###***}$	26.6±0.21$^{###***}$	<0.0001
GPX(IU/L)	0.002±1.5e-005	0.0003±2.9e-005**	0.002± 0.0004$^{##}$	0.002±9.2e-005$^{##}$	0.001± 0.0004	0.002±3.1e-005$^{##}$	0.001± 0.0004	0.017
SOD(IU/L)	3.47± 0.17	1.25± 0.11*	4.701± 1.5$^{##}$	6.81± 0.17$^{###}$	4.51± 0.14$^{##}$	6.11± 0.20$^{###*}$	6.71± 0.24$^{###*}$	0.0001

Values are expressed as mean ±SEM. P- values represent significance level after one-way ANOVA between group comparisons

*Significantly different from the control: *P<0.05, **P<0.01 by Dunnet's test. Significantly different from Dox only: $^{#}$P<0.05, $^{##}$P<0.01 and $^{#}$P<0.0001 by Dunnet's test.*

4.7 Histological Features of the Heart Section of Rats from the Various Experimental Groups

The figure below shows hematoxylin and eosin (H&E) stained images of heart tissue from all seven groups. (A) Photomicrograph of the heart sections of the KI control group showing normal features (x40) (B) Photomicrograph of the heart sections of the Saline group (vehicle) showing normal features (x40). (C) Photomicrograph of the heart sections of Vitamin E group showing normal features (x40). (D) Photomicrograph of the heart sections of the DOX only group showing abnormal hypertrophic myocardial fibres (x40). (E) Photomicrograph of the heart sections of the KI 300 + DOX group showing normal features with a single myocardial fibre showing intracytoplasmic vacuole (x40). (F) Photomicrograph of the heart sections of the KI 600 + DOX group showing normal features. (x40). (G) Photomicrograph of the heart sections of the VE + DOX group showing normal features (x40). In the H&E staining images, the nucleus is blue, and the cytoplasm is red. Collagen fibres show varying red colours.



4.8 DNA Comet Assay

Genotoxicity assay for *Kalanchoe* is shown in Table 4.3.

Table 4.3: The level of DNA damage (% of DNA in the tail) in tissues and organs

tissue, organ / number animal	The epithelium of the rectum (tail DNA, %)	Liver (tail DNA, %)	Bone marrow (tail DNA, %)	Kidney (tail DNA, %)
KI 6000 mg / kg				
1	3.12	2.35	2.15	1.28
2	2.76	2.23	1.11	2.41
3	2.01	1.63	0.14	2.28
4	1.43	2.19	2.16	3.84
5	1.38	2.99	2.17	2.86
X±m	**2.14±0.35**	**2.40±0.15**	**1.55±0.41**	**2.53±0.42**
KI 600 mg / kg				
1	2.75	2.11	**1.95**	1.20
2	2.68	1.39	**2.15**	1.47
3	1.25	0.87	1.66	2.02
4	1.66	1.69	1.61	2.32
5	1.33	1.13	1.20	2.21
X±m	**1.93±0.33**	**1.44±0.22**	**1.71±0.16**	**1.84±0.22**
The negative control, the solvent (2% of starch)				
1	2.54	1.55	1.52	1.60

2	1.57	1.60	2.14	2.29
3	2.15	1.96	1.82	0.97
4	2.54	1.89	1.84	2.30
5	2.07	2.76	3.08	2.12
X±m	**2.17±0.18**	**1.95±0.22**	**2.08±0.27**	**1.86±0.26**
Positive control, methyl methanesulfonate 40 mg / kg				
1	15.14	11.80	11.28	9.05
2	16.00	15.54	16.72	10.91
3	18.77	15.77	17.96	13.59
4	17.11	14.80	17.82	12.46
5	15.90	15.26	16.30	16.46
X±m	**16.58±0.63***	**14.63±0.73***	**16.02±1.23***	**12.49±1.25***

Note - * - differences are significant when compared to the negative control (Wilcoxon-Mann-Whitney test)

X±m = Mean ±SEM

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

Currently, there appear to be a rise in the use of medicinal herbal preparations, probably because of the presence of bioactive compounds in these plant products. Indeed, plant products have been a great source of conventional drugs and potential drugs (Tseng *et al.*, 2007). The aforementioned reason, amongst others, led to the investigation to determine the protective effect of *Kalanchoe integra* in doxorubicin-induced cardiotoxicity.

The ethanolic extract of Kalanchoe integra shows the presence of 13 different peaks with different retention times notably 2 constituents had higher peaks when compared with the other constituents. Their retention times were 3.410 and 24.036. They had a peak area of 1.1754 x10 ^{4} mAU and overall percentage peak area of 19.2% and the highest peak area of 3.223 x10^{4} mAU and overall percentage peak area of 52.63% respectively. This is directly proportional to high concentration of that compound in the plant extract. The rational for performing the HPLC fingerprinting analysis was to ascertain the concentration of bioactive compounds present in the *Kalanchoe integra* extract. It is suggested that a characterization studies be done to determine the corresponding compounds responsible for the observed activity.

During period of experimentation, treated rats showed no physical observable signs of toxicity in movement, salivation, sleep, lethargy and piloerection, in comparison to animals administered distilled water. No mortality was also recorded during the study period. The body weights of the rats were normal throughout the 4 weeks of the study when compared to the vehicle group. There were no changes in the weights of the organs as well. This indicates that the ethanolic extract of

the leaves of *Kalanchoe integra* did not have any significant effect on the body and organ weights of the rats. Changes in body and organ weights according to Bailey and colleagues give an indication that the agent is likely to have toxic effects (Bailey *et al.*, 2004). Michael and others also made a similar report when they mentioned that organ to body weight ratios is an indication of toxicity (Michael *et al.*, 2007). The current study is in line with the reports by Michael *et al.* (2007) and Bailey *et al.* (2004) suggesting that the ethanolic extract of the leaves of *Kalanchoe integra* did not induce toxicity in rats. There were no gross pathological changes in the color, texture and atrophy of the organs when compared with the vehicle. This gives further indication that the extract did not have toxic effects during the study period.

Doxorubicin, an anticancer drug, causes cardiotoxicity which can be detected biochemically through certain blood biomarkers. The biomarkers examined in the current study included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine kinase and lactate dehydrogenase.

Biochemical analysis revealed that when compared to vehicle the doxorubicin only group had increased levels of ALT which was statistically significant. The extract treated groups and group that were administered both doxorubicin and extract did not show any change in ALT levels when compared to the vehicle. Alanine aminotransferase (ALT) is an enzyme that converts α-amino groups from alanine to α- keto groups to generate energy for the cells (mostly the liver). However, when there is damage in a cell, ALT increases in blood serum (Giannini, 2005). A significant increase in aspartate aminotransferase (AST) levels was seen in the doxorubicin only group when compared to the vehicle. The *KI* extract pretreated groups had significantly lower AST levels when compared with doxorubicin only group. AST is another biomarker for tissue damage and may serve as a diagnostic marker for an organ like the heart (Hasan *et al.*, 2018;

Kasarala & Tillmann, 2016). The results from the current study corroborates findings of Khan and colleagues who demonstrated that doxorubicin induced cardiotoxicity increased AST levels in rats (Khan *et al.*, 2014a). A significant increase in alkaline phosphatase (ALP) was observed in doxorubicin only group when compared with vehicle whereas a significant decline was observed in *KI* pre-treated groups when compared to doxorubicin only group. According to McIntyre and Rosalki, high levels of ALP does not give a specific diagnosis (McIntyre & Rosalki, 1991). However, elevated levels of AST and ALT in blood gives an indicates tissue injury (Siddique & Kowdley, 2012).

In the present study, the creatinine kinase (CK) values in the doxorubicin only group were significantly higher when compared to the vehicle. Also, pre-treatment with *KI* extract showed significantly lower CK values when compared with doxorubicin. CK values are biomarkers for muscular damage. CK is available within the body in 3 isoforms namely the skeletal muscle, cardiac muscle and brain tissue types (Ehlers *et al.*, 2002). High CK values in blood do not give a specific diagnosis on the type of muscular damage. Sabeena and colleagues showed that enzymes such as AST, LDH and CK may be biomarkers of cardiotoxicity and as such high levels in serum indicates myocardial necrosis (Sabeena *et al.*, 2004). The current findings on CK agrees with the study by Khan and others who demonstrated that doxorubicin caused myocardial cell membrane damage which resulted in the high CK levels (Khan *et al.*, 2014a). Lactate dehydrogenase (LDH) levels saw a statistically significant increase in the doxorubicin only group when compared with vehicle. Also the *KI* pre-treated groups showing a statistically significant decline in LDH when compared to doxorubicin only group. In the study by Saravanan and others, they showed that LDH levels were high in isoproterenol (ISO)-induced myocardial infarction (Saravanan *et al.*, 2013). The observation was because of myocardial cell damage

which led to the leaking of enzymes such as AST, CK and LDH into blood, hence the observed high values (Saravanan *et al.*, 2013).

Doxorubicin has been found to cause oxidative stress through reactive oxygen species and lipid peroxidation thereby reducing the antioxidant activity in biological systems (Cappetta *et al.*, 2017; Farias *et al.*, 2017). Antioxidant enzymes assayed in the present study were catalase, glutathione peroxidase and superoxide dismutase. There was a statistically significant decline in catalase (CAT) values in the doxorubicin only group when compared with vehicle. However, a significant increase was seen in the *KI* pre-treated groups. The decline in CAT in the doxorubicin only group shows that the doxorubicin treatment induced the development of free radicals in serum which reduced the ability of the catalase to remove reactive oxygen species. However, in the *KI* pre-treated groups the *KI* extract protected the rats against formation of free radicals and hence the enhanced activity of CAT. The protection may be due to the antioxidant activity of *KI* (Asiedu-Gyekye *et al.*, 2012). Alam and others have showed that thymoquinone has the ability to amend doxorubicin induced cardiotoxicity by regulating oxidative damage (Alam *et al.*, 2018). In that study it was shown that doxorubicin caused oxidative stress which further resulted in cardiac muscle pathological changes (Alam *et al.*, 2018). A significant decrease was seen in the doxorubicin only group when glutathione peroxidase (GPX) level was compared with vehicle group with the *KI* pre-treated groups. Doxorubicin-induced reactive oxygen species has the tendency to significantly reduce stores of reduced glutathione (GSH) in cells (Ortiz *et al.*, 2008). Cardiac cells are protected from oxidative stress by antioxidant enzymes such as glutathione peroxidase and glutathione reductase (Vávrová *et al.*, 2011). Mohamed and others reported that adriamycin-induced cardiotoxicity has the tendency to reduce GSH levels (Mohamed *et al.*, 2000). The study further showed that the decline in GSH levels are strongly associated with high

levels of biochemical markers of cardiotoxicity such as the CK, LDH and AST (Mohamed *et al.*, 2000). The study by Alam *et al.* (2018) showed that treatment with doxorubicin reduced the levels of GSH. The current study corroborates these initial reports by Alam and colleagues as well as that of Mohamed and others. The results from the present study showed that there were decreased levels of superoxide dismutase (SOD) in the doxorubicin only group when compared to vehicle. Also, the *KI* pre-treatment groups showed increased SOD levels when compared to the doxorubicin only group. SOD according to Ramesh and Brin, accelerates the dismutation of superoxide radicals resulting in the conversion to peroxide (Ramesh & Brian, 2004). Kwatra and others demonstrated in their study that there was a decrease in SOD levels in doxorubicin only treated group whereas 'naringin' pre-treated groups showed an increase in SOD levels (Kwatra *et al.*, 2016). In the work by Khan and others SOD levels were reduced in the doxorubicin only group (Khan *et al.*, 2014a).

The findings from the biochemical analysis (i.e. ALT, AST, ALP, CK and LDH values) shows that the *KI* pre-treated groups had cardioprotective effect as was observed in the low values recorded. The analysis of the antioxidant enzymes (i.e. CAT, GPX and SOD) also proved that KI pretreated groups had cardioprotective effect.

Histopathological examination of the doxorubicin-only treated groups revealed the presence of abnormal hypertrophic myocardial fibers, small and large vacuoles. However, in the *KI* pre-treated groups, there were no signs of abnormal myocardial fibers: the muscles were of normal shape, size and configuration. It was also observed that there were no vacuolation, neither was there any sign of necrosis or inflammation in the tissues from the KI-treated groups. Kang and others provided evidence that doxorubicin-induced cardiotoxicity caused lesions, myofibrillar myopathy as well as cytoplasmic vacuolization (Kang *et al.*, 2017). Zhang and friends also

showed that doxorubicin treated rats showed morphological changes in heart tissues (Zhang *et al.*, 2017). These changes include myofibrillar breaking and disorganization, necrosis, weakening of cardiac muscles and intracellular edema (Zhang *et al.*, 2017). According to Shaker and colleagues, treatment with doxorubicin was characterized by histopathological evidence of disarrangement of myocardial fibers, swelling of myocardial fibers, myofibrillar loss, cytoplasmic vacuolization and lesions of myocardial vessels (Shaker *et al.*, 2018).

Results from the DNA comet assay test shows that the *KI* extracts did not cause any DNA damage in the epithelium of the intestines, liver, bone marrow and kidney when compared to the negative control in each instance.

It may be postulated that the cardioprotective effect of *Kalanchoe integra* is due to its high antioxidant properties, as earlier reported (Asiedu-Gyekye *et al.*, 2012).

5.2 Conclusion

The current study showed that the ethanolic (70%) leaf extract of *Kalanchoe integra* exhibits cardioprotective effects in rats as seen in the AST, ALT, ALP, CK, LDH, GPX, SOD and CAT values. The leaf extract of *Kalanchoe integra* protected the heart of the rats as seen in the histopathological results. The HPLC fingerprint analysis identified 13 peaks. The first peak with retention time of 3.4min had a peak area of 1.1754 x10 ^{4} mAU and overall percentage peak area of 19.2%. Also peak with retention time of 24.0 min had the highest peak area of 3.223 x10^{4} mAU and overall percentage peak area of 52.63% which is directly proportional to high concentration of that compound in the plant extract. KI also protected the heart of the rats against

histopathological changes such as necrosis, abnormal myocardial fibers, and edema. The study further concludes that *Kalanchoe integra* did not have any genotoxic effects.

5.3 Limitations

1. Other cardiotoxic models could have been used to compliment the experiment.
2. Different animal species could also have been used in this cardiotoxic model.
3. Different extraction methods of *KI* should be used to explore their levels of potencies against cardiotoxicity.

5.4 Recommendations

1. The exact mechanism by which KI shows its cardioprotective effect should be examined in the future.
2. Other markers of cardiotoxicity such as inflammatory markers and apoptotic markers should be examined to further determine the cardioprotective effect of *KI*.
3. A study should be conducted to examine the levels of cardiac troponin I since it is a strong marker for cardiotoxicity.
4. Other oxidative stress markers such as malonaldehyde and total homocysteine should be examined since they play significant roles in lipid peroxidation and cardiotoxicity.
5. Possible electrocardiographic disorders need to be assessed in future studies.

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