EVALUATION OF CARDIOPROTECTIVE EFFECT OF *THESPESIA POPULNEA* WITH SPECIAL REFERENCE TO ANTIOXIDANT ACTIVITY

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

By

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[Enrollment No. 119997290045]

Under the supervision of

Dr. Archana N. Paranjape

GUJARAT TECHNOLOGICAL UNIVERSITY AHMEDABAD

May – 2018

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ABSTRACT

Objective

Cardiac problems are a matter of universal concern. One of the contrivances to find a solution to these problems has been to search for ameliorative measures in plants that have proven or supposed medicinal significance. *Thespesia populnea* (TP), a plant of known medicinal value, is used traditionally, in the coastal regions of India for the treatment of several diseases. The present study was aimed at evaluating the cardioprotective potential of this plant in different models of cardiotoxicity.

Methods

The study was carried out in two models of induced cardiotoxicity. In each model, male adult wistar rats were divided into 10 groups of 8 each. Group I served as vehicle control; group II received adriamycin (15mg/kg i.p. cumulative dose) in model I and ethanol (20%, 2g/kg p.o.) in model II; groups III and IV received TP leaf extract (200 mg/kg and 400 mg/kg) alone; group V and VI received vitamin E (Vit E) (25mg/kg p.o.) and carvedilol (CV) (1mg/kg p.o.) alone. In groups VII, VIII, IX and X all the treatments were administered in combination with adriamycin (for 4 weeks) or ethanol (for 6 weeks) to the rats. After 24 h of last dose of different treatments, electrocardiogram (ECG) was recorded, serum biomarkers of cardiac function such as c-reactive protein (CRP), creatine kinase (CK), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), alanine amino transferase (AlAT), aspartate amino transferase (AST), and serum lipid profile comprising total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL-C), high density lipoprotein (HDL-C) and very low density lipoprotein (VLDL-C) were estimated. Myocardial ATPases such as Na+ ATPase, Ca2+ ATPase and Mg2+ ATPases and antioxidant parameters such as lipid peroxidation, superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione reductase (GR), glutathione S-transferase (GST), and glutathione peroxidase (GPX) were assessed. Cardiac ejection fraction, heart weight and thickness of left ventricle wall were determined. Finally, histopathological examination of the heart was carried out.

Results

Treatment with TP leaf extract, either separately or along with adriamycin or ethanol, significantly ($P<0.001$) increased the food intake and body weight along with a significant ($P<0.001$) improvement in the cardiac function parameters such as heart weight, left ventricle thickness and cardiac ejection fraction. Abnormalities recorded in the ECG with adriamycin or ethanol, such as ST segment elevation, prolongation of QT interval and RR duration were significantly reduced by the leaf extract of TP (400mg/kg). Serum biochemical derangements in cardiac biomarkers that resulted from the administration of adriamycin or ethanol were significantly ($P<0.001$) ameliorated. An improvement in lipid profile with a significant lowering of TC, TG, LDL-C and VLDL-C accompanied by a significant elevation of HDL-C was observed. Cardiac antioxidant status was significantly elevated by TP leaf extract as observed from the antioxidant enzyme activities such as SOD, CAT, GST, GR and GPX, followed by a significant lowering of lipid peroxidation in both the protocols, thereby asserting the cardioprotective potential of TP. GSH levels that were altered by the treatment with adriamycin or ethanol were also brought back to near control status upon treatment with TP leaf extract.

Histopathological studies supported the biochemical findings, with a marked damage to the cardiac architecture with adriamycin or ethanol treatment followed by alleviation of the changes upon treatment with TP leaf extract.

Conclusion

In confirmation of the results, it is suggested that the aqueous extract of Thespesia populnea leaf could provide therapeutic benefit in drug-induced or chemically induced cardiotoxicity to considerable extent by virtue of its antioxidant potential or by way of an indirect mechanism influencing the cardiac function pathways.

Key words: *Thespesia populnea*, cardiotoxicity, oxidative stress, adriamycin, ethanol

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Sangeetha L.A. Rajbanshi

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LIST OF ACRONYMS

AA	Ascorbic acid
AAP	Aminoantipyrine
AAS	Anabolic androgenic steroids
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6sulfonic acid)
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
ADR	Adriamycin
AF	Atrial fibrillation
AIC	Anthracyline-induced cardiotoxicity
ALT	Alanine transaminase
ANOVA	Analysis of Variance
ANP	Atrial natriuretic peptide
AST	Aspartate transaminase
AT1	Angiotensin II type 1 receptor
ATP	Adenosine triphosphate
ATP ase	Adenosine triphosphatase
b	b subunit of the G protein,
b1AR	b1-adrenergic receptor
BHT	Butylated hydroxyl toluene
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CDNB	1-Chloro 2, 4-Dinitro Benzene
CE	Cholesterol esterase
CHOD	Cholesterol oxidase
CK	Creatine kinase
CK-MB	Creatine kinase-MB
CO	Carbon monoxide
CPCSEA	The Committee for the Purpose of Control and Supervision of Experiments on Animals
CRP	C reative protein
CV	Carvedilol
DAG	Diacyl glycerol
DEX	Dexrazoxane
DNA	Deoxy ribonucleic acid
DOX	Doxorubicin
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSBmT	N, N-bis-(4-sulfobutyl)-m-toluidine disodium
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetra-acetate
EEG	Electroencephalogram
EGTA	Ethylene glycol tetra-acetic acid
ETC	Electron transport chain
EtOH	Ethanol
g	g subunit of the G protein
G-6-P	Glucose 6-phosphate

G-6-PDH	Glucose-6-phosphate dehydrogenase
GPX	Glutathione peroxidase
Gq	Gq protein
GR	Glutathione reductase
Gs	a subunit of the stimulatory G protein
GSH	Reduced glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
GTPase	Guanosine triphosphatase
HDL	High density lipoprotein
HF	Heart failure
HK	Hexokinase
HPTLC	High Performance Thin Layer Chromatography
HSF-1	Heat-shock factor1
Hsp 25	Heat- shock protein 25
IAEC	Institutional Animal Ethics Committee
INaL	Inward sodium current
INR	International normalized ratio
IP3	Inositol triphosphate
KHB	Krebs-Henseleit solution
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LP	Lipid peroxidation
LV	Left ventricle
MAPK	Mitogen activated protein kinases
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP (Nicotinamide adenine dinucleotide phosphate)
NBT	Nitroblue tetrazolium
NE	Norepinephrine
NF-KB	Pro-inflammatory nuclear factor-Kappa B
NPSH	Nonprotein thiol compound
PIP_2	Phosphatidylinositol biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMS	Phenazine methosulfate
POD	Peroxidase
RAAS	Renin angiotensin-aldosterone system
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SERCA	Sarcoplasmic reticulum calcium adenosine triphosphatase
SH	Sulfhydryl groups

SNS	Sympathetic nervous system
SOD	Superoxide dismutase
TBA	thiobarbituric acid
TC	Total cholesterol
TG	Triglycerides
TL	Transfer latency
TLC	Thin Layer Chromatography
TNF	Tumor necrosis factor
TP	Thespesia populnea
TRC	Time taken to reach reward chamber
U	Units
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VLDL	Very low density lipoprotein

LIST OF ABBREVIATIONS

%	Percent
w/w	Weight by weight
w/v	Weight by volume
µ	micro
µl	microliter
g	gram
Kg	Kilogram
mg	milligram
ml	Milliliter
mm	Millimeter
min	minute
h	hour
M	molar
nm	nanometer
Rf	Retention factor
mmol	Millimoles
mM	Millimolar
°C	Degrees celsius
cm	centimeter
A/ Abs	absorbance
µm	Micrometer
IU	International unit
wt	weight
s	second

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CHAPTER-1
INTRODUCTION

CHAPTER 1. INTRODUCTION

The human heart with all its associated disorders and dysfunctions is one of most studied organs of the human body. The insults sustained by the heart due to several conditions such as obesity, exposure to environmental pollutants, infections, drugs or chemical agents and even ageing have been the subjects of interest to explore the mechanisms involved in cardiac damage.

Globally, the prevalence of cardiovascular diseases is on the rise with as many as 17.7 million people dying from cardiovascular disease in 2015, representing 31% of all global deaths (http://www.who.int/mediacentre/factsheets/fs317/en/). The major causes of cardiovascular disease are tobacco use, physical inactivity, an unhealthy diet and harmful use of alcohol (http://www.wpro.who.int/mediacentre/factsheets/cardio-vascular_disease /en/). The various factors that predispose to cardiovascular disease and hence cardiac damage range from behavioural risk factors to metabolic and socio-economic-cultural factors. Behavioural risk factors are responsible for about 80% of coronary heart disease and cerebrovascular disease (http://www.wpro.who.int/mediacentre/factsheets/ cardiovascular disease/en/). The effects of unhealthy diet and physical inactivity may be observed in individuals as raised blood pressure, raised blood glucose, raised blood lipids, overweight and obesity. These are called "intermediate risk factors" or metabolic risk factors (http://www.wpro.who.int/mediacentre/factsheets/cardiovascular_disease/en/).

A number of underlying determinants of CVDs are a reflection of the major forces driving social, economic and cultural change – globalization, urbanization, and population ageing (http://www.wpro.who.int/mediacentre/factsheets/cardiovascular_disease/en/). The other determinants of CVDs include poverty, stress and hereditary factors (http://www.wpro.who.int/mediacentre/factsheets/cardiovascular_disease/en/). A variety of

drugs employed in the treatment of different diseases can also generate free radicals in the body, which may cause oxidative stress and other undesirable effects.

Cardiotoxicity is one of the most important adverse reactions of chemotherapy, leading to an increase of morbidity and mortality [1, 2]. Cardiotoxicity can be defined as a condition which develops due to a direct effect of a drug/chemical on the heart and an indirect effect due to enhancement of alterations of hemodynamic flow or due to thrombotic events [3].

The condition can appear early or late in the course of the disease, and may vary from subclinical myocardial dysfunction to irreversible heart failure or even death [4]. Chronic exposures to chemical agents or therapies which are aimed at alleviating diseases such as cancer have carried with them the burden of disease. Many cardiac diseases such as stroke, congestive heart failure, hypertension and coronary artery disease, acute and chronic alcohol exposure, ischemia reperfusion injuries, autoimmune diseases etc have been linked to the generation of free radicals [5]. Development of such heart diseases due to drugs/chemicals is a serious concern that needs to be addressed.

Anthracyclines are an important class of anticancer agents that include adriamycin (doxorubicin), daunorubicin, epirubicin etc, which are widely used in the treatment of solid tumours, hematologic malignancies, breast cancer, lymphomas and so on [6]. Like most of the anticancer drugs, adriamycin also causes various toxic effects, the commonest of which is cardiotoxicity leading to acute and chronic cardiac failure [7]. Cellular damage induced by adriamycin is mediated by the formation of an iron-adriamycin complex that generates free radicals, which in turn causes severe damage to the plasma membrane and interferes with the cytoskeletal structure [8]. Formation of free radicals and superoxides is the most common hypothesis for the mechanism by which anthracyclines can cause cardiotoxicity [9-12].

Studies on adriamycin report congestive heart failure, cardiomyopathy and electrocardiographic changes after cumulative administration of the drug [13]. The mechanisms proposed for cardiotoxic effects of adriamycin include free radical-induced myocardial injury [14], lipid peroxidation [15], decreased activity of Na^+ K^+ ATPase [16], vasoactive amine release [17], impairment in myocardial adrenergic signalling/regulation, increase in serum total cholesterol, triglyceride and low density lipoproteins [18], generation of reactive oxygen species (ROS) like superoxide anion and hydrogen peroxide causing impairment of cell functioning and cytolysis [19]. The drug also causes elevation

of serum enzymes like lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) [20]. It has been suggested that endogenous antioxidant deficits may play a major role in the development of doxorubicin-induced cardiomyopathy and heart failure [21].

Alcohol is the most widely abused substance in the world [22]. WHO also reported that roughly 3.3 million (5.9%) of all global deaths, were attributable to alcohol. Chronic high dose ethanol consumption most commonly causes hepatic, gastrointestinal, nervous and cardiovascular injuries leading to physiological dysfunctions [23]. Alcohol was suggested to increase intracellular Ca^{2+} by direct upregulation of voltage-gated Ca^{2+} channels [24]; cause inhibition of Ca^{2+}-adenosine triphosphatase (Ca^{2+}ATPase) that extrudes Ca^{2+} from the cells [25]; and deplete magnesium ion (Mg^{2+}) that inhibits the sodium ion (Na^{+})-potassium ion (K^{+}) pump (Na^{+}/K^{+}ATPase), causing a build up of intracellular Na^{+} [26]. Alcohol also increases superoxide production through NADPH oxidase activation by effecting an increase in angiotensin II levels in the blood and blood vessels [27]. A direct effect of ethanol in the development of oxidative stress is associated with the generation of free radicals - hydroxyl, hydroxyl-ethyl, and reactive oxygen species (ROS), which react with proteins and lipids and induce their peroxidation [28]. Ethanol itself is a pro-oxidant because it directly generates ROS during its metabolism [29]. Consequences of oxidative stress are damage to DNA, lipids and proteins, resulting in the disruption of cellular homeostasis [30].

Generation of free radicals leading to oxidative stress is believed to be one of the major mechanisms underlying pathogenesis of disease [31]. The specific susceptibility of the cardiac cells to oxidative stress would be due to relatively low levels of antioxidant enzymes in the heart [32]. The pathogenesis of alcoholic fatty liver and alcoholic hyperlipidemia has been known for a long time to be due mainly to a combination of decreased fatty acid oxidation in mitochondria and to increased glycerolipid synthesis [33]. Enhanced hepatic lipogenesis, decreased hepatic release of lipoproteins, lipolysis of peripheral fat are all a result of chronic alcohol consumption [29, 34-38].

Plants are rich source of antioxidants including several phyto-constituents such as flavonoids, phenolics, glycosides, saponins, phytosterols etc, which are capable of terminating free radical reactions, thus preventing the human body from oxidative damage [39]. Phyto-constituents have proved to be beneficial in reducing a number of physiological deviations ranging from altered lipid metabolism, hemodynamics to

oxidative stress etc [39]. A detailed survey of literature shows that flavonoids/phenolics in plants/herbs are responsible for their antioxidant activity. Flavonoids, phenolic compounds, saponins etc are suggested to alleviate the symptoms of organ damage along with the physiological aberrations that can progress into the development of disease [39]. Reports of phyto-constituents such as flavonoids, saponins, polyphenolics etc eliciting cure in disease conditions abound in literature and it is suggested that the antioxidant and free radical scavenging properties of such phytochemicals could be contributing to the amelioration of disease [39].

For the present study, the plant *Thespesia populnea* (TP) belonging to Malvaceae family has been selected owing to its acknowledged medicinal properties [40]. *Thespesia populnea* (L) Sol. ex. *correa* (Malvaceae), is an evergreen shrubby tree, also known as the Indian tulip tree, and is found in the tropical and coastal regions of India [41, 42]. Screening of TP for its phytochemcial constituents has revealed the presence of flavonoids, phytosterols, phenolics, glycosides, saponins and alkaloids [43, 44]. Earlier reports on the pharmacological activities of the plant suggest its usefulness in a number of conditions such as dysentery, piles, diabetes, haemorrhoids and urinary problems [45]. Different parts of the plant have been used to effectively treat various skin disorders such as ulcers, scabies, psoriasis and wounds [43, 46]. The leaves and bark of the tree are used for their oil to treat fracture wounds and for painful joints in southern India and Sri Lanka [47-49]. The bark of the plant has been reported to exhibit memory enhancing effects [50]. Additionally, hepatoprotective [51, 52], analgesic, anti-inflammatory [53], antioxidant [54], anti-diabetic [55] and antidiuretic [56] effects of the leaf and bark of the plant have also been reported.

The leaf of TP is chosen for the present study as its preliminary phytochemical screening revealed the presence of saponins, phenolic compounds and flavonoids. Further, the leaf extract exhibited a greater *in vitro* antioxidant activity and contained a significantly higher percentage of the flavonoid quercetin, as compared to the bark or seed, which was confirmed by HPTLC analysis of the extracts.

In view of the manifold effects that have been reported in literature for this plant, and due to the presence of the above active principles in the leaf it is hypothesized that the ameliorative effect of the leaf extract of TP in countering the cardiotoxic effects caused by adriamycin and ethanol respectively could be explored.

With regard to the drugs used to ameliorate cardiotoxicity, vitamin E and carvedilol deserve attention. Vitamin E refers to a family of compounds that are lipid-soluble antioxidants capable of preventing lipid peroxidation. Naturally occurring forms of vitamin E include four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ) [57]. Biochemically, α-tocopherol functions as a chain-breaking antioxidant, that interrupts the propagation of ROS through lipid membranes by scavenging lipid peroxyl radicals [57]. Tocopherols are exclusively synthesized by photosynthetic organisms, and plant-derived oils are the major sources of vitamin E in the human diet. Vitamin E supplementation is reported to reduce the oxidative stress by elevating antioxidant enzymes and reducing lipid peroxidation [58]. An antioxidant supplementation in atherosclerosis prevention (ASAP) study with 520 subjects demonstrated that combined supplementation with vitamin C and vitamin E (α-tocopheryl acetate) significantly slowed the rate of progression of atherosclerosis in hypercholesterolemic patients [59]. Vitamin E is therefore chosen as one of the reference standards in the present study due to its antioxidative properties and established cardioprotective potential.

Carvedilol is a non-selective third generation β-blocker (β_1 and β_2) as well as a selective α_1-blocker known for its antihypertensive effect [60, 61]. Additionally carvedilol has been shown to possess antioxidant and anti-inflammatory potential [62-64]. Reports on carvedilol increasingly suggest that the drug can significantly preserve left ventricular function, up-regulate Cx43 expression in cardiomyocytes and down-regulate cellular apoptosis in an animal model of dilated cardiomyopathy at least partially through the attenuation of pro-inflammatory cytokines and induction of anti-inflammatory markers including IL-10 and endothelial nitric oxide synthases [65]. In view of the available evidences on carvedilol's beneficial effects on the ailing heart in conditions of drug/chemically-induced oxidative stress and its established therapeutic value in the treatment of various cardiac ailments, this drug too was selected as one of the reference standards in the present study.

The present study comprised investigations into different important aspects of cardiotoxicity in two models namely, (i) cardiotoxicity induced by a therapeutic drug, namely adriamycin and (ii) cardiotoxicity induced by ethanol, and the possibility of counteraction by TP leaf extract to confer cardioprotection. The effects of TP leaf extract on various aspects involved in cardiac injury such as physical changes, cardiac changes, changes in serum cardiac biomarkers, lipid profile, ATPase and oxidative stress markers

such as antioxidant enzymes, and electrocardiographic changes, supported by histopathological data were studied in the present work. Since the project aims at exploring the usefulness of naturally occurring antioxidants from plant sources, its likely impact would be the reduction in oxidative stress and reduction of/reversal from cardiotoxicity to normal cardiac functional status. The project will further pave way for exploring the antioxidant principles from different plant sources and studying their effects for treating free radical-induced cardiac damage and other organ toxicities.

CHAPTER-2
REVIEW OF LITERATURE

CHAPTER 2. REVIEW OF LITERATURE

2.1 Cardiotoxicity

Cardiotoxicity is a known side effect of numerous drugs/chemicals that can be responsible for long-term debilitating effects causing severe morbidity [66]. Cardiotoxicity as a term not only describes a direct effect of the drug on the heart but also indicates an indirect effect due to enhancement of alterations in hemodynamic flow or due to thrombotic events [3]. A variety of drugs or chemicals can target the heart and inflict injury by various mechanisms.

2.1.1 Epidemiology

Cardiovascular diseases such as hypertension, coronary artery disease, ischemia, myocarditis, cardiomyopathy with accompanying heart failure etc have become a growing concern and a global burden that affect millions worldwide. Although the most common causes of cardiac problems direct to aging or underlying metabolic disorders, over the past few decades iatrogenic effects and drug abuse have also been identified as significant contributors of cardiotoxicity leading to increasing morbidity and mortality. This has lead to a withdrawal of as much as 10% of drugs from the clinical market worldwide over the past four decades due to cardiovascular safety concerns [67]. As per the reports, incidences of cardiotoxicity have resulted in around 45% of drug withdrawals between 1994 and 2006, the reason being side effects such as ischemia and arrhythmogenesis (Table 1) due to drugs [68].

Undesirable cardiovascular effects have been the major reason for drug withdrawals that would have otherwise precipitated hazardous conditions such as acute myocardial infarction, cardiac fibrosis or cardiomyopathy etc. These conditions were identified during the post-marketing surveillance for drugs such as rofecoxib (anti-inflammatory drug),

tegaserod (a serotonin 4 receptor agonist), sibutramine (anti-obesity drug), rosiglitazone (antidiabetic drug), saxaglipitin (dipeptidyl peptidase-4 inhibitor; antidiabetic drug) etc, to name a few [69-72].

Cardiotoxicity is the major adverse effect produced by anthracycline antibiotics, but is not limited to such cytotoxic drugs alone, with reports of several other anti-cancer drugs such as paclitaxel and capecitabine [6], 5-fluorouracil [6, 73], vandetanib [74] etc causing significant changes in rhythm and myocardial contractility followed by an increased risk of coronary artery disease.

Table1. Latest drugs discontinued due to cardiotoxicity [68]

Drug Name	Drug Classification	Year of Recall
Fenfluramine	Anorectic	1997
Terfenadine	Antihistamine	1998
Sertindole	Antipsychotic	1998
Astemizole	Antihistamine	1999
Grepafloxacin	Antibiotic	1999
Cisapride	Prokinetic	2000
Droperidol	Tranquilizer	2001
Levomethadyl	Treatment of opiate dependence	2003
Rofecoxib	Nonsteroidal anti-inflammatory agent	2004
Tegaserod	Prokinetic	2007
Benfluorex	Anorectic	2009
Sibutramine	Anorectic	2010
Rosiglitazone	Antidiabetic	2010

In spite of numerous procedures adopted to evaluate the safety profile of drugs during the preclinical stages, acute and chronic cardiotoxic effects of drugs continue to lead the list of safety concerns that need to be addressed [75].

Several factors increase the susceptibility of the heart to toxic effects of drugs [76]. Those which are commonly understood are:

(1) Nutritional deficiencies

(2) Metabolic disorders

(3) Pre-existing cardiac diseases

(4) Obesity

(5) Hyperlipidemia

(6) Alcohol abuse/drug abuse

(7) chronic/inappropriate drug use

(8) Genetic predisposition

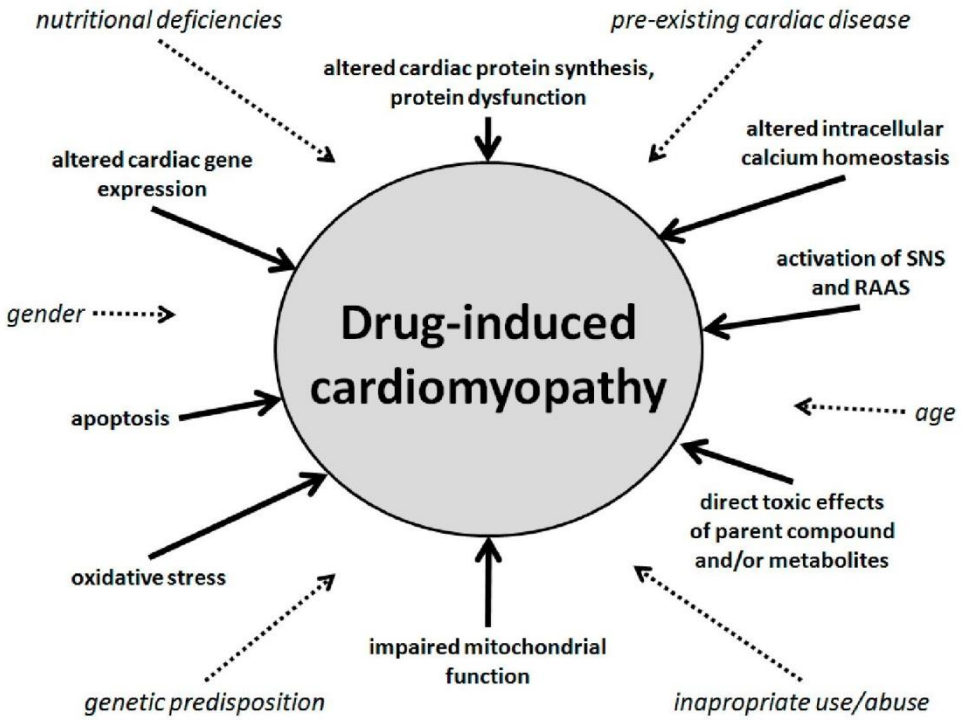

Fig 1. Potential mechanisms (bold) and risk factors (italic) involved in drug-induced cardiomyopathies. (Abbreviations: SNS – sympathetic nervous system, RAAS – renin angiotensin-aldosterone system) [76].

2.2 Pathophysiology of cardiotoxicity

The human heart is highly vulnerable to several toxins, both natural and synthetic. Despite its ability to compensate the changes in myocardial contractility or related physiological mechanisms, the cardiovascular system as a whole suffers from numerous pathophysiological insults, ranging from hypertension to cardiomyopathy and heart failure.

Cardiac toxicants are chemicals responsible for inflicting disturbances in the rhythm and function of the heart. The effects of these substances are multifarious and several mechanisms have been proposed for their negative effects on the heart. The most evident developments upon exposure to these agents are cardiac hypertrophy with heart

failure [77]. Cardiac toxicants can produce hypertrophy in several ways. One of the proposed mechanisms is by activating signalling pathways for hypertrophic changes by agents such as Doxorubicin (adriamycin), or by stimulating the release of endocrine factors, for example. cocaine, acetaldehyde, etc. Toxicant substances may also bring about hemodynamic overload thus leading to subsequent hypertrophy. Example, bleomycin or monocrotaline, or they can cause hypertrophy as a result of ischemia or hypoxia such as due to carbon monoxide, benzopyrene etc [77].

The anticancer antibiotic doxorubicin is reported to produce dilated cardiomyopathy as an end result of cardiotoxicity. Chronic abuse of substances such as alcohol has also been reported to produce dilated cardiac myopathy as a major outcome [78, 79]. Reports suggest that nicotine in cigarettes and benzo[a]pyrene present in the cigarette smoke, may cause development of atherogenic lesions in vascular smooth muscle cells, predisposing the user to atherosclerosis and myocardial ischemia [80].

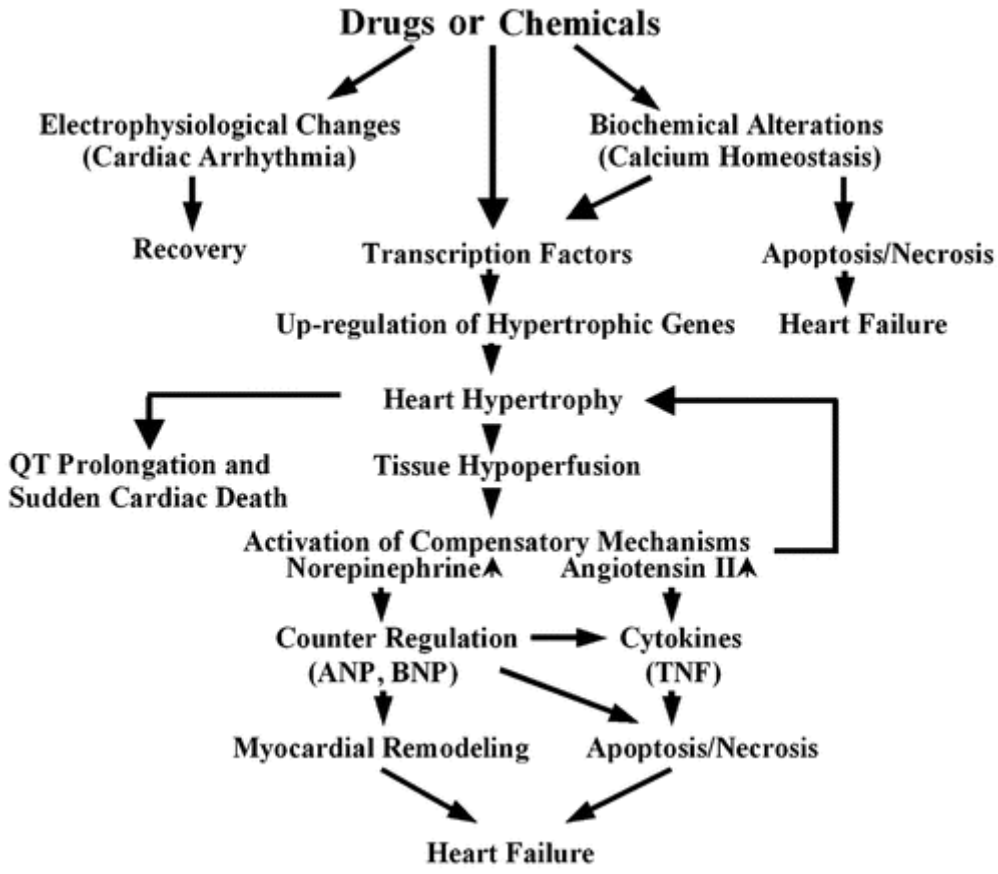

Fig 2. Pathways leading to heart hypertrophy in response to drug or chemical exposure and the transition to heart failure [81].

The role of mitogen activated protein kinases (MAPKs) in cardiotoxicity has also received considerable attention since the past few decades. One of the subfamilies of MAPK super family, the p38 MAPK is suggested to be highly responsive to toxic stress and may be associated with the development of apoptosis underlying ischemia reperfusion injury [82]. The role of P38 MAPK has also been proposed [83] where the studies reported apoptosis in primary cultures of neonatal mouse cardiomyocytes along with activation of p38 MAPK. The involvement of protein kinase C (PKC) in myocardial signalling pathways ultimately resulting in hypertrophy of the heart and its failure has also been extensively studied [84]. Studies report that disturbances in the pathways of PKC signalling may result in disturbances of the cardiac regulatory processes and hence lead to hypertrophy and ultimately heart failure [81] (Kang 2006). One possible mechanism responsible for anthracycline toxicity refers to ROS formation and site-specific DNA damage [85].

Induction of oxidative stress is widely believed to play the main role in anthracycline-induced cardiotoxicity (AIC) [86] by inducing DNA damage, sarcomere damage, mitochondrial dysfunction and loss of pro-survival signalling [87], mediating both survival and death of cardiomyocytes [88]. Another mechanism of AIC comprises the chelation reaction between iron (III) and the α-ketol group of the anticancer drugs doxorubicin (DOX) and epirubicin [89]. The cardiac tissue is susceptible to oxidative damage when exposed to high levels of hydrogen peroxide because these cells have relatively low levels of antioxidant enzymes such as superoxide dismutase and catalase [90]. In addition, cardiomyocytes are rich in mitochondria, which represent up to 50% of cardiomyocyte mass and which serve as both source and target of ROS [91].

The cardiac review and evaluation committee for trials involving the drug trastuzumab (herceptin) defined cardiotoxicity in a way that is tangible to most clinicians [92].

1 Cardiomyopathy characterized by a decrease in left ventricular ejection fraction that is either global or more severe in the septum.

2 Symptoms of congestive heart failure.

3 Signs of heart failure, including but not limited to S3 gallop, tachycardia, or both.

4 Decline in left ventricular ejection fraction of

a. 5% to below 55% with accompanying signs or symptoms of heart failure, or

b. 10% to below 55% without accompanying signs or symptoms.

In brief, cardiotoxicity typically refers to cardiomyopathy, signs and symptoms of heart failure (HF), or clinically significant reductions in left ventricular ejection fraction [93] along with cardiac arrhythmias and cardiomyocyte loss.

2.3 Agents Causing Cardiotoxicity

Several drugs produce cardiotoxicity as one of their major undesirable effects apart from their therapeutic effects. A few of them deserve mention here due to their widespread exposure/use/abuse.

2.3.1 Anabolic steroids

The synthetic derivatives of testosterone, anabolic steroids, that are most commonly used to enhance the athletic performance and muscle development in athletes, were initially developed as adjunct therapy for various medical conditions [94].

Illicit and medical anabolic steroid use has been associated with various defects of the body systems. Hypertension, ventricular remodelling, myocardial ischemia, ventricular fibrillation with sudden cardiac death have been temporarily and causally associated with the use of anabolic steroids [94]. Anabolic androgenic steroids (AAS) like other endogenous steroids influence left ventricular hypertrophic response through the androgen receptor. Androgen receptors are found on skeletal muscle and also on cardiac myocytes. They cause alterations in heart structure, including left ventricular hypertrophy and dilation which can cause impaired contraction and relaxation [95].

TABLE 2. Drugs and substances implicated in cardiomyopathy [96] (Figueredo 2011)

Amphetamine	Ethanol
Anabolic-androgenic steroids	Idarubicin
Anthraquinone	Imatinib
Antipsychotic phenothiazine derivates	Isoproterenol
Arnica herb	Ephedrine
Arsenic	Melarsoprol
Azidothymidine	Methamphetamine
Anagrelide	Methylphenidate
Catecholamines	Minoxidil
Cytarabine	Mitomycin
Clozapine	Mitoxantrone
Cobalt	Paclitaxel
Cocaine	Pentamidine
Chloroquine	Stibogluconate
Cyclophosphamide	Sunitinib
Daunorubicin	Trastuzumab
Diazoxide	Tricyclic antidepressants
Doxorubicin	Zidovudine

Potential manifestations of abuse with anabolic steroids can be dose dependent and result in the development of dilated cardiomyopathy, and heart failure [96-100]. AAS share with endogenous steroid influences on left ventricular hypertrophic response through direct actions on the androgen receptor [101] Androgen receptors are ubiquitously expressed, found not only in skeletal muscle cells but also in cardiac myocytes [102]. AAS can cause hypertension, dyslipidemia, and impaired fasting glucose level as well as alterations in heart structure, including left ventricular hypertrophy and dilation, and impaired contraction and relaxation [96].

2.3.2 Adriamycin

Anthracyclines give rise to myofibrillar disarray and subsequent poor contractility [103, 104]. Specifically, anthracyclines induce degradation of critical components of the sarcomere, like titin, by calcium-dependent kinases like calpain [105, 106]. Anthracyclines prolong the opening time of calcium channels on the sarcoplasmic reticulum, enhance the activity of calcium channels on the cell membrane, and inhibit the uptake of calcium into the sarcoplasmic reticulum, which collectively increase intracellular calcium [107, 108]. Increased intracellular calcium is a major driver of cardiotoxicity in response to anthracyclines. Calcium toxicity eventually results in hypertrophy [109], expression of non-contractile proteins [110] and fibrosis, and consequently sets the stage for the development of HF. Anthracyclines also enter cardiomyocytes via passive diffusion and cause the accumulation of ROS such as semiquinone, oxide and hydrogen peroxide [111].. These ROS also increase intracellular free iron that can lead to direct damage to DNA and the conversion of oxide and hydrogen peroxide to one of the most potent ROS hydroxyls [112]. Finally, anthracyclines impair the mitochondrial production of adenosine triphosphate [112, 113] that is essential for many cardiomyocyte functions that are energy-dependent, like contraction and calcium reuptake.

Adriamycin (also named doxorubicin) is an anthracycline antibiotic that has been used for more than 30 years for the treatment of a wide variety of cancers. It is obtained from *Streptomyces peucetius* [114]. The tumours that respond better to adriamycin are breast and esophageal carcinomas, osteosarcoma, Kaposi's sarcoma, soft-tissue sarcomas, and Hodgkin's and non-Hodgkin's lymphomas [115-117]. In spite of its potential benefit in the treatment of various cancers, its liability to cause cardiotoxicity limits its use. Anticancer drugs of each class individually and in combination of therapies for the treatment of various cancers come with differential risks for the development of HF [6]. In general, however, cardiotoxicity in response to cancer therapies can result from direct cardiomyocyte injury or inflammation, thromboembolic events and subsequent ischemia and/or therapy-induced hypertension [3].

Anthracycline toxicity may proceed in many ways although the one major pathway is via generation of reactive oxygen species and site specific DNA damage [85]. Abnormal changes that may result in the death of cardiomyocytes due to anthracyclines may include

damage to the DNA, sarcomeres, accompanied by mitochondrial dysfunction and diminished pro-survival signalling mechanisms [87]. A second mechanism that is suggested is the chelation reaction that occurs between iron (III) and the α-ketol group of doxorubicin and similar anticancer drugs [89]. Dexrazoxane (DEX) treatment prevents anthracycline cardiotoxicity by this mechanism which supports the involvement of iron chelation in the development of cardiotoxicity [118]. Other factors such as disruption of the sarcomeric structure or elevated levels of inflammatory mediators and accumulation of the end products of oxidative mechanisms may also contribute to the cardiotoxic effects of these drugs [119].

Oxidative stress due to doxorubicin is suggested to activate apoptotic signalling in cardiacmyocytes [120] and studies report interactions between heat-shock factor1 (HSF-1), heat- shock protein 25 (Hsp 25), and P53 leading to the production of pro-apoptotic proteins [121]. Other mechanisms involving the apoptotic effects of doxorubicin mediate alteration of calcium homeostasis that can produce direct effects on the cardiomyocytes such as activation of L-type calcium channels [122] or inhibition of the Na^{+}-Ca^{2+} exchanger [123]. Therefore elevated intracellular calcium is suggested to promote the generation of free radicals and alterations in mitochondrial permeability leading to the release of cytochrome C, an important step implicated in apoptosis [124, 125]. Studies by Kim and co-workers [126] have demonstrated that DOX/ROS-mediated increase of [Ca2+]i plays a critical role in cardiomyocyte apoptosis.

The acute side effects of adriamycin, which may develop within minutes after intravenous administration of the drug are: nausea, vomiting, myelosuppression and arrhythmias. These effects are reversible and clinically manageable [127, 128]. The chronic side effects, which may develop several weeks or months after repetitive doxorubicin administration, include cardiovascular signs indicative of chronic cardiomyopathy and in the last term congestive heart failure. These effects are irreversible and have a grave prognosis [115, 129]. Features associated with chronic cardiomyopathy by adriamycin in patients are marked hypotension (blood pressure 70/50 mmHg), tachycardia, development of cardiac dilatation and ventricular failure. Increase in serum glutamic-oxalacetic transaminase, lactate dehydrogenase, and creatinine phosphokinase enzyme activities have been noted [127]. The manifestations of adriamycin cardiotoxicity can be diverse and may range from prolongation of QT interval to acutely induced cardiac arrhythmias, changes in coronary

vasomotion with consecutive myocardial ischemia, myocarditis, pericarditis, severe contractile dysfunction, and potentially fatal cardiac insufficiency [130].

The ultrastructural events associated with adriamycin cardiomyopathy in patient's biopsy samples are the loss of myofibrils, cytoplasmic vacuolisation, swelling of mitochondria and increased number of lysosomes [131, 132].

2.3.3 Alcohol

Since ancient times, alcoholic and alcohol-containing beverages have been consumed during recreational activities and have been considered symbolic of high society culture worldwide. Alcoholic beverages have been consumed in multiple societies through the centuries and cultures.

Innumerable reports over many decades on the effects of alcohol only suggest the continued interest in exploring the molecular mechanisms of this chemical agent and the subsequent changes it produces in different organ systems.

Ethanol or its metabolites can prompt a sharp increase of free radicals in the human body by acting as a prooxidant or by reducing antioxidant levels and contributing to the progression of a variety of chronic diseases [133]. Alcohol intake increase oxidative stress by compromising the antioxidant defense system. Reactive oxygen species (ROS) are highly reactive and can damage lipids, proteins and DNA [134]. Reactive oxygen species, and reactive nitrogen species, alike are capable of damaging several cellular components such as proteins, lipids and DNA [135].

Alcoholic cardiomyopathy develops as a result of chronic high-dose alcohol consumption and nutritional deficiency in the rat model [136]. Oxidative stress derived from alcohol metabolism has been a major focus in the study of alcohol-induced tissue injury. The metabolism of alcohol produces ROS detrimental to the cellular antioxidant defense system [137], causing cell injury [31]. The response of the body to chronic or acute administration of ethanol has been shown to result in generation of oxygen-derived free radicals to cause alterations in cardiac muscle [137] Ethanol has been shown to interfere with a number of myocardial metabolic steps and cellular mechanisms [138]. The cardiovascular system is a major target for ROS. It has been observed that ROS play an important role in the onset of cardiac toxicity in chronically ethanol-intoxicated animals, causing alterations in the cardiac muscle [137]. Acute cardiovascular changes with acute

harmful consumption of alcohol may manifest as changes in contractility of the myocardium with associated systolic and diastolic disturbances, disturbances of rhythm and sudden death [139]. Such abnormal changes produced by ethanol have been observed with pharmacologic concentrations of ethanol in 1% (by volume) range in studies carried out on isolated myocardium [140]. Reports by Urbano and co-workers suggest that one third of the alcoholics have an ejection fraction of 55 percent or less. These patients show histologically defined changes of cardiomyopathy when analysed using endomyocardial biopsy specimens. Concurrent smoking, hypertension, and malnutrition appear highly associated with the increased risk for developing an alcoholic cardiomyopathy. The incidence of alcohol as a major contributor to cardiomyopathy has been reported to be in the range of 20% to 30%, emphasizing the clinical need to recognize the risk and contribution of alcohol in heart-failure patients [141].

Reports suggest that alcohol might produce its effects on muscles by altering the permeability of the sarcoplasmic reticulum to calcium ions thereby effecting a reduction in the efficiency of calcium activated muscle contraction [142]. Studies by Chen and co-workers suggest that acute alcohol exposure may trigger the process of apoptosis in cell cultures and lead to the induction of pro-apoptotic protein Bax expression and increased caspase-3 enzyme activity [143]. The major target of ethanol-induced oxidative stress is mitochondrial DNA, whose damage leads to impaired function of mitochondria and inhibited synthesis of proteins encoded by mitochondrial DNA [144]. It is proposed that alcohol increases intracellular Ca^{2+} by direct upregulation of voltage-gated Ca^{2+} channels [24]; inhibition of Ca^{2+}-adenosine triphosphatase (Ca^{2+}-ATPase) that extrudes Ca^{2+} from the cells [25]; and magnesium ion (Mg^{2+}) depletion that inhibits the sodium ion (Na^{+})-potassium ion (K^{+}) pump (Na^{+}/K^{+}-ATPase), causing a build up of intracellular Na^{+} [26, 145]. This reaction in turn inhibits the Na^{+}/Ca^{2+} exchanger, thereby increasing the intracellular calcium ions [146-149].

Alcohol also increases superoxide production through NADPH oxidase activation by effecting an increase in angiotensin II levels in the blood and blood vessels [27]. In the endothelium, depletion of NO production or NO reaction with superoxide anion to form toxic peroxynitrite radical has been reported to cause endothelial injury, impairment and hypertension in alcohol-treated rats [150-155]**.** Ethanol itself is a prooxidant because it directly generates reactive oxygen species during its metabolism [29].

The pathogenesis of alcoholic fatty liver and alcoholic hyperlipidemia has been known for a long time to be due mainly to a combination of decreased fatty acid oxidation in mitochondria and to increased glycerolipid synthesis [33], enhanced hepatic lipogenesis, decreased hepatic release of lipoproteins, and lipolysis of peripheral fat [34].

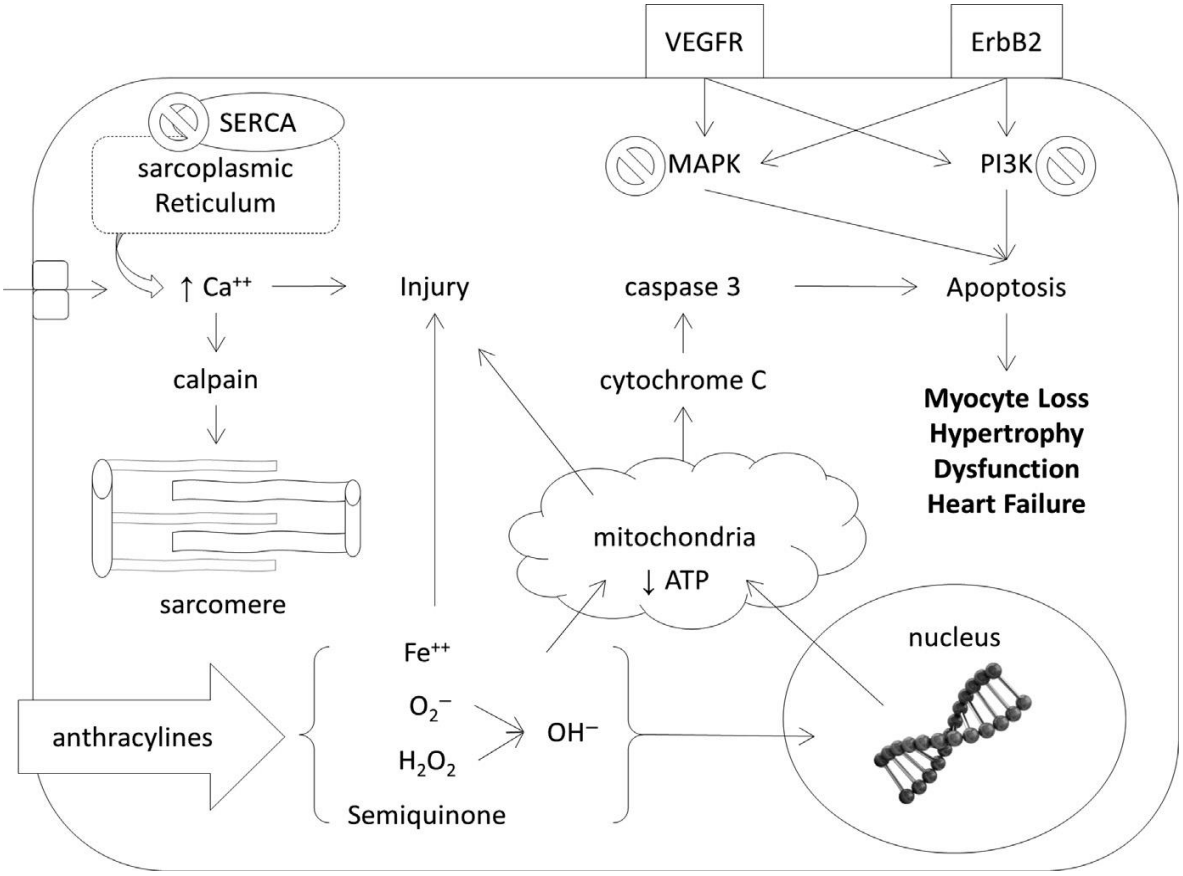

Fig. 3. Mechanisms of direct cardiomyocyte toxicity with cancer drugs. Anthracyclines increase intracellular calcium by several means, including prolonging the opening time of ion channels, such as the ryanodine channel on the sarcoplasmic reticulum, and L-type calcium channels on the cell membrane, and inhibiting the uptake of calcium through the sarcoplasmic reticulum. Increased intracellular calcium stimulates calpain to break down critical components of the sarcomere, like titin, and also results in the expression of non-contractile proteins and fibrosis. In addition to calcium toxicity, another common mechanism of cardiotoxicity is oxidative stress. Anthracyclines, as an example, enter cardiomyocytes via passive diffusion and give rise to several ROS, including semiquinone, O_2 and H_2O_2. These ROS can cause direct cell injury and death but also can give rise to free iron and hyper-ROS, like OH, that can result in direct damage to DNA and subsequent decrease in mitochondrial ATP production. In response to several cancer drugs, mitochondria release cytochrome C that brings about apoptosis through the actions of caspase 3. Cancer drugs that block VEGF or VEGFRs or downstream intracellular processes thereof alter several cell survival signals within cardiomyocytes, including the MAPK and PI3K pathways. ErbB2 receptors are also associated with the

MAPK and PI3K survival pathways. Thus, apoptosis is a common mechanism of cardiotoxicity with cancer drugs that target VEGF or ErbB2 receptors and results in myocyte loss, hypertrophy, ventricular dysfunction, and eventually heart failure. ATP, adenosine triphosphate; Ca11, calcium; ErbB2, ErbB2 (otherwise known as the HER2/ neu receptor); Fe11, iron; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; O_2, oxide; OH, hydroxyl; PI3K, phosphatidylinositide 3-kinase; SERCA, sarcoplasmic reticulum calcium adenosine triphosphatase; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor [142].

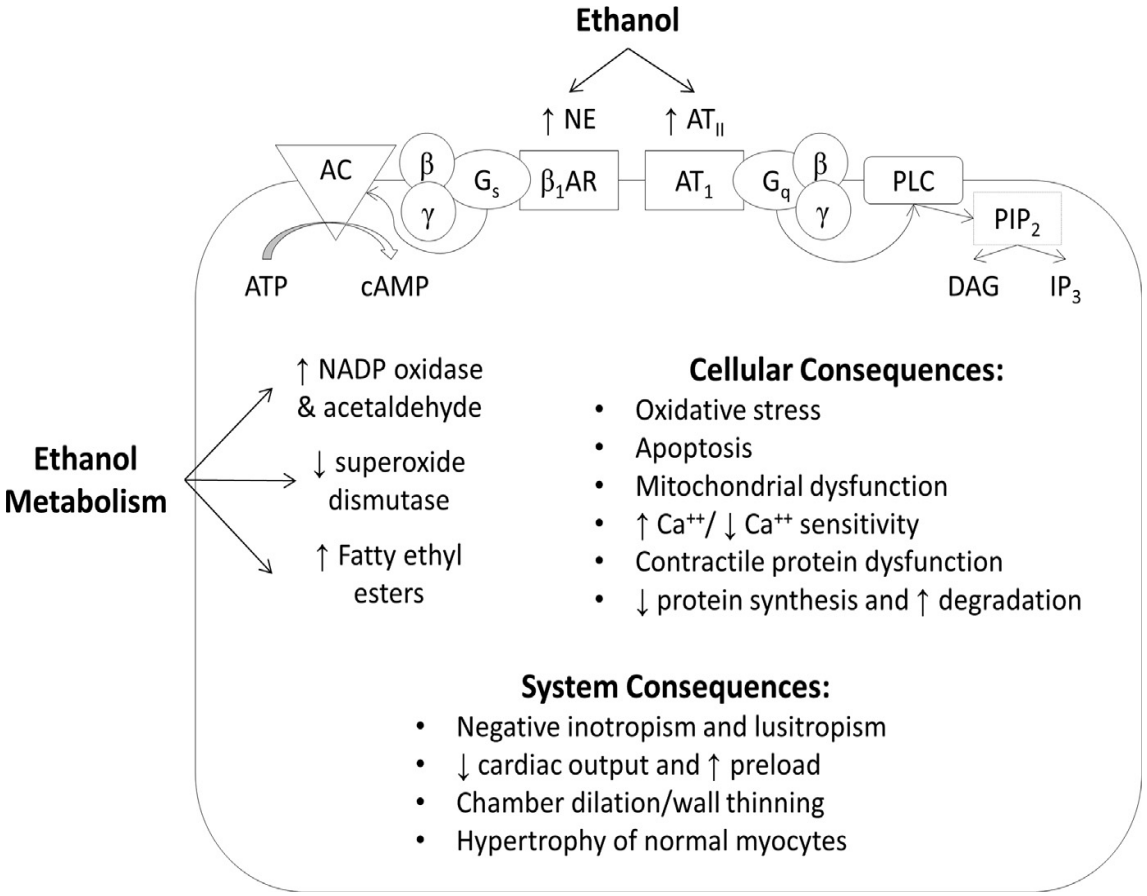

Fig.4. Influence of ethanol on the heart. Long-term and heavy ethanol use increases both NE and AT_{II}. When NE binds with a b_1AR, the 'a' subunit of the stimulatory G protein dissociates and increases the activity of the enzyme AC. AC generates cAMP from ATP. cAMP acts within the cells as the second messenger of NE. When AT_{II} binds with an AT1, the q subunit dissociates from the G-protein complex and activates PLC. PLC in turn hydrolyzes PIP_2 into IP_3 and DAG that act as the second messengers of AT_{II}. Metabolism of ethanol results in several ROS, including NADP oxidase and acetaldehyde and reduces the production of antioxidants like superoxide dismutase. The metabolism of ethanol also results in the accumulation of fatty ethyl esters within cardiomyocytes. Cumulatively, the actions of the intracellular second messengers of NE and AT_{II} (indirect effects) and the end-products of ethanol metabolism

(direct effects) result in a cascade of cellular and system consequences that lead to the development of heart failure. AC, adenylyl cyclase; AT_1, angiotensin II type 1 receptor; AT_{II}, angiotensin II; ATP, adenosine triphosphate; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; Gq, Gq protein; Gs, a subunit of the stimulatory G protein; IP_3, inositol triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; NE, norepinephrine; PIP_2, phosphatidylinositol biphosphate; PLC, phospholipase C; b, b subunit of the G protein; b_1AR, b1-adrenergic receptor; g, g subunit of the G protein [142].

2.3.4 Carbon monoxide

One of the most common air pollutants and causal agents for respiratory disorders, carbon monoxide (CO), is also reported to affect the heart and cause cardiotoxicity, the mechanism of which is incompletely understood [156]. In CO-poisoned patients, an altered balance between ROS and antioxidant levels has been reported [157]. Free radicals and oxidative stress are among factors involved in pathogenesis of acute CO poisoning and particularly appear to have a role in CO-induced cardio-toxicity [158].

2.3.5 CNS Stimulants

CNS stimulants such as cocaine, amphetamines or methamphetamines have been studied for their acute and chronic effects on the myocardium. Abuse with these agents is thought to be secondary to direct cardiac toxicity and indirect amphetamine-induced hypertension, necrosis, and ischemia [159]. It is suggested that 3, 4-methylenedioxymethamphetamine, commonly known as ecstasy, can cause myocardial infarction, arrhythmias, and cardiomyopathy [96, 160]. Animal studies showed that repeated methamphetamine administration may directly induce cellular hypertrophy of cardiomyocytes, myocarditis with inflammatory infiltrates and areas of necrosis, and consequently, may cause eccentric left ventricular dilation and diastolic dysfunction, as well as contractile dysfunction in myocytes [161]. High dose administration may lead to cardiac function disorder with disruption of microtubules and actin [162]. It was suggested that metabolites are responsible for cardiotoxicity [163] as 3,4methylenedioxymethamphetamine (MDMA) is metabolized to catechols and the metabolites of MDMA are reported to undergo redox cycling, producing reactive oxygen and nitrogen species [164].

2.3.6 Cocaine

Cocaine, a widely known substance of abused, has been reported to be frequently associated with acute cardiovascular illness. Cardiovascular complaints, particularly chest pain, are common among cocaine users [165, 166]. The effects of cocaine on the cardiovascular system are suggested to be occurring via the inhibition of norepinephrine reuptake into the synaptic cleft by sympathetic neurons [166]. Cocaine use has also been associated with an increase in platelet count [167], increased platelet activation [168] and platelet hyper-aggregability [169]. Reports suggest that chronotropic effects of cocaine use are intensified in the setting of alcohol use [170]. It is reported that cocaine administration can reduce left ventricular function and increase end-systolic wall stress [171]. Studies have also suggested that cocaine users have elevated levels of C-reactive protein, von Willebrand factor, and fibrinogen that may also contribute to thrombosis [172].

2.4 Available Treatments for Cardiotoxicity:

TABLE 3. Treatments available for anthracycline cardiotoxicity [173]

Drug	**Actions**
Dexrazoxane (75)	Antioxidant agent against development of AIC, without increasing non-cardiac and non-hematologic toxicity
Selenium(78)	Antioxidant agent
Probucol(83)	Antioxidant agent preserves cardiac function
Ranolazine(85)	Selective inhibitor of the cardiomyocyte late inward sodium current (INaL), with anti-ischemic, antiarrhythmic and ATP-sparing actions
Statins(86)	Inhibition of the Ras-homologous GTPase Rac1
β-blockers(88)	Antioxidant and anti-apoptotic effects

TABLE 4. Treatment of alcoholic cardiomyopathy [174]

Medication	Treatment goal	Dosage	Adverse reaction	Evidence
ACE Inhibitors	HF + Prognosis	As tolerated	-	High in HF
β-blockers	HF + Prognosis	As tolerated	-	High in HF
Diuretics	HF + Prognosis	As needed	-	High in HF
Digitalis	Rate Control	According to digoxin or digitoxin level	Avoid dosage	Moderate in atrial fibrillation (AF)
Anticoagulants	Avoid stroke	INR 1.8-2.2 in AF	Bleeding	High in HF

Treatment of alcoholic cardiomyopathy follows the usual regimen for therapy of heart failure, including ACE inhibitors, beta-blockers, diuretics including spironolactone or eplerinone, and digitalis in atrial fibrillation for rate control together with anticoagulation, whenever appropriate [174].

2.5 Oxidative Stress and Endogenous Antioxidants

Living beings are equipped with natural cellular defences which help in countering the harmful effects of exogenous insults. This is because the cells have developed several protective mechanisms to prevent free radical formation or to detoxify the free radicals [31].

Endogenous oxidative stress can be the result of normal cellular metabolism and oxidative phosphorylation [175]. ROS are generated mainly by the mitochondrial electron transport chain (ETC). Almost all cells and tissues continuously convert a small proportion of molecular oxygen to ROS in ETC [175] Reactive oxygen species (ROS) are produced by other pathways, including the respiratory burst taking place in activated phagocytes, due to ionizing effect of radiation on components of cell membranes, and as byproducts of several cellular enzyme (NADPH oxidases, xanthine oxidase, nitric oxide synthase) mediated reactions [175]. An intracellular increase in ROS has been shown to induce damage to mitochondrial respiratory chain complex proteins and also to mitochondrial DNA [176].

So, a lack of adequate energy for intracellular metabolism would likely contribute towards a defective antioxidant defense [176].

Exogenous sources of ROS can also have an impact on the overall oxidative status of a cell. Drugs, hormones, and other xenobiotic chemicals can produce ROS by either direct or indirect mechanisms [177-179]. Alternatively, oxidative stress can also occur when there is a decrease in the antioxidant capacity of a cell. Non-enzymatic antioxidant levels (vitamin E, vitamin C, glutathione etc) and enzymatic antioxidant levels (superoxide dismutase, glutathione peroxidase, and catalase) in the cell can be decreased through modification in gene expression, decreased in their uptake in the diet, or can be overloaded in ROS production, which creates a net increase in the amount of oxygen free radicals present in the cell [180, 181].

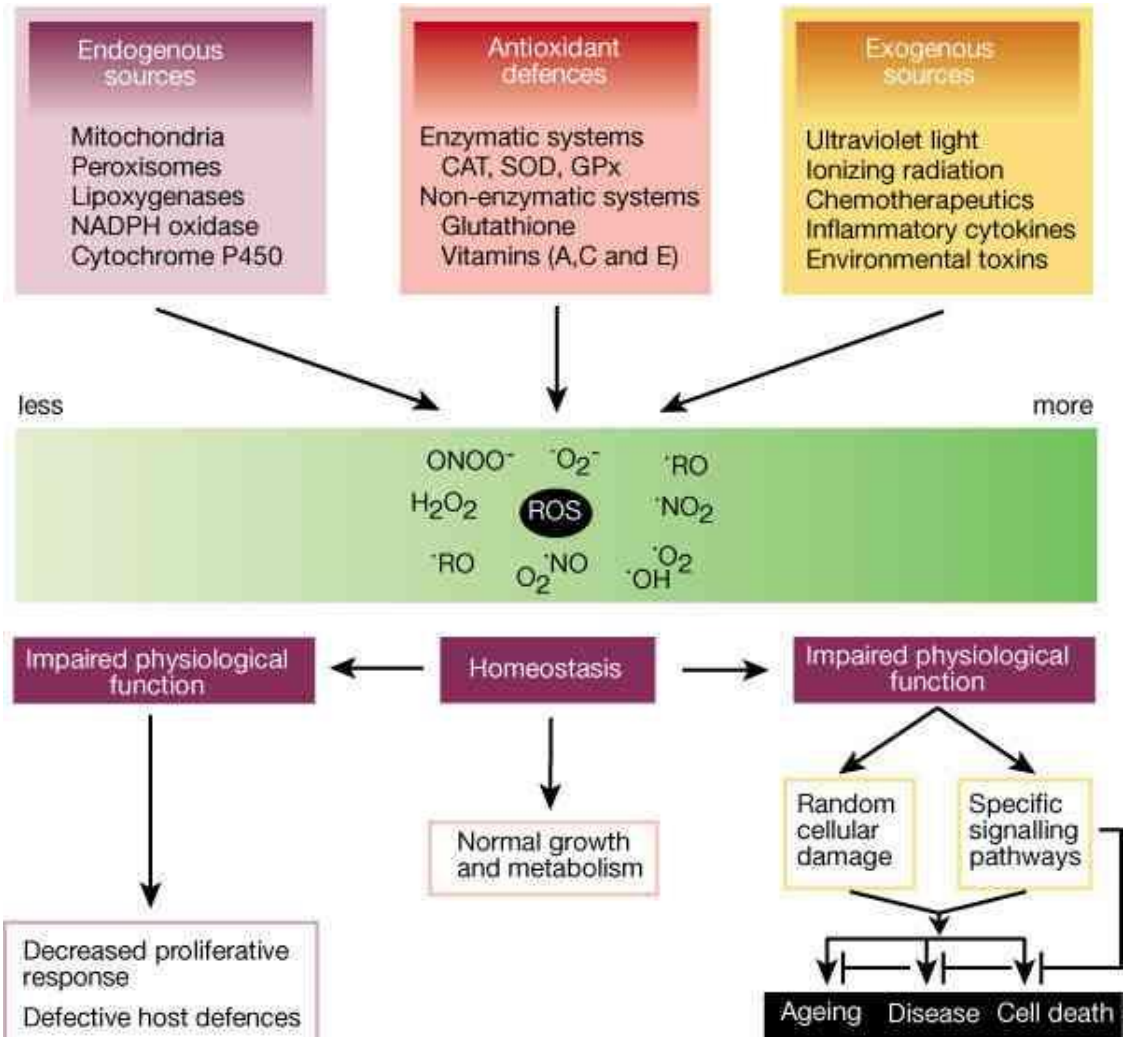

Fig 5. Sources of ROS, antioxidant defences, and subsequent biological effects depending on the level of ROS production [182]

Free radicals play an important role in tissue injury by altering the oxidant-antioxidant equilibrium [183, 184]. Superoxide dismutase (SOD) is an important endogenous antioxidant enzyme and can exist in several common forms. It contains copper and zinc, or manganese, iron or nickel and acts as the first line defense system against ROS by scavenging superoxide radicals. SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. Humans contain three forms of SOD: SOD_1 is located in the cytoplasm, SOD_2 in the mitochondria, and SOD_3 is extracellular. Catalase (CAT) is a common, highly efficient antioxidant enzyme found in cells. It is a tetramer of four polypeptide chains and contains four porphyrin heme groups which allow the enzyme to react with H_2O_2. Each CAT molecule can decompose millions of H_2O_2 molecules to water and oxygen every second. Glutathione peroxidase (GPX) present in the cytoplasm of the cells protects the cell against oxidative injury caused by H_2O_2 and prevents the formation of hydroxyl radical from H_2O_2. It consists of four protein subunits, each of which contains one atom of the element selenium at its active site. GPX removes H_2O_2 by coupling its reduction to H_2O with oxidation of GSH. Glutathione reductase (GR) is a flavoprotein enzyme and an important cellular antioxidant necessary for the conversion of GSH. The oxidised glutathione is glutathione disulphide (GSSG) which is reduced back to GSH in the presence of the enzyme GR which uses NADPH as an electron donor. The ratio of GSH/GSSG is an important general measure of oxidative stress of an organism. Very high concentration of GSSG may damage many enzymes oxidatively [185-187]. Glutathione is a tripeptide and a powerful antioxidant which is highly abundant in the cytosol and is the major soluble, non-enzymatic antioxidant in these cell compartments. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are essential for DNA repair and expression. It is the major intracellular non-protein thiol compound (NPSH) synthesized intracellularly from cysteine, glycine and glutamate. GSH is important in maintaining -SH groups in other molecules including proteins, regulating thiol-disulfide status of the cell, and detoxifying foreign compounds and free radicals. GSH is capable of scavenging hydroxyl radical and singlet oxygen directly, or detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of GPX. GSH is also involved in amino acid transport through the plasma membrane, regeneration of some important antioxidants, and regulation of vitamin C and vitamin E. For example, glutathione can reduce the tocopherol radical of vitamin E directly or indirectly via reduction of semi-dehydro-ascorbate to ascorbate [185-187, 188, 189].

In recent years there has been increased interest in the therapeutic use of antioxidants in the treatment of disease associated with oxidative stress [188, 190, 191]. Several studies reported that low antioxidant intake or low blood levels of antioxidants increases the risk of different diseases, causes low dietary intake of fruits and vegetables, and doubles the risk of cancer [188]. In normal physiological condition the generation of ROS is tightly regulated by different enzymatic and non-enzymatic antioxidants. Overproduction of ROS results in oxidative stress, which is important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA [186, 187]. Antioxidants are defined as the substances which at low concentration significantly inhibit or delay the oxidative process, while often being oxidized themselves. Endogenous and exogenous antioxidants are used to neutralize free radicals and protect the body from free radicals by maintaining redox balance [185, 187, 192-194]. The term reactive oxygen intermediate (ROI) describes the chemical species formed upon incomplete reduction of molecular oxygen, namely superoxide radical anion (O_2•−), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•), while ROS includes both ROI and ozone (O_3) and singlet oxygen ($^1O_2$) [195]. A somewhat more inclusive definition also includes within ROS, compounds such as hypochlorous (HOCl), hypobromous (HOBr), and hypoiodous acids (HOI). Incorporation of peroxyl (ROO•), alkoxyl (RO•), semi-quinone (SQ•−) and carbonate (CO_3•−) radicals and organic hydroperoxides (ROOH) is also frequently encountered within the definition of ROS [190]. ROS may also be classified as free radicals and nonradical species [188, 191]. Reactive nitrogen species (RNS) that bear oxygen atoms include nitric oxide radical (NO or NO•), nitrogen dioxide radical (NO2•), nitrite (NO2−), and peroxynitrite ($ONOO^-$) [196]. ROS, in particular hydroxyl and peroxyl radicals, hydrogen peroxide and superoxide radical anion, have long been implicated in oxidative damage inflicted on fatty acids, DNA and proteins as well as other cellular components [186]. ROS overproduction is associated with numerous disorders.

Oxidative stress caused by the imbalance between excessive formation of ROS and limited antioxidant defences is connected to many pathologies including age-related disorders, cancer, cardiovascular, inflammatory, and neuro-degenerative diseases such as Parkinson's and Alzheimer's diseases [193, 197-199].

2.6 Plant Profile

Thespesia populnea (L.) Soland. ex Correa (TP) is an evergreen tree growing up to a height of 20 m, with a dense crown. The bark is greyish brown in colour and the twigs are densely covered with glabrescent, brown to silvery scales [200].

The tree belongs to the family Malvaceae and blossoms and bears fruit all through the year. It is pantropic in distribution and is a native of Australia, China and India.

Local names [200]

Bengali: palaopipal; **English:** Indian tulip tree; **French:** motel debou; **Gujarati:** paarsapeepla; **Hindi:** paras-pipal; **Sanskrit:** gardha-bhanda; **Tamil:** poovarasam; **Telugu:** Gangaraavi.

Fig 6. *Thespesia populnea* tree with flowers

2.6.1 Phytoconstituents of *Thespesia populnea*

Gossypol was found to be the major component of TP [201] producing anti-fertility effects in rats [202, 203] as well as in human beings [204]. Four naturally occurring quinones viz. thespone, thespesone, mansonone-D, and mansonone-H have been extracted from

heartwood of the plant [205]. The phytochemical study of bark reveals the presence of gossypol, tannin and coloring matter [206] and leaf extract indicates the presence of lupeol, lupenone, β-sistosterol [207] and also acacetin, quercetin, and vanillic, syringic, melilotic, and ferulic acids [206].

Phytochemical analysis of TP leaf claims the presence of lupeol, lupenone, β-sitosterol, acacetin, quercetin, and vanillic, syringic, melilotic and ferulic acids [55, 205, 206].

Bark of TP has been reported to contain four naturally occurring quinones, viz. thespone, thespesone, mansonone-D and mansonone-H. Gossypol, tannins, and coloring matter have also been reported to be present in the heartwood of the plant [55, 205, 206].

Seeds have been reported for the presence of β-sitosterol, ceryl alcohol, and a yellow pigment thespesin [203, 208, 209].

Flowers have been claimed to be possessing kaempferol, kaempferol-7-glucoside, gossypetin and herbacetin [203, 208, 209].

2.6.2 Update of Pharmacological Studies Reported

The heartwood has a healing property useful in treating pleurisy and cholera, colic and high fevers; it is carminative. The cooked fruit crushed in coconut oil provides a salve, which, if applied to the hair, kills lice [200]. The sap of the leaves and decoctions of most parts of the plant are used externally to treat various skin diseases. Juices from the pounded fruits mixed with pounded leaves are ingredients of a poultice to treat headaches and itches [200]. A decoction of the astringent bark is used to treat dysentery and haemorrhoids, and a maceration of it is drunk for colds. The fruit contains an antibiotic and the juice is used to treat herpes. Other extracts of the plant have significant anti-malarial activity. Leaf and bark decoctions are taken for high blood pressure [200]. Leaf tea is taken for rheumatism and urinary retention. Seeds are purgative. An ayurvedic preparation namely “panchvalkala” contains TP and is suggested to possess free radical scavenging activity [210].

α-Amylase inhibitory activity was reported for ethyl acetate and methanolic extracts of the leaves of TP [211]. This effect was correlated to the presence of phenolic acids such as ferulic acid, vanillic acid, ellagic acid, coumaric acid, and gallic acid.

Analgesic and anti-inflammatory properties of leaf extracts of TP for different animal models of chemical, mechanical and thermally induced pain have been studied [53]. It was reported in the study that after oral administration of aqueous and ethanol extracts of TP leaf in the doses of 100, 200 and 400mg/kg body wt, significant reduction in carageenan-induced paw edema was observed in rats.

Several studies have demonstrated hepatoprotective and nephroprotective effects of different extracts of TP leaf [52] using ethanol as the inducing agent for organ damage. The studies involved assessment of antioxidant biomarkers supported by histopathological evidence indicating amelioration from tissue damage due to oxidative stress.

Methanolic extract of leaves were studied for nephroprotective effect in cisplatin-induced nephrotoxicity and hepatotoxicity [212]. The study suggests an increase in biochemical parameters such as urea, creatinine, bilirubin, ALT (alanine transaminase) and AST (aspartate transaminase) due to cisplatin and these biochemical changes were effectively ameliorated by the leaf extract of TP.

Ethanolic extracts of bark and leaves of TP have been evaluated for their anti-hyperglycemic and antioxidant effects in streptozotocin-induced diabetes [55]. The study demonstrated that the bark and leaf extracts exhibit significant anti-hyperglycaemic activity due to their antioxidant potential.

The antibacterial and antifungal activity of TP leaves were determined [213] by disc diffusion method and estimating the minimum bactericidal and minimum fungicidal concentration of the crude extracts of TP leaves. Two gram-positive (*Staphylococcus aureus* MTCC 7443 & 737) and eight gram-negative bacterial strains (*Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus pyogenes*, *Salmonella typhimurium*, *Shigella flexneri*, *Proteus mirabilis*, *Proteus vulgaris* and *Vibrio cholerae*) were used in the study to evaluate the antibacterial activity of hexane, chloroform, ethyl acetate and methanol crude extracts of leaves of TP. The study suggests that the chloroform extract of TP exhibited the highest antibacterial activity and the methanol extract showed the highest activity against *Aspergillus fumigatus* and *Microsporum gypseum*.

TP bark has been evaluated for memory enhancing activity [270] with a reversal of scopolamine- or diazepam-induced amnesia in doses of 200 and 400mg/kg and also a reduction in transfer latency (TL) and time taken to reach reward chamber (TRC).

Anti-steroidogenic activity was determined [214] in methanolic extract of TP bark in the doses of 100, 250 and 400mg/kg. Serum cholesterol, AST and ALT activities, urea, uric acid, ovarian protein content, 3β-hydroxysteroid dehydrogenase and 17 β-hydroxysteroid dehydrogenase activities, estradiol and progesterone concentrations in the serum were estimated. The study revealed a decrease in ovarian cholesterol content along with serum estradiol due to the inhibition of the above mentioned steroidogenic enzymes, hence exhibiting a steroidogenic effect of TP.

Different extracts of TP bark such as petroleum ether, butanolic, ethyl acetate and successive alcoholic extracts were evaluated for antipsoriatic activity in perry scientific mouse tail model [215]. The extract was studied for its potential to cause orthokeratotic cell differentiation which was expressed as number of scale regions per section and hence was suggested to be of potential use in the treatment of psoriasis.

Methanolic extract of flowers was evaluated for antiviral effect as minimal inhibitory concentration (MIC50) required to reduce virus-induced cytopathogenicity by 50% in confluent cell cultures [216]. Cytotoxic effect was determined from minimal cytotoxic concentration that was sufficient to alter the normal cell morphology of the confluent cell cultures that were exposed to the extracts.

The foregoing review of literature explains cardiotoxicity and the different agents that could affect the functioning of the heart. It is apparent that a reasonable amount of research has been carried out on plant components and the phytoconstituents thereof in an attempt to find ameliorative measures for cardiotoxicity. *Thespesia populnea* is one of the plants examined by researchers for its potential to treat different ailments. These researches have met with a fair degree of success. However, no investigations are available on cardio-protective effects of *Thespesia*, as can be seen from this review of literature. In the present study, the protective effects of *Thespesia* have been examined during cardiotoxicity induced by adriamycin and ethanol, taking physical parameters, biochemical parameters, EEG and histopathology as indices.

CHAPTER-3
MATERIAL AND METHODS

CHAPTER 3. MATERIAL AND METHODS

3.1 Experimental animals and their maintenance

Wistar strain, male albino rats, weighing 200 ± 20 g were obtained from Sun Pharma Advanced Research Company Pvt. Ltd., Tandalja, Vadodara. The rats were housed in clean polypropylene cages, maintained in a temperature-controlled room (25 ± 2°C) with a photoperiod of 12 h light and 12 h dark cycle. The rats were provided with standard pellet diet (VRK Nutritional Solutions, Laboratory Animal Diets, Pune, India) and water *ad libitum* throughout the experimental period. The protocol for this study was approved by the Institutional Animal Ethics Committee (Regd. No. 1029/PO/ERe/S/07/CPCSEA) in its proposal number BIP/IAEC/2015/07 dated 3rd July 2015.

3.2 Selection of plant material and preparation of crude extract

Fresh leaves of *Thespesia populnea* (TP) were collected. The plant material was taxonomically identified and authenticated by the Botanical Survey of India, Jodhpur. A voucher specimen (No.: BSI/AZRC/I.1202/Tech/2012-13 (PI.Id.)/719) was deposited in the herbarium for future reference.

Leaves for the study were selected out of the collected lot by handpicking those that were not mottled, crumpled, rusty or discoloured. They were then dried under shade.

Sufficient quantity of leaves was used to obtain 100g of dried leaf powder. After thorough cleaning, the leaves were dried under shade and were then powdered in an electric grinder, sieved using a 24 mesh sieve to obtain 100 g of fine leaf powder as the final weight and used for extraction. This powder was initially defatted with petroleum ether (40%-60%). Out of the defatted leaf powder, 30g was taken each time, soaked in 250 ml of water and allowed for percolation for 24hr. The solvent was filtered using a moist muslin cloth. 100ml water was added to the residual leaf powder again and the extraction was continued. This process was repeated three to four times until a colourless extract was obtained. Finally, the extract was distilled and

concentrated under reduced pressure in a Buchi Rotavapor (R-114) to yield a dark coloured semisolid residue, which was then dried in a vacuum desiccator to remove any remaining water. The percent yield of the aqueous extract obtained was 13.6%. The same procedure was followed each time for the preparation of extract as and when required for the study.

The aqueous extract (AQ-E) was suspended in 5% gum acacia for obtaining two concentration doses equivalent to 200 mg/kg and 400 mg/kg, calculated according to the body weight, and was used in all experiments. [217].

3.3 Preliminary phytochemical screening of the extract

The aqueous extract of TP leaf was subjected to phytochemical screening as per standard experimental procedures and evaluated for the presence of flavonoids, phenolic compounds, alkaloids, glycosides, saponins, steroids and carbohydrates [218, 219].For the estimation of flavonoids and phenolic compounds, the concentration of the extract taken was 1mg/ml. From this 1mg/ml TP extract, 100 µg/ml was taken for the estimation of flavonoids.

3.4 Estimation of total flavonoid content of the extract

Procedure for calibration curve for quercetin:

Calibration curve was obtained by taking different concentrations of quercetin (10-100 µg/ml) in methanol. To 1 ml of each concentration of quercetin solution, 2.5 ml distilled water was added. 150 µl of 5% $NaNO_2$ solution was then added. After 6 minutes, 150 µl of 10% $AlCl_3$ was added to the solution. After 5 minutes, 1ml of 1M NaOH was added and the absorbance was measured immediately at 510 nm against the blank and the calibration curve was prepared.

The total flavonoid content was determined by the method of Zhishen *et al*. [220]. Quercetin was used as the reference standard in this estimation. 100 µl of TP leaf extract and 2.5ml of distilled water were added to 150µl of 5% sodium nitrite solution. After 6min, 150µlof 10% aluminium chloride was added to the mixture. The solution was allowed to stand for 5 min, and 1ml of 1M NaOH was added and the absorbance was read immediately at 510 nm against the reagent blank. The reagent blank contained all reagents except the extract. The total flavonoid content was expressed as mg of quercetin equivalents per gram of dry extract. The total flavonoid content of the extract was 16.4 µg/ml

expressed as 164 µg/mg quercetin equivalents (QE). From this the percentage w/w of flavonoids in the extract was determined.

3.5 Estimation of total phenolic content of the extract

Procedure for calibration curve for gallic acid:

Calibration curve was obtained by taking different concentrations of gallic acid (10-100µg/ml). To 1ml of each concentration of gallic acid solution, 1ml of Folin Ciocalteau phenol reagent was added. After 3 min, 1 ml of saturated sodium carbonate (35%) was added to the mixture, and it was made up to 10 ml by adding deionised distilled water. The mixture was kept for 90 min at room temperature in dark. The absorbance was measured at 725 nm against the blank and the calibration curve was prepared.

Total phenolic content was determined by the method of Singleton *et al*. [221] with some modifications, using the Folin Ciocalteu reagent. From 1mg/ml of TP extract, 1ml of 100 µg/ml was taken for the estimation of total phenolics.1 ml of TP leaf extract was mixed with 1 ml of Folin Ciocalteu phenol reagent. After 3 min, 1 ml of saturated sodium carbonate (Na_2CO_3) (35%) was added to the mixture, and it was made up to 10 ml by adding deionised distilled water. The mixture was kept for 90 min at room temperature in the dark. The absorbance was measured at 725 nm against the blank. The total phenolic content was expressed as mg of gallic acid equivalents per gram of dry extract. Gallic acid was used as the reference standard. The content of total phenolics was 56 µg/ml expressed as 560 µg/mg gallic acid equivalents (GAE), and from this the percentage w/w of total phenolics in the extract was determined.

3.6 High Performance Thin Layer Chromatography (HPTLC) analysis of TP extract

A TLC fingerprint profile of the leaf, seed and bark extracts of the plant was established using HPTLC. For the development of the TLC fingerprint, 3g of the extract obtained was suitably diluted with methanol and along with it quercetin (used as reference standard) was spotted on a pre-coated Silica Gel G60 F254 TLC plate (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter, and the plate was developed in the solvent system of toluene: ethyl acetate: formic Acid (5:4:1). The plate was dried at room temperature, scanned using CAMAG TLC Scanner 3 at UV 320 nm, and the Rf values and peak area of

the resolved bands were recorded. Relative percentage area of each band was calculated from the peak areas. The TLC plate was developed by spraying 5% methanolic ferric chloride ($FeCl_3$) solution for the detection of flavonoids. In a pilot study it was observed that the leaf extract contained greater amount of quercetin as compared to bark and seed extracts. Hence the leaf extract was selected for further study. The cardio-protective effects of this extract in adriamycin and ethanol models of cardiac injury were investigated.

3.7 *In vitro* free radical scavenging activity

For all *in vitro* antioxidant assay protocols, the concentration of the extract solution used was 1mg/ml (from which the concentrations of 50, 100, 150, 200, 250 µg/ml were used for the assay).

3.7.1 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of TP was determined by the method of Halliwell *et al.* [222]. The incubation mixture in a total volume of 1 ml contained 0.1 of buffer, varying volumes of aqueous extract of TP leaf (50, 100, 150, 200, 250 µg), 0.2 ml of $FeCl_3$, 0.1 ml of ascorbic acid, 0.1 ml of ethylene diamine tetra-acetate (EDTA), 0.1 ml of hydrogen peroxide (H_2O_2)and 0.2 ml of 2-deoxyribose. The contents were mixed thoroughly and incubated at room temperature for 60 min and then 1 ml of thiobarbituric acid (TBA) and 1 ml of trichloroacetic acid (TCA) were added. All the tubes were kept in a boiling water bath for 30 min. The absorbance of the supernatant was read in a spectrophotometer at 535 nm against reagent-blank containing water in the place of extract. The efficiency of various TP extracts was compared between different concentrations (50, 100, 150, 200, 250 µg) of standard antioxidant α-tocopherol. Decreased absorbance of the reaction mixture indicated increased hydroxyl radical scavenging activity.

3.7.2 Superoxide anion scavenging activity

Superoxide anion scavenging activity of TP was determined by the method of Nishimiki *et al.* [223]. To 1 ml of nitroblue tetrazolium (NBT), 1 ml of nicotinamide adenine dinucleotide (NADH) solution and varying volumes of aqueous extract of TP leaf (50, 100,

150, 200, 250 µg) were added and mixed well. The reaction was started by the addition of 100 µl of phenazine methosulfate (PMS). The reaction mixture was incubated at 30°C for 15 min. The absorbance was measured at 560 nm in a spectrophotometer. The blank contained water in the place of TP leaf extract. Ascorbic acid was used as standard for comparison. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

3.7.3 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging assay

The DPPH assay was done according to the method of Brand-Williams *et al*. (1995) with some modifications. The stock solution was prepared by dissolving 24 mg of DPPH in 100 ml methanol and then stored at -20˚C until needed. 1 ml of this solution would contain 2.4 mg of DPPH. The working solution was obtained by mixing 10 ml stock solution with about 45 ml methanol to obtain an absorbance of 1.1 ± 0.02 units at 517 nm using the spectrophotometer.

For studying the *in vitro* antioxidant activity of plant extract, the antioxidant property of the extract was compared with the standard antioxidant compounds like ascorbic acid and BHT, using the same concentrations of the extract and the standard compounds. The reaction mixture in a total volume of 3 ml contained 1 ml of $DPPH^{\bullet}$, various concentrations of aqueous extract of TP leaf (50, 100, 150, 200, 250 µg) and made up to 3 ml with water. The tubes were incubated for 10 min at 37°C. The absorbance of the blue colour chromophore formed was measured at 517 nm. Ascorbic acid and butylated hydroxyl toluene (BHT) were used as standards for comparison.

3.7.4 Evaluation of total antioxidant activity - 2, 2'-azino-bis (3-ethylbenzothiazoline-6sulfonic acid) ($ABTS^{\bullet+}$) radical cation decolourization assay

The assay involves the measurement of the total antioxidant activity of solutions of pure substances [225].In the ABTS assay, 10 mg of ABTS was dissolved in water to obtain a concentration of 7mM (stock solution). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 1ml of ABTS stock solution with 1ml of 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use for complete oxidation of $ABTS^{\bullet+}$. Prior to assay, this solution was diluted in ethanol (about 1.89ml v/v) and equilibrated at 30°C to give an absorbance of 0.70±0.02 at 734 nm.

This solution was used in the assay for further estimation. 2.8ml of the final diluted ABTS solution was added to 0.2 ml each of the concentrations of 50 µg, 100 µg, 150 µg, 200 µg, 250 µg each of TP extract, BHT and ascorbic acid in ethanol. The blank contained water in the place of TP extract or standard. The absorbance was measured at 30°C exactly 1minute after the initial mixing up to 6 min. Triplicate analyses were made at each dilution of the sample and the standard and the percent inhibition was evaluated at 734 nm. The percent inhibition was plotted against the concentration. The percent inhibition was calculated according to the equation [1-(At/Ac)] x 100, where Ac = absorbance of the of the control reaction and At = absorbance of the of the test compound.

3.7.5 Reducing power

The reducing power of TP leaf extract was determined by the method of Oyaizu [226]. Various concentrations of the aqueous extract of leaf (0.2 ml) were mixed with 1.0 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium ferricyanide. The mixture was shaken well and incubated at 50°C for 30 min. After incubation, 1.0 ml of 10% TCA was added to stop the reaction. The mixture was centrifuged at 3000 rpm for 10 min. 1.5 ml supernatant, 1.5 ml deionized water and 0.1 ml $FeCl_3$ (0.1%) were mixed and incubated for 10 min, and the absorbance was read at 700 nm on spectrophotometer. The control was prepared using distilled water instead of extract. Ascorbic acid was used as the standard. Higher absorbance indicated higher reducing power.

3.7.6 Scavenging of hydrogen peroxide

The ability of TP leaf extract to scavenge H_2O_2 was determined according to the method of Ruch *et al.* [227]. A 40 mM solution of H_2O_2 was prepared in phosphate buffer (pH 7.4). H_2O_2 concentration was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer. Aqueous extract of TP leaf in varying concentrations (50, 100, 150, 200, 250 µg) was added to H_2O_2 solution (0.6 ml, 40 mM). The absorbance of H_2O_2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 by TP leaf extract and standard was calculated.

3.8 EXPERIMENTAL DESIGN: ADRIAMYCIN-INDUCED CARDIOTOXICITY MODEL (PROTOCOL-I)

The rats were divided into 10 groups of eight rats each (6 rats were used for the experiments), and the experimental solutions were administered everyday via orogastric tube for 28 days. The treatment schedule of 28 days was fixed as per the established procedure [228].

Group I: The rats received 5% gum acacia only (5 ml/kg per day p.o.) for 28 days and served as vehicle control (VC).

Group II: The rats received adriamycin (ADR) (15mg/kg during 3rd and 4th weeks in 6 equally divided doses of 2.5mg/kg i.p. on alternate days).

Group III: The rats were given only aqueous leaf extract (TP200, 200 mg/kg) for 28 days.

Group IV: The rats were given only aqueous leaf extract (TP400, 400 mg/kg) for 28 days.

Group V: The rats received TP200 for 28 days followed by ADR during the 3^{rd} and 4^{th} weeks (TP200 + ADR).

Group VI: The rats received TP400 for 28 days followed by ADR during the 3^{rd} and 4^{th} weeks (TP400 + ADR).

Group VII: The rats received vitamin E (VIT E, 25 mg/kg, p.o.) for 28 days and served as drug control or reference control.

Group VIII: The rats received vitamin E for 28 days, followed by ADR during the 3^{rd} and 4^{th} weeks (VIT E + ADR).

Group IX: The rats received carvedilol (CV, 1mg/kg, p.o.) for 28 days and served as drug control or reference control.

Group X: The rats received CV for 28 days, followed by ADR during the 3^{rd} and 4^{th} weeks (CV + ADR).

3.9 EXPERIMENTAL DESIGN: ETHANOL-INDUCED CARDIOTOXICITY MODEL (PROTOCOL-II)

The rats were divided into 10 groups of eight rats each (6 rats were used for the experiments), and the treatment was given daily via orogastric tube for 6 weeks.

Group I: The rats received only 5% gum acacia (5 ml/kg per day p.o.) for 6 weeks and served as vehicle control (VC).

Group II: The rats received ethanol (EtOH, 20%, 2g/kg, p.o.) for 6 weeks.

Group III: The rats were given only aqueous TP leaf extract (TP200, 200mg/kg) for 6 weeks.

Group IV: The rats were given only aqueous TP leaf extract (TP400, 400mg/kg) for 6 weeks.

Group V: The rats received TP200 plus EtOH for 6 weeks.

Group VI: The rats received TP400 plus EtOH for 6 weeks.

Group VII: The rats received vitamin E (VIT E, 25 mg/kg, p.o.) for 6 weeks. This group served as drug control or reference control.

Group VIII: The rats received Vitamin E plus EtOH for 6 weeks.

Group IX: The rats received carvedilol (CV, 1mg/kg, p.o.) for 6 weeks. This served as drug control or reference control.

Group X: The rats received CV plus EtOH for 6 weeks

3.10 Induction of cardiotoxicity by adriamycin

Adriamycin was injected in the dose of 2.5 mg/kg (15 mg/kg cumulative dose) every alternate day during the 3rd and 4th weeks of the 28-day treatment protocol [228] in the adriamycin group (disease control) and in the groups receiving TP leaf extract (200 & 400 mg/kg respectively), vitamin E and carvedilol. Individual control groups were maintained for all the groups under treatment.

3.11 Induction of cardiotoxicity by ethanol:

Ethanol-cardiotoxicity was induced following Husain and Somani [229]. Ethanol 20% (2 g/kg, p.o.) was given via orogastric tube daily for 6 weeks in the disease control group. In the combination treatment groups TP leaf extract (200 & 400 mg/kg, respectively), vitamin E (25 mg/kg, p.o.) or carvedilol (1mg/kg, p.o.), were administered prior to ethanol

treatment, via orogastric tube, daily for 6 weeks. Individual control groups were maintained for all the groups under treatment.

3.12 Electrocardiography

On the 29th day the rats were anaesthetized with light ether anaesthesia and electrocardiographic recording was carried out according to the method of Danesi *et al*. [230]. Lead II ECG was recorded in the anaesthetized animals using needle electrodes. Electrodes were inserted under the skin in the right upper limb, right lower limb and the left lower limb. ECG was recorded using student's physiograph (Biodevice) and the changes in different wave patterns were analyzed.

3.13a. Measurement of cardiac ejection fraction *in vitro*:

For the assessment of cardiac % ejection fraction, rats were heparinized (500 IU/100g body weight), and anaesthetised with diethyl ether. The hearts were rapidly excised and briefly soaked in Krebs-Henseleit solution (KHB) at 4°C, containing (in mmol): NaCl 118; KCl 4.7; $CaCl_2$ 2.4; $MgSO_4 \cdot 7H_2O$ 1.5; KH_2PO_4 1.2; $NaHCO_3$ 20 and glucose 10, with pH at 7.3–7.4. The solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C [231]. Coronary perfusion was initiated through a short cannula in the aortic root with perfusion speed of 14 ml/min. The time required for initial stabilization was 20 minutes.

3.13b. Thickness of left ventricle wall:

In order to measure the thickness, the heart was cut horizontally and the thickness of left ventricle wall was measured using a vernier caliper (Aerospace Digimatic) with a sensitivity of 0.01.

3.14 Assay of ATPase activities:

ATPase activities were assayed by the method of Fritz and Hamrick [232] as reported by Desaiah and Ho [233].

Na^+/K^+ and Mg^{2+}ATPase activities were estimated in the mitochondrial fraction. The reaction mixture in a final volume of 3.0 ml contained 3 mmol ATP, 3 mmol magnesium chloride ($MgCl_2$), 100 mmol sodium chloride ($NaCl_2$), 20 mmol potassium chloride (KCl), 135 mmol imidazole-hydrochloric acid buffer (pH 7.5), and 0.3ml of mitochondrial suspension as the enzyme source. The reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 0.1 ml of 50% TCA. The samples were then assayed for inorganic phosphate using the method of Lowry and Lopez [234] as modified by Phillips and Hayes [235]. The colour was read at 620 nm in a spectrophotometer. Mg^{2+}ATPase activity was measured in the presence of 1 Mm ouabain, a specific inhibitor of Na^+/K^+ATPase [236]. Ouabain-sensitive Na^+/K^+ATPase activity was obtained from the difference between total ATPase activity and Mg^{2+}ATPase activity. The enzyme activity was expressed as µmoles of inorganic phosphate formed/mg protein/h.

Ca^{2+}ATPase activity was determined by measuring the inorganic phosphate liberated during the hydrolysis of ATP. The activity was estimated in the mitochondrial fraction. The reaction mixture in a volume of 3.0 ml contained 135 mmol imidazole-hydrochloric acid buffer (pH 7.5), 5 mmol $MgCl_2$, 0.05 mmol $CaCl_2$, 4 mmol ATP, and 0.3 ml of mitochondrial suspension as the enzyme source. The mixture was incubated at 37°C for 30 minutes and then the reaction was stopped by the addition of 0.1 ml of 50% TCA. The inorganic phosphate formed was estimated by the method of Lowry and Lopez [234] as modified by Phillips and Hayes [235]. The colour was read at 620 nm in a spectrophotometer.

Mg^{2+}ATPase activity was measured in the presence of 0.5 mM ethylene glycol tetra-acetic acid (EGTA), and this value was subtracted from the total ATPase activity to obtain Ca^{2+} ATPase activity.

Determination of Inorganic Phosphate

Inorganic Phosphorus (Pi) was estimated by the method described by Lowry and Lopez [234].

1) 2.5 g of ammonium molybdate was dissolved in 100 ml with 3 M sulphuric acid.

2) 1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent (0.25 %):

(a) 0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite was prepared. (b) Accurately weighed 15 g of sodium metabisulphite and 20 g of sodium sulphite were dissolved in 50 ml of distilled water separately. 250 mg of ANSA was dissolved in 50 ml of sodium metabisulphite and 50 ml of 20% w/v of sodium sulphite, mixed well and stored at room temperature.

Procedure:

1ml of the supernatant was taken and the volume was made up to 5.0 ml with distilled water. To this, 1ml of 2.5% ammonium molybdate reagent and 0.5 ml of ANSA reagent were added. The color developed in 20 minutes was read using blank containing water instead of sample at 620 nm. The enzyme activity was expressed as µmoles of inorganic phosphorus liberated/ mg protein/h.

For determining Na^+/K^+ATPase Mg^{2+}ATPase activity was measured in the presence of 10 µl of 1 mM ouabain, a specific inhibitor of Na^+/K^+ATPase. Ouabain-sensitive Na^+/K^+ATPase activity was obtained from the difference between total ATPase activity and Mg^{2+}ATPase activity. The enzyme activity was expressed as µmoles of inorganic phosphate formed/mg protein/h.

For determining Ca^{2+}ATPase activity, Mg^{2+}ATPase activity was measured in the presence of 0.5 mM ethylene glycol tetra-acetic acid (EGTA), and this value was subtracted from the total ATPase activity to obtain Ca^{2+}ATPase activity. The enzyme activity was expressed as µmoles of inorganic phosphate formed/mg protein/h.

$$\text{ATPase activity} = \frac{1}{1.586 \times 0.5} \times \frac{60}{15} \times \frac{1000}{\text{amt. of protein in the tissue}} \times \text{Absorbance}$$

3.15 Myocardial antioxidant parameters

At the end of the dosing schedules for all the experimental groups, the heart was excised under euthanasia in chilled Tris buffer (10mM, pH 7.4) and used to prepare homogenates for enzyme assays.

The hearts were homogenized in 5% (w/v) phosphate buffer (pH 7.0), and centrifuged at 10,000 rpm at 4°C for 10 min, using a Remi 24 high speed cooling centrifuge to separate

the homogenate. Isolation of subcellular organelles like mitochondria and peroxisomes was done using the methods of Cotman and Matthews [237], Dodd *et al.* [238] and Kodavanti *et al.* [239]. These fractions were used for the assay of marker enzymes. All the activities of antioxidant enzymes were estimated in the mitochondrial fraction, except for catalase which was estimated in the peroxisomal fraction.

ASSAY OF CARDIAC OXIDATIVE STRESS MARKERS

3.15.1 MDA content [lipid peroxidation (LP)]:

MDA content was estimated as described by Ohkawa *et al.* [240]. The heart tissue was homogenized (5% w/v) in 50 mM phosphate buffer (pH 7.0) containing 0.1 mmol EDTA. The homogenate was centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was used for the estimation. 200 µl of the supernatant was added to 50 µl of 8.1% sodium dodecyl sulphate (SDS), vortexed and incubated for 10 min at room temperature. 375 µl of 20% acetic acid (pH 3.5) and 375 µl of thiobartbituric acid (0.6%) were added and placed in a boiling water bath for 60 min and then the sample was allowed to cool at room temperature. A mixture of 1.25 ml of butanol: pyridine (15:1) was added, vortexed and centrifuged at 10,000 rpm for 5 min. The colored layer (500µl) was measured at 532 nm in a Hitachi U-2000 spectrophotometer, using 1, 1, 3, 3-tetraethoxypropane as the standard. The values were expressed in µmoles of malondialdehyde formed/ g wet weight of the tissue.

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \frac{1}{\text{tissue taken in mg}} \times 1000$$

3.15.2 Reduced glutathione (GSH) content:

Reduced glutathione content was determined according to the method of Akerboom *et al.* [241]. The tissue was homogenized in 0.1 M ice-cold phosphate buffer (pH 7.0) containing 0.001M EDTA and the protein was precipitated with 1 ml of 5% (w/v) sulfosalicylic acid. The contents were centrifuged at 10,000 rpm for 15 min at 4ºC. The resulting supernatant was used as the enzyme source. The reaction mixture in a total volume of 2.5 ml contained 2.0 ml of 0.1M potassium phosphate buffer, 0.05 ml of NADPH (4 mg/ml of 0.5%

$NaHCO_3$), 0.02 ml of DTNB (1.5 mg / ml), 0.02 ml of glutathione reductase (6 units/ml) and 0.41 ml of enzyme source. The reaction was initiated by adding the enzyme source and the change in absorbance was recorded at 425 nm against the reagent blank. The glutathione content was expressed in nmol/g wet weight of the tissue.

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of the std}} \times \frac{0.097}{\text{wet weight of the tissue taken in mg}} \times 1000$$

3.15.3 Superoxide dismutase (SOD, EC: 1.15.1.6):

SOD activity was determined according to the method of Misra and Fridovich [242]. The heart tissue was homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM ethylene diamine tetra-acetic acid (EDTA) to give a 5% homogenate (w/v). The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was separated and used for enzyme assay. 100 µl of tissue extract was added to 880 µl carbonate buffer (0.05 M, pH 10.2, containing 0.1mmol EDTA). 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and the optical density was measured at 480 nm for 4 min in a Hitachi U-2000 Spectrophotometer. The enzyme activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit/mg protein.

$$\frac{X}{Y} \times \frac{1}{\text{amount of proteins present in the sample}}$$

X= Abs of the control-Abs of the sample; Y= 50% inhibition in control value

3.15.4 Catalase (CAT, EC: 1.11.1.6):

Catalase activity was measured by a slightly modified version of Aebi [243]. The heart tissue was homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mmol EDTA to give a 5% (w/v) homogenate. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The resulting supernatant was used as the enzyme source. 10 µl of 100% EtOH were added to 100 µl of tissue extract and then placed in an ice-bath for 30 min. Following this, the tubes were kept at room temperature and 10 µl of Triton X-100 RS were added. In a cuvette containing 200 µl of phosphate buffer and 50 µl of tissue extract, 250 µl of 0.066 M H_2O_2 (in phosphate buffer) were added, and the decrease in optical

density was measured at 240 nm for 60 s in a UV spectrophotometer. The molar extinction coefficient of 43.6 M cm^{-1} was used to determine CAT activity. One unit of activity is equal to the moles of H_2O_2 degraded/mg protein/min.

$$\frac{\Delta \text{ Absorbance}}{43.6} \times \frac{\text{volume of assay}}{\text{volume of enzyme source}} \times \frac{1}{\text{mg of protein in enzyme source}}$$

3.15.5 Glutathione reductase (GR, EC: 1.6.4.2):

Glutathione reductase activity was determined by a slightly modified method of Carlberg and Mannervik [244] at 37°C. The heart tissue was homogenized (5% w/v) in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The separated supernatant part was used as the enzyme source. NADPH (50 µl, 2 mM) in 10 mM Tris buffer (pH 7.0) was added to the cuvette containing 50 µl of GSSG (20 mM) in phosphate buffer (0.5 M, pH 7.0 containing 0.1 mM EDTA) and 800 µl of phosphate buffer. Tissue extract (100 µl) was added to the NADPH-GSSG buffered solution and measured at 340 nm for 3 min. Molar extinction coefficient of 6.22 X 10^3 M cm^{-1} was used to determine GR activity. One unit of activity is equal to the mM of NADPH oxidized/mg protein/min. The enzyme activity was expressed in µ moles of NADPH oxidized/mg protein/min.

$$\frac{\Delta \text{ Absorbance}}{6.22} \times \frac{\text{volume of assay mixture}}{\text{volume of enzyme source in ml}} \times \frac{1}{\text{mg protein in enzyme source}}$$

3.15.6 Glutathione –S- tranferase (GST, EC: 2.5.1.18):

GST activity was measured with its conventional substrate, 1-Chloro 2, 4-Dinitro Benzene (CDNB) at 340 nm as per the method of Habig *et al.* [245]. The heart tissue was homogenized in 50 mM ice-cold Tris-HCl buffer (pH 7.4) containing 0.2 M sucrose and centrifuged at 10,000 rpm for 45 min at 4°C and the resulting supernatant was again centrifuged at 30,000 rpm for 1 hour at 4°C. The pellet was discarded and the supernatant was used as the enzyme source. The reaction mixture in a total volume of 3 ml contained 2.4 ml of 0.3 M potassium phosphate buffer (pH 6.9), 0.1 ml of 30 mM CDNB, 0.1 ml of 30 mM GSH and 0.4 ml of enzyme source. The reaction was initiated by the addition of glutathione and the absorbance was read at 340 nm against the reagent blank. The activity was expressed as µ mol of thioether formed/mg protein/min.

$$\frac{\Delta \text{ Absorbance}}{9.6} \times \frac{\text{total volume of assay mixture}}{100 \times \text{mg protein in enzyme source}} \times 1000$$

3.15.7 Glutathione peroxidase (GPX, EC: 1.11.1.9):

Glutathione peroxidase activity was determined by a modified version of Flohe and Gunzler [246] at 37ºC. Tissue homogenate (5% w/v) of heart was prepared in 50 mM phosphate buffer (pH 7.0) containing 0.1 mmol EDTA. The homogenate was centrifuged at 10,000 rpm for 10 min at 4ºC. The resulting supernatant was used as the enzyme source. The reaction mixture consisted of 500 µl of phosphate buffer, 100 µl of glutathione reductase (GR) (0.24 units), and 100 µl of tissue extract. The mixture was incubated at 37ºC for 10 min. Then 50 µl of 12mM t-butyl hydroperoxide was added to 450 µl of tissue reaction mixture and measured at 340 nm for 3 min. Molar extinction coefficient of 6.22 X 10^{-3} M cm^{-1} was used to determine the activity. One unit of activity is equal to mmol of NADPH oxidized/mg protein/min. The enzyme activity was expressed as µmol of NADPH oxidized/mg protein/min.

$$\frac{\Delta \text{ Absorbance}}{6.22} \times \frac{\text{total volume of assay mixture}}{\text{volume of enzyme source}} \times \frac{1}{\text{mg protein}}$$

3.16 SERUM BIOCHEMICAL PARAMETERS

Blood samples were collected at the end of the experiment and dispensed into clean plain glass test tubes. They were allowed to stand for 30 min at room temperature to clot. Serum for the assays was then separated from the clot by centrifugation at 5000 rpm for 10 min and used for the estimation of cardiac biomarker enzymes and lipid profile parameters. Erba Mannheim test kits (Erba, Germany) and a biochemistry auto-analyzer (Microlab, India) and Incubator Dual Block (Microlab, India) were used for all the determinations. Standard procedures were followed for all the serum biochemical assays.

3.16.1 Estimation of C-reactive protein (CRP):

C-reactive protein was determined by latex turbidimetry [247, 248]. A change in absorbance is observed when latex particles coated with a specific anti-human CRP exhibit

agglutination when they come in contact with samples containing CRP. The change in absorbance depends on the CRP content in the sample. The procedure is as follows:

10 µl of sample was mixed well with 1000 µl of working reagent. Working reagent was prepared by mixing 9 ml of reagent-1 (tris buffer 20 mmol/l, pH 8.2, sodium azide 0.95g/l) + 1 ml reagent-2 (latex particles coated with goat IgG anti-human CRP, pH 8.2). The absorbance was read immediately (A1) and at 2 minutes (A2) after the addition of the sample. Quantity of CRP in the samples was determined as follows:

$$\text{CRP (mg/L)} = \frac{\text{(A2-A1) Sample}}{\text{(A2-A1) calibrator}} \times \text{calibrator concentration (75mg/L)}$$

3.16.2 Estimation of creatine kinase-MB (CK-MB):

Creatine kinase-MB activity was measured by the CK (NAC act) kinetic method (Young *et al*., 1975).

The sample was incubated in the CK-MB reagent which contained the anti CK-M antibody. The activity of the non-inhibited CK-B was then determined based on the following series of reactions:

$$\text{ADP + Creatine Phosphate} \xrightarrow{\text{CK}} \text{Creatine + ATP}$$

$$\text{ATP + Glucose} \xrightarrow{\text{HK}} \text{ADP + Glucose-6-Phosphate}$$

$$\text{G-6-P + NAD}^{+} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate + NADH + H+}$$

In the sample mixture, CK-B is involved in the catalysis of reversible phosphorylation of ADP in the presence of creatine phosphate, leading to the formation of ATP and creatine. Further, phosphorylation of glucose by hexokinase (HK) to form ADP and glucose-6-phosphate (G-6-P) occurs leading to the oxidation of glucose-6-phosphate to 6-phosphogluconate along with the production of NADH. The rate of NADH formed is directly proportional to the CK-MB activity which is measured at 340 nm. The procedure is as follows:

1 ml of CK-MB reagent was pipetted into appropriate test tubes and pre-warmed at 37°C for 2 minutes. CK-MB reagent consisted of 4 parts of buffer (4 ml) and an antibody to CK-M monomer and 1 part of hexokinase (1ml). 50 µl of sample was added to the reagent

mix and incubated at 37°C for five minutes. The absorbance was then read every minute for two minutes and the average absorbance difference per minute was calculated.

Average absorbance was multiplied by factor 3376 which yielded CK-B expressed as IU/L.

CK-MB activity was calculated from CK-B activity as follows:

CK-MB Activity (U/L) = CK-B Activity U/L x 2

3.16.3 Estimation of creatine kinase (CK):

Creatine kinase (CK) activity was measured by the CK (NAC act) kinetic method [249, 250].

The reaction between creatine phosphate and ADP is catalyzed by creatine kinase (CK) giving creatine and ATP. This results in the conversion of ATP and glucose to ADP and glucose 6-phosphate in the presence of the enzyme hexokinase. Glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) which further reduces NAD to NADH. The rate of NADH formation is proportional to the CK activity in the sample and is determined photometrically at 340 nm. The procedure is as follows:

1ml of CK reagent [consisting of 4 parts of buffer (4 ml) and 1 part (1ml) of hexokinase and glucose-6-phosphate dehydrogenase (G-6-PDH)] was pipetted into appropriate test tubes and pre-warmed at 37°C for 2 minutes. 20 µl of sample was added to the reagent mix and after 2 minutes the change in absorbance was measured. The initial absorbance was read and the timer started simultaneously followed by noting subsequent readings every minute for three minutes. The average absorbance difference per minute was calculated (ΔAbsorbance/min).

CK U/L = ΔA/min. x 8200

3.16.4 Estimation of lactate dehydrogenase (LDH) activity:

Lactate dehydrogenase (LDH) activity was measured by diagnostic reagent for quantitative *in vitro* determination of LDH in human serum and plasma (LDH-SLR Kinetic Test) method. The diagnostic reagent uses pyruvate and is based on the method of Henry *et al*. [251]. The estimation involves the catalysis of reduction of pyruvate to lactate by LDH, thereby oxidizing reduced nicotinamide adenine dinucleotide (NADH) to NAD. The activity of LDH was determined by the rate of decrease in absorbance at 340 nm as NAD was produced.

Pyruvate + NADH → Lactate + NAD^+

In the assay, the 25 µl of sample was added to 1 ml of working reagent, mixed and incubated at 37°C for 1 min. The working reagent was prepared by mixing 9 ml of buffer reagent with 1 ml of enzyme reagent. After incubating for 1 min the change in absorbance per min. (Δ A/min.) against reagent blank was measured for the next 2 minutes.
LDH activity was calculated as follows:
LDH Activity (U/L) = Δ A/min x 6592

3.16.5 Aspartate aminotransferase (AST):

Aspartate aminotransferase activity was determined by Dynamic Extended Stability, Modified IFCC Method [251-255].

The estimation involves the transfer of an amino group from L-aspartate to α- ketoglutarate to yield oxaloacetate and L-glutamate. Oxaloacetate is reduced along with simultaneous oxidation of NADH to NAD in the presence of malate dehydrogenase (MDH). The AST activity is determined as a direct measure of decrease in absorbance at 340 nm.

$$\text{L-Aspartate} + \alpha\text{-ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H+} \xrightarrow{\text{MDH}} \text{Malate} + NAD^+$$

In the assay, 25µl of sample was mixed with 500 µl of working reagent and aspirated. Working reagent was prepared by mixing 4 parts of reagent-1 (20 mmol/L tris buffer, pH 7.8, 230 mmol/L L-Aspartate, >33.3 µkat/L, 13.21mmol/L 2-Oxoglutarate and >3.33µkat/L MDH) with 1 part of reagent-2 per assay tube (1.51 mmol/L NADH). The absorbance was read at 340 nm and the AST activity was calculated as follows:
AST activity (IU/L) = ΔA/min. x Factor (3376)

3.16.6 Alanine aminotransferase (ALT):

Alanine aminotransferase activity was determined by Dynamic Extended Stability, modified IFCC method [256-258].
ALT in the sample catalyzes the transfer of the amino group from L-alanine to α-ketoglutarate leading to the formation of pyruvate and L-glutamate. Pyruvate is reduced

further to lactate in the presence of lactate dehydrogenase (LDH) and NADH resulting in the oxidation of NADH TO NAD^+. This oxidation step is monitored by measuring the rate of decrease in absorbance at 340 nm.

$$\text{L-Alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H+} \xrightarrow{\text{LDH}} \text{L-Lactate} + NAD^+$$

In the assay procedure, 25µl of sample was mixed with 500 µl of working reagent and aspirated. Working reagent was prepared by mixing 4 parts of reagent-1 (100 mmol/L Tris Buffer, 440 mmol/L, LDH > 4U/ml and 13.20 mmol/L α-ketoglutarate) with 1 part of reagent-2 (1.52 mmol/L β-NADH). The absorbance was read at 340 nm and the ALT activity was calculated as follows:

ALT activity (IU/L) = ΔA/min. x Factor (3376)

3.17 Estimation of protein:

Soluble proteins content of the heart tissue was estimated for the purpose of expressing the enzyme activities per mg protein. The estimation was done by the method of Lowry *et al.,* [370]. Heart tissue was weighed accurately, and 1% homogenate was prepared in distilled water. 2 ml of 1% crude homogenate was centrifuged at 2500 rpm for 15 minutes, and the supernatant was used for estimation of soluble proteins. To the supernatant 2 ml of the 10% trichloroacetic acid (TCA) was added and centrifuged at 2500 rpm for 10 minutes. The residue was dissolved in 1 ml of 1N NaOH. To 0.1 ml of the dissolved residue 4 ml of the alkaline copper reagent was added followed by 0.4 ml of Folin Ciocalteau phenol reagent. After 30 minutes the colour developed was read at 600 nm in a spectrophotometer against a reagent blank. The amount of soluble proteins present in the sample was calculated as follows and the values were expressed as mg/g wet weight of the tissue.

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \frac{\text{amount of protein present in the standard}}{\text{wet weight of the tissue}} \times 1000$$

3.18 LIPID PROFILE ASSAYS (total cholesterol, triglycerides, HDL, LDL, VLDL)

3.18.1 Estimation of total cholesterol:

Total cholesterol in the serum was determined by CHOD-PAP end point method based on Trinders methodology [259-261].

Cholesterol ester is hydrolyzed to cholesterol and fatty acids in the present of cholesterol esterase (CHE). Cholesterol is oxidized to choles-4-en-3-one liberating hydrogen peroxide (H_2O_2). The liberated H_2O_2 reacts with 4-aminoantipyrine (4AAP) and phenol in the presence of peroxidase (POD) to form a quinoneimine dye. The absorbance of the quinoneimine dye is directly proportional to cholesterol concentration of the sample.

1. Cholesterol ester + H_2O $\xrightarrow{CHE}$ Cholesterol + Fatty acids

2. Cholesterol + O_2 $\xrightarrow{CHO}$ Cholest-4-en-3-one + H_2O_2

3. 2 H_2O_2 + 4AAP + Phenol $\xrightarrow{POD}$ Quinoneimine dye + 4 H_2O

In the assay, 10 µl of sample was mixed with 1000 µl of working reagent and incubated for 5 minutes at 37°C. The working reagent consisted of 100 mmol/L Goods Buffer, pH 6.4, >100U/L cholesterol oxidase, >200U/L Cholesterol esterase, >3000U/L peroxidase, 0.3 mmol/L 4-amino antipyrine, 5 mmol/L phenol). Concentration of standard was 200 mg/dl. The absorbances of the test and standard were read against reagent blank at 505 nm. The standard sample contained 10 µl of standard in 1000 µl of working reagent, whereas the blank consisted of 10 µl of distilled water in 1000 µl of working reagent.

Total cholesterol in the sample was calculated as:

$$\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}$$

3.18.2 Estimation of Triglycerides:

Serum triglycerides were estimated by GPO-Trinder Method, end point based on the method of Wako with modifications [262,263].

The assay involves the conversion of triglycerides in the serum to glycerol and free fatty acids by lipoprotein lipase. Glycerol is phosphorylated to glycerol-3-phosphate which then undergoes oxidation to DAP with the liberation of H_2O_2.The liberated H_2O_2 reacts with 4-aminoantipyrine (4AAP) and TOOS in the presence of peroxidase (POD) to form a quinoneimine dye. The intensity of the quinoneimine dye is proportional to the triglycerides concentration of the sample.

1. Triglycerides + H_2O $\xrightarrow{\text{LPL}}$ Glycerol + free fatty acids

2. Glycerol + ATP $\xrightarrow[Mg^{+2}]{\text{GK}}$ Glycerol-3-phosphate + ADP

3. Glycerol-3-phosphate + O_2 $\xrightarrow{\text{GPO}}$ DAP + H_2O_2

4. H_2O_2 + 4AAP + TOOS $\xrightarrow{\text{Peroxidase}}$ Quinoneimine dye + 4 H_2O

In the assay procedure, 10 µl of sample was mixed with 1000 µl of working reagent and incubated for 5 minutes at 37°C. The working reagent consisted of 40 mmol/L Buffer pipes, pH7.0, 0.4 mmol/L 4-aminoantipyrine (4AAP), 2.0 mmol/L ATP, 2.5 mmol/L Mg^{2+}, 0.2 mmol/L, 0.2mmol/L TOOS, 1500 U/L glycerol kinase (GK), 4000 U/L glycerol-3-phosphate oxidase (GPO), 2200 U/L peroxidase and 4000 U/L lipoprotein lipase (LPL),.The concentration of standard was 200 mg/dl. The absorbance of the test and standard were read against reagent blank at 546 nm. The standard sample contained 10 µl of standard in 1000 µl of working reagent, whereas blank consisted of 10 µl of distilled water in 1000 µl of working reagent.

Triglycerides in the sample were calculated as:

$$\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}$$

3.18.3 HDL Cholesterol:

HDL Cholesterol concentration in the serum samples were determined by direct enzymatic colorimetric method [264-266].

HDL estimation includes two steps. The first step involves acceleration of the reaction of cholesterol oxidase (CHOD) with non-HDL unesterified cholesterol resulting in the

formation of peroxide. The peroxide thus formed is consumed by N, N-bis-(4-sulfobutyl)-m-toluidine disodium (DSBmT) in the reagent. This reaction is catalyzed by peroxidase enzyme, resulting in a colorless product. The next step in the estimation is the precipitation of LDL and VLDL on the addition of a detergent that dissolves the HDL in the sample, specifically cholesterol esterase (CE) and chromagenic coupler to develop colour for determining the HDL-C quantitatively.

$$\text{Cholesterol esters} \xrightarrow{\text{CHE}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{CHOD}} \text{4-Cholestenone + } H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{peroxidase}} 2H_2O + O_2 - 2O$$

In the assay, 10 µl of sample was mixed with 750 µl of reagent-I [enzyme reagent, consisting of cholesterol oxidase, peroxidase enzyme and N, N-bis-(4-sulfobutyl)-m-toluidine disodium (DSBmT)] and incubated for 5 minutes at 37°C. Immediately 250 µl of reagent-II (developer reagent consisting of detergent and chromagenic coupler) was added to the mixture and after 5 seconds the absorbance (A1) was read at 546 nm. After 5 minutes, the absorbance (A2) was read.

The concentrations of HDL in the samples were calculated as follows.

$$\text{HDL cholesterol (mg/dl)} = \frac{\text{(A2-A1) of Unknown}}{\text{(A2-A1) of calibrator}} \times \text{calibrator value (65mg/dL)}$$

The concentrations of LDL and VLDL in the samples were calculated according to Friedewald's formula (Friedewald *et al*., 1972).

$$\text{LDL cholesterol (mg/dl)} = \text{total cholesterol} - \frac{\text{triglyceride}}{5} - \text{HDL cholesterol}$$

$$\text{VLDL cholesterol (mg/dl)} = \frac{\text{triglyceride}}{5}$$

3.19 Histopathological changes:

The heart tissue was fixed for 48 h in 4% paraformaldehyde, dehydrated by passing successively in different grades of ethyl alcohol, cleared in xylene, and embedded in paraffin. Sections of 5 μm thickness were prepared, stained with haematoxylin and eosin and mounted using neutral DPX mountant for microscopic observation. Slides mounted with sections were later visualized under a trinocular microscope (Primostar - Carl Zeiss). The histopathological study was done under blinded conditions.

3.20 Statistical analysis:

Values were expressed as mean ± SEM from six animals. For all observations in the study, comparisons were made for all single drug treatments with vehicle control and all combination treatments with adriamycin or ethanol treatments. The significance of differences was determined by using either one-way or two-way ANOVA followed by post-hoc test using Graphpad Prism 5 computer package software. P values of <0.05 were considered as statistically significant.

CHAPTER-4

RESULTS

CHAPTER 4. RESULTS

Before examining its ameliorative effect on various parameters in the present study, the *Thespesia* leaf extract was subjected to preliminary phytochemical screening to find out the important constituents. This was then followed by testing its effectiveness in combating the adriamycin- and alcohol-induced changes in physical and physiological parameters in albino rats.

4.1 PHYTOCHEMICAL EVALUATION OF *THESPESIA POPULNEA* LEAF EXTRACT

4.2 Preliminary phytochemical screening:

The yield of the aqueous extract was 13.6% w/w.

Phytochemical screening of the aqueous extract of *T. populnea* (TP) revealed the presence of various chemical constituents (Table.5). The constituents present in the extract were flavonoids, phenolic compounds, saponins, carbohydrates and cardiac glycosides.

Table 5: Chemical constituents identified in the aqueous extract of *Thespesia* leaf.

Test	Chemical constituent	Presence (+)/Absence (--)
Shinoda test Lead acetate test Alkali test	Flavonoids	++
$FeCl_3$ test	Phenolic compounds and tannins	++
Lead acetate test		
Foam test	Saponins	++
Legal's test Keller-Killiani's test	Cardiac glycosides	++
Bontrager's test	Anthraquinone glycosides	++
Leibermann-Buchard reaction	Triterpenoids	--
Fehling's test Benedict's test	Carbohydrates	++
Mayer's test Dragendorff's test Hager's test	Alkaloids	--
Spot test	Fixed oils and fats	--

4.3 Quantification of flavonoids and phenolic compounds in the extract:

The TP leaf extract was found to contain 16.4% w/w of flavonoids and 56% w/w of phenolic compounds.

4.4 HPTLC analysis of TP leaf extract (densitogram of the aqueous extract):

Standard (Quercetin) **Leaf extract**

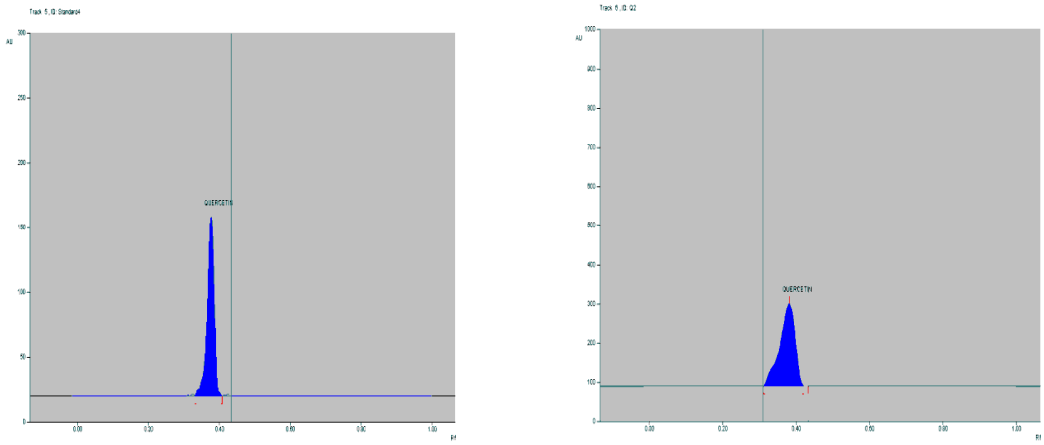

Fig. 7: Densitograms representing HPTLC analysis for the detection of flavonoids (quercetin) in the TP leaf extract

HPTLC analysis of *Thespesia populnea* leaf aqueous extract was carried out by comparing with quercetin as standard. A peak in the densitogram of the extract with Rf value 0.38 corresponded with that of standard quercetin. The result indicated the presence of quercetin as one of the constituents in the leaf extract.

4.5 FREE RADICAL SCAVENGING ACTIVITY OF AQUEOUS EXTRACT OF *THESPESIA POPULNEA* LEAF:

***In vitro* antioxidant studies of leaf extract**:

Free radical scavenging property of TP leaf aqueous extract was assessed *in vitro*.

The % scavenging activities of the extract and the standards at fixed concentrations (50, 100, 150, 200, 250 µg/ml) were determined and the concentration at which 50% scavenging effect is observed for aqueous extract was compared with that of the standards. EC50 values were not determined as the maximum scavenging activity was not measured and only the scavenging activities for fixed concentrations were determined.

TP extract in the doses of 50, 100, 150, 200 and 250µg/ml exhibited a dose-dependent increase in free radical scavenging activity in comparison to standard antioxidants ascorbic acid (AA) and butylated hydroxytoluene (BHT), thus confirming the antioxidant property of the extract *in vitro*. Higher the absorbance, greater was the radical scavenging activity.In general it was observed that the effect of the extract was lower in comparison to those of the standards.

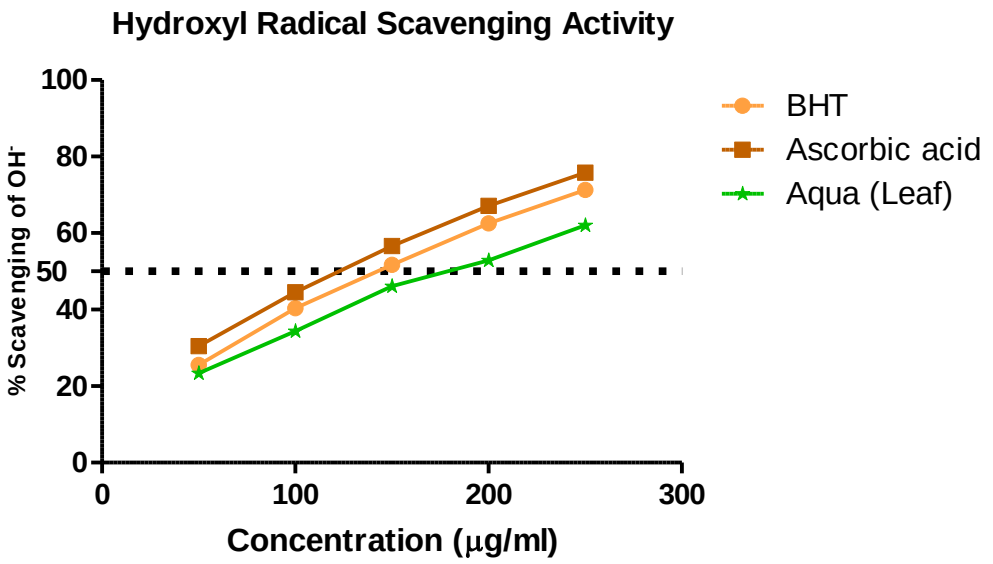

Fig 8a. Hydroxyl radical scavenging activity of *Thespesia populnea* leaf aqueous extract with butylated hydroxytoluene (BHT) and ascorbic acid (AA) as standards.

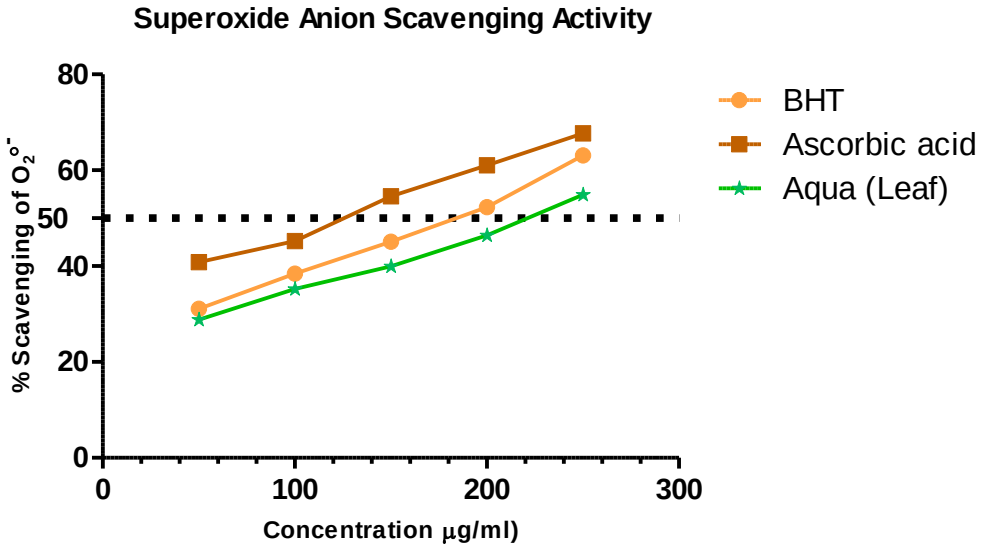

Fig 8b. Superoxide anion radical scavenging activity of *Thespesia populnea* leaf aqueous extract with butylated hydroxytoluene (BHT) and ascorbic acid (AA) as standards.

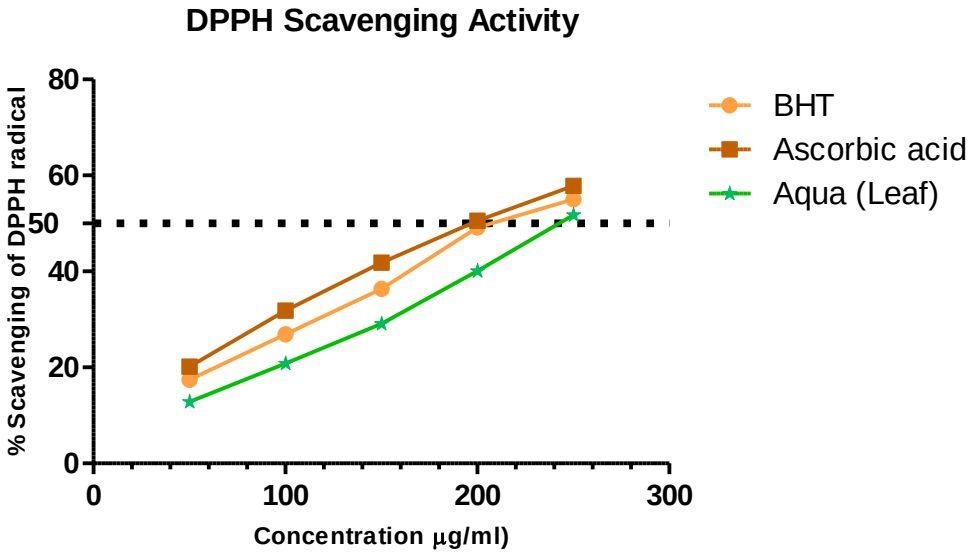

Fig 8c. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of *Thespesia populnea* leaf aqueous extract with butylated hydroxytoluene (BHT) and ascorbic acid (AA) as standards.

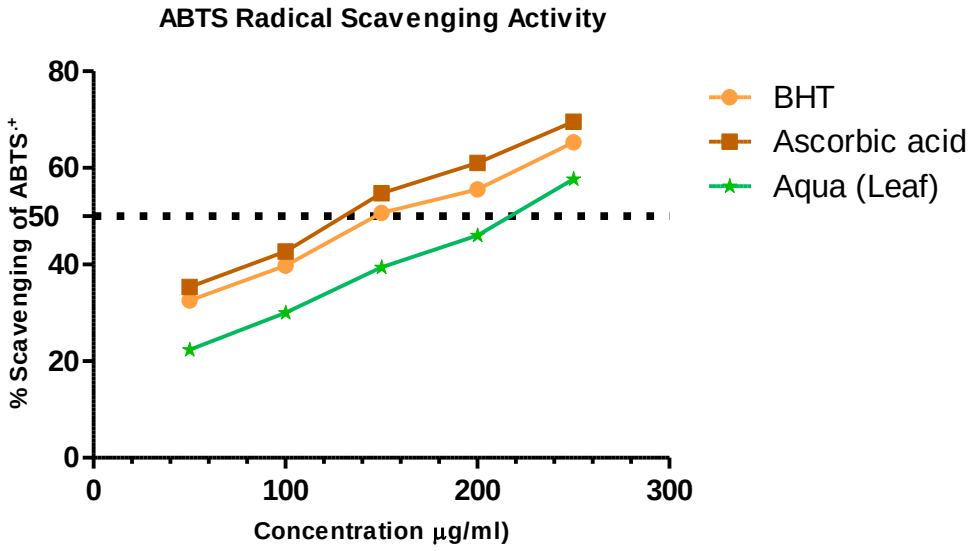

Fig 8d. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity of *Thespesia populnea* leaf aqueous extract with butylated hydroxytoluene (BHT) and ascorbic acid (AA) as standards.

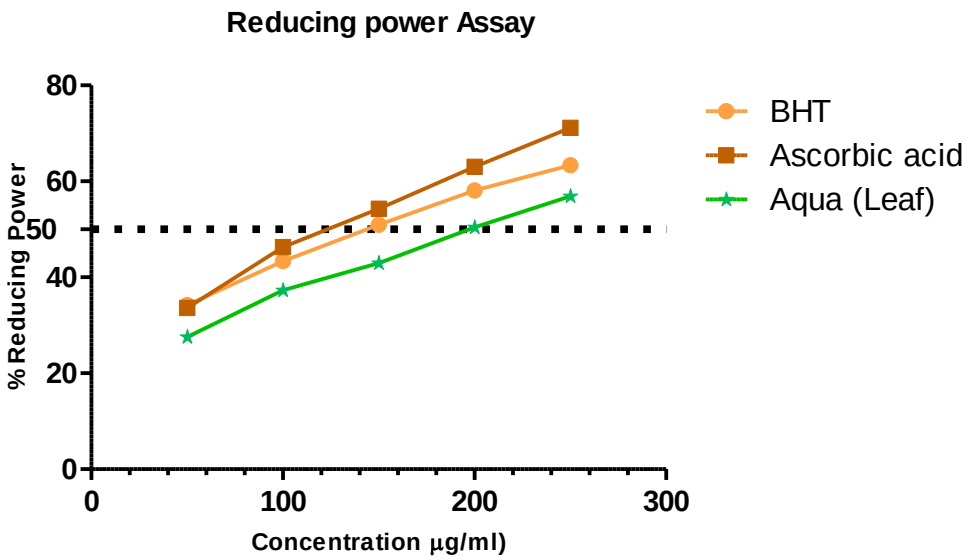

Fig 8e. Reducing power of *Thespesia populnea* leaf aqueous extract with butylated hydroxytoluene (BHT) and ascorbic acid (AA) as standards.

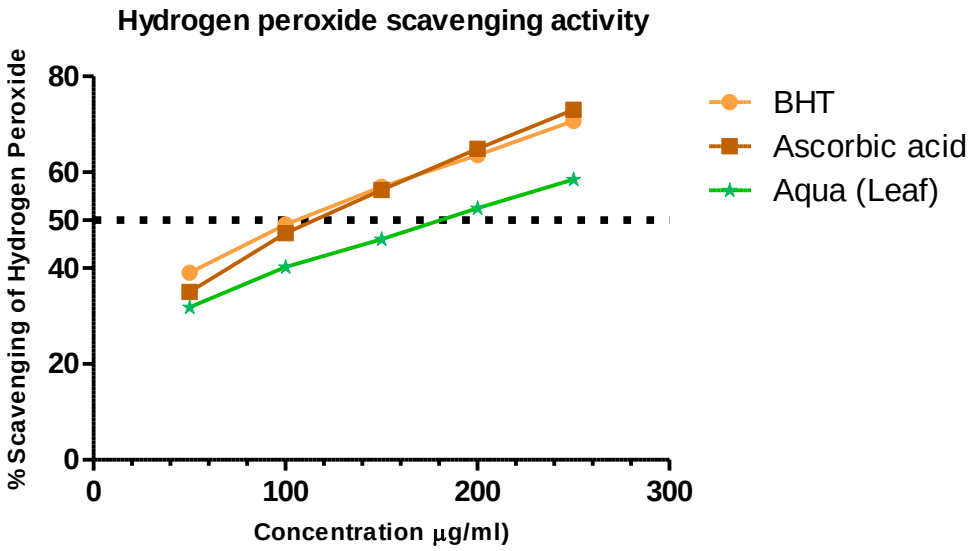

Fig 8f. Hydrogen peroxide radical scavenging activity of *Thespesia populnea* leaf aqueous extract with butylated hydroxytoluene (BHT) and ascorbic acid (AA) as standards.

Table 6. *In vitro* % antioxidant scavenging effects of the standards (BHT and ascorbic acid) and aqueous extract of *Thespesia populnea* leaf.

ANTIOXIDANT Scavenging effect	Concentrations exhibiting 50% scavenging effect (µg/ml)		
	BHT	Ascorbic acid	Aqua (Leaf)
Hydroxyl Radical	148.7	128.8	182.5
Superoxide anion	175.7	122.3	217.4
DPPH radical	216.92	201.06	248.43
$ABTS^{\cdot+}$ radical	158.02	135.02	213.37
Reducing Power	150.0	130.32	201.78
Hydrogen Peroxide	112.70	121.60	181.98

4.6 PROTOCOL I: EVALUATION OF *THESPESIA POPULNEA* LEAF EXTRACT IN ADRIAMYCIN INDUCED CARDIOTOXICITY

4.6.1 Effect of TP leaf extract on physical parameters:

General changes were observed in the rats treated with adriamycin that were exclusively observed only in this group of animals. The changes included development of gradual roughness of body fur that acquired a pinkish tinge. The nose and eyes had exudates around them that appeared like red droplets. The feces gradually developed diarrheal characteristics with increased water content, followed by reduced mobility of animals as the treatment schedule progressed. These changes were effectively reduced to normal by TP200, TP400, vitamin E and carvedilol treatments.

4.6.2 Effect of TP leaf extract on food-intake and body weight:

The changes in food-intake (Table 7, Fig 9 and Fig 10) and body weight (Table 8, Fig 11 and Fig 12) for all the experimental groups were recorded weekly throughout the 28-day study period.

During the treatment period, rats administered with adriamycin showed reduced activity, decreased appetite and progressive physical exhaustion (indicated by reduced alertness and motor movements) compared to the other treatment groups. Rats receiving adriamycin alone showed a consistent decrease in food-intake and weight-loss over the treatment

period, whereas the groups receiving TP leaf extract, vitamin E or carvedilol did not show perceptible weight loss in comparison to the vehicle (gum acacia) control group. Rats receiving carvedilol recorded a significant increase in body weight compared to other treatments. The reduction in food intake and body weight due to adriamycin treatment was significantly countered by TP extract (400mg/kg dose), vitamin E and carvedilol treatments when they were administered prior to the administration of adriamycin.

Table 7. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on food intake during adriamycin-induced cardiotoxicity in rats.

Experimental Group	FOOD INTAKE (g)			
	1st week	2nd week	3rd week	4th week
Vehicle control (5% Gum acacia)	17.83 ± 1.32	26.33 ± 1.17	23.16 ± 1.27	24.16 ± 1.16
Disease control (adriamycin, 15 mg/kg cumulative dose)	20.17 ± 1.08 NS	18.33 ± 0.843$^{###}$	14.50 ± 0.95$^{###}$	10.17± 0.60$^{###}$
Thespesia leaf extract (200 mg/kg)	16.33 ± 0.76 NS	19.17 ± 0.79$^{##}$	21.33 ± 0.88 NS	26.17 ± 1.70 NS
Thespesia leaf extract (400 mg/kg)	19.50 ± 0.71 NS	23.83 ± 0.79 NS	24.33 ± 1.50 NS	26.50 ± 1.31 NS
Thespesia leaf extract (200 mg/kg) + adriamycin	15.33 ± 1.14***	22.50 ± 1.17*	19.16 ± 0.90***	14.66 ± 0.71*
Thespesia leaf extract (400 mg/kg) + adriamycin	17.66 ± 1.2 NS	23.30 ± 0.80*	21.0± 1.15***	19.83 ± 1.19***
Standard (Vitamin E, 25 mg/kg, p.o.)	15.33 ± 0.88 NS	21.66 ± 0.98$^{#}$	24.50 ± 1.28 NS	26.17 ± 1.07 NS
Vitamin E (25 mg/kg, p.o.) + adriamycin	19.50 ± 0.99 NS	20.88 ± 1.62 NS	23.00 ± 1.46***	20.67 ± 1.20 ***
(Carvedilol, 1mg/kg, p.o.)	21.83 ± 1.04 *	27.16 ± 0.94 NS	32.67 ± 1.31$^{###}$	35.00± 1.71$^{###}$
Carvedilol(1mg/kg, p.o.) + adriamycin	19.60 ± 1.42 NS	23.00 ± 1.51*	26.17 ± 0.70***	22.33 ± 1.20 ***

Note: The values are expressed are mean ± SEM from 6 rats, followed by significance.

NS – Not significant.

#: Value differs significantly from vehicle control group $^{#}$**P<0.05,** $^{##}$**P<0.01,** $^{###}$**P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

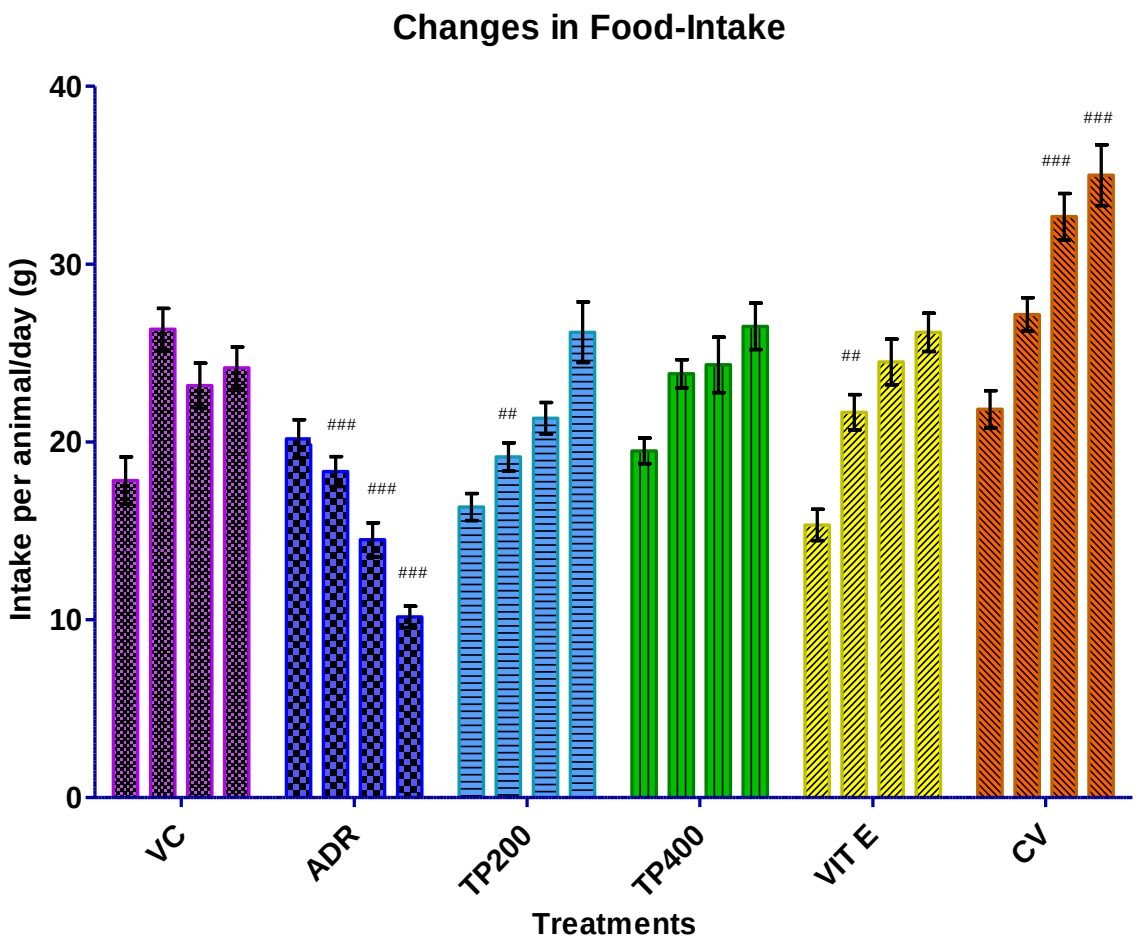

Fig 9. Changes in food-intake as compared to vehicle control

Histograms representing the weekly changes in food-intake over a period of 28 days in control rats and those receiving different treatments. Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

$^{\#}$: Value differs significantly from vehicle control group $^{\#}$**P< 0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* leaf extract 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* leaf extract 400mg/kg dose (p.o.); **vit E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg).

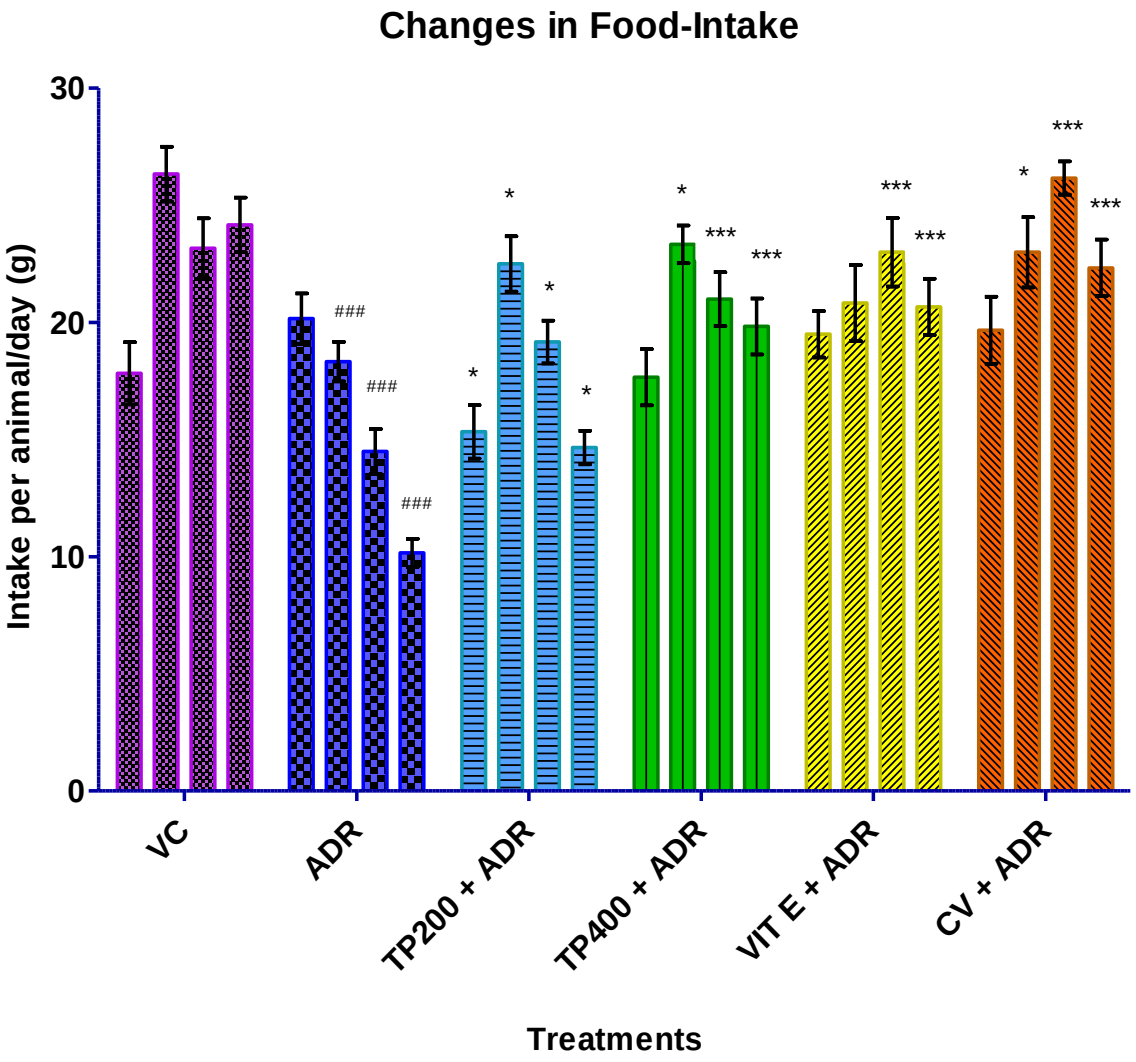

Fig 10. Changes in food-intake as compared to disease control

Histograms representing the weekly changes in food-intake over a period of 28 days in control rats and those receiving different treatments. Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC** - Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **vit E + ADR** - rats treated with vit E + adriamycin; **CV + ADR** rats treated with carvedilol + adriamycin.

Table 8. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in body weight in adriamycin-induced cardiotoxicity in rats.

Experimental Group	CHANGES IN BODY WEIGHT (g)			
	1st week	**2nd week**	**3rd week**	**4th week**
Vehicle control (5% Gum acacia)	199.17 ± 5.06	216.67 ± 3.57	220.00 ± 2.23	225.83 ± 2.71
Disease control (Adriamycin, 15 mg/kg cumulative dose)	215.83± 2.00 ###	206.67± 4.01#	190.00± 2.58 ###	158.33± 2.78 ###
Thespesia leaf extract (200 mg/kg)	209.16 ± 2.38#	217.5± 2.81 NS	224.17± 2.01 NS	232.50± 1.71 NS
Thespesia leaf extract (400 mg/kg)	211.67± 3.07 ##	220.00± 1.82 NS	229.17± 2.01 NS	245.00± 2.23 ###
Thespesia leaf extract (200 mg/kg) + adriamycin	215.00 ± 2.23 NS	237.5± 2.14 ***	215.8± 3.00***	176.7± 1.05 ***
Thespesia leaf extract (400 mg/kg) + adriamycin	220.00± 2.58NS	245.00± 1.82***	227.5± 2.14 ***	204.17± 1.53 ***
Standard (Vitamin E, 25 mg/kg, p.o.)	198.33± 4.01 NS	220.00± 1.82 NS	228.33± 3.07 NS	234.17± 1.53 NS
Vitamin E (25 mg/kg, p.o.) + adriamycin	214.16 ± 2.38 NS	238.30 ± 2.30 ***	225.00± 2.23 ***	199.5± 2.56***
(Carvedilol, 1mg/kg, p.o.)	201.67± 2.10 NS	222.50± 1.11 NS	237.50± 1.11 ###	262.5± 1.71 ###
Carvedilol (1mg/kg, p.o.) + adriamycin	219.70 ± 1.53 NS	238.33± 3.07***	225.00 ± 2.23***	211.67 ± 1.66 ***

Note: The values are expressed are mean ± SEM from 6 rats, followed by significance.

NS – Not significant.

#: Value differs significantly from vehicle control group **#P<0.05, ##P<0.01, #P<0.001**

***:** Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

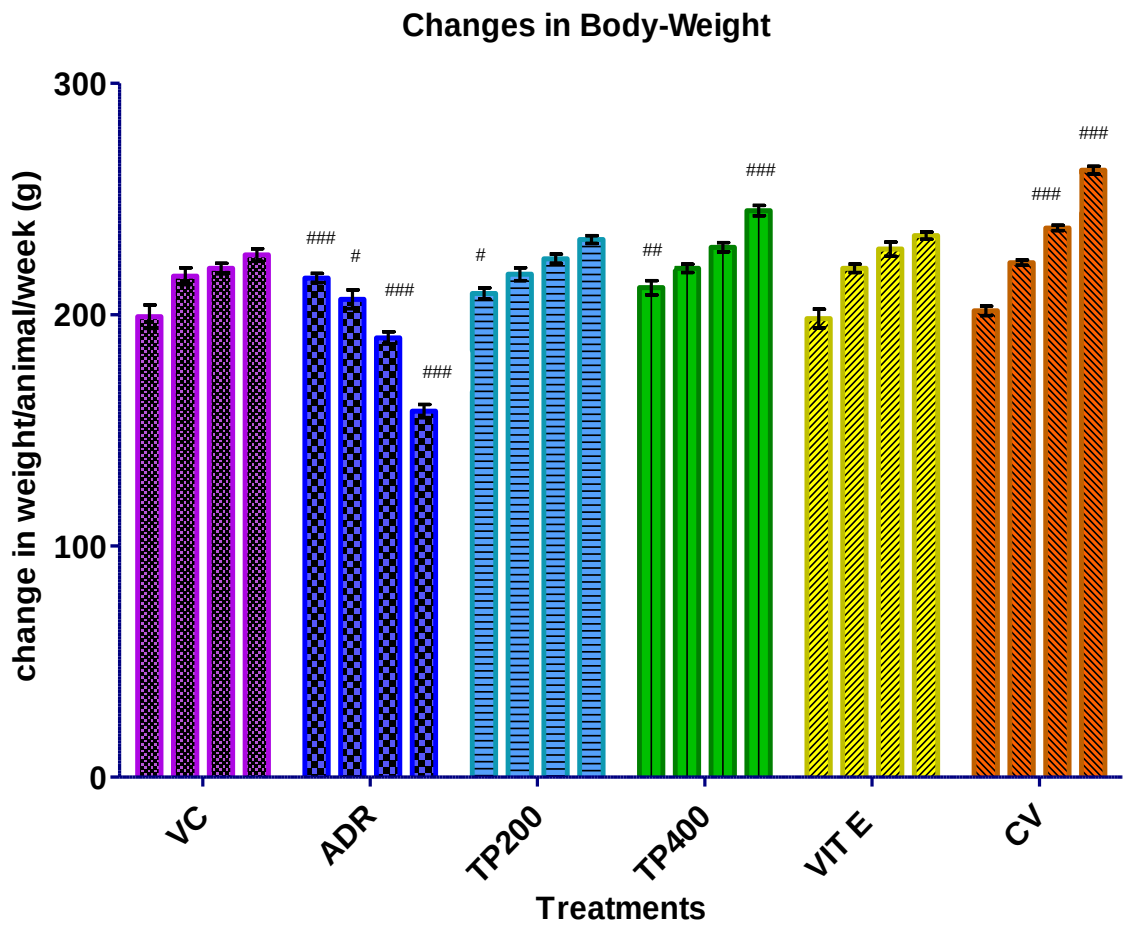

Fig 11. Changes in body weight as compared to vehicle control

Histograms representing the weekly changes in body weight over a period of 28 days in control rats and those receiving different treatments. Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

$^{\#}$: Value differs significantly from vehicle control group $^{\#}$**P< 0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* leaf extract 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* leaf extract 400mg/kg dose (p.o.); **vit E** - rats receiving vitamin E (25mg/kg); **CV**- rats receiving carvedilol (1mg/kg).

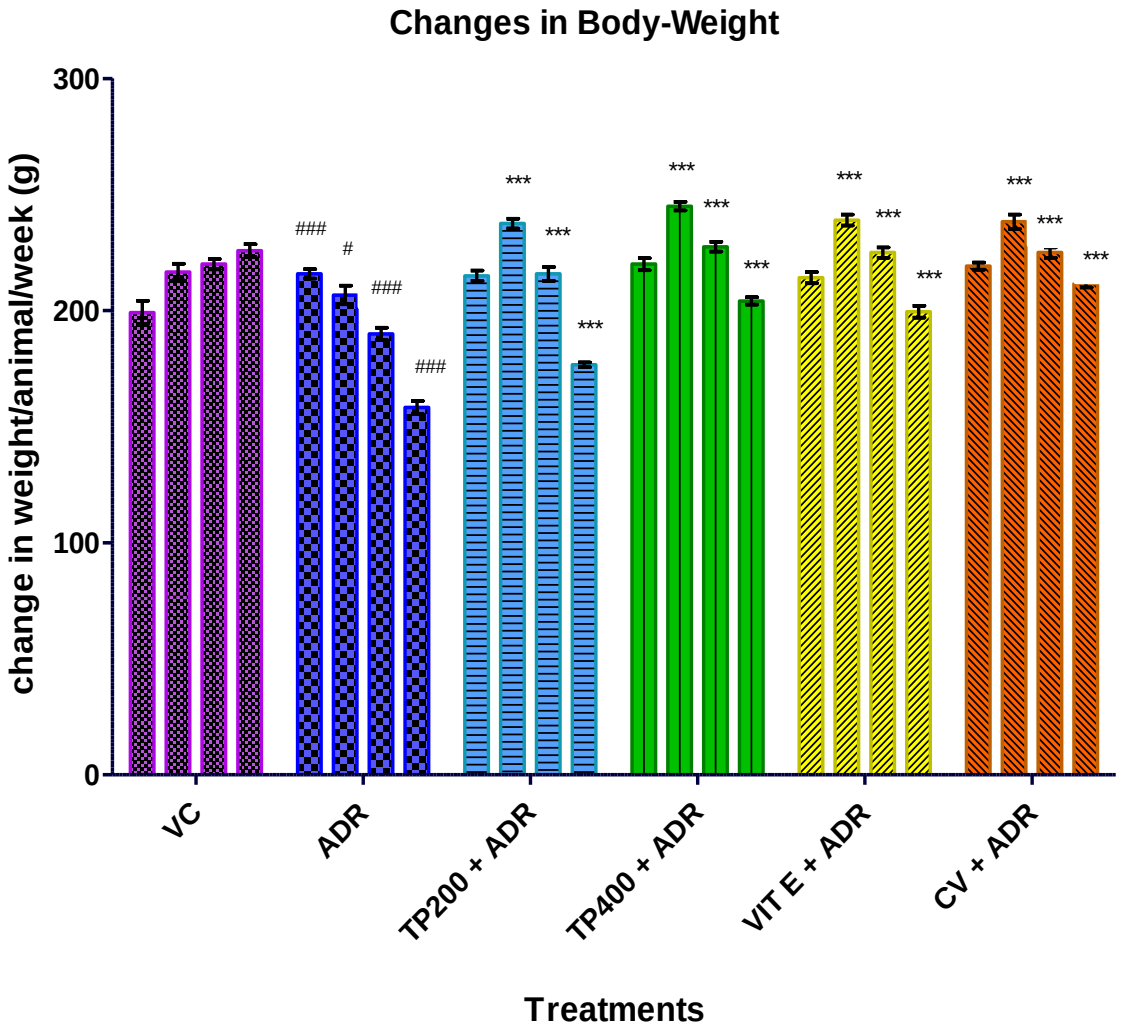

Fig 12. Changes in body weight as compared to disease control

Histograms representing the weekly changes in food-intake over a period of 28 days in control rats and those receiving different treatments. Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR -** rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **vit E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** rats treated with carvedilol + adriamycin.

4.7 Effect of *T. populnea* leaf extract on selected cardiac parameters and *in vitro* cardiac function in adriamycin-induced cardiotoxicity:

4.7.1 Effect of *T. populnea* leaf extract on heart weight:

The heart weight (Table 9; Fig. 13) significantly increased in the adriamycin-treated rats (disease control) when compared to the vehicle control group. The groups receiving only TP leaf extract (200mg/kg and 400 mg/kg) or vitamin E (25 mg/kg) showed heart weights similar to the vehicle control, and those receiving carvedilol (1 mg/kg) showed an increase in heart weight. When adriamycin was administered following the administration of TP extract, vitamin E or carvedilol, the rats showed a slight increase in the heart weight, but the increase was significantly less compared to the rats treated with adriamycin alone. No significant difference in heart weight was observed in rats treated with TP200 plus adriamycin compared to those receiving adriamycin alone.

4.7.2 Effect of *T. populnea* leaf extract on thickness of left ventricle wall:

The left ventricular wall thickness (Table 9; Fig.14) significantly decreased in the disease control group compared to the vehicle control. In all the other treatment groups, the thickness of the ventricular wall significantly increased as compared to the disease control group. The group receiving only carvedilol recorded the maximum increase in ventricle wall thickness.

4.7.3 Effect of *T. populnea* leaf extract on cardiac ejection fraction:

A strikingly significant low cardiac ejection fraction (Table 9; Fig. 15) was recorded in the disease control group compared to the vehicle control, while the rats treated individually with TP leaf extracts, vitamin E and carvedilol showed more or less similar percent ejection fractions as the vehicle control. When TP leaf extract, vitamin E or carvedilol were administered prior to the administration of adriamycin, there was a significant increase of the cardiac ejection fraction compared to the level in disease control.

Table 9. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in heart weight, thickness of the left ventricle wall and cardiac ejection volume during adriamycin-induced cardiotoxicity in rats after a treatment period of 28 days.

Experimental Group	Heart weight (g)	Thickness of left ventricle wall (mm)	Cardiac ejection volume or effluent collected (ml)/min	% Ejection Fraction
Vehicle Control (5% Gum acacia)	1.06 ± 0.03	2.53 ± 0.13	14.0 ± 0.03	100
Disease Control (Adriamycin, 15 mg/kg cumulative dose)	1.34 ± 0.05$^{\#\#\#}$	0.88 ± 0.09$^{\#\#\#}$	2.03 ± 0.06$^{\#\#\#}$	14.3
Thespesia Leaf Extract (200 mg/kg)	1.08 ± 0.06 NS	2.32 ± 0.13 NS	13.80 ± 0.05 NS	98.6
Thespesia Leaf Extract (400 mg/kg)	1.07 ± 0.02 NS	2. 33 ± 0.15 NS	13.90 ± 0.04 NS	99.3
Thespesia Leaf Extract (200 mg/kg) + Adriamycin	1.27 ± 0.02 NS	1.60 ± 0.06*	10.20 ± 0.05**	72.9
Thespesia Leaf Extract (400 mg/kg) + Adriamycin	1.12 ± 0.03**	2.50 ± 0.20***	12.60 ± 0.09***	88.6
Standard (Vitamin E, 25 mg/kg, p.o.)	1.05 ± 0.02 NS	2.63 ± 0.15 NS	13.80 ± 0.09 NS	98.6
Vitamin E (25 mg/kg, p.o.) + Adriamycin	1.15 ± 0.02**	2.58 ± 0.16***	12.20 ± 0.08***	87.1
(Carvedilol, 1mg/kg, p.o.)	1.16 ± 0.02 NS	3.80 ± 0.13$^{\#\#\#}$	12.90 ± 0.08 NS	92.1
Carvedilol(1mg/kg, p.o.) + Adriamycin	1.14 ± 0.02**	2.9 ± 0.031***	10.10 ± 0.12***	72.1

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.

NS – Not significant.

#: Value differs significantly from vehicle control group $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$

*: Value differs significantly from disease control group *P< 0.05, **P<0.01, ***P<0.001

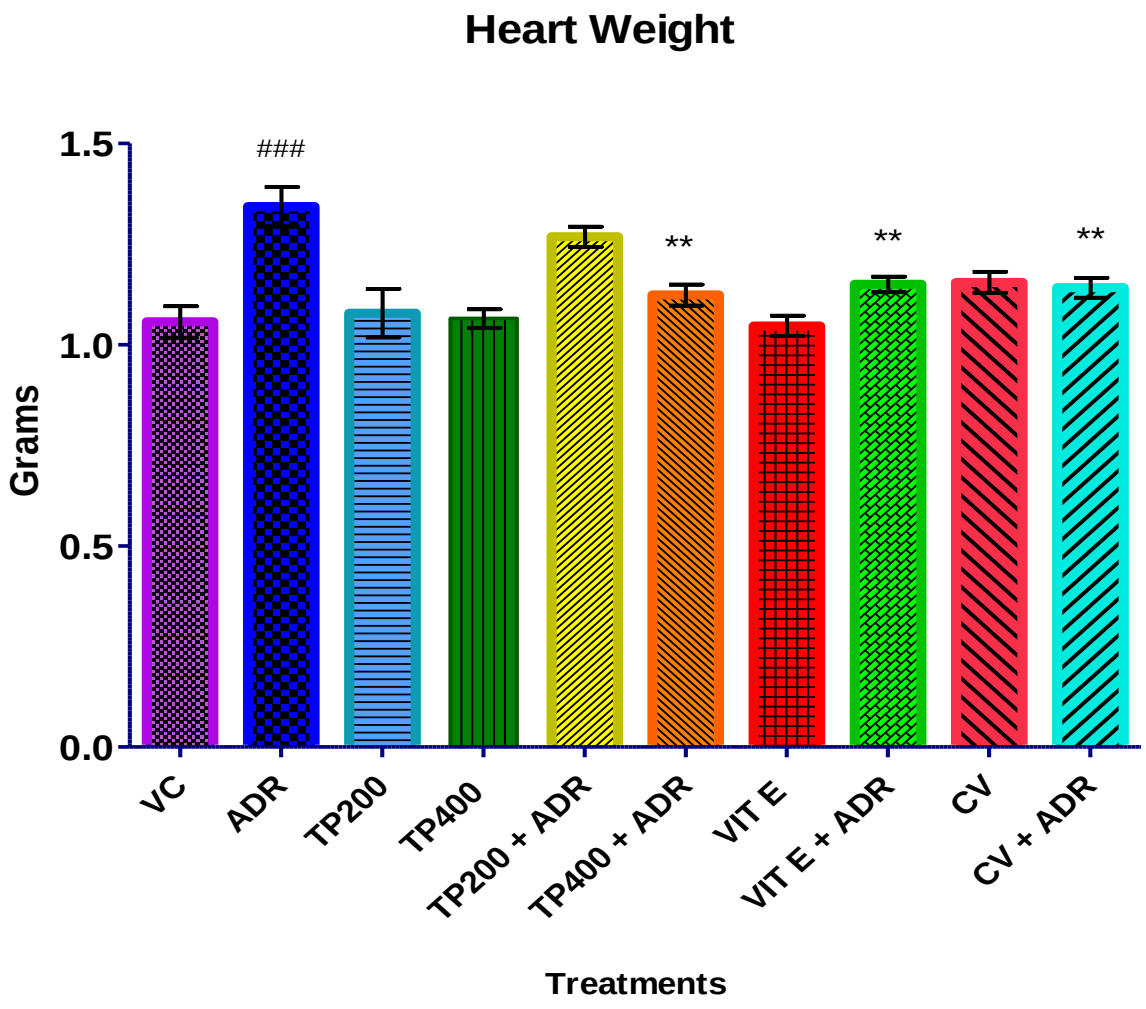

Fig. 13: Histograms representing the changes in heart weight after a treatment period of 28 days in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC** - Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

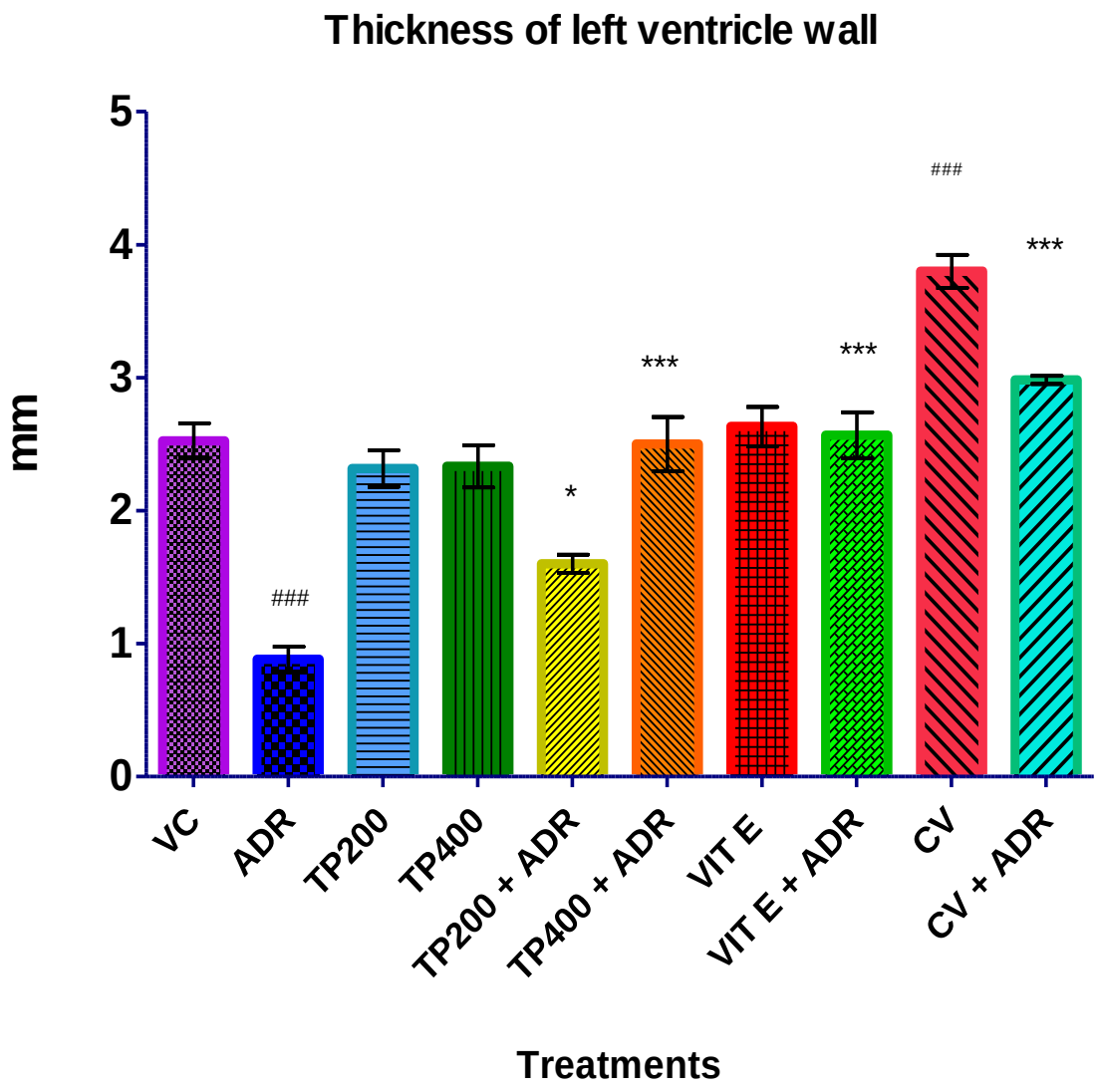

Fig. 14: Histograms representing the changes in thickness of left ventricle after a treatment period of 28 days in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR -** rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

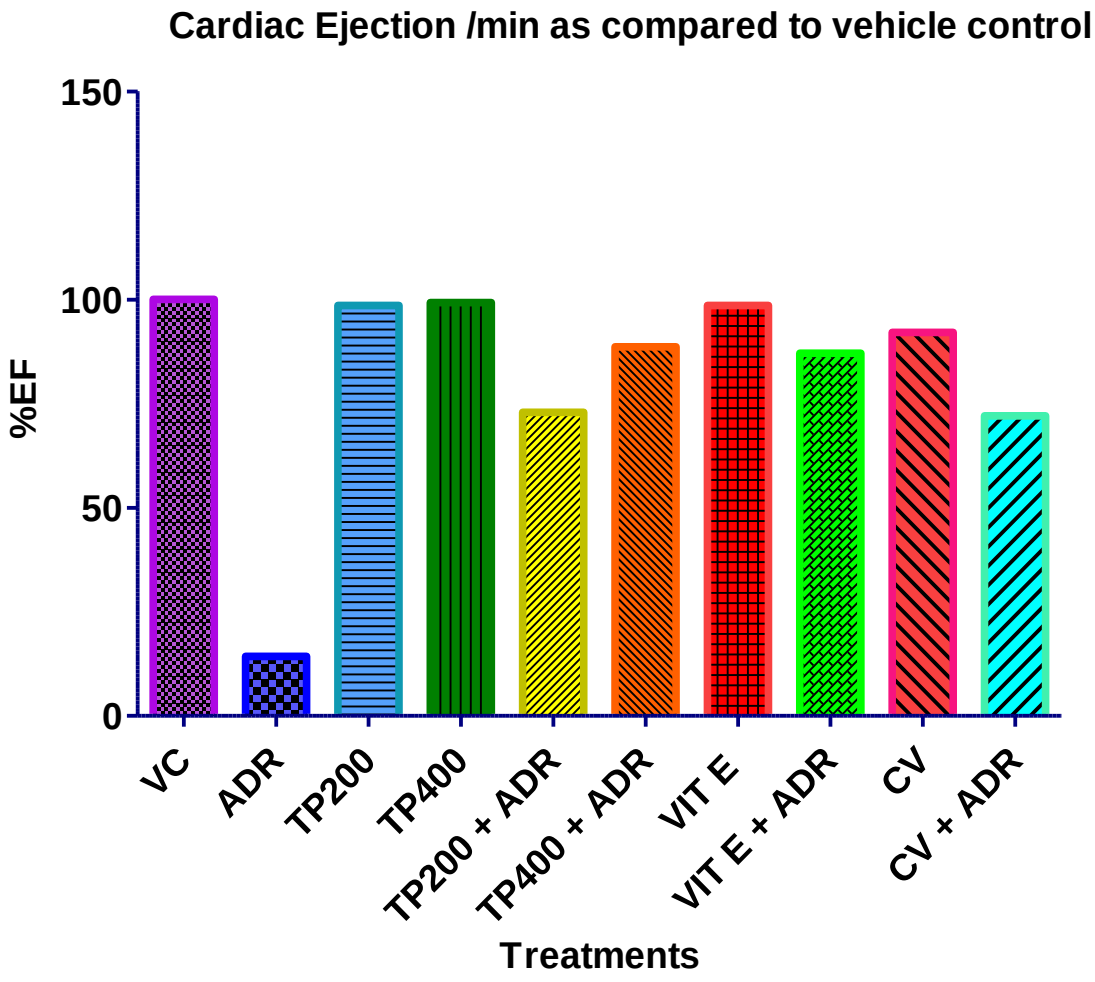

Fig. 15: Histograms representing the changes in thickness of cardiac ejection fraction after a treatment period of 28 days in control rats and those receiving different treatments.

Data representing percent change in cardiac ejection fraction, with each point representing data obtained from 6 rats.

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose(p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

Note: The contents in the figure are shown in terms of percent ejection fraction (%EF). Since the representation is in the form of '%' ejection fraction, points of significance are not mentioned in the graph. The same data is mentioned in the form of ejection volumes in tables nos. 9 and 17 where the points of significance are shown.

4.8 Effect of *T. populnea* leaf extract on changes in ECG in adriamycin-induced cardiotoxicity:

The effects of adriamycin, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on alterations in electrocardiogram (ECG) after the treatment period of 28 days are illustrated in Table 10 and Fig. 16a-j**.** The parameters examined were QT-interval, QRS-complex duration and R-R interval.

QT-interval:

Administration of adriamycin caused a significant ($P<0.001$) prolongation (increase) of the QT-interval in the disease control compared to the vehicle control. Individual administration of TP200 or TP400 did not cause any change in the QT-interval from the vehicle control, while individual administration of vitamin E or carvedilol prolonged the QT-interval moderately, that was nevertheless statistically significant compared to the vehicle control. Normal PQRST wave pattern was recorded with individual administration of TP200, TP400, vitamin E and carvedilol. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin caused a reduction of the prolongation of the QT-interval from the disease control towards the vehicle control. The reversals were partial and not complete, although statistically significant ($P<0.001$) (Table 10; Fig. 16a-j). This was truer with carvedilol. Pre-treatment with carvedilol did not correct the abnormality in the ECG caused due to adriamycin as indicated by the persistent prolongation of QT interval.

QRS-complex duration:

Administration of adriamycin caused a significant (over 2-fold) increase of the QRS-complex duration in the disease control compared to the vehicle control. It also caused ST segment elevation compared to the vehicle control. Individual administration of TP200 or TP400 did not cause any change in the QRS-complex duration from the vehicle control, while individual administration of vitamin E or carvedilol increased the duration slightly compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin caused a reduction in the QRS-complex duration compared to the disease control. The reductions in QRS duration were partial and not complete, although statistically significant (($P<0.001$) with reference to the disease control (Table 10; Fig. 16a-j).

R-R Interval:

Administration of adriamycin caused a prolongation ($P<0.001$) of the R-R interval in the disease control compared to the vehicle control. Individual administration of TP200, TP400, vitamin E or carvedilol caused slight non-significant increases or decreases in the R-R interval compared to the vehicle control. Administration of TP200, TP400 or vitamin E prior to the administration of adriamycin caused a reduction in the prolongation of the R-R interval in comparison to the disease control. The reductions were not statistically significant (Table 10; Fig. 16a-j). However, quite interestingly administration of carvedilol prior to the administration of adriamycin caused a further significant ($P<0.001$) prolongation of the R-R interval as compared to that of the disease control (Table 10; Fig 16a-j).

Table 10. Effect of *T. populnea* leaf extract on changes in ECG in adriamycin-induced cardiotoxicity after a treatment period of 28 days.

Experimental Group	QT Interval (s)	QRS Complex duration (s)	R-R Interval (s)
Vehicle Control (5% Gum acacia)	0.26 ± 0.50	0.15± 0.04	0.90 ± 0.07
Disease Control (Adriamycin, 15 mg/kg cumulative dose)	0.60 ± 0.07$^{###}$	0.35 ± 0.04$^{###}$	1.24 ± 0.06$^{###}$
Thespesia Leaf Extract (200 mg/kg)	0.29± 0.05 NS	0.18± 0.05 NS	0.93 ± 0.06 NS
Thespesia Leaf Extract (400 mg/kg)	0.27 ± 0.06 NS	0.16 ± 0.06 NS	0.85 ± 0. 07 NS
Thespesia Leaf Extract (200 mg/kg) + Adriamycin	0.48 ± 0.06***	0.23 ± 0.06***	0.92 ± 0. 08 **
Thespesia Leaf Extract (400 mg/kg) + Adriamycin	0.35 ± 0.06***	0.22 ± 0.05***	0.89 ± 0. 09 ***
Standard (Vitamin E, 25 mg/kg, p.o.)	0.34 ± 0.05$^{###}$	0.21 ± 0.06 ###	0.92 ± 0. 09 NS
Vitamin E (25 mg/kg, p.o.) + Adriamycin	0.42 ± 0.06***	0.16 ± 0.05***	0.75 ± 0. 04***
(Carvedilol, 1mg/kg, p.o.)	0.38± 0.09$^{###}$	0.25 ± 0.05$^{###}$	0.99 ± 0.07NS
Carvedilol(1mg/kg, p.o.) + Adriamycin	0.48 ± 0.07***	0.22 ± 0.06***	1.48 ± 0. 12***

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.

NS – Not significant.

#: Value differs significantly from vehicle control group $^{#}P<0.05$, $^{##}P<0.01$, $^{###}P<0.001$

*: Value differs significantly from disease control group *P< 0.05, **P<0.01, ***P<0.001

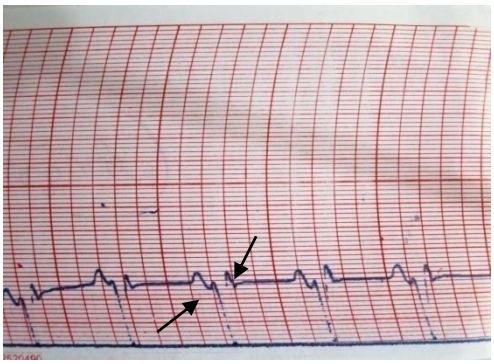

Fig. 16a. ECG of vehicle control
Normal wave pattern displaying PQRST waves

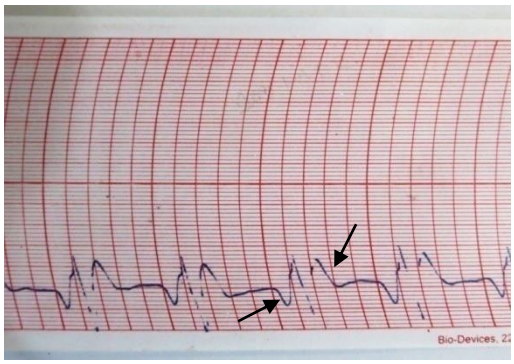

Fig. 16b. ECG of adriamycin treatment
-ST segment elevation, increased T wave amplitude, prolonged QT interval, QRS complex duration and RR interval (suggesting abnormal ventricular depolarization and repolarization)

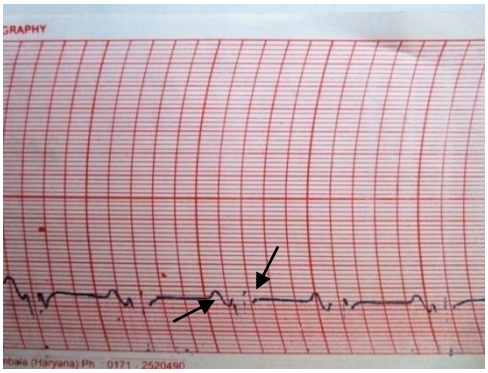

Fig. 16c. ECG of TP200
Non-significant increase in QRS and RR intervals compared to vehicle control

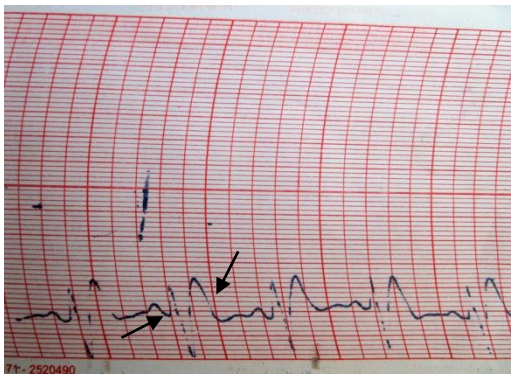

Fig. 16d. ECG of TP200+ ADR
-Reduction in QT interval and QRS complex duration; significant reduction in RR interval

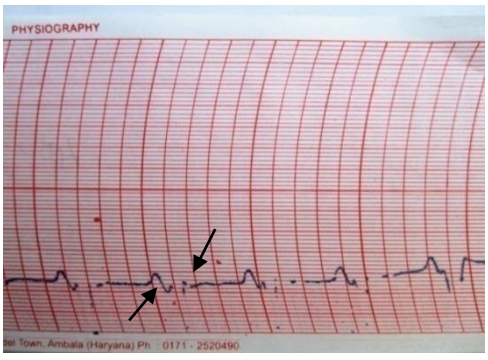

Fig. 16e. ECG of TP400
Non-significant increase in QRS and RR intervals compared to vehicle control

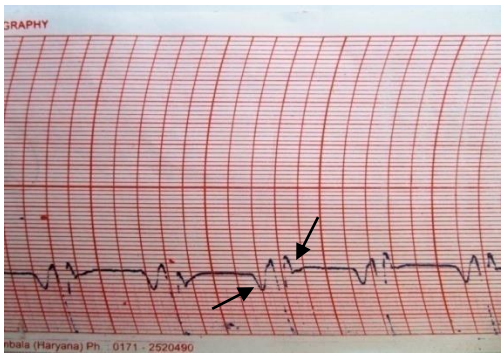

Fig. 16f. ECG of TP400+ ADR
-Reduction in QT interval and QRS complex duration; significant reduction in RR interval

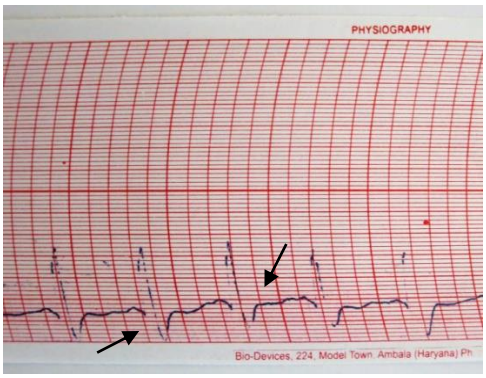

Fig. 16g. ECG of vitamin E
-Significant increase in QT and QRS intervals

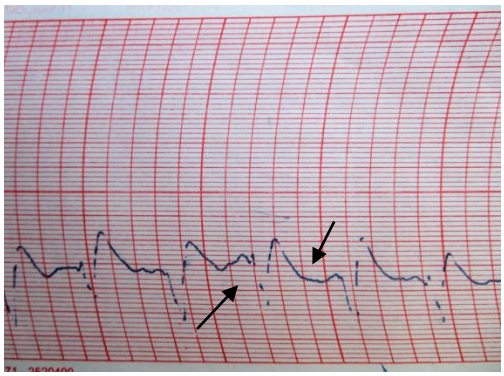

Fig. 16h. ECG of vitamin E + ADR
-Partial reduction in QRS complex duration; significant reduction in RR interval

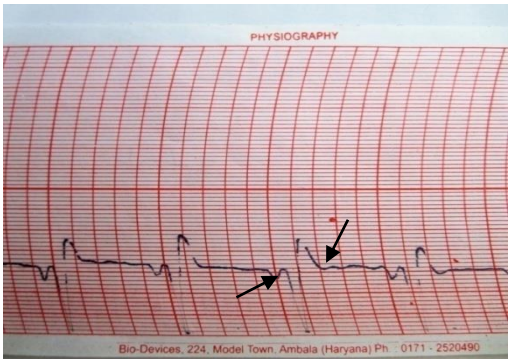

Fig. 16i. ECG of carvedilol

Significant increase in QT, QRS and RR intervals

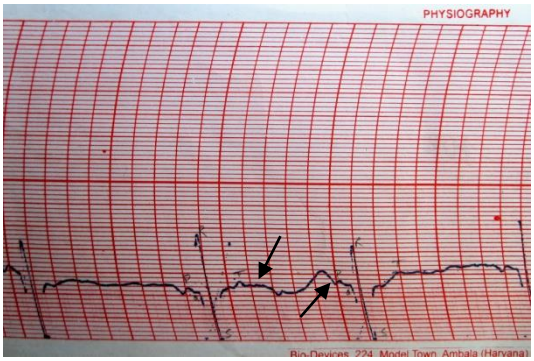

Fig. 16j. ECG of carvedilol+ ADR

-Persistent prolongation of QT interval; significant increase in RR interval, suggesting prolonged repolarization and increased time taken between each cardiac cycle

Fig.16a-16j: Electrocardiographic recordings representing the changes in QT interval, QRS complex and RR interval durations after a treatment period of 28 days in control rats and those receiving different treatments (Adriamycin-induced cardiotoxicity).

4.9 Effect of *T. populnea* leaf extract on alterations in cardiac ATPase activities in adriamycin-induced cardiotoxicity:

The effects of adriamycin, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on alterations in cardiac ATPase activities after the treatment period of 28 days are illustrated in Table 11 and Figures 17 to 19**.** The parameters examined were sodium-potassium ATPase (Na^+/K^+ATPase), calcium ATPase (Ca^{2+}ATPase) and magnesium ATPase (Mg^{2+}ATPase).

4.9.1 Sodium-potassium ATPase (Na^+/K^+ATPase):

Administration of adriamycin caused a significant ($P<0.001$) decrease in the Na^+/K^+ATPase activity in the disease control compared to the vehicle control. Individual administration of TP200 did not cause any change in the ATPase activity from the vehicle control, while individual administration of TP400, vitamin E or carvedilol significantly elevated the ATPase activity compared to the vehicle control. Administration of TP200 prior to the administration of adriamycin caused an increase in the ATPase activity compared to the level in disease control. In contrast, administration of TP400, vitamin E or carvedilol prior to the administration of adriamycin caused an increase in the ATPase activity in comparison to the level in disease control. The elevation in Na^+/K^+ATPase activity was recorded to be above the level in the vehicle control (Table 11; Fig. 17).

4.9.2 Calcium ATPase (Ca^{2+}ATPase):

Administration of adriamycin caused a significant ($P<0.001$) decrease in the Ca^{2+}ATPase activity in the disease control from the activity in vehicle control. Individual administration of TP200, TP400 or vitamin E caused significant ($P<0.001$) increases in the Ca^{2+}ATPase activity of the vehicle control, while individual administration of carvedilol did not cause any effect on the enzyme activity. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin effectively increased the Ca^{2+}ATPase activity compared to that observed with the disease control (Table 11; Fig. 18).

4.9.3 Magnesium ATPase (Mg^{2+}ATPase):

Administration of adriamycin caused a decrease ($P<0.001$) in the Mg^{2+}ATPase activity from the vehicle control to the disease control. Individual administration of TP200, TP400 and vitamin E caused slight and non-significant variations in the Mg^{2+}ATPase activity from the vehicle control, while individual administration of carvedilol caused a decrease in enzyme activity from the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin caused an increase in Mg^{2+}ATPase activity in comparison to the disease control. Administration with TP200 was least effective on Mg^{2+}ATPase, bringing about a partial, although significant ($P<0.001$), increase (Table 11; Fig. 19).

Table 11. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the levels of sodium-potassium ATPase (Na^+/K^+ATPase), calcium ATPase (Ca^{2+}ATPase) and magnesium ATPase (Mg^{2+}ATPase) in rat heart tissue during adriamycin-induced cardiotoxicity in rats after a treatment period of 28 days.

Experimental Group	Na^+/K^+ ATPase	Ca^{2+} ATPase	Mg^{2+} ATPase
Vehicle Control (5% Gum acacia)	1.49 ± 0.11	1.58 ± 0.06	3.52 ± 0.11
Disease Control (Adriamycin, 15 mg/kg cumulative dose)	0.80 ± 0.03$^{\#\#\#}$	0.64 ± 0.1$^{\#\#\#}$	2.38 ± 0.14$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg)	1.50 ± 0.12 NS	1.70 ± 0.07$^{\#\#\#}$	3.14 ± 0.08 NS
Thespesia Leaf Extract (400 mg/kg)	2.18 ± 0.17$^{\#\#}$	2.12 ± 0.05$^{\#\#\#}$	3.49 ± 0.15 NS
Thespesia Leaf Extract (200 mg/kg) + Adriamycin	1.09 ± 0.05 NS	1.16 ± 0.014***	2.81 ± 0.11
Thespesia Leaf Extract (400 mg/kg) + Adriamycin	1.67 ± 0.12***	1.70 ± 0.08***	3.81 ± 0.18***
Standard (Vitamin E, 25 mg/kg, p.o.)	2.13 ± 0.12$^{\#\#}$	1.88 ± 0.06$^{\#\#\#}$	3.75 ± 0.13NS
Vitamin E (25 mg/kg, p.o.) + Adriamycin	1.87 ± 0.09 ***	1.33 ± 0.03***	3.32 ± 0.15***
(Carvedilol, 1mg/kg, p.o.)	3.12 ± 0.11$^{\#\#\#}$	1.56 ± 0.04 NS	2.23 ± 0.09$^{\#\#\#}$
Carvedilol(1mg/kg, p.o.) + Adriamycin	1.66 ± 0.12***	1.21 ± 0.05***	3.60 ± 0.11***

Note: The values are expressed are mean ± SEM from 6 rats, followed by significance. NS – Not significant.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

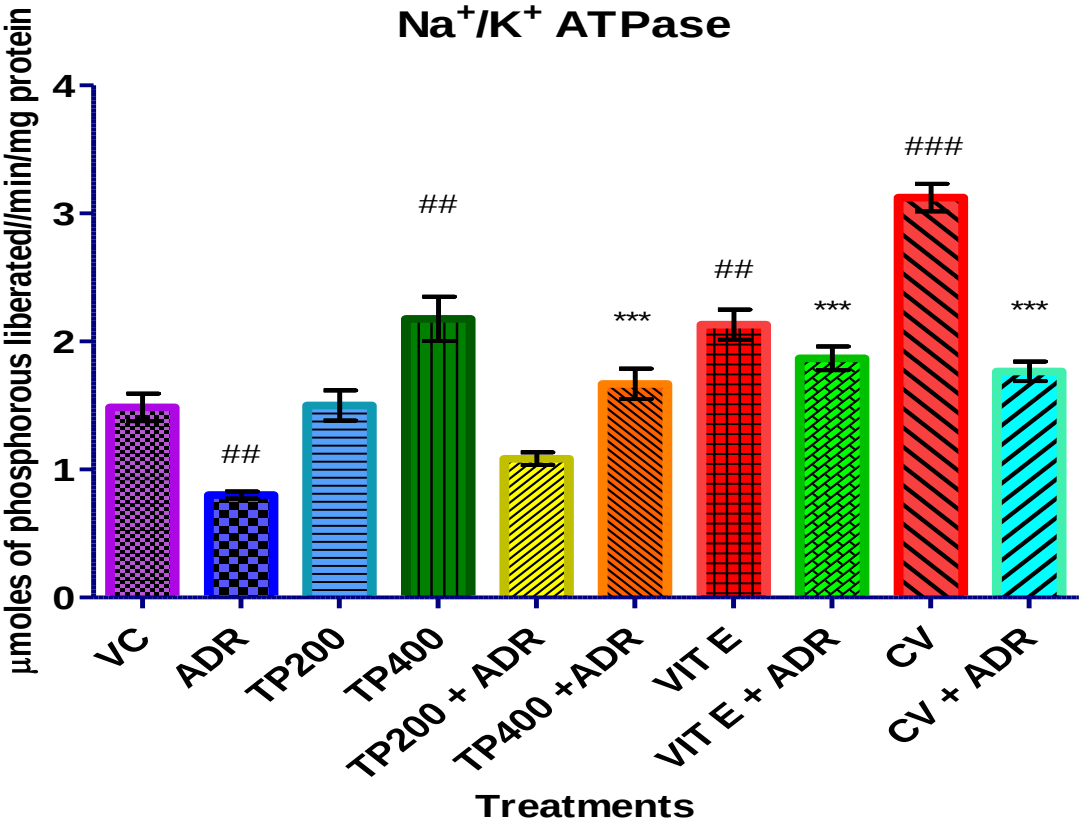

Fig. 17: Histograms representing the changes in sodium-potassium ATPase (Na^+/K^+ATPase) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

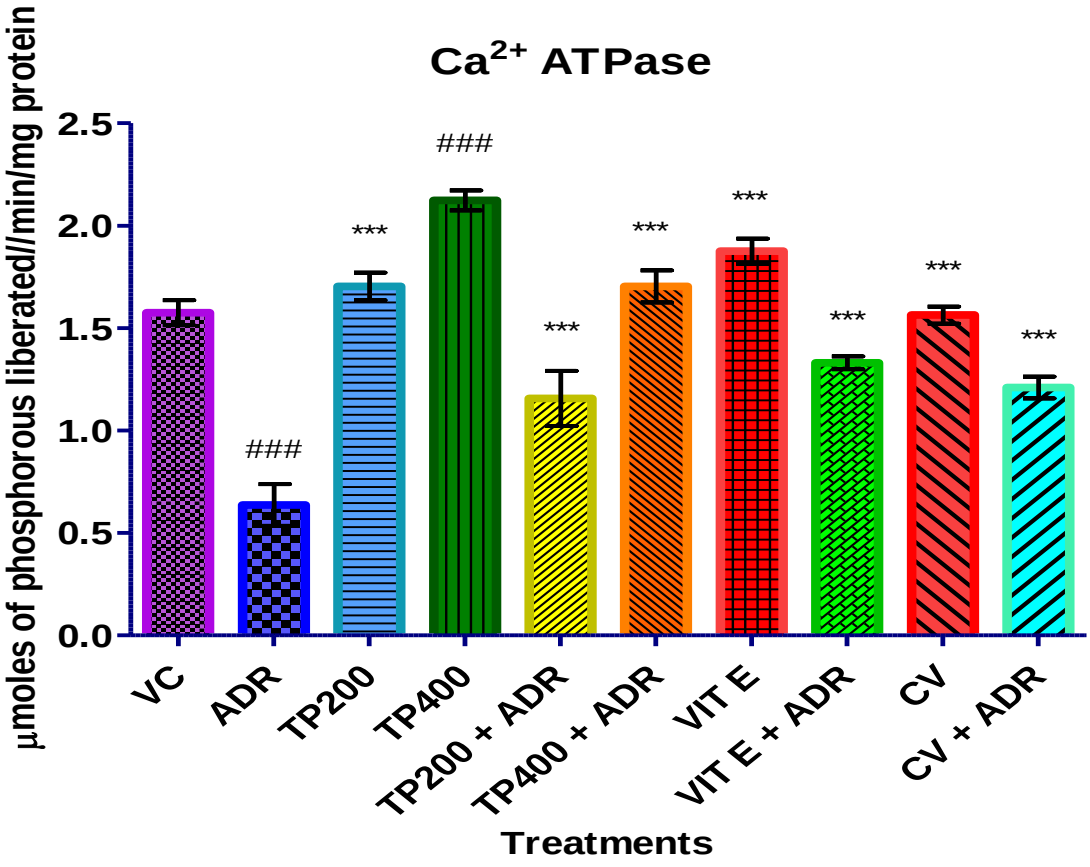

Fig. 18: Histograms representing the changes in calcium ATPase (Ca^{2+}ATPase) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC** - Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

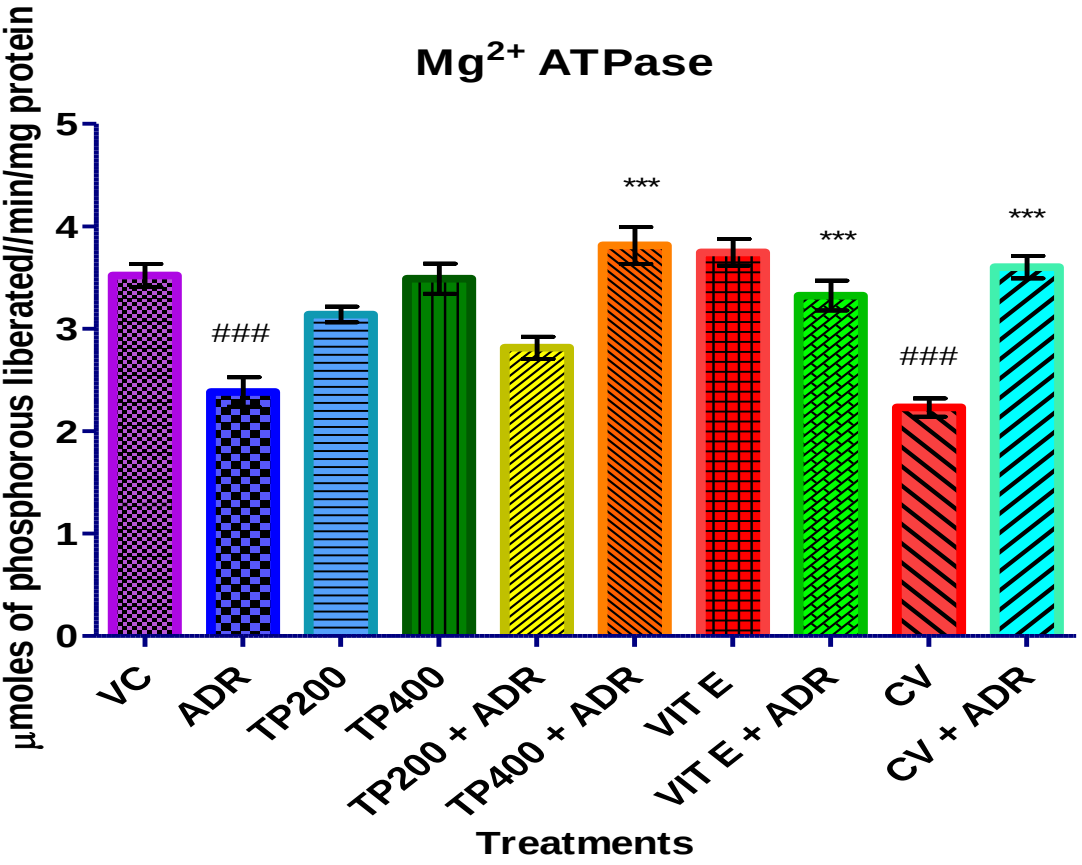

Fig. 19: Histograms representing the changes in magnesium ATPase (Mg^{2+}ATPase) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

4.10 Effect of *T. populnea* leaf extract on alterations in myocardial antioxidant parameters in adriamycin-induced cardiotoxicity:

The effects of adriamycin, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on alterations in myocardial antioxidant parameters after the treatment period of 28 days are illustrated in Table 12 and Figures 20 to 26**.** The parameters examined were lipid peroxidation (LP, in terms of malondialdehyde, MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST).

4.10.1 Lipid peroxidation (LP):

Administration of adriamycin (disease control) caused a significant increase ($P<0.001$) in LP (indicated by the MDA level) in comparison to the vehicle control. Individual treatments with TP200, TP400 and vitamin E or carvedilol caused slight decreases in LP in comparison to the vehicle control that was nevertheless statistically significant. Prior administration of TP200, TP400, vitamin E or carvedilol before the administration of adriamycin caused a decrease in MDA levels as compared to the level in disease control. TP200 and carvedilol were found to cause less reduction in lipid peroxidation compared to TP400 and vitamin E (Table 12; Fig. 20).

4.10.2 Reduced glutathione (GSH):

Administration of adriamycin caused a significant (over 4-fold) decrease in the GSH level compared to the vehicle control. Individual administration of TP200, TP400 or vitamin E caused only minor variations in the GSH level in comparison to the vehicle control, but individual administration of carvedilol caused a perceptible decrease. Administration of TP400 or vitamin E prior to the administration of adriamycin effected an increase in the GSH level in comparison to the disease control, but administration of TP200 or carvedilol prior to adriamycin administration caused only partial, albeit significant ($P<0.001$), increases as compared to the disease control. (Table 12; Fig. 21).

4.10.3 Superoxide dismutase (SOD) activity:

Administration of adriamycin caused a significant (over 2-fold) decrease in the SOD activity in the disease control compared to the vehicle control. Administration of TP200, TP 400, vitamin E or carvedilol individually caused only minor variations in SOD activity with respect to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin caused an increase in the SOD activity in comparison to the disease control (Table 12; Fig. 22).

4.10.4 Catalase (CAT) activity:

Administration of adriamycin caused a significant (5-fold) decrease in the CAT activity in the disease control compared to the vehicle control. Administration of TP200, TP400, vitamin E and carvedilol individually caused slight, non-significant, decreases in the CAT activity compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin caused effective increases in the CAT activity compared to the disease control. Of these, TP400 and vitamin E caused significant increase in the enzyme activity comparable to the vehicle control, while TP200 and carvedilol showed partial improvement in the enzyme activity. (Table 12; Fig. 23).

4.10.5 Glutathione peroxidase (GPX) activity:

Administration of adriamycin caused a significant (over 4-fold) decrease in the GPX activity in the disease control compared to the vehicle control. In comparison, individual administration of TP200, TP400, vitamin E or carvedilol caused only small fluctuations in the enzyme activity compared to the vehicle control. Administration of TP400 or vitamin E prior to the administration of adriamycin increased the GPX activity effectively in comparison to the disease control. In comparison, administration of TP200 or carvedilol prior to the administration of adriamycin effected partial, although significant, increase of the enzyme activity. (Table 12; Fig. 24).

4.10.6 Glutathione reductase (GR):

Administration of adriamycin caused a significant (over 2-fold) decrease in GR activity as compared to the vehicle control. Individual treatment with TP200 or carvedilol caused decreases in GR activity in comparison to the vehicle control, while individual treatments with TP400 or vitamin E caused increases in activity as compared to the vehicle control. Administration of TP400 or vitamin E prior to the administration of adriamycin caused a substantial increase in the enzyme activity in comparison to the disease control. Administration of TP200 or carvedilol prior to the administration of adriamycin caused only a partial, albeit significant, increase in GR activity compared to that of the disease control (Table 12; Fig. 25).

4.10.7 Glutathione-S-transferase (GST):

Administration of adriamycin caused a significant (over 3-fold) decrease in the GST activity in the disease control compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol individually caused significant ($P<0.001$) increases in the GST activity as compared to the vehicle control. Administration of TP200 or carvedilol prior to the administration of adriamycin caused a decrease of GST activity as compared to that in disease control. In contrast, administration of TP400 or vitamin E prior to the administration of adriamycin caused an increase in GST activity compared to the disease control. This increase was significantly elevated above the level of activity in the vehicle control (Table 12; Fig. 26).

Table 12. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the levels of lipid peroxidation (LP), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) during adriamycin-induced cardiotoxicity in rats after a treatment period of 28 days.

Experimental Group	LP	GSH	SOD	CAT	GPX	GR	GST
Vehicle Control (5% Gum acacia)	117.5 ± 0.45	31.51 ± 0.62	16.45 ± 0.36	0.76 ± 0.05	8.57 ± 0.12	50.38 ± 1.27	0.98 ± 0.02
Disease Control (Adriamycin, 15 mg/kg cumulative dose)	270.79 ± 0.80$^{###}$	6.95 ± 0.15$^{###}$	6.21 ± 0.68$^{###}$	0.15 ± 0.05$^{###}$	1.88 ± 0.29$^{###}$	19.22 ± 0.84$^{###}$	0.28 ± 0.05$^{###}$
Thespesia Leaf Extract (200 mg/kg)	111.00 ± 1.30 $^{#}$	27.30 ± 1.08$^{#}$	14.09 ± 0.12$^{##}$	0.69 ± 0.04 NS	6.96 ± 0.47$^{###}$	42.34 ± 1.19$^{###}$	1.6 ± 0.03$^{###}$
Thespesia Leaf Extract (400 mg/kg)	106.5 ± 1.13$^{###}$	34.33 ± 0.78 NS	15.94 ± 0.10 NS	0.72 ± 0.15 NS	8.09 ± 0.15 NS	62.64 ± 1.21$^{###}$	2.25 ± 0.08$^{###}$
Thespesia Leaf Extract (200 mg/kg) + Adriamycin	178.42 ± 1.31***	20.11 ± 0.79***	12.29 ± 0.52***	0.48 ± 0.02***	6.17 ± 0.14***	30.42 ± 0.79***	0.7 ± 0.02***
Thespesia Leaf Extract (400 mg/kg) + Adriamycin	125.5± 1.11***	31.21 ± 0.6***	15.66 ± 0.55***	0.75 ± 0.02***	8.79 ± 0.09***	49.58 ± 0.96***	1.59 ± 0.02***
Standard (Vitamin E, 25 mg/kg, p.o.)	104.42± 1.50$^{###}$	32.4 ± 0.40 NS	16.09 ± 0.10 NS	0.79 ± 0.05 NS	8.52 ± 0.11 NS	59.13 ± 1.02$^{###}$	2.08 ± 0.07$^{###}$
Vitamin E (25 mg/kg, p.o.) + Adriamycin	124.33± 0.95***	32.01 ± 0.66***	15.90 ± 0.57***	0.71± 0.02***	8.65 ± 0.13***	49.61 ± 0.96***	1.44 ± 0.04***
(Carvedilol, 1mg/kg, p.o.)	115.63 ± 1.60$^{###}$	23.79 ± 0.63$^{###}$	14.09 ± 0.51$^{##}$	0.72 ± 0.03 NS	7.33 ± 0.12$^{###}$	46.30 ± 0.96 NS	1.17 ± 0.02$^{#}$
Carvedilol(1mg/kg, p.o.) + Adriamycin	166.17 ± 1.40***	23.66 ± 1.81***	11.61 ± 0.42***	0.65 ± 0.01***	6.05 ± 0.11***	33.86 ± 1.03***	0.81 ± 0.03***

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.

NS – Not significant.

#: Value differs significantly from vehicle control group **$^{#}$P<0.05, $^{##}$P<0.01, $^{###}$P<0.001**

*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

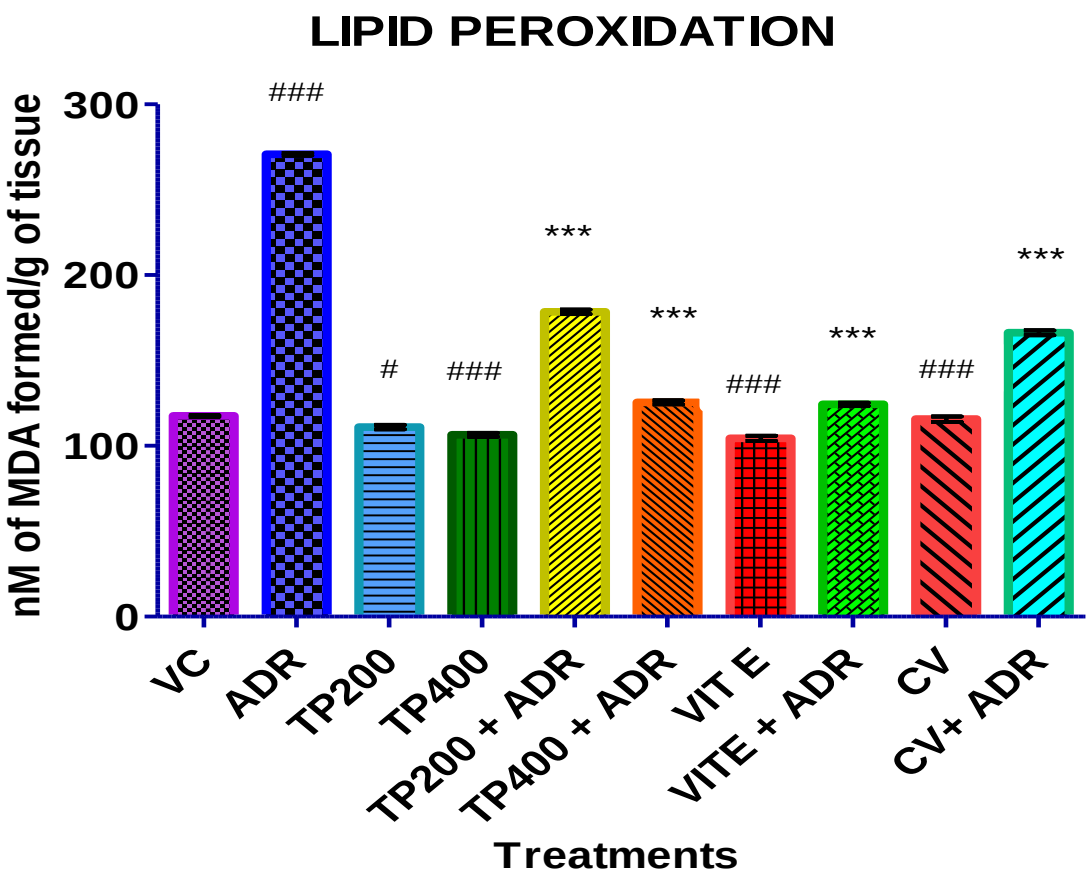

Fig. 20: Histograms representing the changes in lipid peroxidation (LP, in terms of malondialdehyde, MDA) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

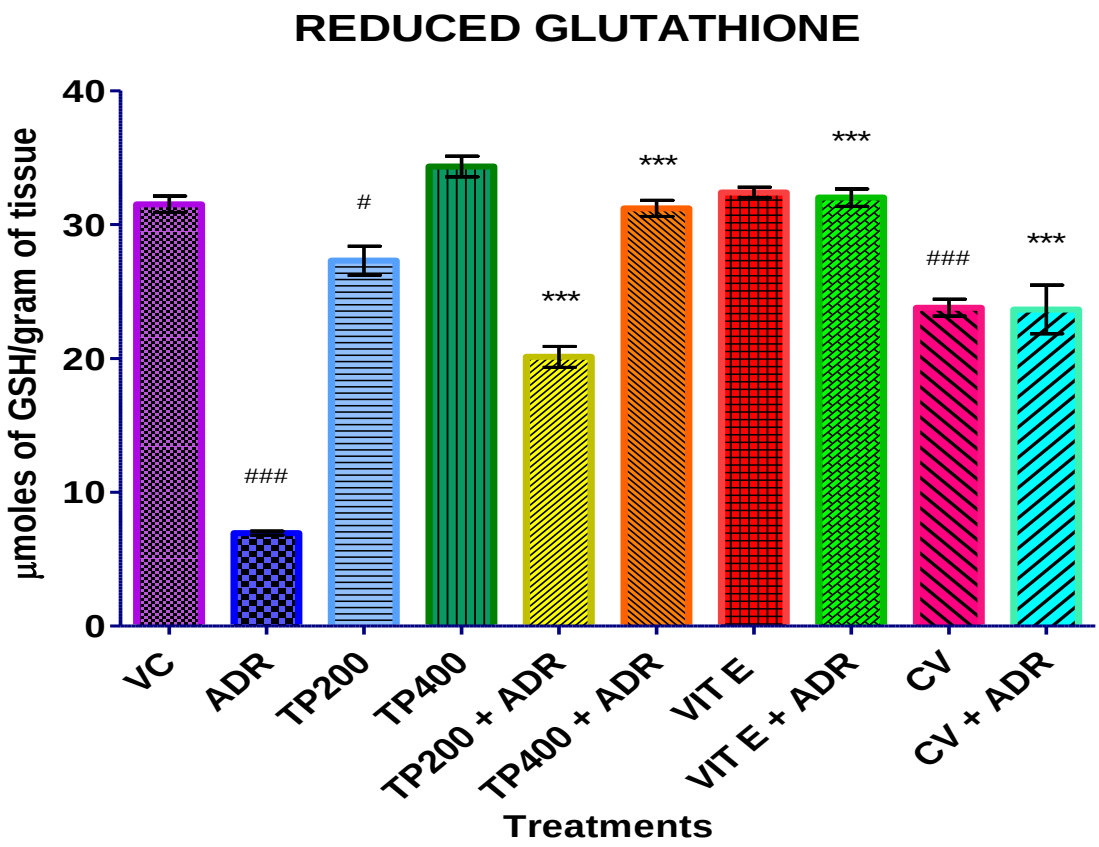

Fig. 21: Histograms representing the changes in reduced glutathione (GSH) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR -** rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

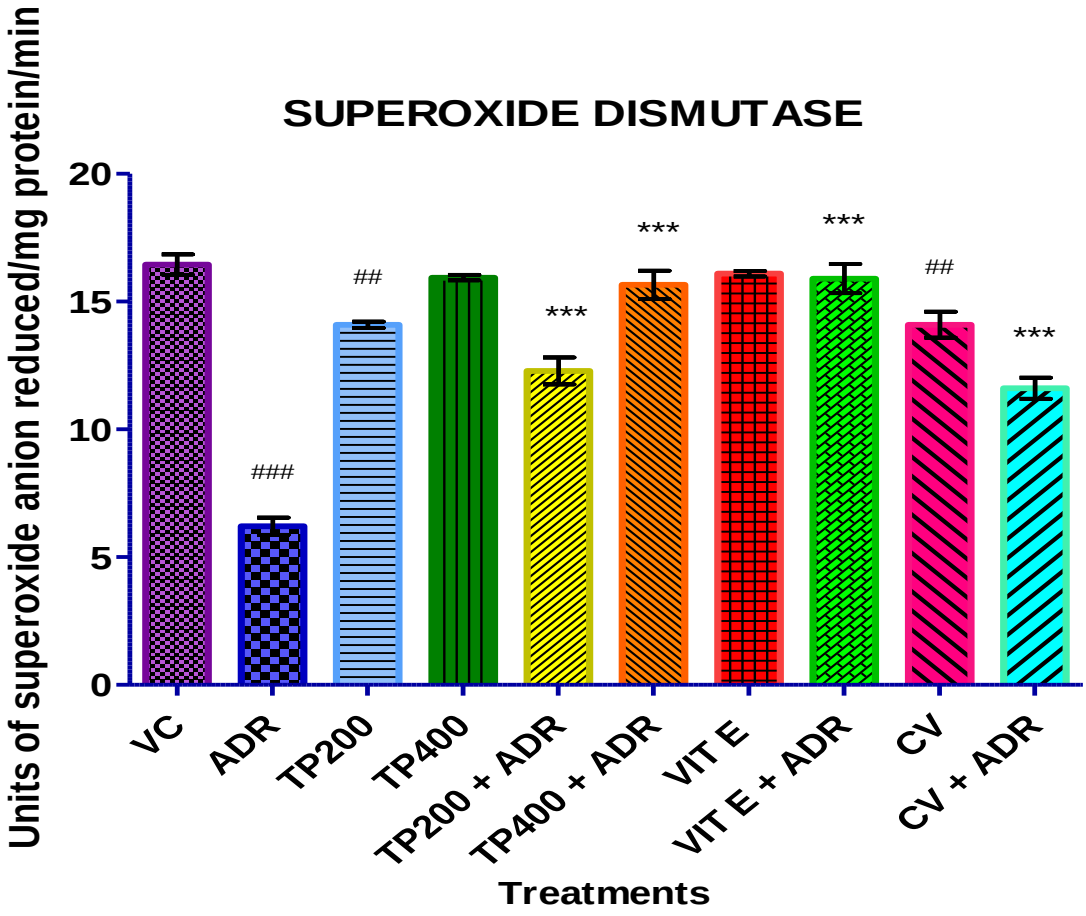

Fig. 22: Histograms representing the changes in superoxide dismutase (SOD) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

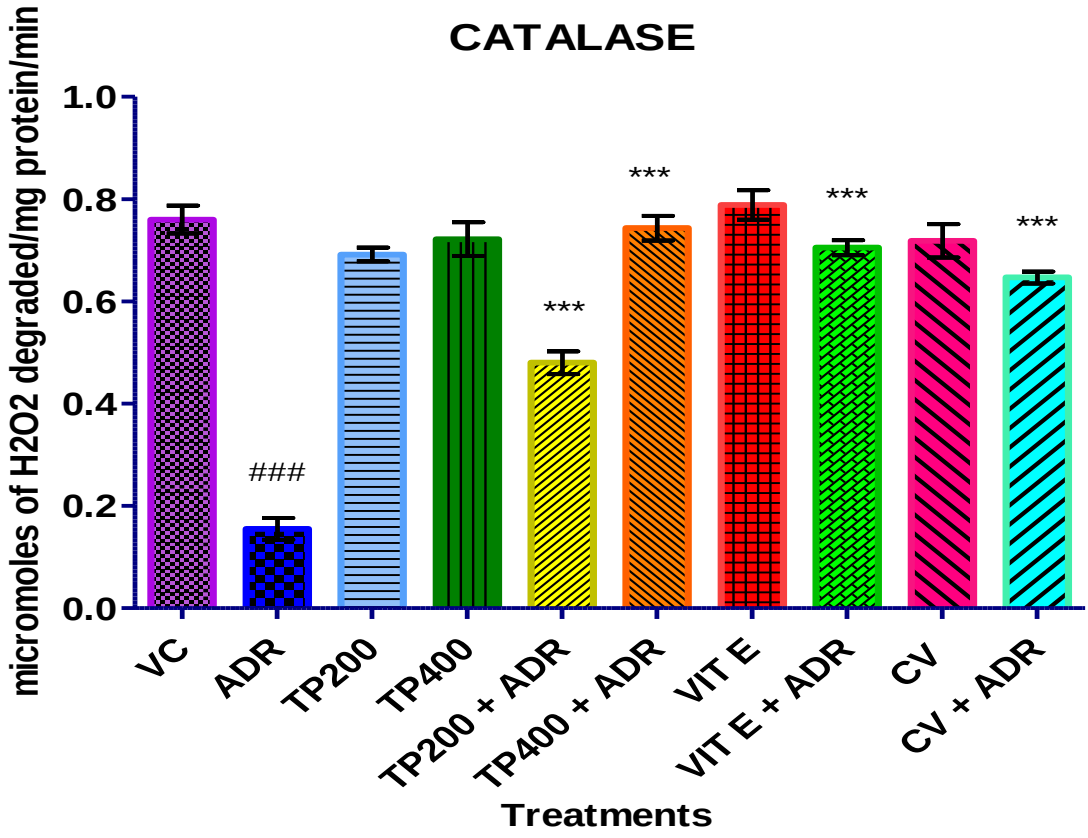

Fig. 23: Histograms representing the changes in catalase (CAT) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR -** rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

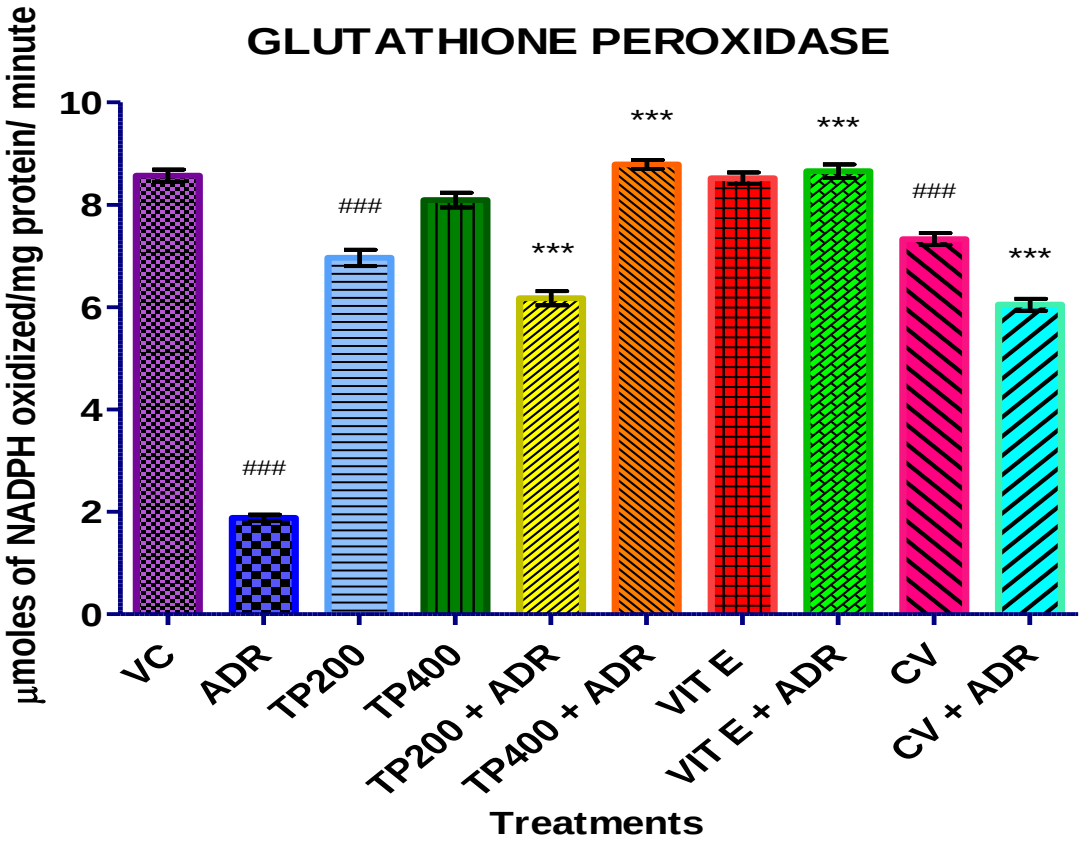

Fig. 24: Histograms representing the changes in glutathione peroxidase (GPX) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

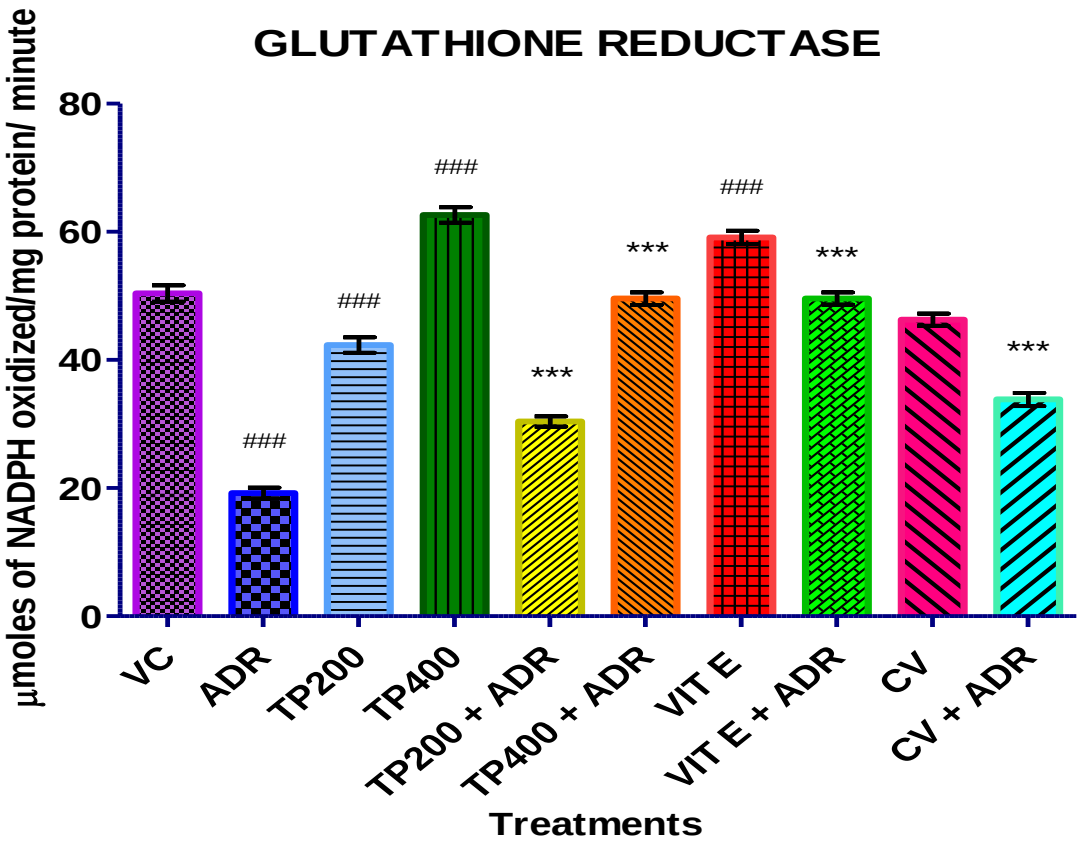

Fig. 25: Histograms representing the changes in glutathione reductase (GR) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

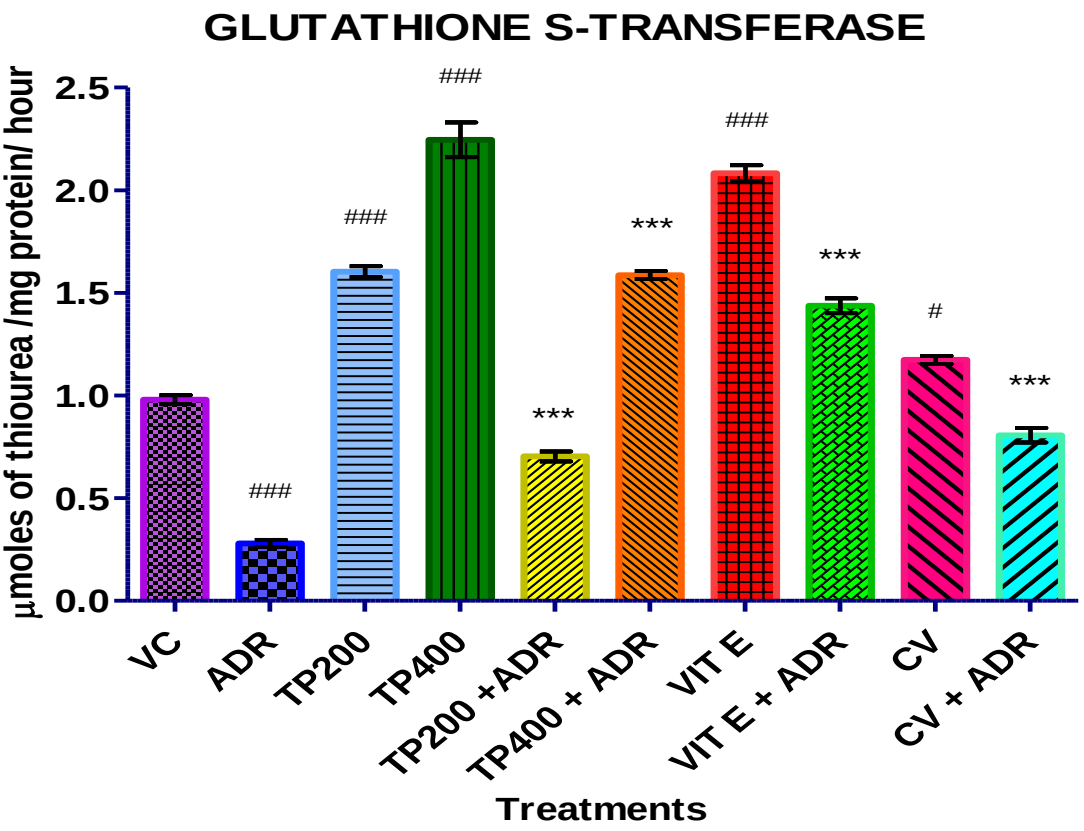

Fig. 26: Histograms representing the changes in glutathione-S-transferase (GST) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR -** rats treated with carvedilol + adriamycin.

4.11 Effect of *T. populnea* leaf extract on alterations in serum biochemical parameters in adriamycin-induced cardiotoxicity:

The effects of adriamycin, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on serum biochemical parameters (cardiac biomarkers) after the treatment period of 28 days are illustrated in Table 13 and Figures 27 to 32**.** The parameters examined were serum C-reactive protein (CRP), and activities of creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

4.11.1 C-reactive protein (CRP):

Administration of adriamycin (disease control) caused a very significant increase ($P<0.001$) in serum CRP compared to the vehicle control. Individual treatments with TP200 and vitamin E caused slight decreases in CRP, while TP400 and carvedilol caused an increase. Administration of TP200, TP400, Vitamin E or carvedilol prior to the administration of adriamycin prevented the rise in CRP caused by adriamycin. The results obtained were comparable to that of the vehicle control. TP400 was effective in preventing the elevation in CRP level. The lowering of CRP levels with TP400 was comparable to that of the vehicle control (Table 13; Fig. 27).

4.11.2 Creatine kinase-MB (CK-MB) activity:

Administration of adriamycin caused a striking increase (over 13-fold) in the CK-MB activity in comparison to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol individually caused slight increases or decreases in the activity level compared to that of the vehicle control. Administration of TP200, TP400 or carvedilol prior to the administration of adriamycin brought down the activity effectively as compared to the disease control. The activity of CK-MB upon administration of vitamin E prior to adriamycin significantly lowered the activity compared to the disease control towards the vehicle control level (Table 13; Fig. 28).

4.11.3 Creatine kinase (CK) activity:

Administration of adriamycin caused a very significant increase (over 8-fold) in the CK-MB activity in comparison to the vehicle control. Administration of TP200 or TP400 separately caused little or no elevation in the enzyme activity, while individual

administration of vitamin E or carvedilol caused a decrease. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin caused a significant decrease in the enzyme activity compared to the level of the disease control (Table 13: Fig. 29).

4.11.4 Lactate dehydrogenase (LDH) activity:

Administration of adriamycin caused a 3-fold increase in the LDH activity in the disease control compared to the vehicle control. Administration of TP200 individually caused a decrease in the enzyme activity, while administration of TP 400, vitamin E or carvedilol separately effected moderate elevations in the enzyme activity in comparison to the disease control. Administration of TP200, vitamin E or carvedilol prior to the administration of adriamycin brought down the activity effectively in comparison to the disease control. The decrease in LDH activity was similar to that of the vehicle control. Administration of TP400 prior to adriamycin administration caused the enzyme activity to decrease significantly in comparison to that of the disease control (Table 13; Fig. 30).

4.11.5 Aspartate aminotransferase (AST) activity:

Administration of adriamycin caused a significant (over 4-fold) increase in the AST activity in the disease control compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol individually caused only slight increases or decreases in the activity compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin prevented the rise in the enzyme activity effectively in comparison to the disease control. Of these, TP200 was less effective in causing reduction in the enzyme activity compared to the disease control (Table 13; Fig. 31).

4.11.6 Alanine aminotransferase (ALT) activity:

Like AST, administration of adriamycin also caused a significant (about 4-fold) increase in the ALT activity in the disease control compared to the vehicle control. Administration of TP200 or TP400 separately caused slight elevation in the enzyme activity, while individual administration of vitamin E or carvedilol caused a greater elevations (about 2-fold) in ALT activity compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin prevented the elevation in the enzyme activity effectively in comparison to the disease control. Of these, with TP200 and

carvedilol the enzyme activity remained slightly above the vehicle control level, while with TP400 and vitamin E the increase in ALT activity due to adriamycin was significantly prevented in comparison to the disease control (Table 13; Fig. 32).

Table 13. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the levels of serum C-reactive protein (CRP), and activities of creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) during adriamycin-induced cardiotoxicity in rats after a treatment period of 28 days.

Experimental Group	CRP	CKMB	CK	LDH	AST	ALT
Vehicle Control (5% Gum acacia)	1.22 ± 0.08	32.43 ± 1.17	115.17 ± 5.31	903.16 ± 10.43	40.34 ± 2.39	30.03 ± 2.02
Disease Control (Adriamycin, 15 mg/kg cumulative dose)	28.35 ± 1.40$^{\#\#\#}$	433.95 ± 7.04$^{\#\#\#}$	990.85 ± 9.49$^{\#\#\#}$	2695.03 ± 17.90 $^{\#\#\#}$	179.31 ± 4.53$^{\#\#\#}$	113.59 ± 2.10$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg)	0.88 ± 0.22 NS	43.20 ± 11.9 NS	126.27 ± 9.83 NS	606.33 ± 1.11$^{\#\#\#}$	44.13 ± 3.38 NS	37.90 ± 2.31 NS
Thespesia Leaf Extract (400 mg/kg)	1.83 ± 0.38 NS	24.61 ± 3.29 NS	111.77 ± 6.25 NS	476.30 ± 9.75**	47.01 ± 7.83 NS	40.88 ± 3.73 NS
Thespesia Leaf Extract (200 mg/kg) + Adriamycin	10.08 ± 0.87***	96.57 ± 5.38***	366.53 ± 4.45***	1660.90 ± 4.37***	77.83 ± 2.87***	34.68 ± 1.17***
Thespesia Leaf Extract (400 mg/kg) + Adriamycin	3.73 ± 0.39***	47.2 ± 1.39***	154.00 ± 3.54***	1256.20 ± 12.7$^{\#\#\#}$	50.90 ± 2.45***	22.22 ± 1.34***
Standard (Vitamin E, 25 mg/kg, p.o.)	1.23 ± 0.26 NS	23.38 ± 3.81 NS	70.24 ± 5.29 NS	1088.30 ± 16.27$^{\#\#\#}$	38.82 ± 3.38 NS	28.21 ± 2.49 NS
Vitamin E (25 mg/kg, p.o.) + Adriamycin	3.66 ± 0.54***	31.27 ± 0.78***	147.17 ± 2.95***	1061.20 ± 14.10***	41.60 ± 3.13***	59.04 ± 1.42***
Carvedilol, (1mg/kg, p.o.)	2.67 ± 1.42 NS	29.03 ± 1.95 NS	31.23 ± 3.41 NS	1062.10 ± 11.50$^{\#\#\#}$	48.12 ± 3.18 NS	38.71 ± 1.86 NS
Carvedilol (1mg/kg, p.o.) + Adriamycin	4.52 ± 0.78***	58.26 ± 1.93***	171.33 ± 2.68***	1316.30 ± 12.40***	44.71 ± 2.18***	63.16 ± 1.66***

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance, NS – Not significant.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

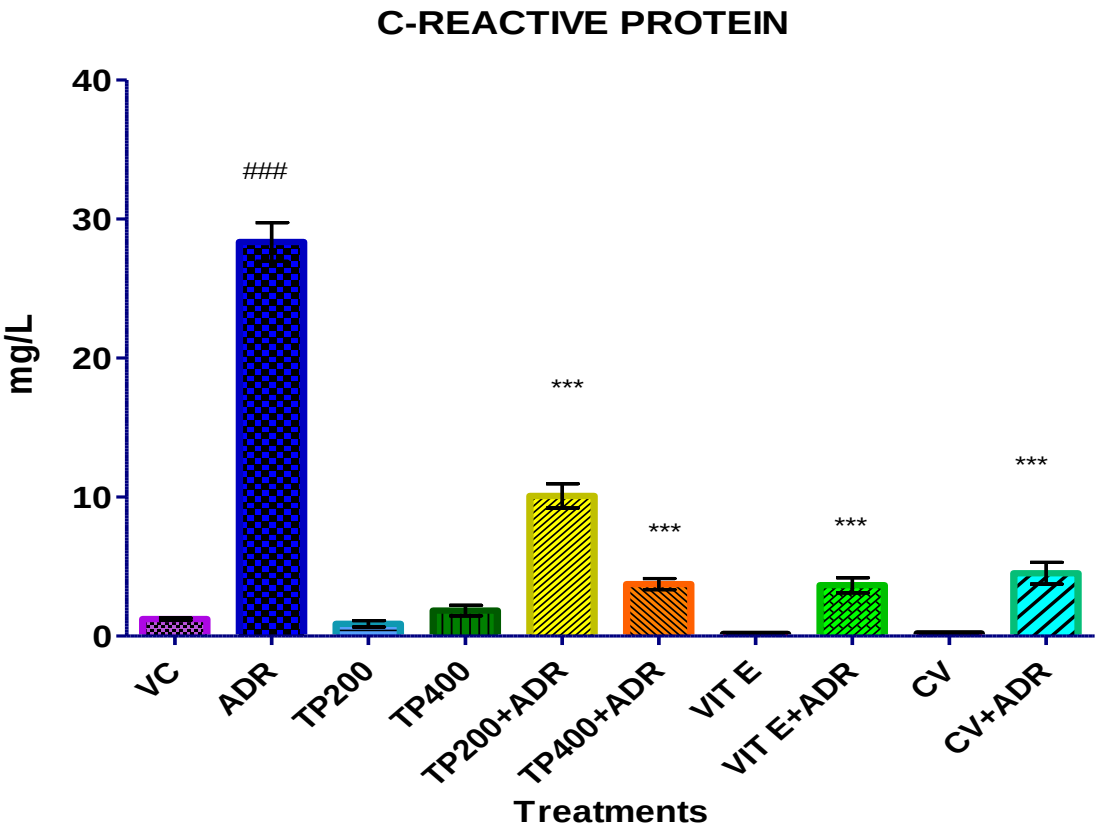

Fig. 27: Histograms representing the changes in serum C-reactive protein (CRP) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR -** rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

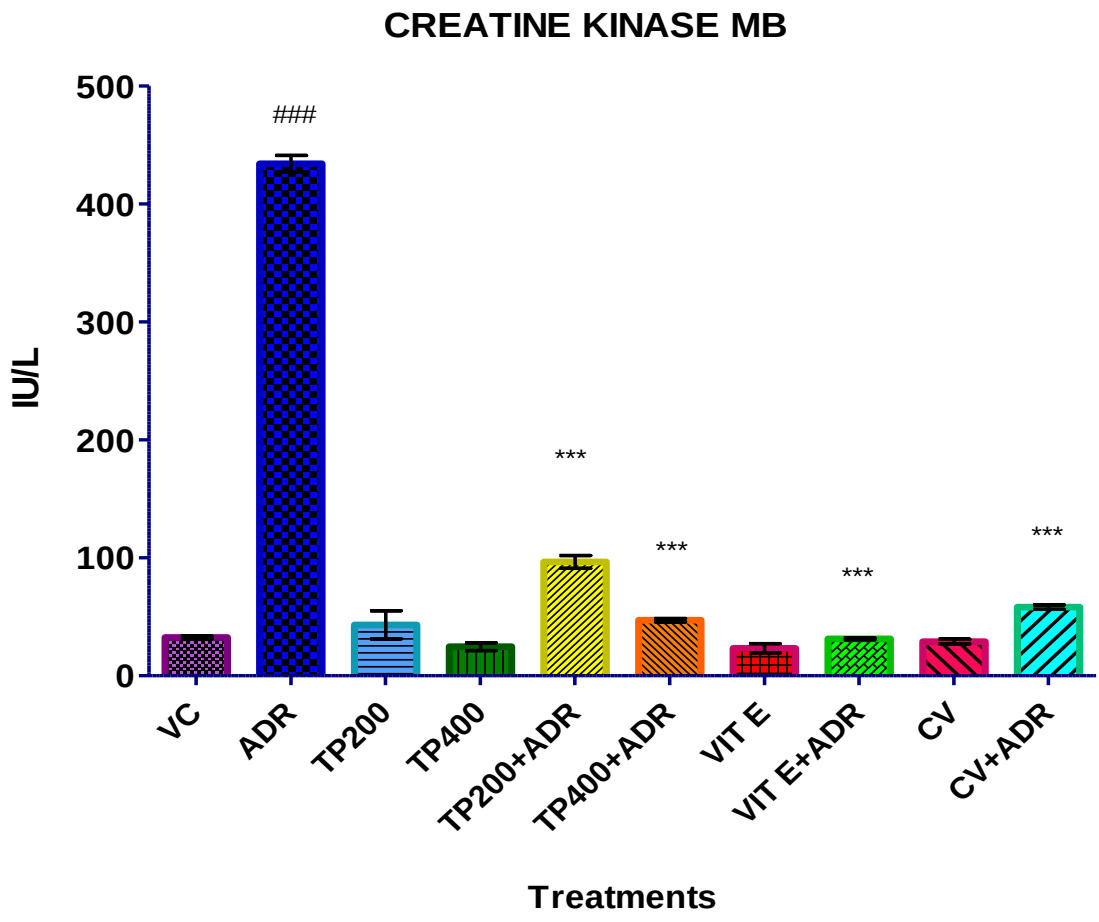

Fig. 28: Histograms representing the changes in serum creatine kinase-MB (CKMB) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

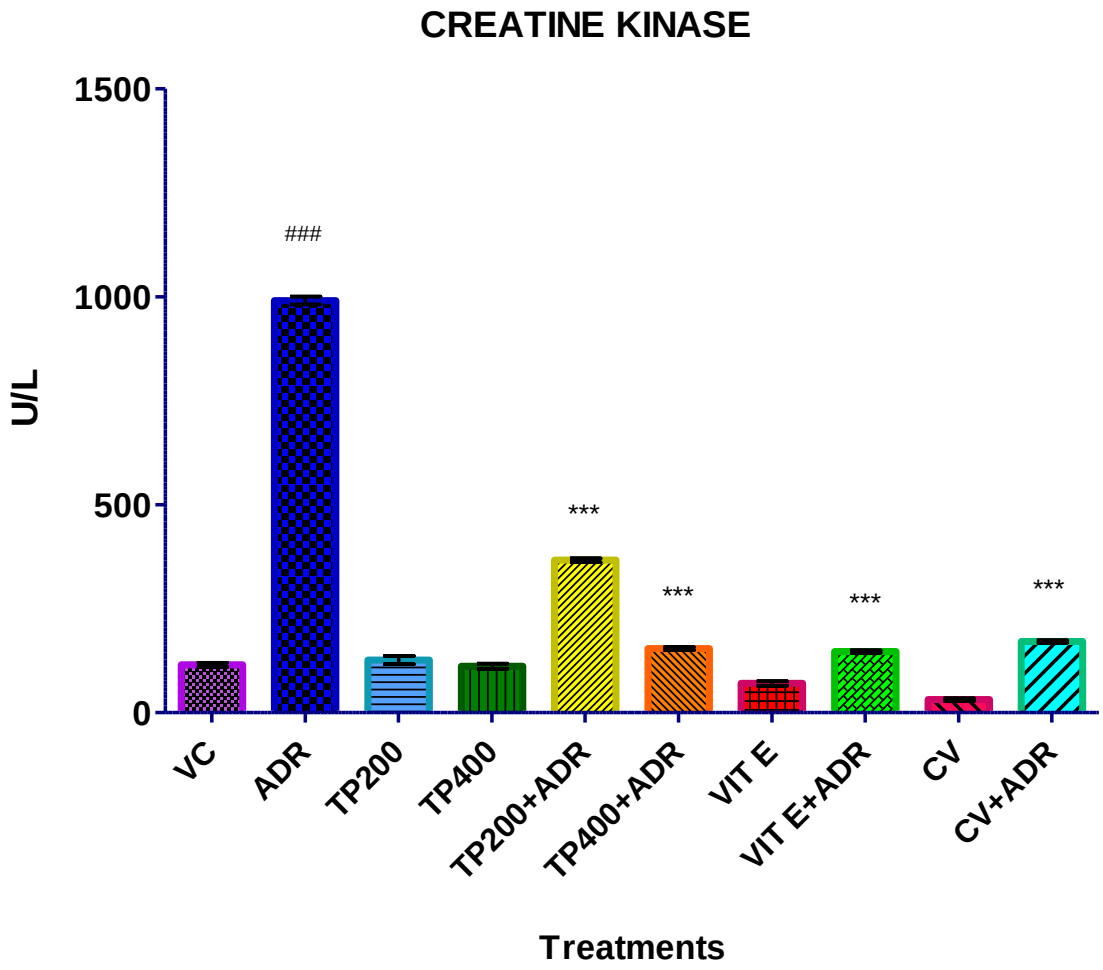

Fig. 29: Histograms representing the changes in serum creatine kinase (CK) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

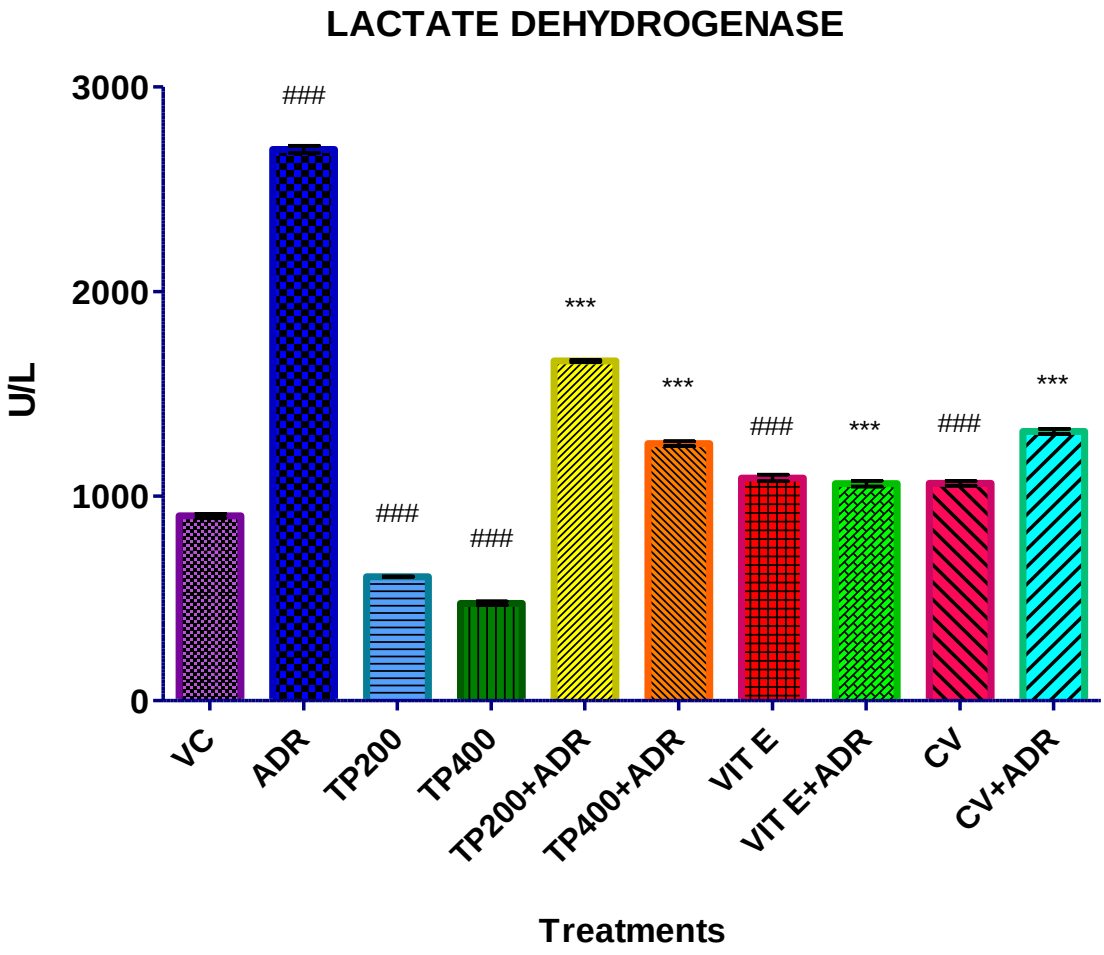

Fig. 30: Histograms representing the changes in serum lactate dehydrogenase (LDH) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

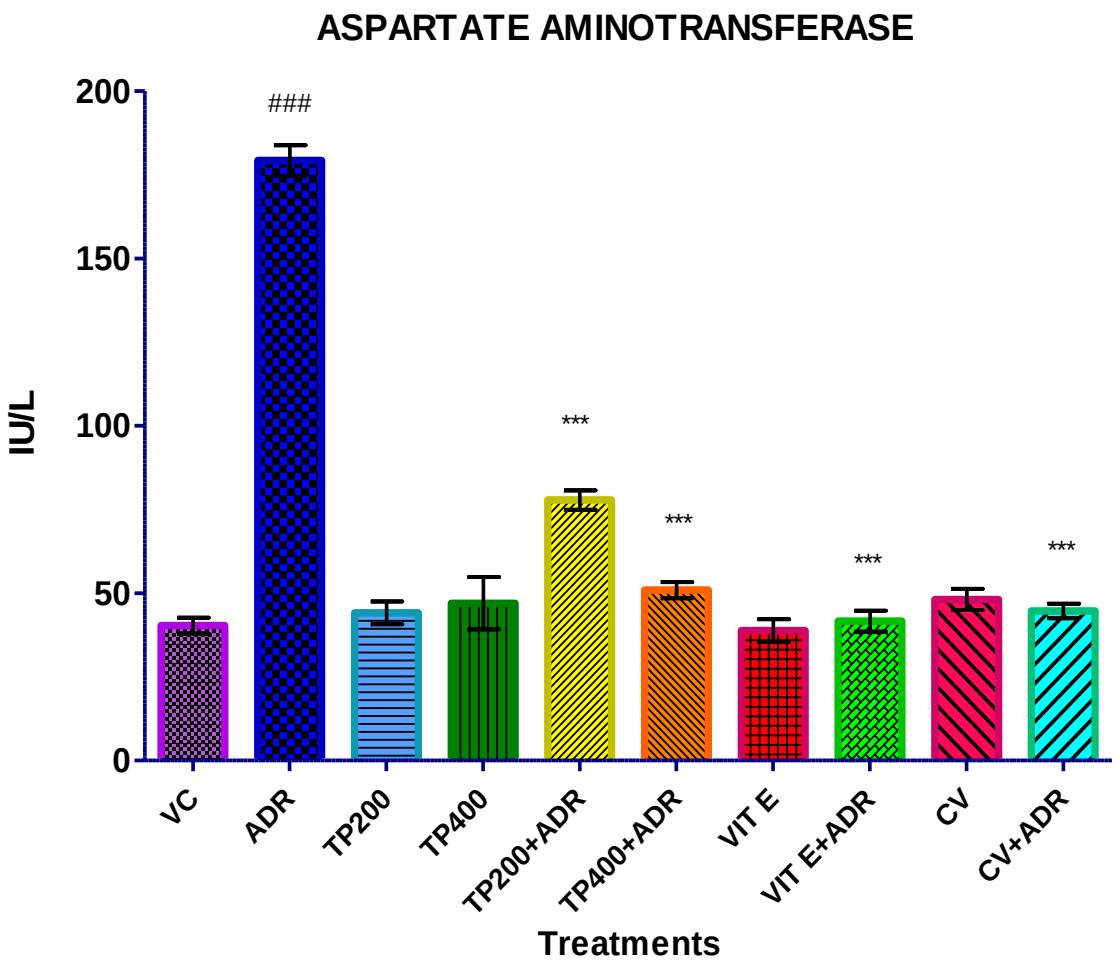

Fig. 31: Histograms representing the changes in serum aspartate aminotransferase (AST) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC** - Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

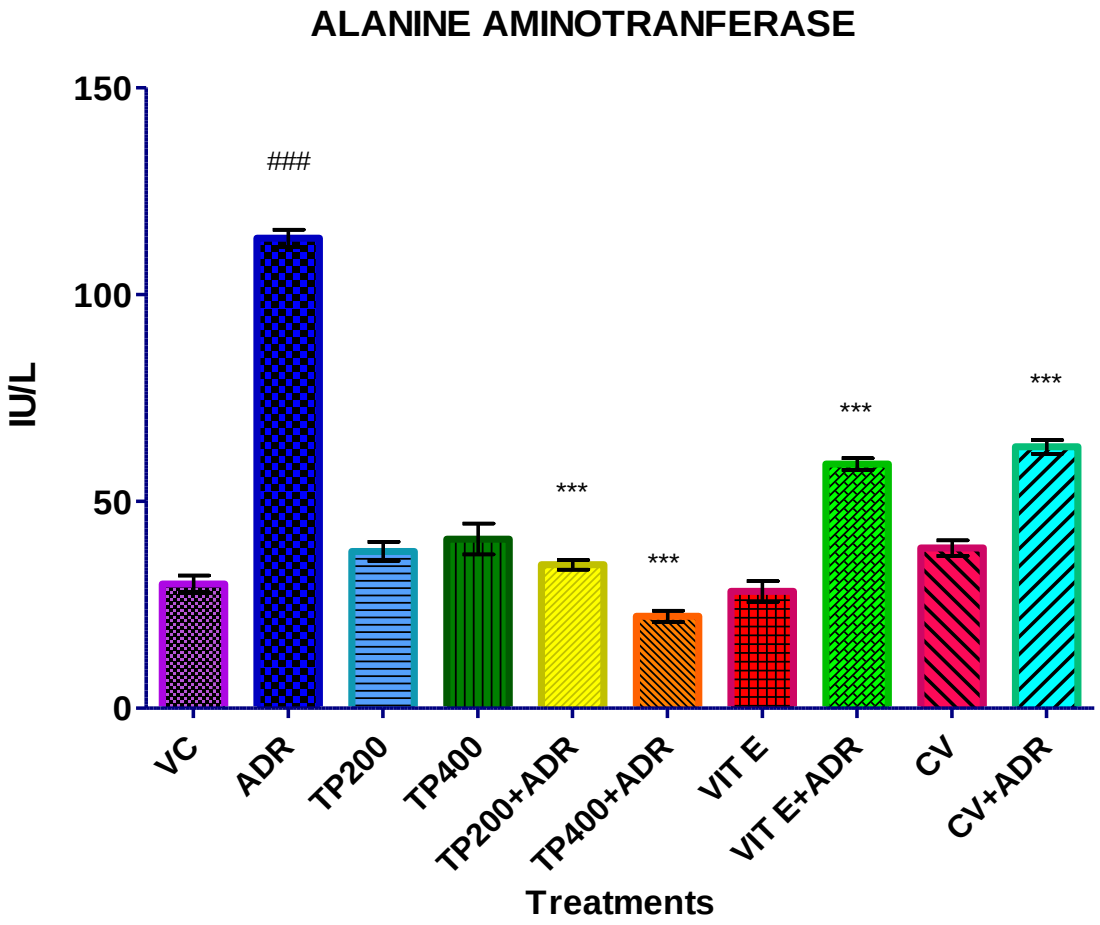

Fig. 32: Histograms representing the changes in serum alanine aminotransferase (ALT) activity levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

4.12 Effect of *T. populnea* leaf extract on alterations in serum lipid profile parameters in adriamycin-induced cardiotoxicity.

The effects of adriamycin, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on serum lipid profile parameters after the treatment period of 28 days are illustrated in Table 14 and Figures 33 to 37**.** The parameters examined were total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL).

4.12.1 Total cholesterol (TC):

Administration of adriamycin (disease control) caused a significant increase ($P<0.001$) in TC compared to the vehicle control. Individual treatments with TP200, TP400, vitamin E and carvedilol caused decreases in TC level compared to the vehicle control, with TP400 causing a greater decrease than the other three individual treatments. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin prevented the abnormal rise in cholesterol levels in comparison to the disease control. In all the four cases, the TC level was significantly decreased and was below the vehicle control levels. Maximal decrease compared to the disease control level was effected by TP400 (Table 14; Fig. 33).

4.12.2 Triglycerides (TG):

Administration of adriamycin caused a significant (2-fold) increase in the TG level compared to the vehicle control. Individually, administration of TP400, vitamin E or carvedilol caused slight decreases in the TG level as compared to the vehicle control, while TP200 caused an increase. Administration of TP400, vitamin E or carvedilol prior to the administration of adriamycin prevented the rise in the TG level effectively in comparison to the disease control. With TP200, only a moderate decrease in comparison to the disease control was observed. (Table 14; Fig. 34).

4.12.3 High density lipoprotein (HDL):

Administration of adriamycin caused a significant decrease ($P<0.001$) in the HDL level in the disease control compared to the vehicle control. Administration of TP200, TP 400, vitamin E or carvedilol individually also caused non-significant decreases in the HDL levels. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin caused an increase in the HDL content compared to that of the disease control. (Table 14; Fig. 35).

4.12.4 Low density lipoprotein (LDL):

Administration of adriamycin caused a significant (over 3-fold) increase in the LDL content in the disease control compared to the vehicle control. Administration of TP200, TP400 and vitamin E individually caused slight decreases in the LDL level of the vehicle control, while individual administration of carvedilol caused a slight increase. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin lowered the LDL level effectively in comparison to that of the disease control. The decrease in LDL levels on pre-treatment with TP200, TP400, vitamin E or carvedilol were comparable to the level in vehicle control. Of these, TP200 and TP400 caused a decrease to below the vehicle control level, vitamin E reduced the LDL content to the vehicle control level, but carvedilol kept it at a higher level compared to the vehicle control (Table 14; Fig. 36).

4.12.5 Very low density lipoprotein (VLDL):

Administration of adriamycin caused an increase ($P<0.001$) in the VLDL level in the disease control compared to the vehicle control. Individual administration of TP200 did not cause any change in the VLDL level of the vehicle control, while TP400, vitamin E and carvedilol individually caused decreases ($P<0.001$) in the VLDL level. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of adriamycin reduced the VLDL content effectively, as compared to the disease control. Of these, with TP200 the reduction in the VLDL content was not statistically significant, while with TP400, vitamin E and carvedilol the VLDL content was decreased significantly compared to the level of disease control (Table 14; Fig. 37).

Table 14. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the serum lipid profile parameters comprising levels of serum total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) during adriamycin-induced cardiotoxicity in rats.

Experimental Group	**TC (mg/dl)**	**TG (mg/dl)**	**HDL (mg/dl)**	**LDL (mg/dl)**	**VLDL (mg/dl)**
Vehicle Control (5% Gum acacia)	166.4 ± 5.84	126.65 ± 3.69	78.93 ± 4.21	53.33 ± 4.23	33.06 ± 0.86
Disease Control (Adriamycin, 15 mg/kg cumulative dose)	257.01 ± 9.92$^{\#\#\#}$	250.85 ± 9.31$^{\#\#\#}$	27.56 ± 2.98$^{\#\#\#}$	179.28 ± 9.9$^{\#\#\#}$	50.17 ± 1.86$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg)	146.45 ± 6.6 NS	168.92 ± 17.09 NS	69.34 ± 5.07 NS	43.32 ± 8.19 NS	33.7 ± 3.41 NS
Thespesia Leaf Extract (400 mg/kg)	111.40 ± 9.95$^{\#\#\#}$	101.44 ± 7.69 NS	54.36 ± 6.92 NS	36.74 ± 7.51 NS	20.28 ± 1.53$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg) + Adriamycin	133.69 ± 5.16 ***	206.79 ± 7.63 NS	68.04 ± 7.11***	24.28 ± 5.01***	41.35 ± 1.52 NS
Thespesia Leaf Extract (400 mg/kg) + Adriamycin	119.33 ± 5.7 ***	135.65 ± 5.31***	66.9 ± 3.38***	25.3 ± 6.3***	27.13 ± 1.06***
Standard (Vitamin E, 25 mg/kg, p.o.)	126.05 ± 9.31$^{\#\#}$	112.31 ± 8.51 NS	61.14 ± 3.82 NS	42.44 ± 9.5 NS	22.46 ± 1.7$^{\#\#}$
Vitamin E (25 mg/kg, p.o.) + Adriamycin	146.15 ± 5.71***	136.49 ± 11.86***	68.42 ± 8.59***	50.42 ± 10.9***	27.29 ± 2.37***
(Carvedilol, 1mg/kg, p.o.)	143.87 ± 3.46 NS	121.12 ± 6.73 NS	60.78 ± 5.0 NS	58.8 ± 5.79 NS	24.22 ± 1.34 NS
Carvedilol(1mg/kg, p.o.) + Adriamycin	154.5 ± 3.98***	135.8 ± 9.61***	53.96 ± 7.37NS	73.37 ± 4.85***	27.16 ± 1.92***

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.

NS – Not significant.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

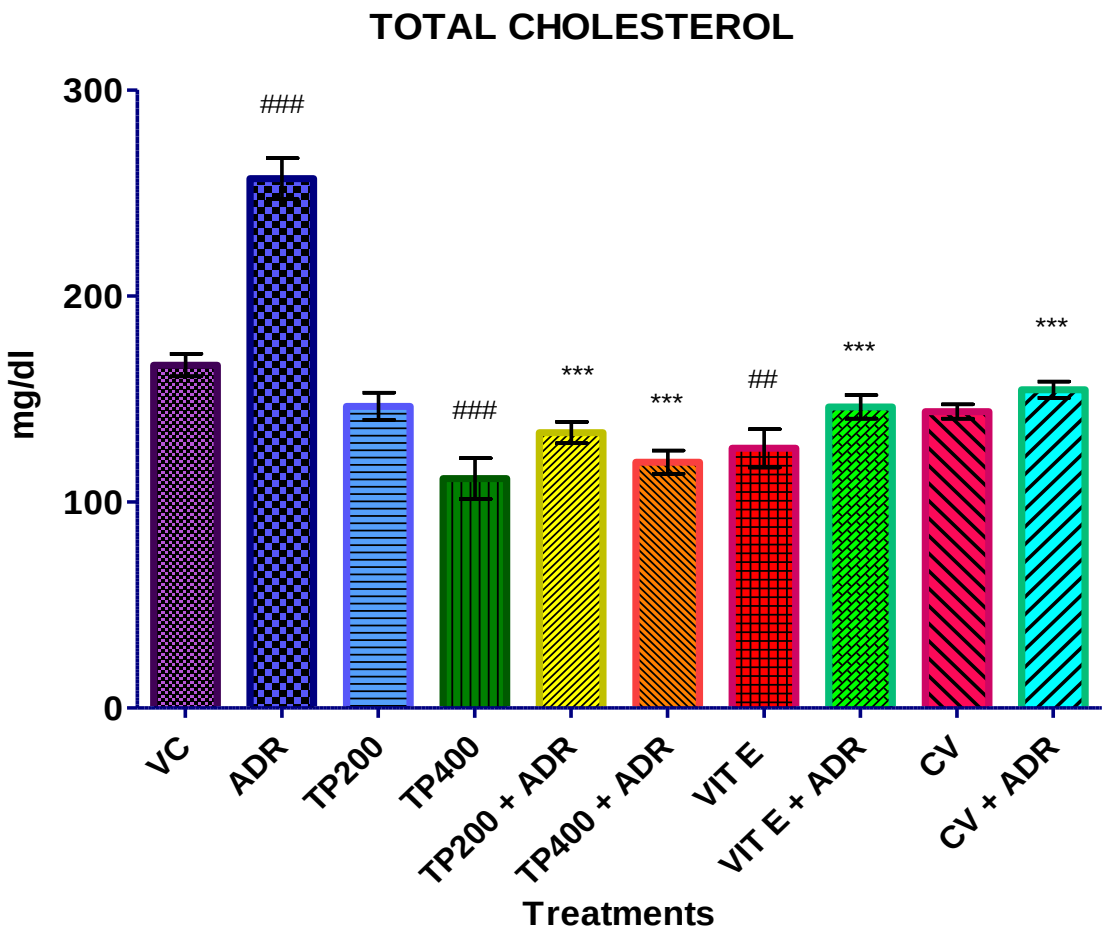

Fig. 33: Histograms representing the changes in serum total cholesterol (TC) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC** - Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

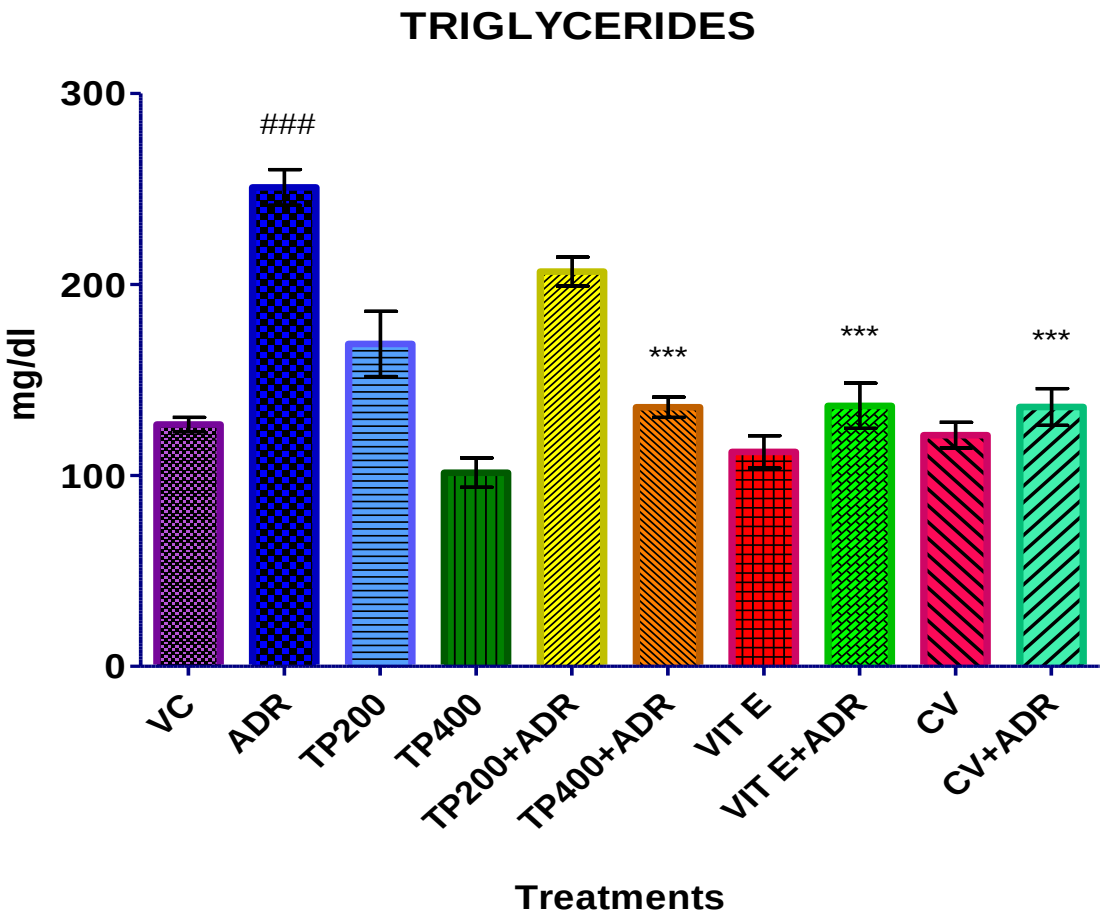

Fig. 34: Histograms representing the changes in serum triglyceride (TG) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

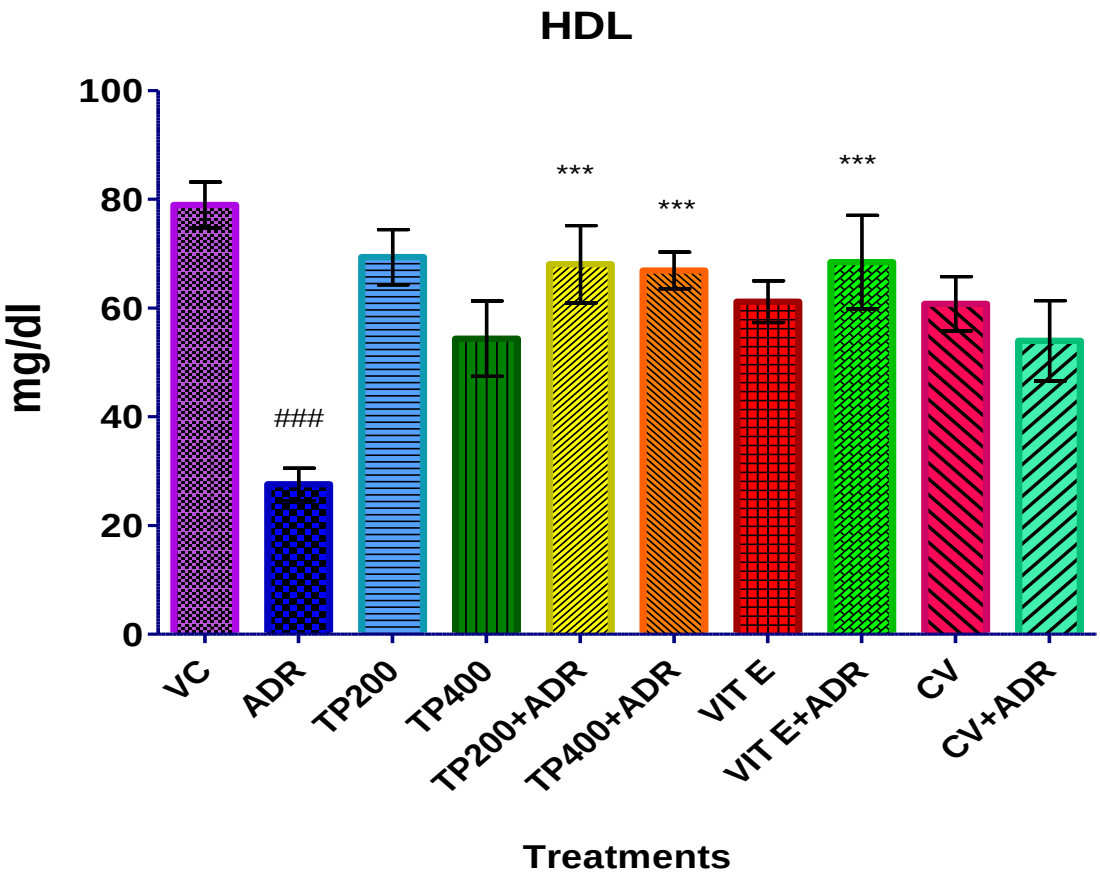

Fig. 35: Histograms representing the changes in serum high density lipoprotein (HDL) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

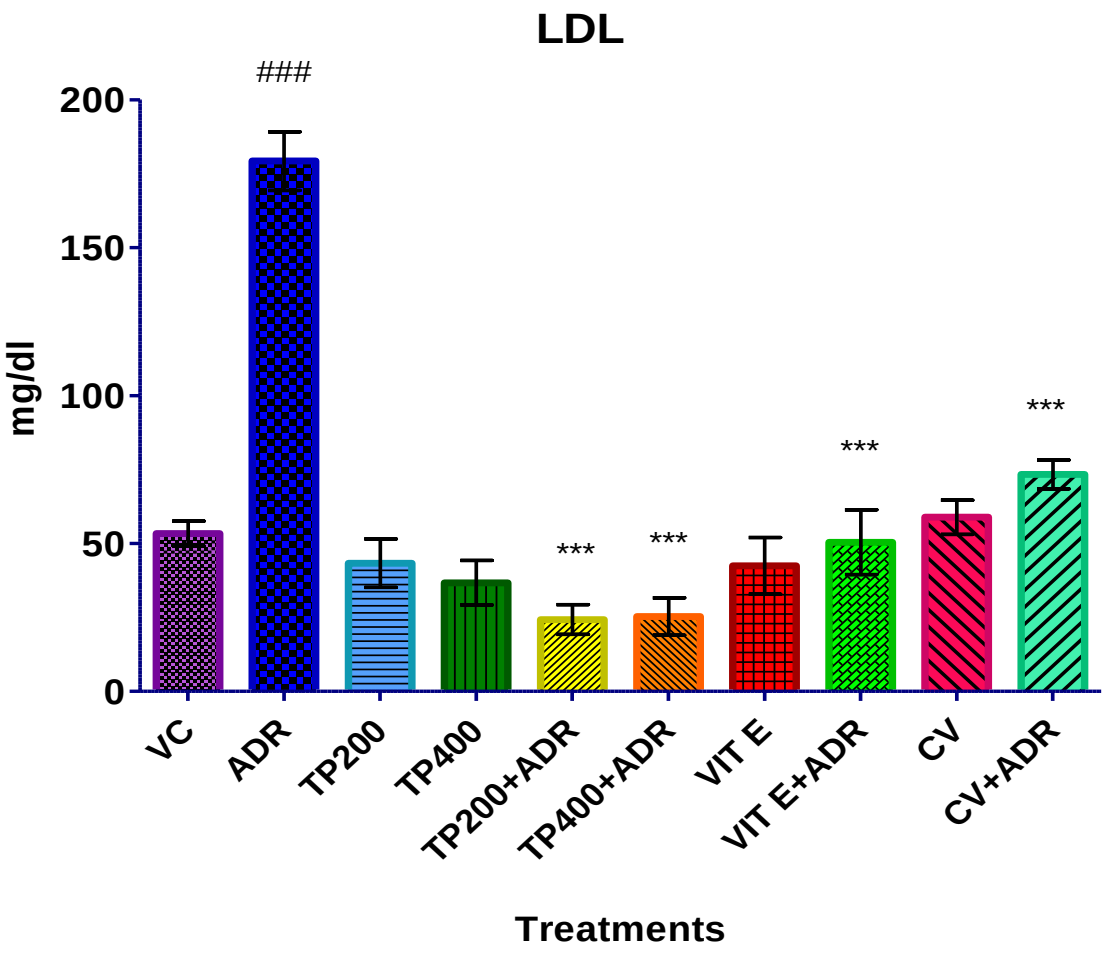

Fig. 36: Histograms representing the changes in serum low density lipoprotein (LDL) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

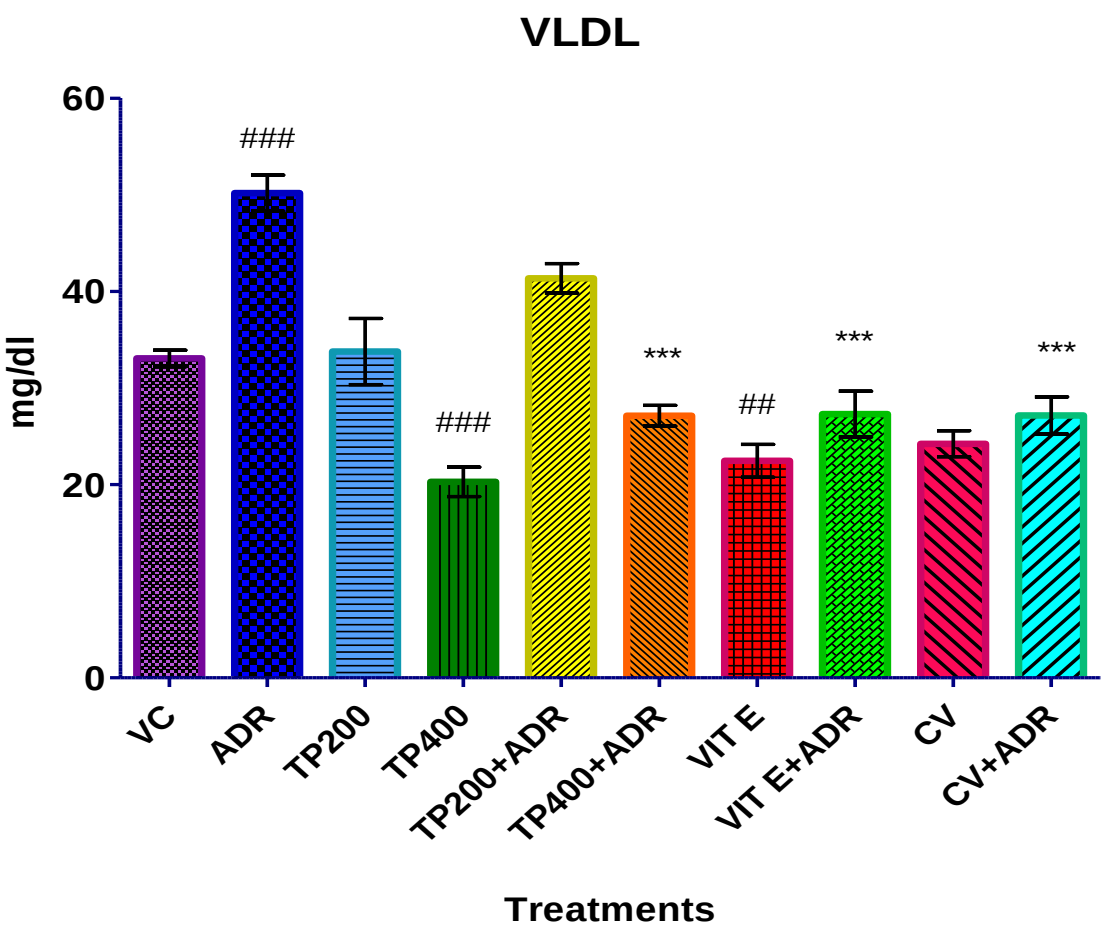

Fig. 37: Histograms representing the changes in serum very low density lipoprotein (VLDL) levels after a treatment period of 28 days in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001.**

*: Value differs significantly from adriamycin group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **ADR** - rats administered with adriamycin (15mg/kg cumulative dose, i.p.); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + ADR** - rats treated with TP200 + adriamycin; **TP400 + ADR** - rats treated with TP400 + adriamycin; **VIT E** - rats receiving vitamin E (25mg/kg); **CV** - rats receiving carvedilol (1mg/kg); **VIT E + ADR** - rats treated with vit E + adriamycin; **CV+ ADR** - rats treated with carvedilol + adriamycin.

4.13 Effect of *Thespesia populnea* on histopathological changes in adriamycin-induced cardiotoxicity:

Histopathology of cardiac tissue in sections showed a normal architecture in the vehicle control group (Fig. 38a). Sections of cardiac tissue from adriamycin-treatment showed dilation of cardiac muscle fibers with breaks in the muscle strands accompanied by loss of cardiac muscle fibers (Fig.38b). Cardiac sections of treatment groups receiving *Thespesia* (200mg/kg and 400 mg/kg respectively), vitamin E or carvedilol individually (Figs. 38c, e, g, i) showed no notable abnormal histopathological changes compared to adriamycin. TP200 + adriamycin (Fig. 38d) showed lesser damage to the structural integrity of the cardiac muscle fibers with mild areas of congestion and vacuolization, whereas TP400 + adriamycin (Fig. 38f) exhibited an improvement in the structural integrity in section with lesser vacuolization and no damage to the muscle fibers.

Vitamin E alone did not alter the appearance of the cardiac tissue and displayed normal, intact myofibrils. Vitamin E + Adriamycin (Fig. 38h) showed decreased myofibrillar vacuolization with mild areas of congestion. The overall architecture of the cardiac musculature was observed to be preserved by vitamin E-treatment.

Individual treatment with carvedilol showed no significant alterations in the cardiac tissue as indicated by the absence of edema and presence of intact myofibrillar structure. However, a few areas of congestion were observed. Treatment with carvedilol plus adriamycin (Fig. 38j) displayed partial preservation of myofibrils, marked by the presence of breaks in the muscle fibers, myofibrillar vacuolization and a disruption of normal cardiac architecture.

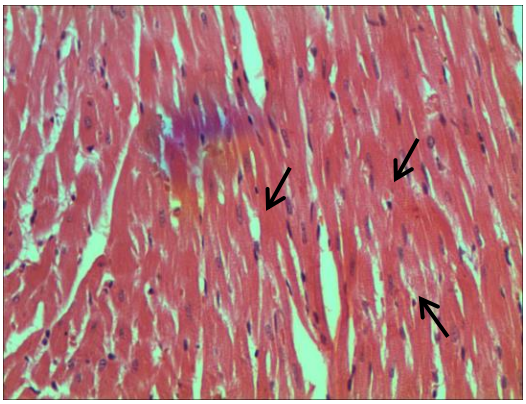

Fig. 38a. Vehicle Control

-Normal cardiac myofibrils.
- Intact tissue architecture.

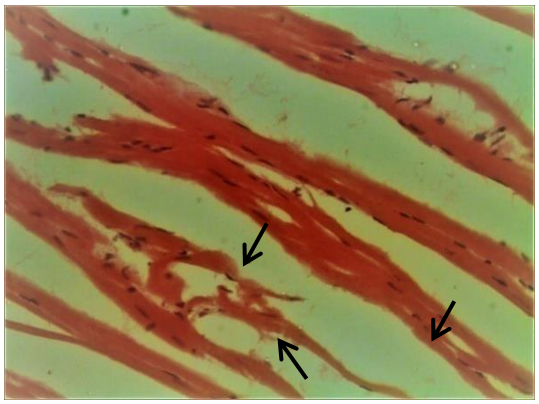

Fig. 38b. Adriamycin (ADR)

-Extensive myofibril loss and separation of muscle fibres indicative of dilated cardiomyopathy.
-Damage to tissue architecture with breaks in the muscle fibres and large spaces in-between.

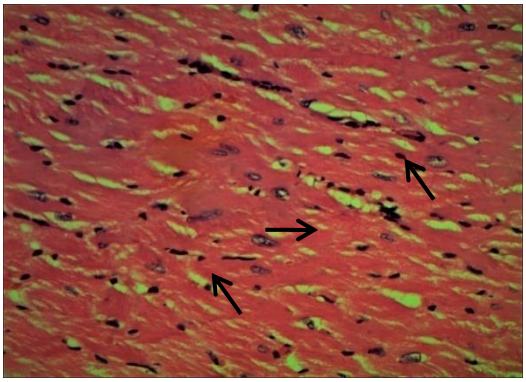

Fig. 38c. TP200

-Normal cardiac architecture.
-No disruption of muscle fibres.

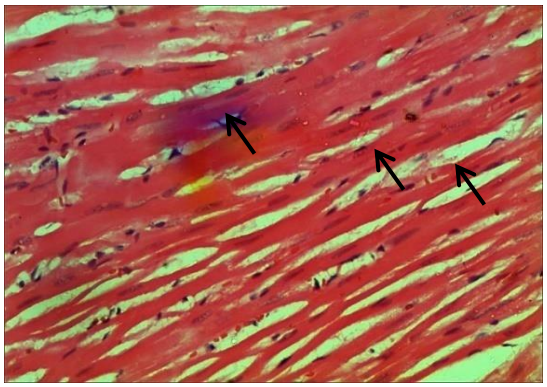

Fig. 38.TP200 + ADR

-Myofibrillar vacuolization.
-Mild areas of congestion and edema.

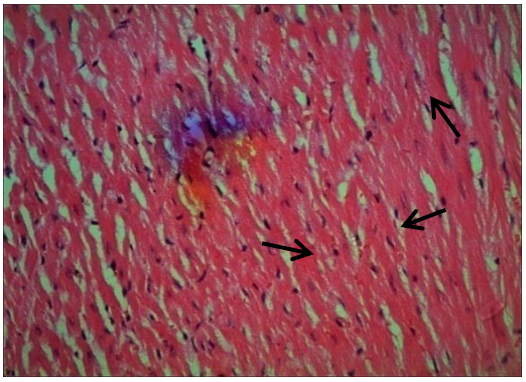

Fig. 38e.TP400

Preserved myocardial architecture.

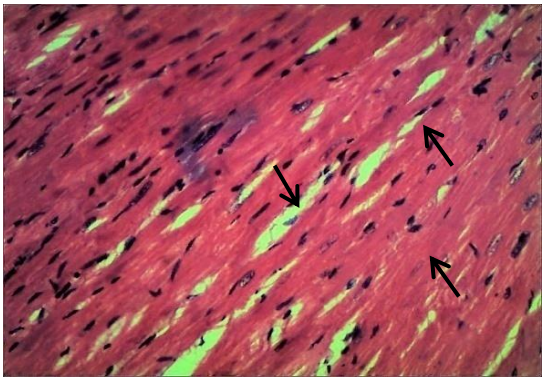

Fig. 38f.TP400+ ADR

- Minimal Myofibrillar vacuolization.
- Absence of necrosis/ Mild areas of congestion and edema.

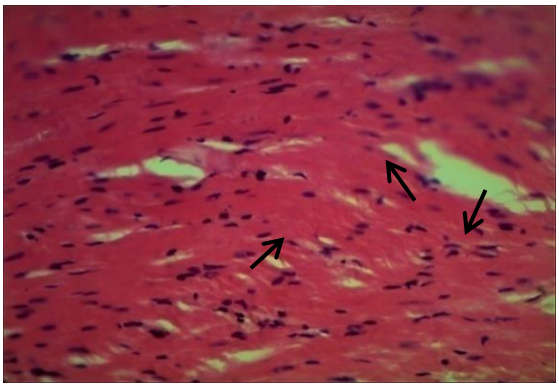

Fig. 38g. vitamin E

-Normal architecture of cardiac Myofibrils.
-Absence of congestion and edema

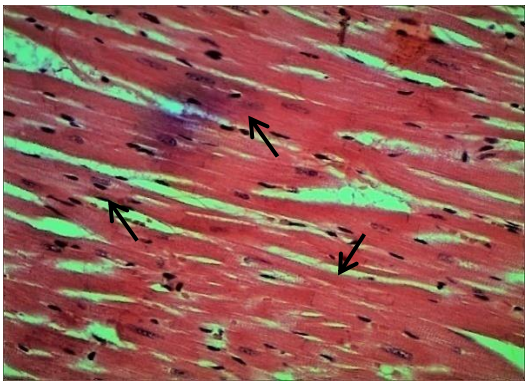

Fig. 38h. vitamin E + ADR

-Mild Myofibrillar vacuolization.
-Decreased damage to muscle. fibres with few areas of mild congestion.

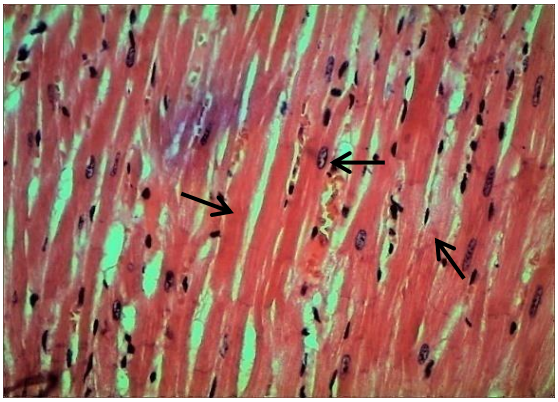

Fig. 38i Carvedilol

-Mild appearance of cytoplasmic organelles and congestion.
-absence of fibrillar changes.

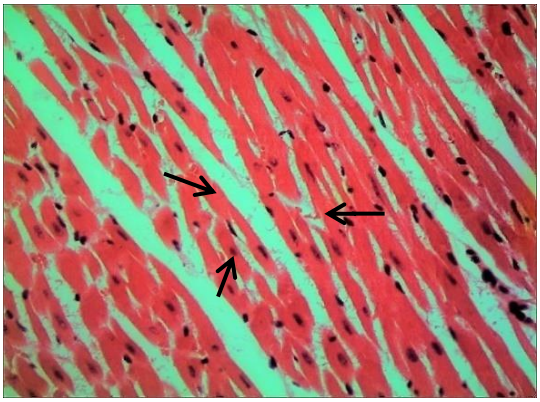

Fig. 38j Carvedilol+ ADR

- frequent damage to myofibril with breaks in the muscle strand length.
- Partial preservation of myofibrils.

Fig.38a-38j: Electrocardiographic recordings representing the changes in QT interval, QRS complex and RR interval durations after a treatment period of 28 days in control rats and those receiving different treatments.

4.14 PROTOCOL-II: EVALUATION OF *THESPESIA POPULNEA* LEAF EXTRACT IN ETHANOL-INDUCED CARDIOTOXICITY

4.14.1 Effect of TP leaf extract on physical parameters:

General changes observed in the rats receiving ethanol were development of gradual roughness of body fur with no discoloration of the fur. The animals were observed to show increased wetting of the cages in comparison to other groups. The animals displayed prolonged periods of inactivity compared to other treatments and the feces also had diarrheal features similar to that seen with adriamycin treatment. These changes were strikingly significant with ethanol treatment alone as compared to all the other treatment groups.

4.14.2 Effect of TP leaf extract on food-intake and body weight:

In variation of the adriamycin dosing schedule, in ethanol-induced cardiotoxicity the rats received ethanol (20%, 2g/kg, p.o.) daily for 6 weeks.

Changes in food-intake and body weight were recorded every week from the first week to the 6^{th} week in all the treatments. Ethanol-treated animals exhibited a significant decrease in food-intake from the 4^{th} week followed by a consistent decline till the end of the 6^{th} week. Single drug-treatment groups did not exhibit significant changes in food-intake in the case of TP200 and TP400. However, vitamin E-treated rats showed marginal increases during 3^{rd} to sixth weeks and carvedilol-treated rats showed significant increases in food-intake right from the 2^{nd} week up to the 6^{th} week in comparison to the respective vehicle control groups. When TP200 was administered prior to the administration of ethanol, slight increases in food-intake were observed from the 3^{rd} week onwards. When TP400, vitamin E or carvedilol was administered prior to ethanol, a significant increase in food-intake was observed during the 5^{th} and 6^{th} weeks of the study (Table 15; Figs. 39&40).

Body weights significantly increased in the ethanol-treated group from the first week till the 3rd week, followed by a significant decline during the 4^{th}, 5th and 6^{th} weeks when compared to the vehicle control group. Individual administration of TP200 extract caused slight and non-significant changes in the body weights compared to the respective vehicle controls during all weeks of the treatment. Significant increases in body weights were observed in comparison to vehicle control group with individual TP400 or vitamin E

treatment from the 4th week onwards, Individual carvedilol treatment increased the body weights from the 3rd to the 6th week. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol induced a reversal of the body weights from the disease control towards the vehicle control, with carvedilol taking the weights to above the vehicle controls during all weeks of the treatment period (Table 16; Figs.41& 42).

Table 15. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on food-intake during ethanol-induced cardiotoxicity in rats.

Experimental Group	CHANGES IN FOOD-INTAKE (g)					
	1st week	2nd week	3rd week	4th week	5th week	6th week
Vehicle control (5% Gum acacia)	17.83± 1.32	24.33± 0.99	23.17± 1.27	24.17± 1.16	26.67± 1.33	29.83± 1.27
Ethanol (20%)	18.17± 0.74 NS	24.67± 1.3 NS	23.00± 1.09 NS	19.00± 0.85 #	15.33± 0.98 ###	11.67± 0.61 ###
Thespesia leaf extract (200 mg/kg)	16.33± 0.76 NS	19.17± 0.79 #	21.33± 2.81NS	26.17± 1.70 NS	25.33± 0.98 NS	26.83± 0.91 NS
Thespesia leaf extract (400 mg/kg)	19.50± 1.94 NS	23.83± 0.79 NS	24.33± 1.56 NS	26.5± 1.31 NS	24.67± 0.91 NS	27.00± 1.39 NS
Thespesia leaf extract (200 mg/kg) + Ethanol (20%)	18.16± 0.70 NS	21.33± 0.49 NS	23.16± 1.01 NS	22.67± 1.85 NS	18.33± 0.76 NS	16.67± 1.54 *
Thespesia leaf extract (400 mg/kg) + Ethanol (20%)	16.50± 1.38 NS	22.67± 1.17 NS	24.17± 1.64 NS	23.17± 0.70 NS	24.00± 0.93 ***	25.17± 1.4 ***
Standard (Vitamin E, 25 mg/kg, p.o.)	20.33± 0.76 NS	21.67± 0.98 NS	24.50± 1.28 NS	25.17± 1.95 NS	23.67± 1.02 NS	25.83± 1.42 NS
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	16.00± 0.70 NS	20.83± 1.01 NS	23.50± 0.42 NS	20.67± 1.25 NS	23.83± 1.16***	21.17± 1.72 ***
(Carvedilol, 1mg/kg, p.o.)	21.83± 1.04 NS	27.17± 0.94 NS	35.00± 1.71 ###	32.67± 1.31 ###	34.17± 1.72###	39.00± 0.85 ###
Carvedilol (1mg/kg, p.o.) + Ethanol 20%)	15.83± 1.27 NS	20.67± 0.66 NS	22.33± 1.56 NS	18.33± 1.47 NS	21.33± 0.88**	22.83± 0.60 ***

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.
NS – Not significant.
#: Value differs significantly from vehicle control group **#P<0.05, ##P<0.01, ###P<0.001**

*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

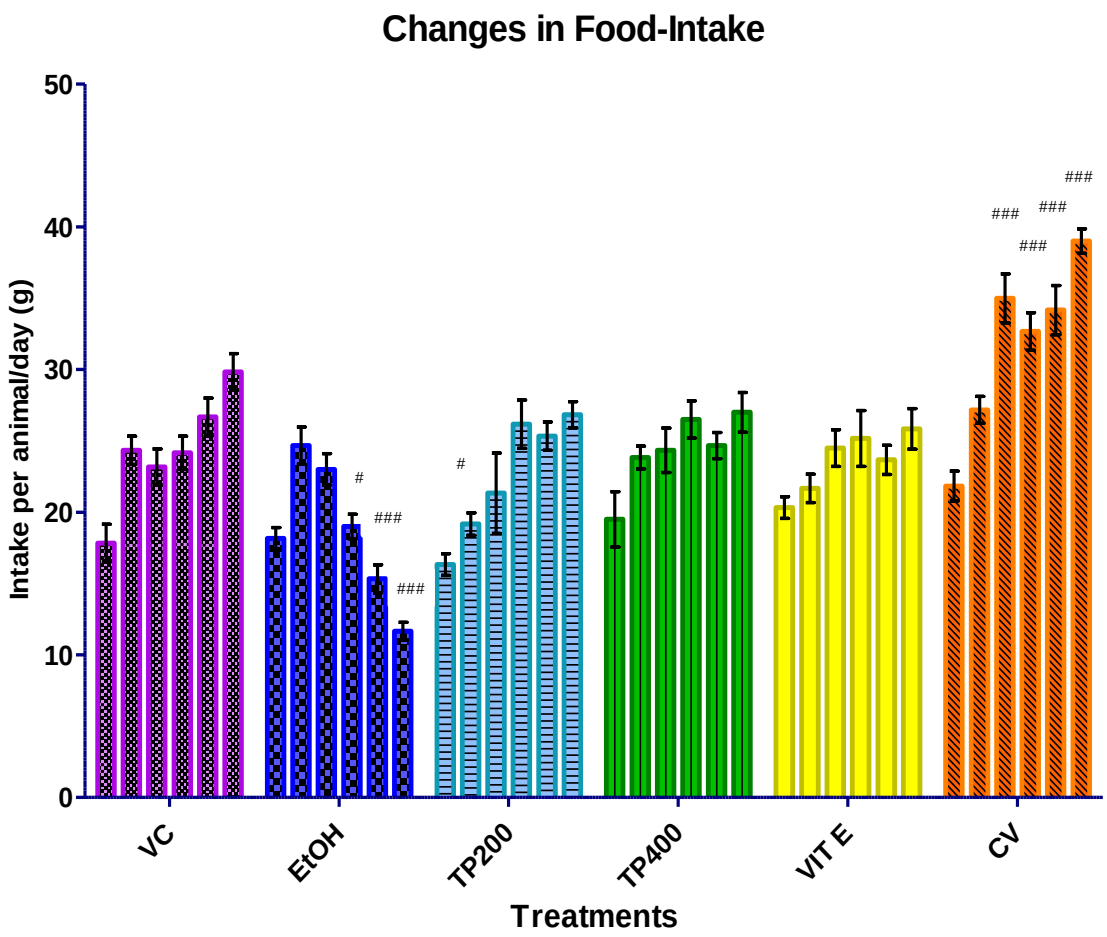

Fig. 39: Histograms representing the weekly changes in food-intake over a period of 6 weeks in vehicle control rats and those receiving different individual treatments.

Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

$^{\#}$: Value differs significantly from vehicle control group $^{\#}$**P< 0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200** - rats receiving *T. populnea* leaf extract 200mg/kg (p.o); **TP400** - rats receiving *T. populnea* leaf extract 400mg/kg (p.o); **vit E** - rats receiving vitamin E (25mg/kg, p.o); **CV**- rats receiving carvedilol (1mg/kg, p.o).

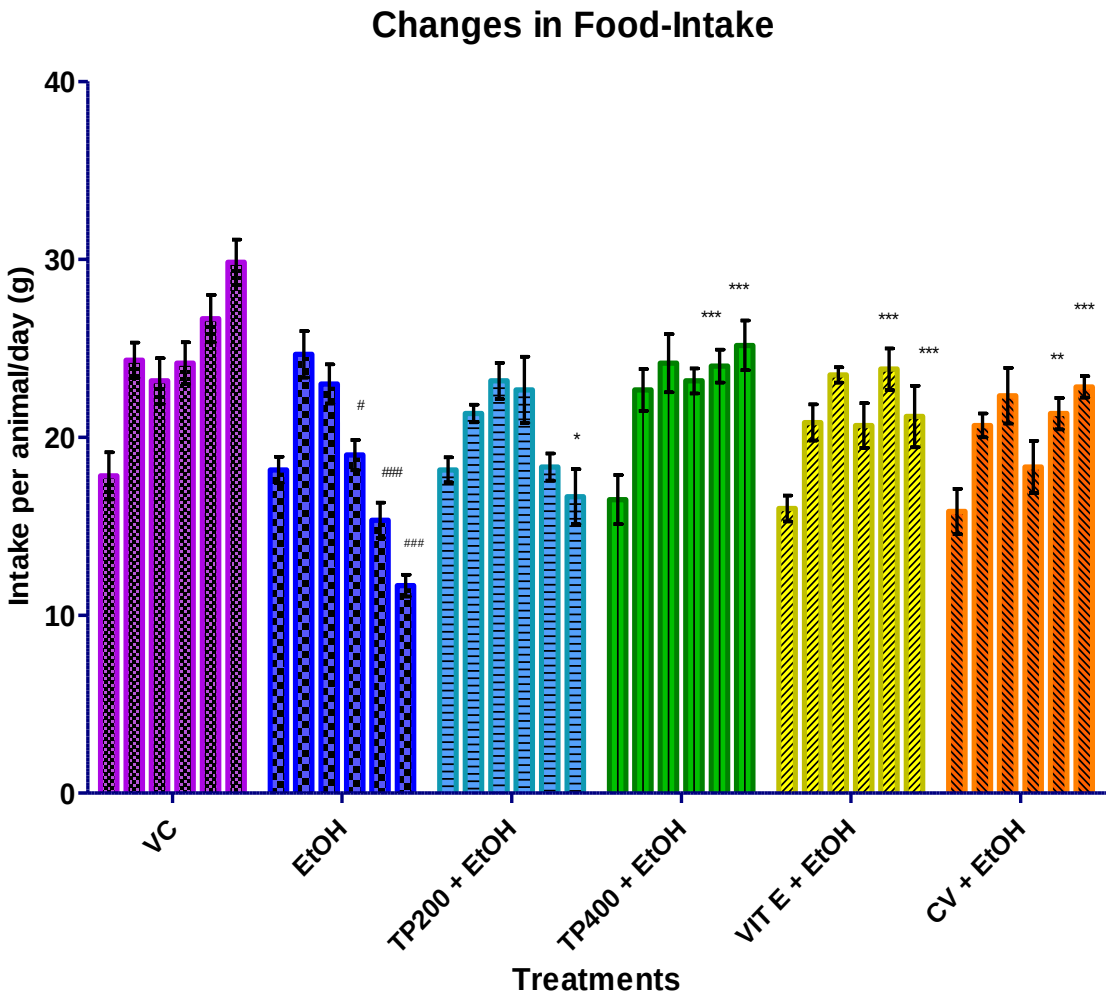

Fig. 40: Histograms representing the weekly changes in food-intake over a period of 6 weeks in vehicle control rats and those receiving different treatments.

Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

$^{\#}$: Value differs significantly from vehicle control group $^{\#}$**P< 0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**

*****: Value differs significantly from ethanol (disease control) group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + ethanol** - rats treated with vit E + ethanol; **CV+ ethanol -** rats treated with carvedilol + ethanol.

Table 16. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in body weight in adriamycin-induced cardiotoxicity in rats.

Experimental Group	CHANGES IN BODY WEIGHT (g)					
	1st week	**2nd week**	**3rd week**	**4th week**	**5th week**	**6th week**
Vehicle control (5% Gum acacia)	199.17± 5.06	216.67± 3.57	220.00± 2.23	225.83± 2.71	229.17± 2.01	242.50± 2.14
Ethanol (20%)	218.33± 5.86$^{\#\#\#}$	235.83± 6.24$^{\#\#\#}$	250.00± 3.16$^{\#\#\#}$	241.66± 3.33$^{\#\#}$	214.16± 2.38 ##	185.83± 4.16$^{\#\#\#}$
Thespesia leaf extract (200 mg/kg)	209.16± 2.38 NS	217.50± 2.81 NS	224.17± 2.01 NS	232.50± 1.71 NS	230.00± 1.82 NS	241.67± 2.78 NS
Thespesia leaf extract (400 mg/kg)	211.67± 3.07$^{\#}$	220.00± 1.82 NS	229.17± 2.01 NS	245.00± 2.23$^{\#\#\#}$	240.83± 2.00 #	255.83± 2.01$^{\#\#}$
Thespesia leaf extract (200 mg/kg) + Ethanol (20%)	202.50± 2.81****	216.67± 3.07*****	223.33± 2.47*****	234.17± 2.38 NS	224.1± 1.53 NS	217.5± 2.14*****
Thespesia leaf extract (400 mg/kg) + Ethanol (20%)	205.00± 2.88 *	213.33± 3.57***	222.50± 1.71***	235.00± 2.23 NS	220.83± 2.01 NS	215.83± 1.53***
Standard (Vitamin E, 25 mg/kg, p.o.)	198.33± 4.01 NS	220.00 ±1.82 NS	228.33± 3.07 NS	234.17 ±1.53 NS	243.33± 4.59$^{\#\#}$	265.83± 2.01$^{\#\#\#}$
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	207.50± 2.14 NS	214.17± 3.00***	225.83± 3.00***	245.00± 2.88NS	234.17± 2.38*	221.67± 2.10*****
(Carvedilol, 1mg/kg, p.o.)	201.67± 2.10 NS	222.50± 1.11 NS	237.50± 1.11$^{\#\#\#}$	262.5± 1.71$^{\#\#\#}$	275.83± 1.53$^{\#\#\#}$	289.17± 2.01$^{\#\#\#}$
Carvedilol (1mg/kg, p.o.) + Ethanol (20%)	213.33± 1.66 NS	230.83± 1.53 NS	244.17± 1.53 NS	255.83± 2.01****	266.67± 3.57*****	253.33± 2.47*****

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.
NS – Not significant.
#: Value differs significantly from vehicle control group $^{\#}$**P<0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**

*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

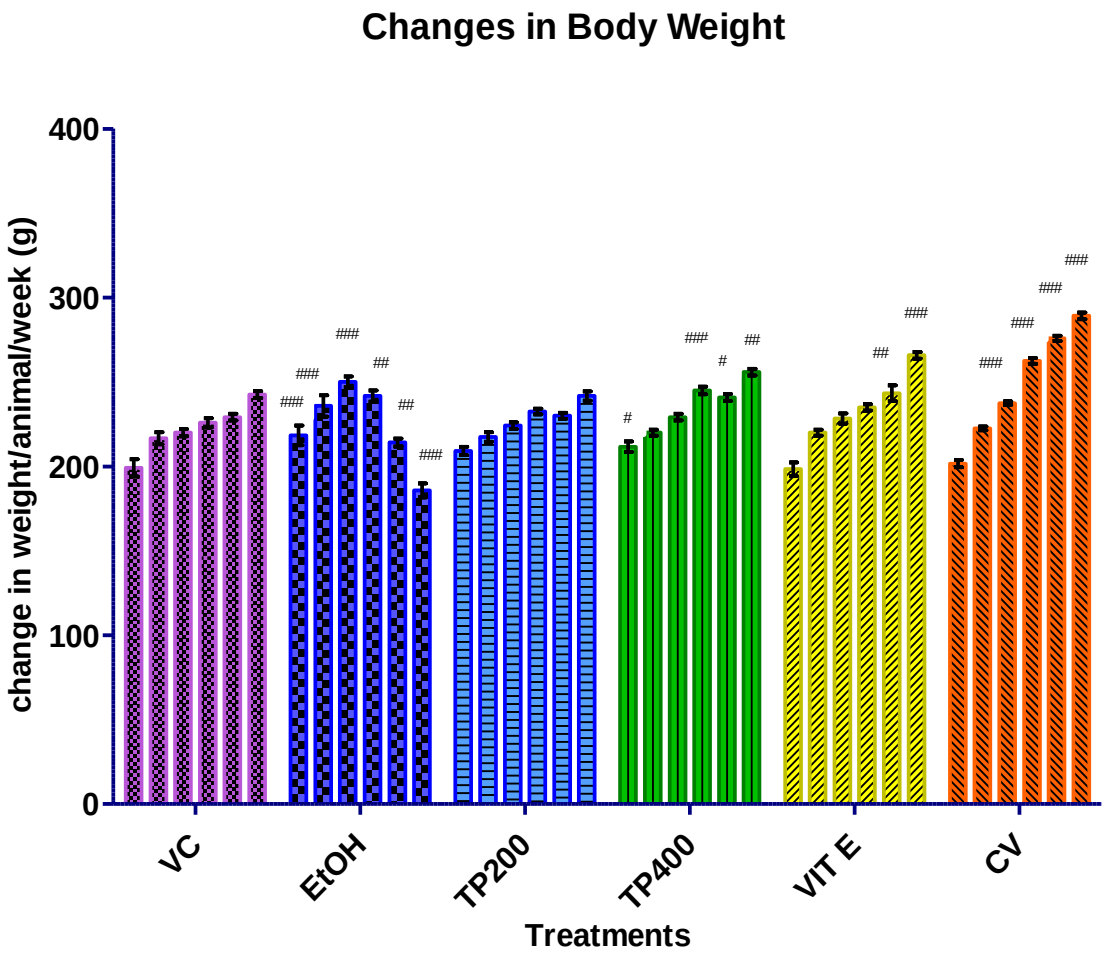

Fig. 41: Histograms representing the weekly changes in body weight over a period of 6 weeks in vehicle control rats and those receiving different individual treatments.

Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

$^{\#}$: Value differs significantly from vehicle control group $^{\#}$**P< 0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200** - rats receiving *T. populnea* leaf extract 200mg/kg (p.o); **TP400** - rats receiving *T. populnea* leaf extract 400mg/kg (p.o); **vit E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o).

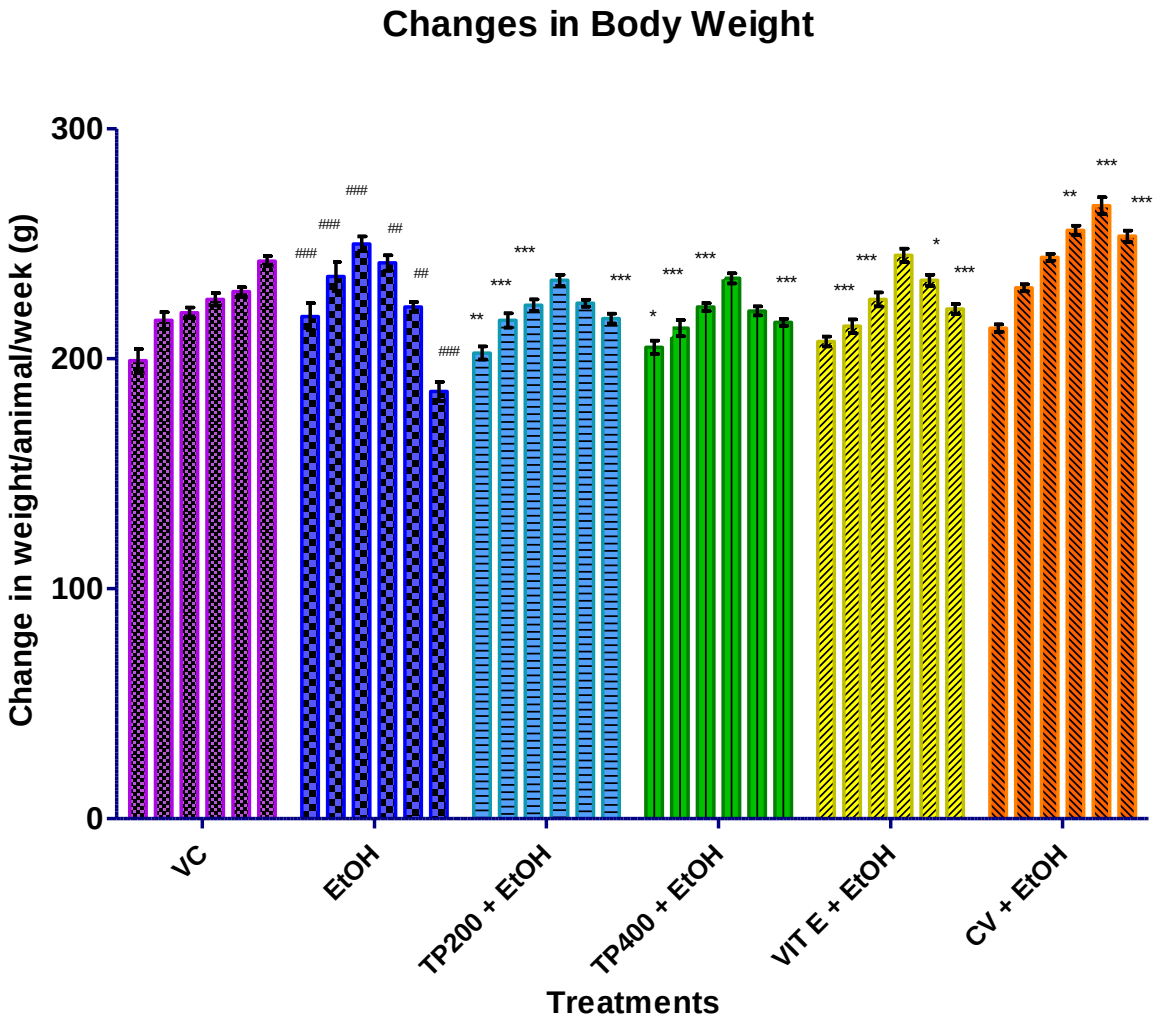

Fig. 42: Histograms representing the weekly changes in body weight over a period of 6 weeks in vehicle control rats and those receiving different treatments.

Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

$^{\#}$: Value differs significantly from vehicle control group $^{\#}$**P< 0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**

*****: Value differs significantly from ethanol (disease control) group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

4.15. Effect of *T. populnea* leaf extract on selected cardiac parameters and *in vitro* cardiac function in ethanol-induced cardiotoxicity:

4.15.1 Effect of *T. populnea* leaf extract on heart weight:

The heart weight significantly increased in the ethanol-treated rats compared to the vehicle control group. No significant changes in heart weight were observed in the groups treated individually with TP leaf extract (200mg/kg and 400 mg/kg) or vitamin E (25 mg/kg) in comparison to vehicle control, while those receiving carvedilol (1 mg/kg) showed a slight increase in heart weight over the vehicle control. When TP extracts, vitamin E or carvedilol were administered prior to ethanol, the rats showed an increase in body weight in comparison to the disease control. While with TP 400 and vitamin E the increase was more effective and the body weights were comparable to vehicle control level, TP200 and carvedilol effected only partial increments by taking the body weights to midway between the disease control and vehicle control (Table 16; Fig. 43).

4.15.2 Effect of *T. populnea* treatment on thickness of left ventricle wall:

Thickness of the left ventricle wall significantly decreased in ethanol-treated rats compared to the vehicle control group. No significant change was observed in the thickness of left ventricle wall in individual treatments with TP200, TP400 or vitamin E in comparison to the vehicle control. In contrast, Individual administration of carvedilol induced significant increase in the ventricle wall thickness compared to the vehicle control. Administration of TP200, TP400 or vitamin E prior to the administration of ethanol induced an increase in the left ventricle wall thickness from the disease control level towards the vehicle control. With carvedilol this increase was very striking, and the ventricle thickness was increased significantly to above the vehicle control level (Table 16; Fig. 44).

4.15.3 Effect of *T. populnea* extract on cardiac ejection fraction:

The cardiac ejection volume was significantly lowered from the vehicle control when ethanol was administered individually. Individual administration of TP200, TP400, vitamin E or carvedilol caused no change in the cardiac ejection fraction from the vehicle control level. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol caused a significant increase in the cardiac ejection volume compared to the disease control level to near vehicle control level (Table 16; Fig. 45).

Table 17. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in heart weight, thickness of the left ventricle wall and cardiac ejection volume during ethanol-induced cardiotoxicity in rats after a treatment period of 6 weeks.

Experimental Group	**Heart weight (g)**	**Thickness of left ventricle (mm)**	**Cardiac ejection volume/(effluent collected (ml)**	**% Ejection Fraction**
Vehicle Control (5% Gum acacia)	1.06 ± 0.03	2.53 ± 0.13	14.00 ± 0.03	100
Disease Control (Ethanol 20%)	1.42 ± 0.01$^{\#\#\#}$	1.14 ± 0.03$^{\#\#\#}$	4.50 ± 0.58$^{\#\#\#}$	32.1
Thespesia Leaf Extract (200 mg/kg)	1.08 ± 0.06 NS	2.32 ± 0.13 NS	13.80 ± 0.05 NS	98.6
Thespesia Leaf Extract (400 mg/kg)	1.07 ± 0.02NS	2. 33 ± 0.15 NS	13.90 ± 0.04 NS	99.3
Thespesia Leaf Extract (200 mg/kg) + Ethanol (20%)	1.32 ± 0.03 NS	1.83 ± 0.05 NS	11.40 ± 0.24NS	81.4
Thespesia Leaf Extract (400 mg/kg) + Ethanol (20%)	1.13 ± 0.016***	2.50 ± 0.20***	13.30 ± 0.13***	95.0
Standard (Vitamin E, 25 mg/kg, p.o.)	1.05 ± 0.02 NS	2.63 ± 0.15 NS	13.80 ± 0.09 NS	98.6
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	1.18 ± 0.03***	2.58 ± 0.16***	13.40 ± 0.07***	95.7
(Carvedilol, 1mg/kg, p.o.)	1.16 ± 0.02 NS	3.80 ± 0.23$^{\#\#\#}$	12.90 ± 0.08 NS	92.1
Carvedilol (1mg/kg, p.o.) + Ethanol (20%)	1.25 ± 0.02*	4.46 ± 0.13***	12.40 ± 0.10***	88.6

Note: The values are expressed are mean ± SEM from 6 rats, followed by significance

NS – Not significant.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**

***:** Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

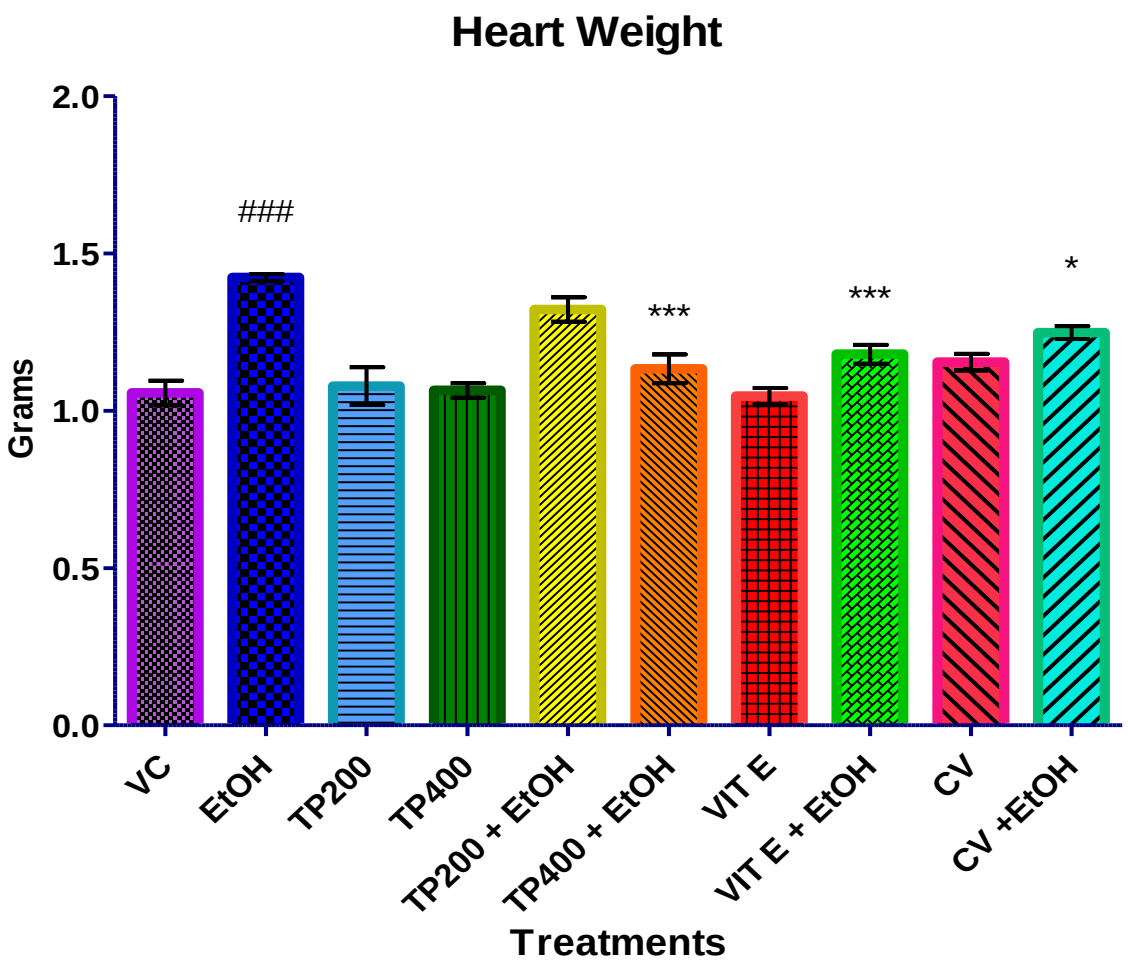

Fig. 43: Histograms representing the changes in heart weight in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol (disease control) group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

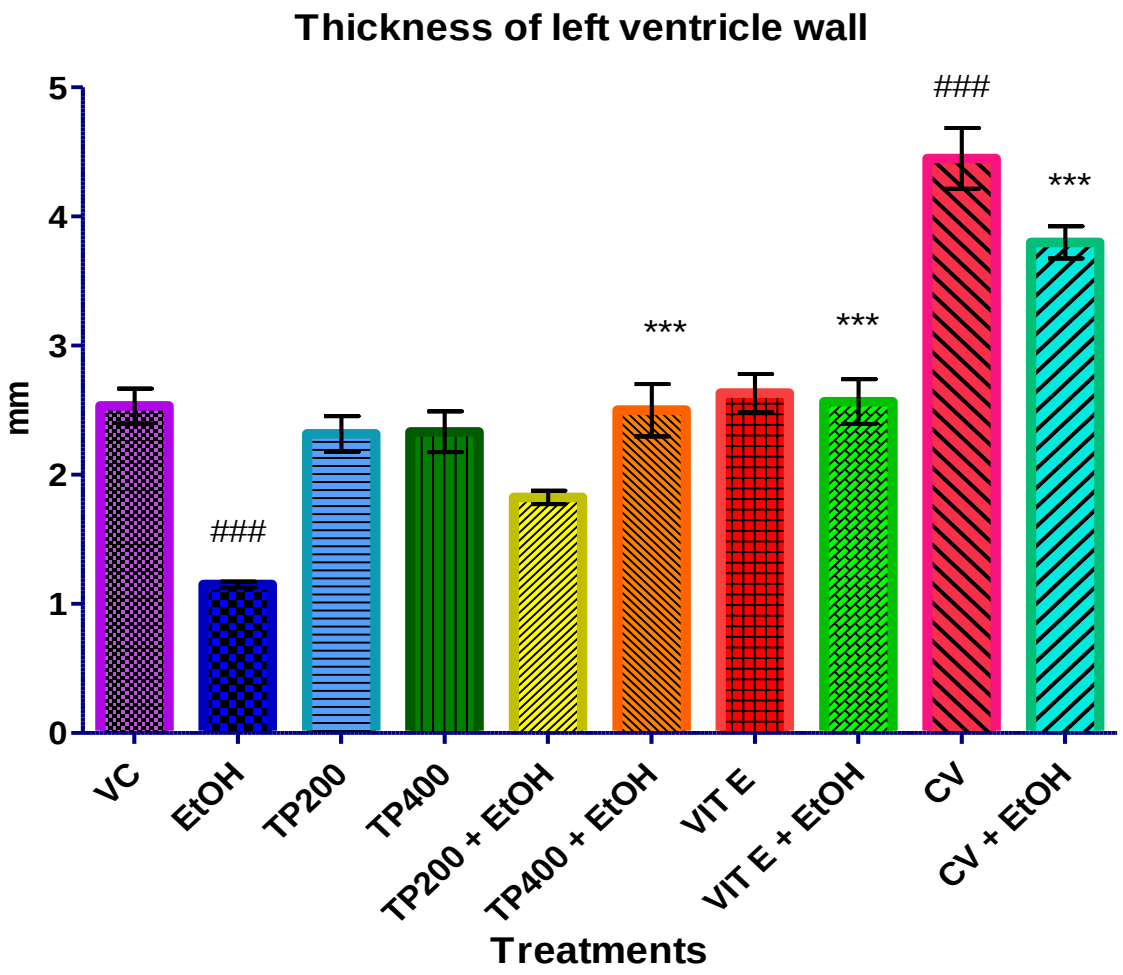

Fig. 44: Histograms representing the changes in thickness of left ventricle wall in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol (disease control) group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

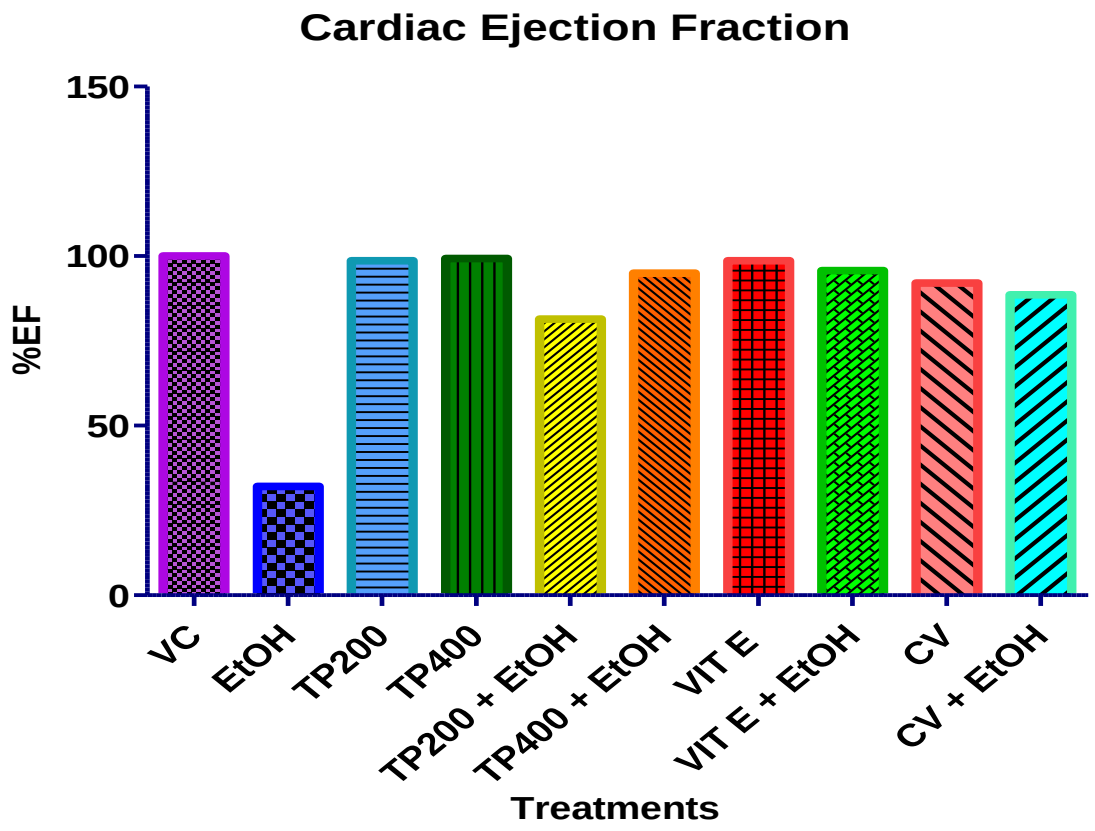

Fig. 45: Histograms representing the changes in cardiac ejection fraction in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

***: Value differs significantly from ethanol (disease control) group *P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

Note: The contents in the figure are shown in terms of percent ejection fraction (%EF). Since the representation is in the form of '%' ejection fraction, points of significance are not mentioned in the graph. The same data is mentioned in the form of ejection volumes in table nos. 9 and 17, in which the points of significance are shown.

4.16 Effect of *T. populnea* leaf extract on changes in ECG in ethanol-induced cardiotoxicity in rats.

The effects of ethanol, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on alterations in electrocardiogram (ECG) after the treatment period of 6 weeks are illustrated in Table 18 and Figures 46a–46j. The parameters examined were QT-interval, QRS-complex duration and R-R interval.

QT-interval:

Administration of ethanol caused a significant ($P<0.001$) prolongation (increase) of the QT-interval in the disease control compared to the vehicle control. Individual administration of TP200 or TP400 did not cause any change in the QT-interval from the vehicle control, while individual administration of vitamin E or carvedilol prolonged the QT-interval moderately compared to the vehicle control, which was statistically significant. Normal PQRST wave pattern was recorded with individual administration of TP200, TP400, vitamin E and carvedilol. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol caused a decrease in the prolongation of the QT-interval from the disease control towards the vehicle control. The reductions were partial and not complete, although statistically significant ($P<0.001$) (Table 18; Fig. 46a-46j). This was truer with vitamin E and carvedilol. Pre-treatment with vitamin E and carvedilol did not correct the abnormality in the ECG caused due to ethanol as indicated by the persistent prolongation of QT interval.

QRS-complex duration:

Administration of ethanol caused a significant (over 2-fold) increase of the QRS-complex duration in the disease control compared to the vehicle control. It also caused ST segment elevation compared to the vehicle control. Individual administration of TP200 or TP400 did not cause any change in the QRS-complex duration from the vehicle control, while individual administration of vitamin E or carvedilol increased the duration slightly compared to the vehicle control. Also, increased amplitude of S wave was observed with carvedilol treatment. Administration of TP200 and TP400 prior to the administration of ethanol reduced the prolongation of the QRS-complex duration as compared to the disease control. Administration of vitamin E prior to the administration of ethanol also caused a reduction in increase of the QRS-complex duration caused by ethanol but the reversal were

partial and not complete, although statistically significant ((P<0.001) with reference to the disease control (Table 18; Fig. 46a-46j). Administration of carvedilol prior to the administration of ethanol did not cause any significant change in the QRS-complex duration.

R-R Interval:

Administration of ethanol caused a prolongation (P<0.001) of the R-R interval in the disease control compared to the vehicle control. Individual administration of TP200, TP400, vitamin E or carvedilol caused slight non-significant increases or decreases in the R-R interval from the vehicle control. Administration of TP200, TP400 or vitamin E prior to the administration of ethanol reduced the prolongation of the R-R interval compared to the disease control. The reductions were not statistically significant (Table 18; Fig. 46a-46j).

Overall, the abnormal changes brought about by ethanol treatment were significantly ameliorated to a greater extent by pretreatment with *T. populnea* extract in the dose of 400 mg, vitamin E and carvedilol respectively. Pre-treatment with TP200 did not correct the abnormality in the ECG caused by ethanol administration, which could be observed from the persistent prolongation of QT interval, RR interval and ST segment elevation in the group. QRS duration with TP200 pretreatment was significantly reduced though, as compared to ethanol treatment alone.

Table 18. Effect of *T. populnea* leaf extract on changes in ECG of rat heart during ethanol-induced cardiotoxicity after a treatment period of 6 weeks.

Experimental Group	QT Interval (s)	QRS Complex duration (s)	R-R Interval (s)
Vehicle Control (5% Gum acacia)	0.26 ± 0.05	0.15± 0.04	0.90 ± 0.07
Disease Control (Ethanol 20%)	0.52 ± 0.06$^{\#\#\#}$	0.33 ± 0.05$^{\#\#\#}$	1.08 ± 0. 06$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg)	0.29± 0.05 NS	0.18± 0.05^{NS}	0.93 ± 0.06^{NS}
Thespesia Leaf Extract (400 mg/kg)	0.27 ± 0.06 ^{NS}	0.16 ± 0.06 ^{NS}	0.85 ± 0. 07 ^{NS}
Thespesia Leaf Extract (200 mg/kg) + Ethanol (20%)	0.33 ± 0.05***	0.16 ± 0.08***	1.03 ± 0. 06 ^{NS}
Thespesia Leaf Extract (400 mg/kg) + Ethanol (20%)	0.29 ± 0.05***	0.17 ± 0.07***	0.71 ± 0. 06***
Standard (Vitamin E, 25 mg/kg, p.o.)	0.34 ± 0.05$^{\#\#\#}$	0.21 ± 0.06***	0.92 ± 0. 09 ^{NS}
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	0.40 ± 0.05***	0.26 ± 0.05***	0.98 ± 0.06***
(Carvedilol, 1mg/kg, p.o.)	0.38± 0.09***	0.25 ± 0.05***	0.99 ± 0.07NS
Carvedilol (1mg/kg, p.o.) + Ethanol (20%)	0.43 ± 0.05***	0.31 ± 0.05NS	0.94 ± 0. 07**

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.

NS – Not significant.

#: Value differs significantly from vehicle control group $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$

*: Value differs significantly from disease control group *P< 0.05, **P<0.01, ***P<0.001

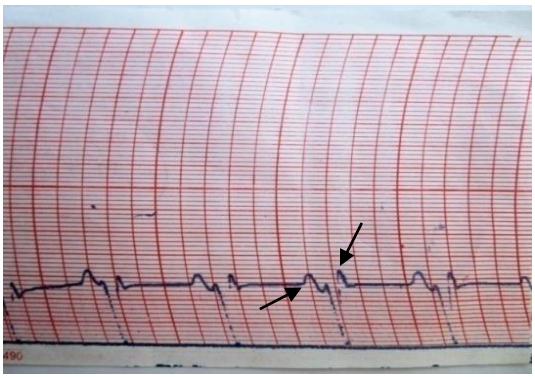

Fig. 46a. ECG of vehicle control
-Normal wave pattern displaying PQRST waves

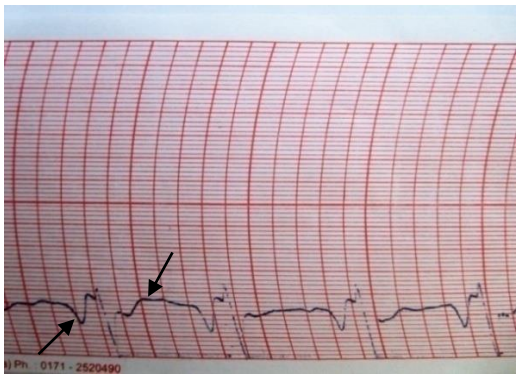

Fig. 46b. ECG of Ethanol treatment
-Abnormal QRS complex, typified by a deep Q-wave, ST segment elevation, prolonged QT interval, QRS complex duration and RR interval (abnormal ventricular depolarization and repolarization).

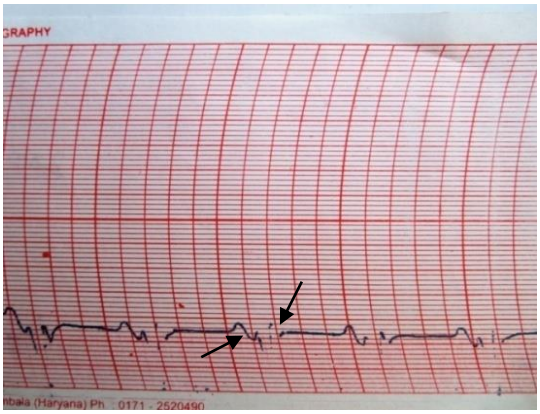

Fig. 46c. ECG of TP200
-Non-significant increase in QRS and RR intervals

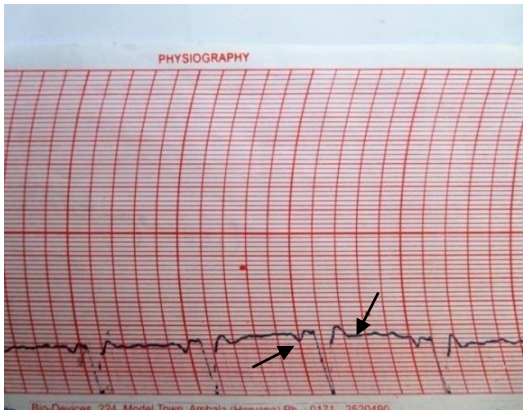

Fig. 46d. ECG of TP200+ EtOH
-Reduction in Q wave depth, ST segment elevation and duration of QT interval and RR interval compared to disease control

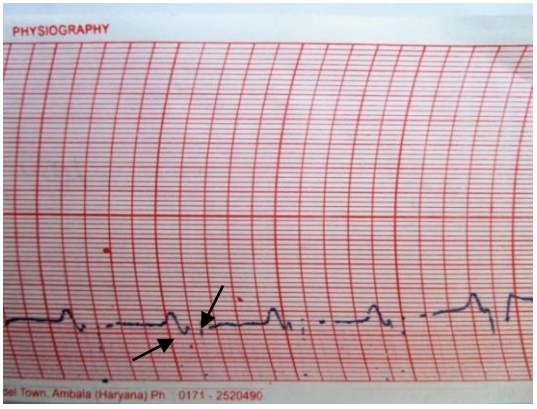

Fig. 46e. ECG of TP400
-Non-significant increase in QRS and RR intervals

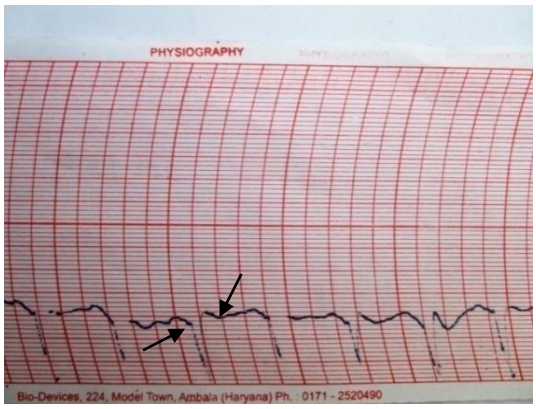

Fig. 46f. ECG of TP400+ EtOH
-Significant reduction in Q wave depth, ST segment elevation and duration of QT, QRS and RR intervals compared to disease control

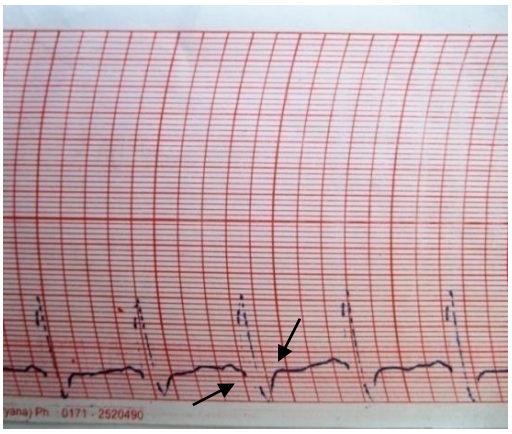

Fig. 46g. ECG of vitamin E
-Significant increase in QT and QRS intervals

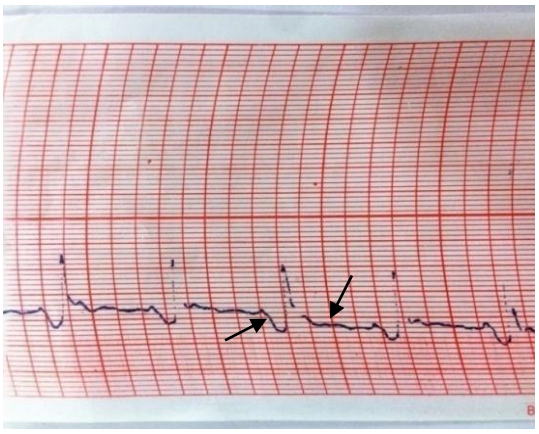

Fig. 46h. ECG of vitamin E + EtOH
-moderate reduction in the duration of QT interval, QRS complex and RR interval compared to disease control.
-Moderate reduction in Q wave depth, absence of ST segment elevation

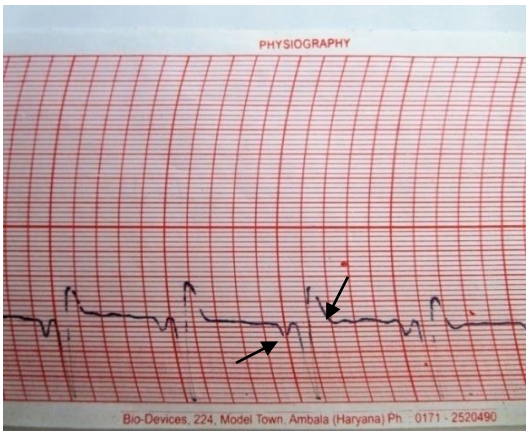

Fig. 46i. ECG of carvedilol
-Significant increase in QT and QRS intervals

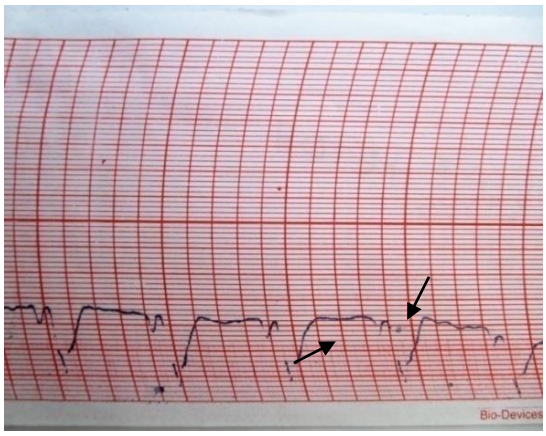

Fig. 46j. ECG of carvedilol+ EtOH
-mild reduction in the duration of QT interval, and RR interval compared to disease control.
-Absence of ST segment elevation

Fig.46a-46j: Electrocardiographic recordings representing the changes in QT interval, QRS complex and RR interval durations after a treatment period of 6 weeks in control rats and those receiving different treatments (Ethanol-induced cardiotoxicity).

4.17 Effect of *T. populnea* leaf extract on alterations in cardiac ATPase activities in ethanol-induced cardiotoxicity:

The effects of ethanol, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on alterations in cardiac ATPase activities after the treatment period of 6 weeks are illustrated in Table 19 and Figures 47 to 49**.** The parameters examined were sodium-potassium ATPase (Na^+/K^+ATPase), calcium ATPase (Ca^{2+}ATPase) and magnesium ATPase (Mg^{2+}ATPase).

4.17.1 Sodium-potassium ATPase (Na^+/K^+ATPase):

Administration of ethanol caused a significant ($P<0.001$) decrease in the Na^+/K^+ATPase activity in the disease control compared to the vehicle control. Individual administration of TP200 did not cause any change in the ATPase activity from the vehicle control, while individual administration of TP400, vitamin E or carvedilol significantly elevated the ATPase activity over the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol caused an increase in the ATPase activity in comparison to the disease control. This increase was observed above the level in the vehicle control (Table 19; Fig. 47).

4.17.2 Calcium ATPase (Ca^{2+}ATPase):

Administration of ethanol caused a two-fold decrease in the Ca^{2+}ATPase activity in the disease control from the activity in vehicle control. Individual administration of TP400 caused a significant ($P<0.001$) increase in the Ca^{2+}ATPase activity of the vehicle control, while individual administration of TP200, vitamin E and carvedilol did not cause any effect on the enzyme activity. Administration of TP200 or TP400 prior to the administration of ethanol effectively elevated the Ca^{2+}ATPase activity in comparison to the disease control level to above the vehicle control level, while prior administration of vitamin E or carvedilol increased ($P<0.001$) the Ca^{2+}ATPase activity compared to the disease control level. This increase in the enzyme activity was near to the vehicle control level (Table 19; Fig. 48).

4.17.3 Magnesium ATPase (Mg^{2+}ATPase):

Administration of ethanol caused a decrease ($P<0.001$) in the Mg^{2+}ATPase activity from the vehicle control to the disease control. Individual administration of TP200, TP400 and vitamin E caused minor elevations in the Mg^{2+}ATPase activity compared to the vehicle control, while individual administration of carvedilol caused a decrease in enzyme activity in comparison to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol caused an elevation in the Mg^{2+}ATPase activity when compared to the disease control. TP200 was the least effective in causing this elevation, bringing about a partial, although significant ($P<0.001$), prevention of decrease in enzyme activity (Table 19; Fig.49).

Table 19. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the levels of sodium-potassium ATPase (Na^+/K^+ATPase), calcium ATPase (Ca^{2+}ATPase) and magnesium ATPase (Mg^{2+}ATPase) in rat heart tissue during adriamycin-induced cardiotoxicity in rats after a treatment period of 6 weeks.

Experimental Group	**Na^+/K^+ ATPase**	**Ca^{2+} ATPase**	**Mg^{2+} ATPase**
Vehicle Control (5% Gum acacia)	1.49 ± 0.07	1.58 ± 0.06	3.52 ± 0.11
Disease Control (Ethanol 20%)	0.79 ± 0.05$^{###}$	0.79 ± 0.07$^{###}$	2.49 ± 0.1$^{###}$
Thespesia Leaf Extract (200 mg/kg)	1.50 ± 0.07 NS	1.70 ± 0.07 NS	3.66 ± 0.08 NS
Thespesia Leaf Extract (400 mg/kg)	1.67 ± 0.08NS	2.12 ± 0.05$^{###}$	3.78 ± 0.11 NS
Thespesia Leaf Extract (200 mg/kg) + Ethanol (20%)	1.60 ± 0.05 ***	1.64 ± 0.04***	3.09 ± 0.14*****
Thespesia Leaf Extract (400 mg/kg) + Ethanol (20%)	1.90 ± 0.06***	1.82 ± 0.06***	3.69 ± 0.13***
Standard (Vitamin E, 25 mg/kg, p.o.)	2.13 ± 0.12$^{###}$	1.88 ± 0.06 ##	3.75 ± 0.13 NS
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	1.74 ± 0.08***	1.51 ± 0.08***	3.24 ± 0.11***
(Carvedilol, 1mg/kg, p.o.)	3.12 ± 0.11$^{###}$	1.61 ± 0.04 NS	3.23 ± 0.09NS
Carvedilol (1mg/kg, p.o.) + Ethanol (20%)	1.95 ± 0.09***	1.42± 0.03 ***	3.15 ± 0.07*

Note: The values are expressed are mean ± SEM from 6 rats, followed by significance.
NS – Not significant.
#: Value differs significantly from vehicle control group $^{\#}$**P<0.05,** $^{\#\#}$**P<0.01,** $^{\#\#\#}$**P<0.001**
***:** Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

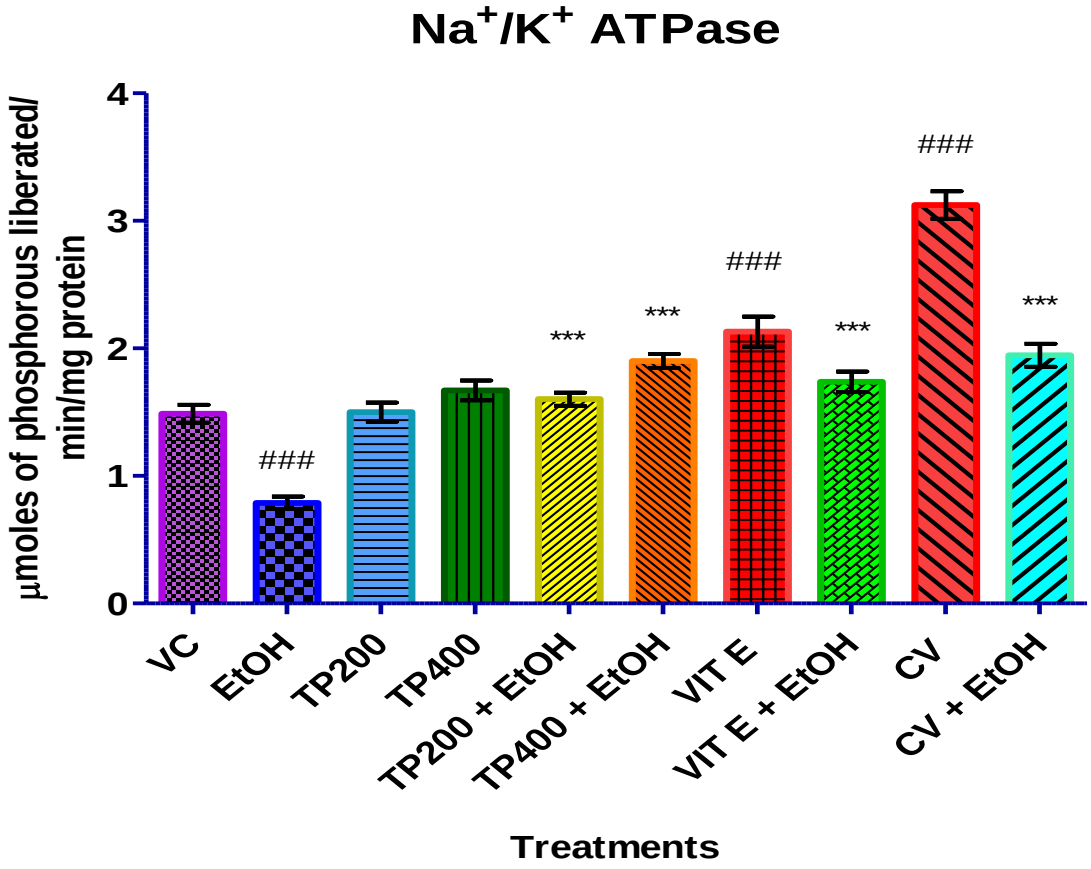

Fig. 47: Histograms representing the changes in sodium-potassium ATPase (Na^+/K^+ ATPase) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

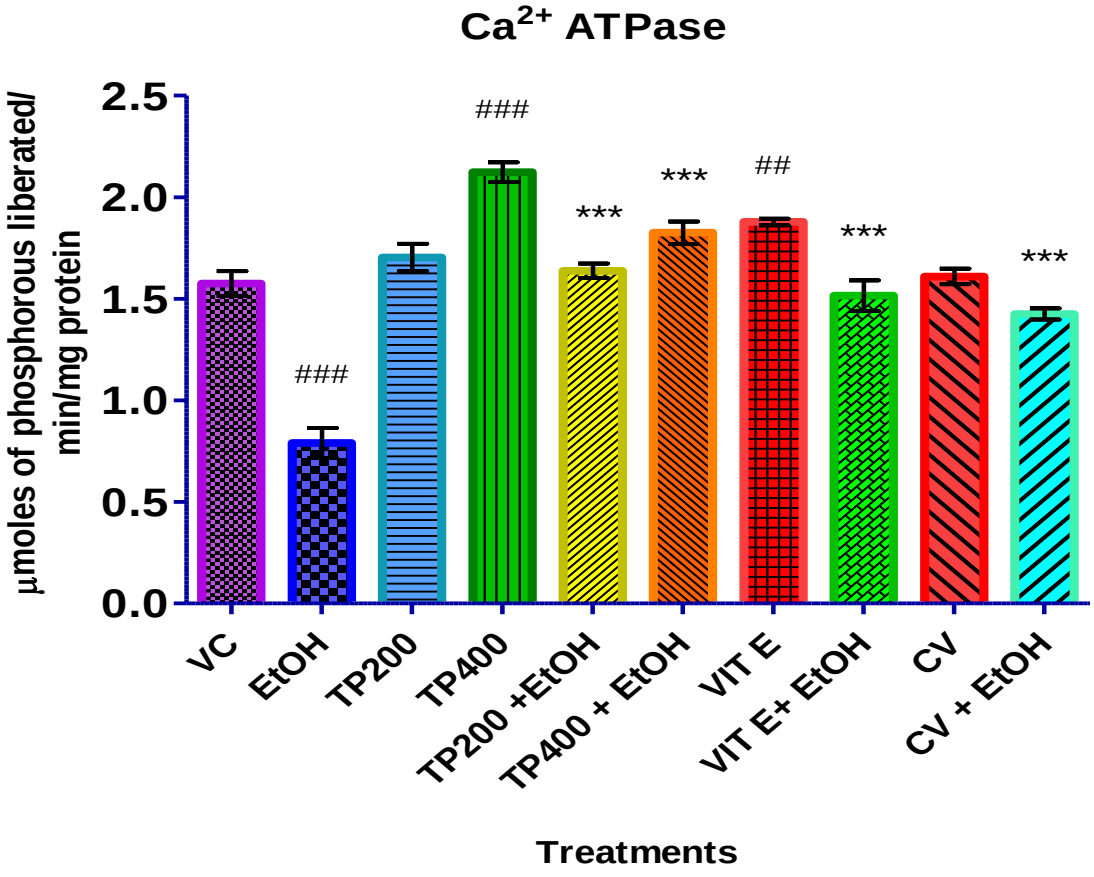

Fig. 48: Histograms representing the changes in calcium ATPase (Ca^{2+}ATPase) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

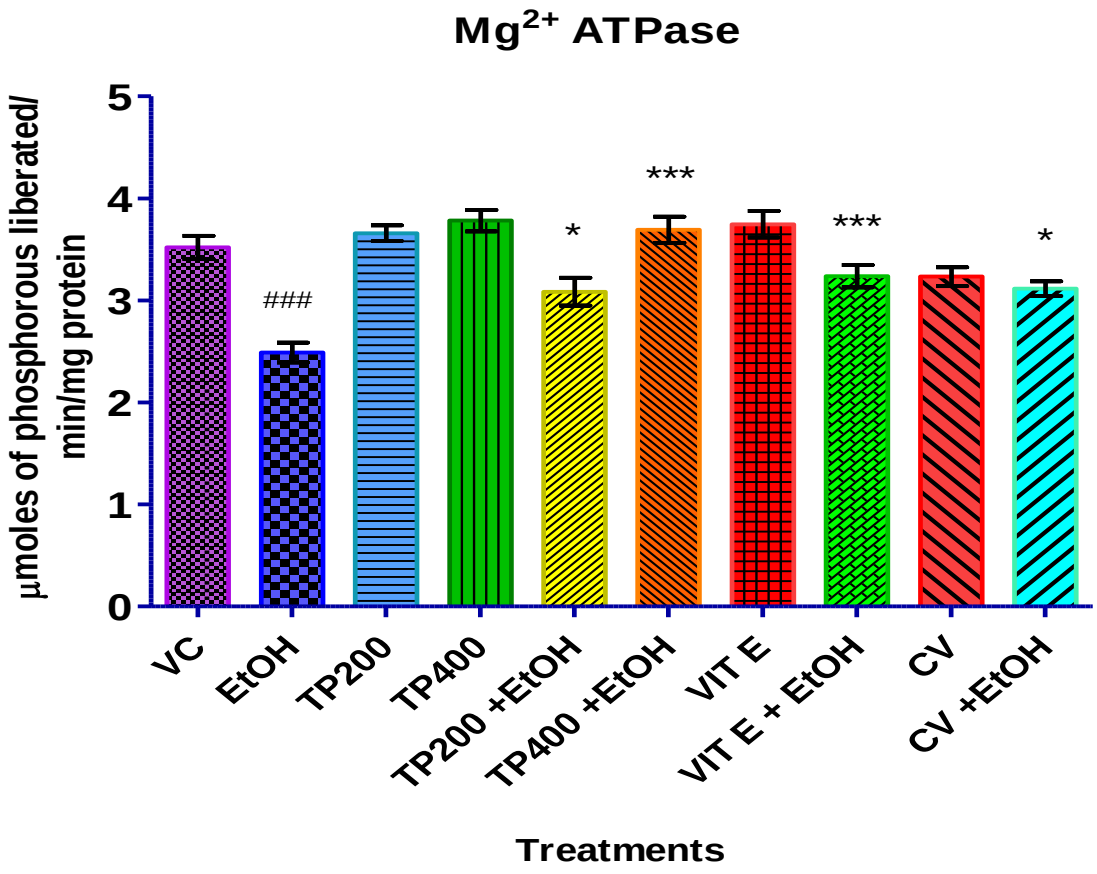

Fig. 49: Histograms representing the changes in magnesium ATPase (Mg^{2+}ATPase) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

4.18 Effect of *T. populnea* leaf extract on alterations in myocardial antioxidant parameters in ethanol-induced cardiotoxicity:

The effects of ethanol, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on alterations in myocardial antioxidant parameters after the treatment period of 6 weeks are illustrated in Table20 and Figures 50 to 56**.** The parameters examined were lipid peroxidation (LP, in terms of malondialdehyde, MDA), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST).

4.18.1 Lipid peroxidation (LP):

Administration of ethanol (disease control) caused a 2-fold increase ($P<0.001$) in LP over the vehicle control. Individual treatments with TP200, TP400 and vitamin E or carvedilol caused slight decreases in LP from the vehicle controls. Prior administration of TP200, TP400, vitamin E or carvedilol before the administration of ethanol caused a reduction of LP level in comparison to the level in disease control. These lowering of LP were partial, and TP200 and carvedilol were found to cause less recovery compared to TP400 and vitamin E (Table 20; Fig. 50).

4.18.2 Reduced glutathione (GSH):

Administration of ethanol caused a significant (around 4-fold) decrease in the GSH level from the vehicle control. Individual administration of TP400 or vitamin E did not cause any perceptible variations in the GSH level from the vehicle control, but individual administration of TP200 and carvedilol caused perceptible decreases that were statistically significant. Administration of TP400 or vitamin E prior to the administration of ethanol effected greater elevation of GSH content in comparison to the disease control level, but administration of TP200 or carvedilol prior to ethanol administration caused only partial, albeit significant ($P<0.001$), elevations compared to the disease control (Table 20; Fig. 51).

4.18.3 Superoxide dismutase (SOD) activity:

Administration of ethanol caused a significant (over 2-fold) decrease in the SOD activity in the disease control compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol individually caused only minor variations in SOD activity from the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the

administration of ethanol caused an elevation in the SOD activity compared to the disease control level. The elevations were near to the vehicle control level (Table 20; Fig. 52).

4.18.4 Catalase (CAT) activity:

Administration of ethanol caused a significant (over 3-fold) decrease in the CAT activity in the disease control from the vehicle control. Individual administration of TP200, TP400, vitamin E and carvedilol caused slight, non-significant decreases in CAT activity from the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol induced elevation of CAT activity in comparison to the disease control. The elevations in CAT activity were nearer to the vehicle control. Of these, TP400 and vitamin E caused a greater preventive effect reflected by the elevation of enzyme activity (Table 20; Fig. 53).

4.18.5 Glutathione peroxidase (GPX) activity:

Administration of ethanol caused a significant (3-fold) decrease in the GPX activity in the disease control compared to the vehicle control. Individual administration of TP200, TP400, vitamin E or carvedilol caused only minor fluctuations in the enzyme activity from the vehicle control. Administration of TP400 or vitamin E prior to the administration of ethanol induced a significant increase in GPX activity compared to the disease control, bringing the enzyme activity closer to the vehicle control. Prior administration of carvedilol before ethanol administration elevated the enzyme activity to near the vehicle control, but the elevation with TP200 was only partial (Table 20; Fig. 54).

4.18.6 Glutathione reductase (GR):

Administration of ethanol caused about 2-fold decrease in GR activity from the vehicle control. Individual treatment with TP200 or carvedilol caused decreases in GR activity from the vehicle control, while individual treatments with TP400 or vitamin E caused increases in activity over the vehicle control. Administration of TP400 or vitamin E prior to the administration of ethanol caused an increase in the enzyme activity compared to the level in disease control. In contrast, prior administration of TP200 or carvedilol caused only a partial, albeit significant, elevation in GR activity compared to the disease control (Table 20; Fig. 55).

4.18.7 Glutathione-S-transferase (GST):

Administration of ethanol caused a 2-fold decrease in the GST activity in the disease control compared to the vehicle control. Individual administration of TP200, TP400 or vitamin E caused significant ($P<0.001$) increases in the enzyme activity from the vehicle control, while carvedilol caused a non-significant elevation. Administration of TP200 or carvedilol prior to the administration of ethanol caused an elevation of GST activity compared to the level in disease control. The elevation was observed to be near to the level in vehicle control, while prior administration of TP400 or vitamin E caused an increase in GST activity compared to that of disease control to above the level of vehicle control (Table 20; Fig. 56).

Table 20. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the levels of lipid peroxidation (LP) and reduced glutathione (GSH) levels, and activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) during adriamycin-induced cardiotoxicity in rats after a treatment period of 6 weeks.

Experimental Group	LP	GSH	SOD	CAT	GPX	GR	GST
Vehicle Control (5% Gum acacia)	117.50± 0.45	31.51± 0.62	16.45 ± 0.36	0.76 ± 0.05	8.57 ± 0.12	50.38 ± 1.27	0.98 ± 0.02
Disease Control (Ethanol 20%)	239.92± 0.51$^{\#\#\#}$	8.03± 0.39$^{\#\#\#}$	7.54 ± 0.77$^{\#\#\#}$	0.22 ± 0.02$^{\#\#\#}$	2.83 ± 0.08$^{\#\#\#}$	25.69 ± 1.50$^{\#\#\#}$	0.49 ± 0.02$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg)	111.00± 1.29 $^{\#\#}$	27.30± 1.08$^{\#}$	14.09 ± 0.12$^{\#\#}$	0.69 ± 0.04 NS	6.96 ± 0.47$^{\#\#\#}$	42.34 ± 1.19$^{\#\#\#}$	1.60 ± 0.03$^{\#\#\#}$
Thespesia Leaf Extract (400 mg/kg)	106.50± 1.13$^{\#\#\#}$	34.33 ± 0.78 NS	15.94 ± 0.10 NS	0.72 ± 0.15 NS	8.09 ± 0.15 NS	62.64 ± 1.21$^{\#\#\#}$	2.25 ± 0.08$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg) + Ethanol (20%)	193.50± 0.66***	18.98 ± 0.88***	10.04 ± 0.73***	0.64 ± 0.03***	4.89 ± 0.13***	37.30 ± 0.72***	0.82 ± 0.06***
Thespesia Leaf Extract (400 mg/kg) + Ethanol (20%)	131.21± 1.03***	28.03 ± 1.20***	13.78 ± 0.35***	0.71 ± 0.06***	9.88 ± 0.09***	54.32 ± 0.73***	1.37 ± 0.06***
Standard (Vitamin E, 25 mg/kg, p.o.)	104.42± 1.50$^{\#\#}$	32.40 ± 0.40 NS	16.09 ± 0.10 NS	0.79 ± 0.05 NS	8.52 ± 0.11 NS	59.13 ± 1.02$^{\#\#\#}$	2.08 ± 0.07$^{\#\#\#}$
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	120.21± 0.57***	29.25 ± 0.89*	14.47 ± 0.89***	0.73± 0.09***	9.76 ± 0.27***	55.22 ± 0.86***	1.27 ± 0.03***
(Carvedilol, 1mg/kg, p.o.)	115.63± 1.56 NS	23.79 ± 0.63$^{\#\#\#}$	14.09 ± 0.51$^{\#\#\#}$	0.72 ± 0.03 NS	7.33 ± 0.12$^{\#\#\#}$	46.30 ± 0.96 NS	1.17 ± 0.02NS
Carvedilol (1mg/kg, p.o.) + Ethanol (20%)	166.75± 0.90***	19.74 ± 0.93***	13.79 ± 0.63***	0.67 ± 0.04***	7.08 ± 0.10***	39.29 ± 1.30***	0.81 ± 0.03***

Note: The values are expressed are mean ± SEM from 6 rats, followed by significance.
NS – Not significant.
#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**
*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

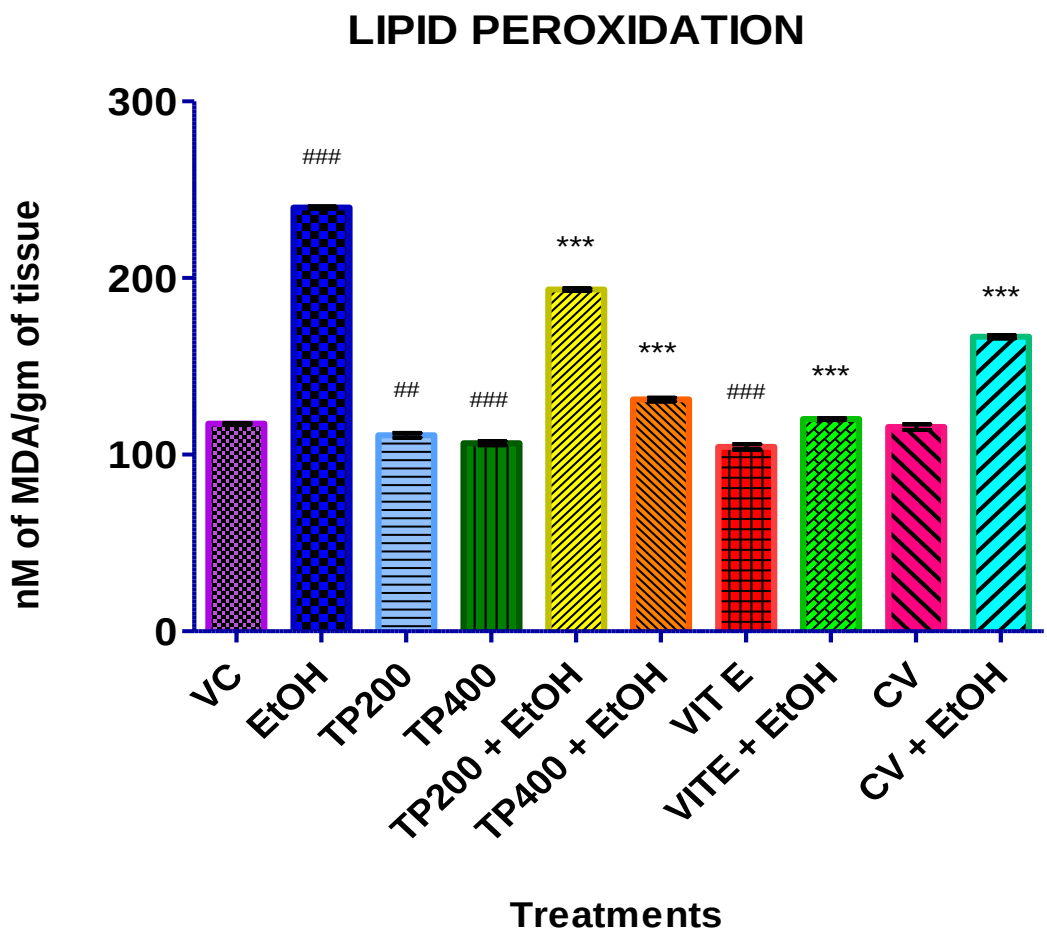

Fig. 50: Histograms representing the changes in lipid peroxidation (LP) in terms of malondialdehyde (MDA) content in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

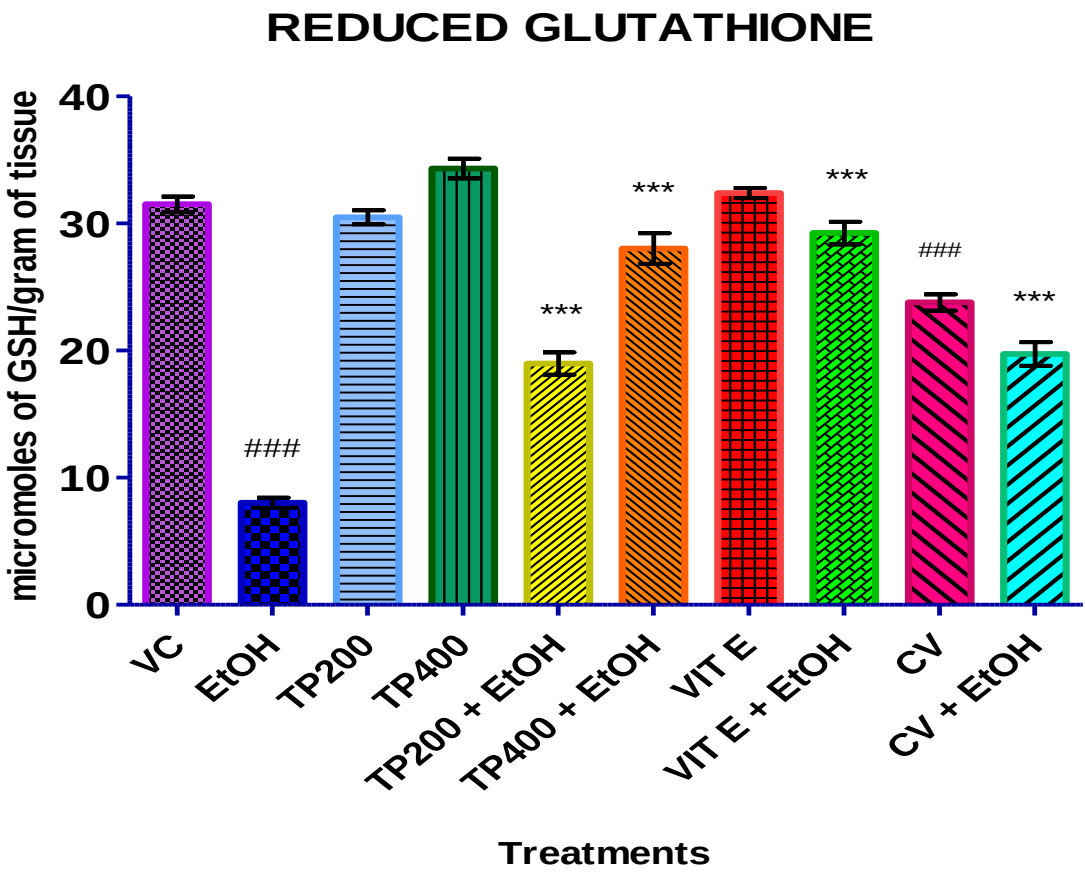

Fig. 51: Histograms representing the changes in reduced glutathione (GSH) in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

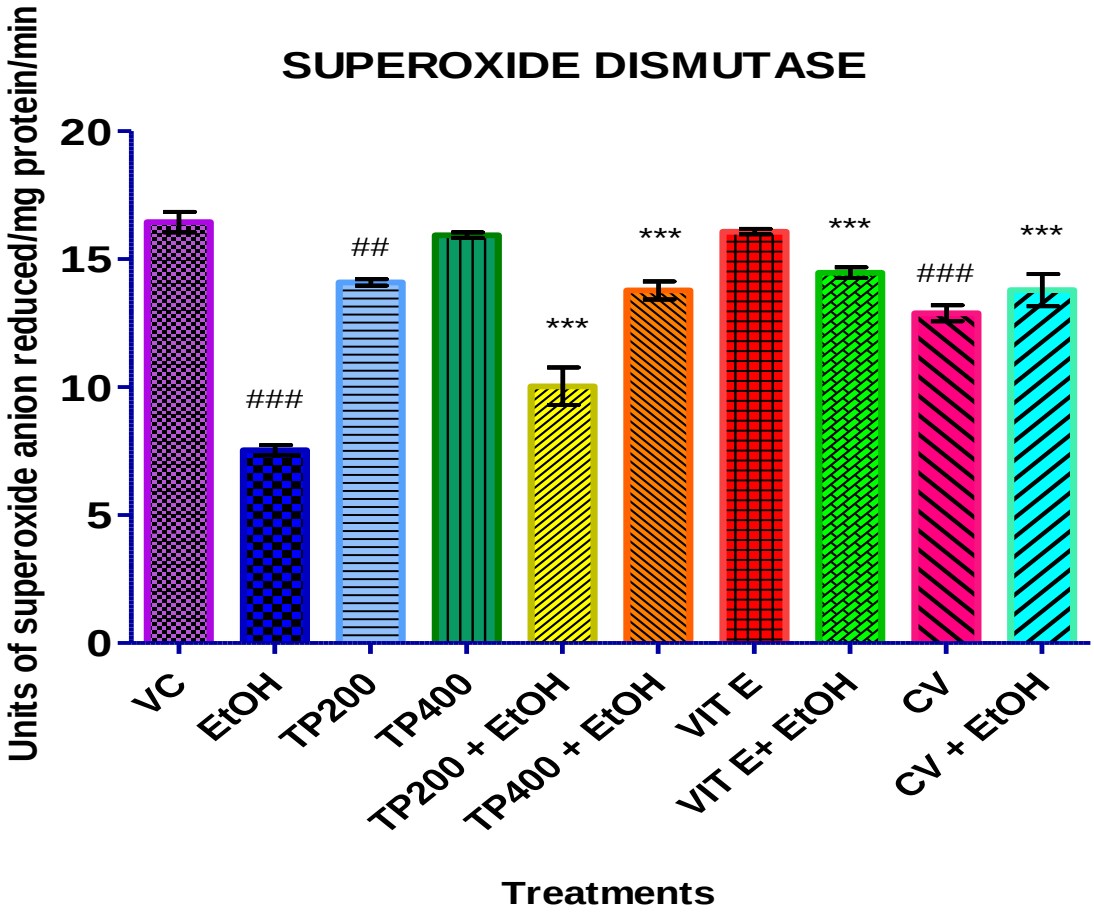

Fig. 52: Histograms representing the changes in superoxide dismutase (SOD) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

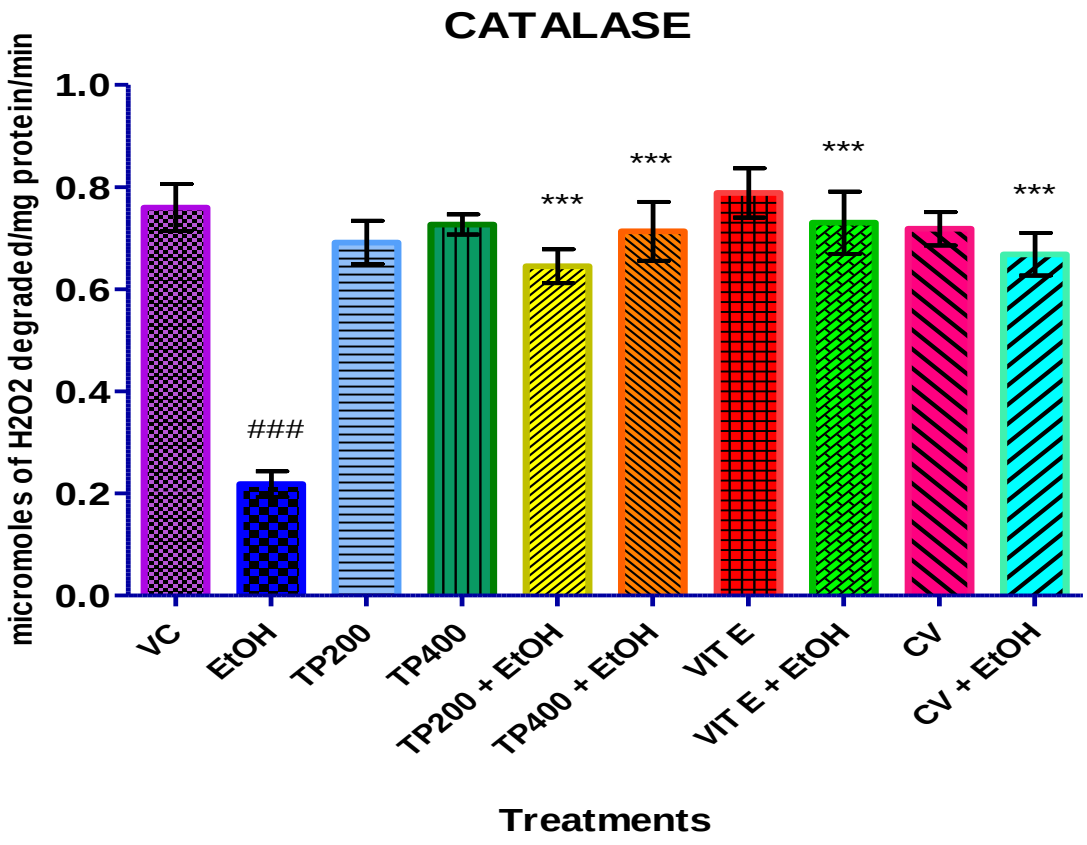

Fig. 53: Histograms representing the changes in catalase (CAT) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

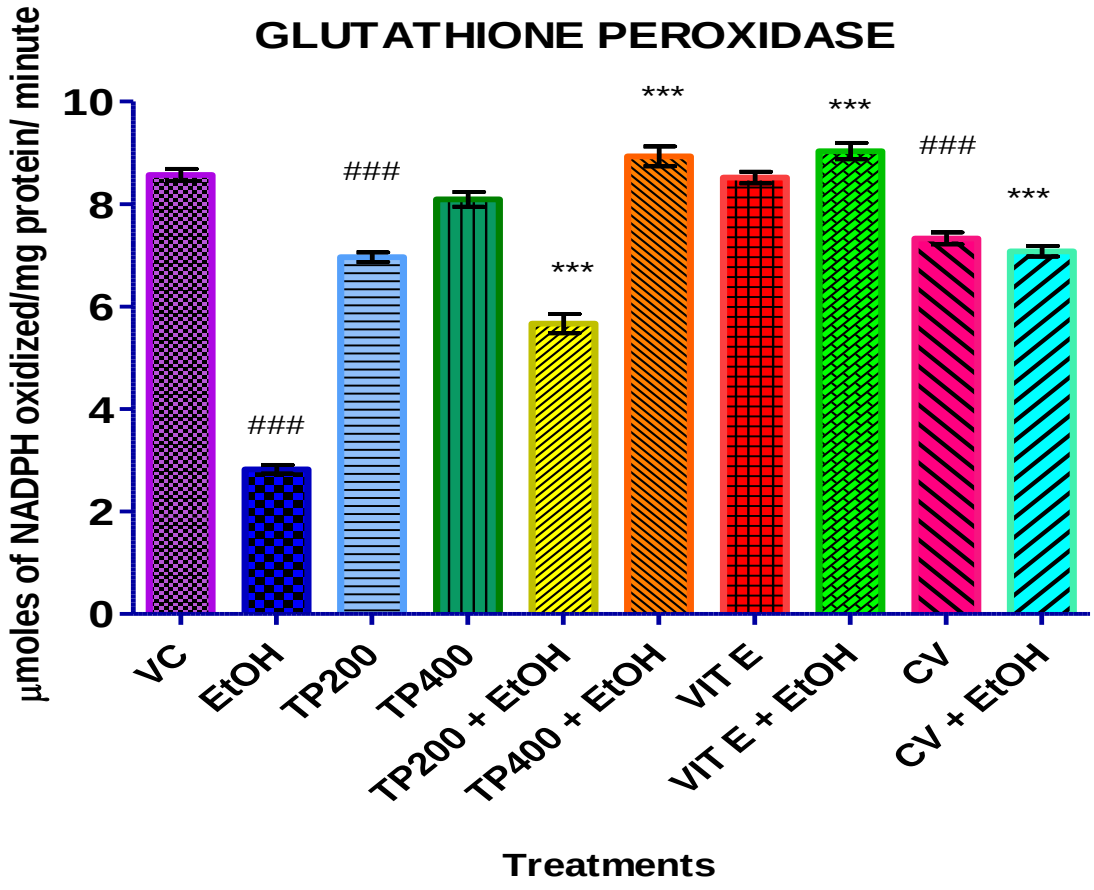

Fig. 54: Histograms representing the changes in glutathione peroxidase (GPX) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

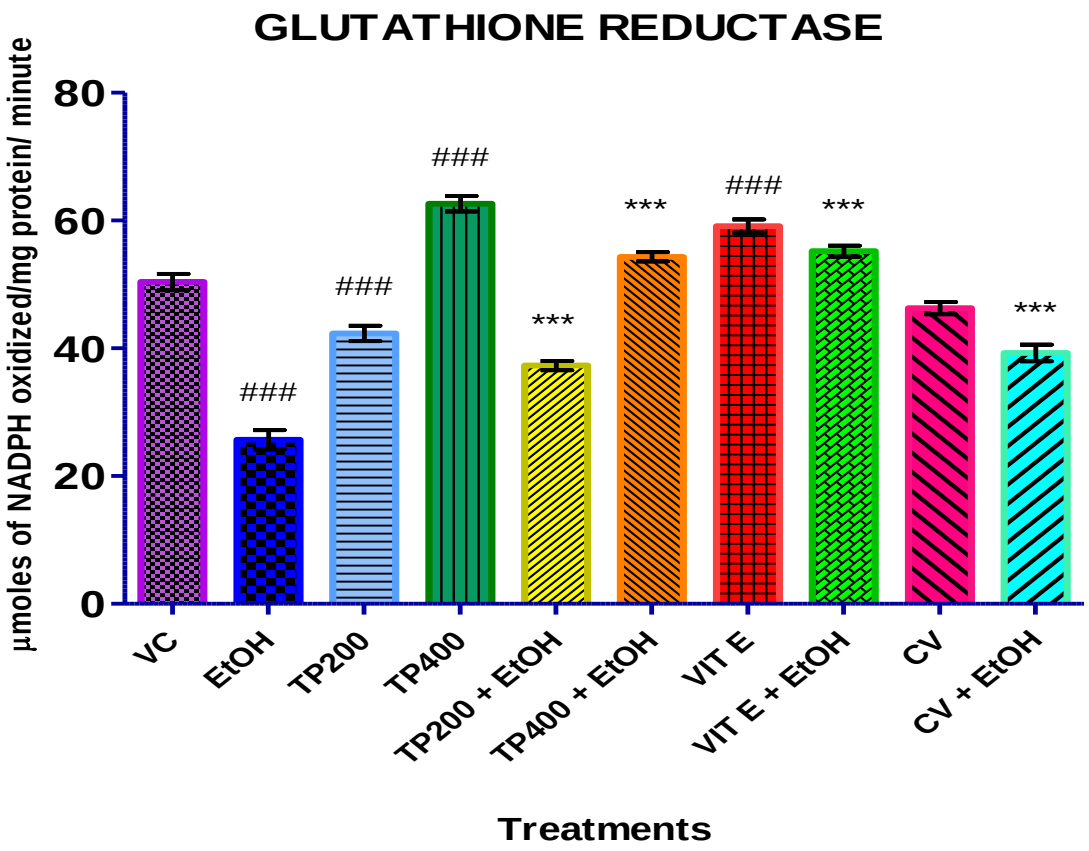

Fig. 55: Histograms representing the changes in glutathione reductase (GR) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

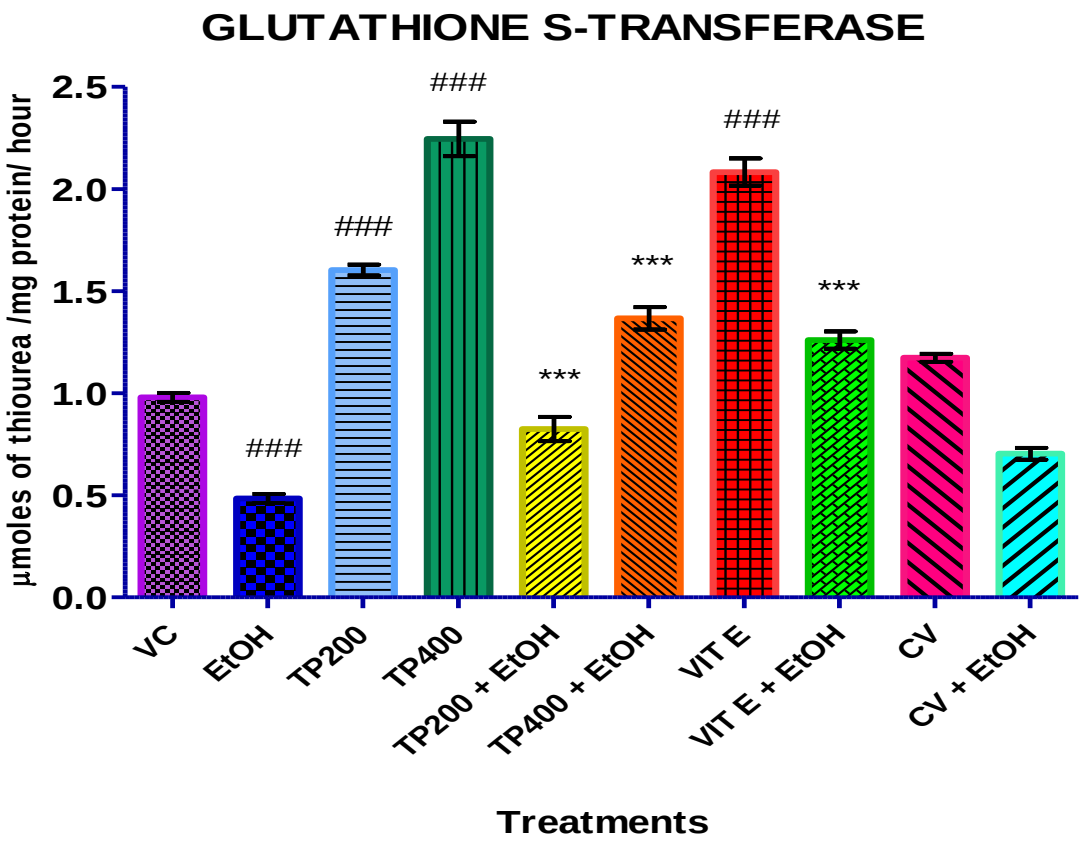

Fig. 56: Histograms representing the changes in glutathione-S-transferase (GST) activity in heart tissue after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

4.19 Effect of *T. populnea* leaf extract on alterations in serum biochemical parameters in ethanol-induced cardiotoxicity:

The effects of ethanol, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination with ethanol, on serum biochemical parameters (cardiac biomarkers) after the treatment period of 6 weeks are illustrated in Table 21 and Figures 57 to 62**.** The parameters examined were serum C-reactive protein (CRP), and activities of creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

4.19.1 C-reactive protein (CRP):

Administration of ethanol (disease control) caused a very significant increase ($P<0.001$) in serum CRP from the vehicle control. Individual treatments with TP200 and vitamin E caused slight decreases in CRP, while TP400 and carvedilol effected slight increases. Administration of TP200, TP400, Vitamin E or carvedilol prior to the administration of adriamycin caused a reduction in CRP content compared to that of the disease control. Greater protection was effected by carvedilol than TP200, TP400 and vitamin E, by bringing down the CRP level nearer to the vehicle control (Table 21; Fig. 57).

4.19.2 Creatine kinase-MB (CK-MB) activity:

Administration of ethanol caused a striking increase (over 9-fold) in the CK-MB activity over the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol individually caused slight increases or decreases in the activity level of the vehicle control. Administration of TP200, TP400 or carvedilol prior to the administration of ethanol caused partial preventive effect by bringing down the CK-MB activity in comparison to the disease control level, towards the vehicle control in the order of carvedilol>TP200>TP400. Administration of vitamin E prior to ethanol decreased the enzyme activity in comparison to the disease control level. The decrease in CK-MB activity was comparable to that of the vehicle control (Table 21; Fig. 58).

4.19.3 Creatine kinase (CK) activity:

Administration of ethanol caused a very significant increase (over 4-fold) in the CK activity over the vehicle control. Administration of TP200 or TP400 separately caused little or no elevation in the enzyme activity, while individual administration of vitamin E or carvedilol caused a decrease. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol caused partial elevations in the enzyme activity as compared to the disease control. The elevations in enzyme activity were brought towards that of the vehicle control in the order of carvedilol>TP200>vitamin E>TP400 (Table 21; Fig. 59).

4.19.4 Lactate dehydrogenase (LDH) activity:

Administration of ethanol caused an increase (over 4-fold) in the LDH activity in the disease control compared to the vehicle control. Administration of TP200 and TP400 individually caused a decrease in the enzyme activity, while administration of vitamin E or carvedilol separately effected moderate elevations in the enzyme activity compared to that in the vehicle control. Administration of TP200, TP400 or vitamin E prior to the administration of ethanol prevented the rise in LDH activity in comparison to the level in disease control. This reduction in LDH activity was comparable to the level of vehicle control, with vitamin E taking the activity down to below the level off the vehicle control. In contrast, treatment with carvedilol prior to ethanol administration did not cause any reduction in the enzyme activity but elevated it to slightly above the level of the disease control (Table 21; Fig. 60).

4.19.5 Aspartate aminotransferase (AST) activity:

Administration of ethanol caused a significant (over 6-fold) increase in the AST activity in the disease control compared to the vehicle control. Administration of TP200, TP400, vitamin E or carvedilol individually caused only slight increases or decreases in the activity as compared to the vehicle control. Administration of TP400 prior to the administration of ethanol prevented the rise in AST effectively in comparison to the in disease control group, to the level in vehicle control. Prior treatment with TP200, vitamin E and carvedilol were less effective and caused only partial decreases as compared to the disease control, in the order of carvedilol>TP200>vitamin E (Table 21; Fig. 61).

4.19.6 Alanine aminotransferase (ALT) activity:

Like AST, administration of ethanol also caused a significant (about 6-fold) increase in the ALT activity in the disease control compared to the vehicle control. Administration of TP200 or TP400 separately caused slight elevation in the enzyme activity, while individual administration of vitamin E or carvedilol caused a greater elevations (about 2-fold) in ALT activity compared to the vehicle control. Administration of TP400 prior to the administration of ethanol brought down the enzyme activity effectively from the level in disease control to the level in vehicle control. As in the case of AST activity, prior treatment with TP200, vitamin E and carvedilol were less effective and caused only partial reversals from the disease control, in the order of carvedilol>TP200>vitamin E (Table 21; Fig. 62).

Table 21. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the levels of serum C-reactive protein (CRP), and activities of creatine kinase-MB (CK-MB), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) during ethanol-induced cardiotoxicity in rats after a treatment period of 6 weeks.

Experimental Group	CRP	CKMB	CK	LDH	AST	ALT
Vehicle Control (5% Gum acacia)	1.22 ± 0.08	32.43 ± 1.17	115.17 ± 5.31	903.16 ± 10.43	40.34 ± 2.39	30.03 ± 2.02
Disease Control (Ethanol 20%)	27.25 ± 1.49$^{\#\#\#}$	307.99 ± 7.15$^{\#\#\#}$	521.12± 9.20$^{\#\#\#}$	4028.80± 58.36$^{\#\#\#}$	262.61± 4.73$^{\#\#\#}$	177.42 ± 2.58$^{\#\#\#}$
Thespesia Leaf Extract (200 mg/kg)	0.88 ± 0.08 NS	43.20 ± 1.02NS	126.27 ± 4.82 NS	606.33 ± 1.11$^{\#\#\#}$	44.13 ± 3.38 NS	37.90 ± 2.31NS
Thespesia Leaf Extract (400 mg/kg)	1.83 ± 0.16 NS	24.61 ± 1.89 NS	111.77± 3.49 NS	476.3 ± 9.75$^{\#\#\#}$	47.01 ± 2.22 NS	40.88 ± 3.73 NS
Thespesia Leaf Extract (200 mg/kg) + Ethanol (20%)	9.33 ± 0.03***	221.59 ± 4.02***	256.91± 5.81***	1289.30 ± 5.95***	133.75 ±5.10***	97.15 ± 3.57***
Thespesia Leaf Extract (400 mg/kg) + Ethanol (20%)	4.00 ± 0.36***	94.89 ± 2.01***	149.14± 3.30***	1189.20 ± 5.18***	42.11± 3.38***	27.81 ± 2.68***
Standard (Vitamin E, 25 mg/kg, p.o.)	1.23 ± 0.04 NS	23.38 ± 1.32 NS	70.24 ± 2.98 $^{\#\#\#}$	1088.30 ± 16.27 NS	38.82 ± 1.80 NS	59.04 ± 1.42$^{\#\#\#}$
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	3.54 ± 0.11***	35.25 ± 1.31***	237.65± 10.39***	520.00 ± 39.25***	98.63 ± 3.34***	61.46 ± 4.46***
(Carvedilol, 1mg/kg, p.o.)	2.67 ± 0.17 NS	29.03 ± 1.95 NS	31.23 ± 2.11$^{\#\#\#}$	1062.10 ± 11.5 NS	48.12 ± 3.18 NS	63.16 ± 4.39$^{\#\#\#}$
Carvedilol (1mg/kg, p.o.) + Ethanol (20%)	2.52 ± 0.20***	265.09 ± 5.29***	292.72± 7.75***	4380.50 ±48.17***	181.91 ±4.47***	100.18 ± 4.27***

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.
NS – Not significant.
#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#}$P<0.001**
***:** Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

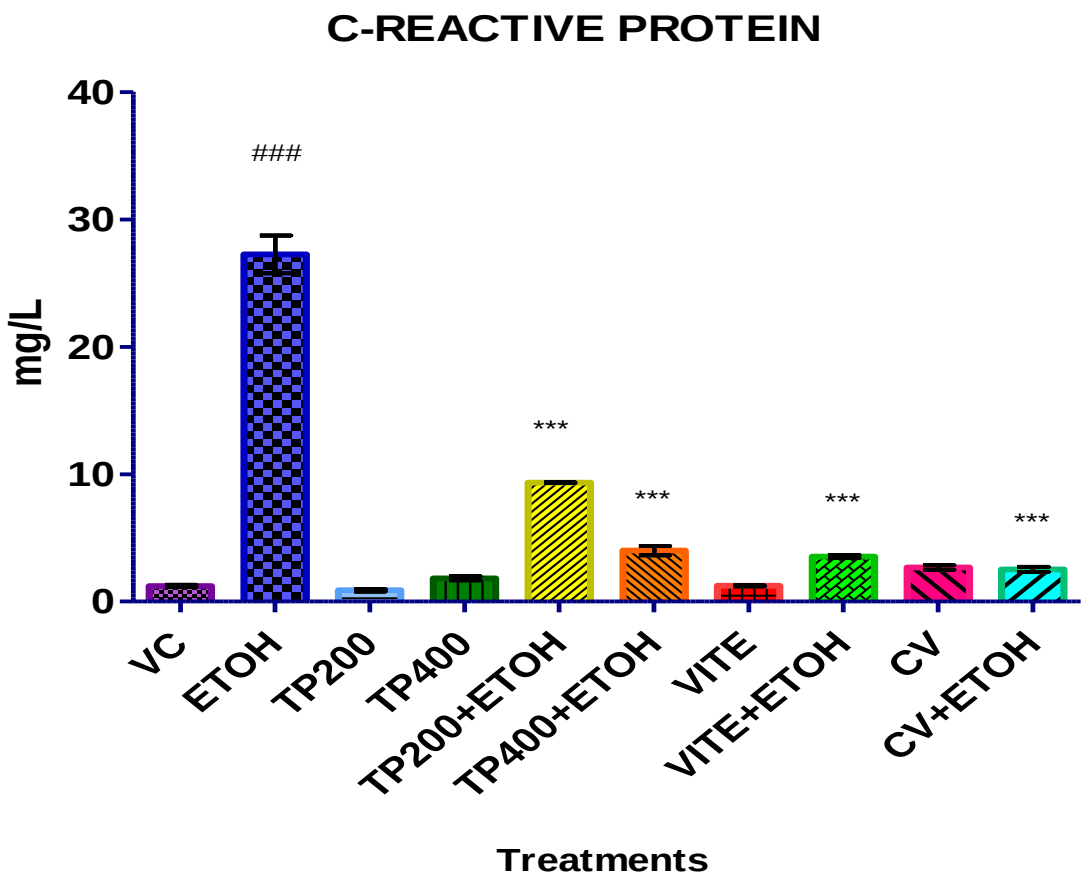

Fig. 57: Histograms representing the changes in serum C-reactive protein (CRP) in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

***:** Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

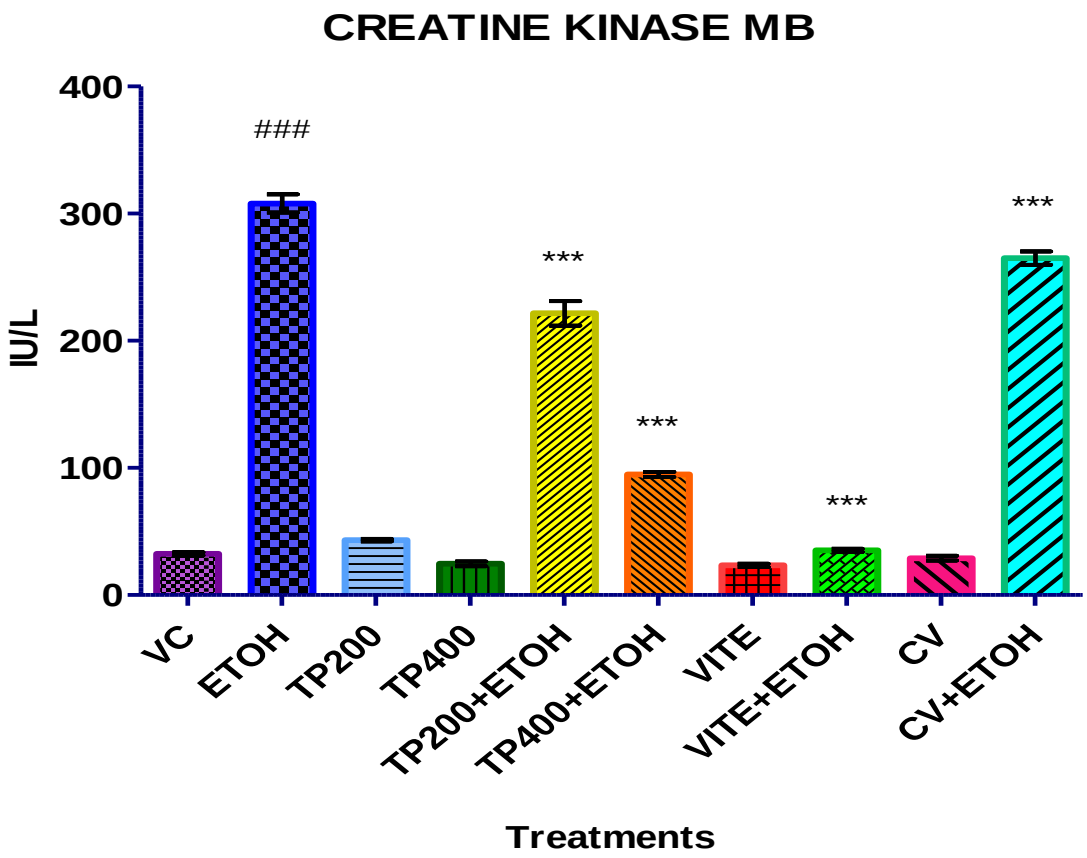

Fig. 58: Histograms representing the changes in serum creatine kinase MB (CK-MB) activity in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

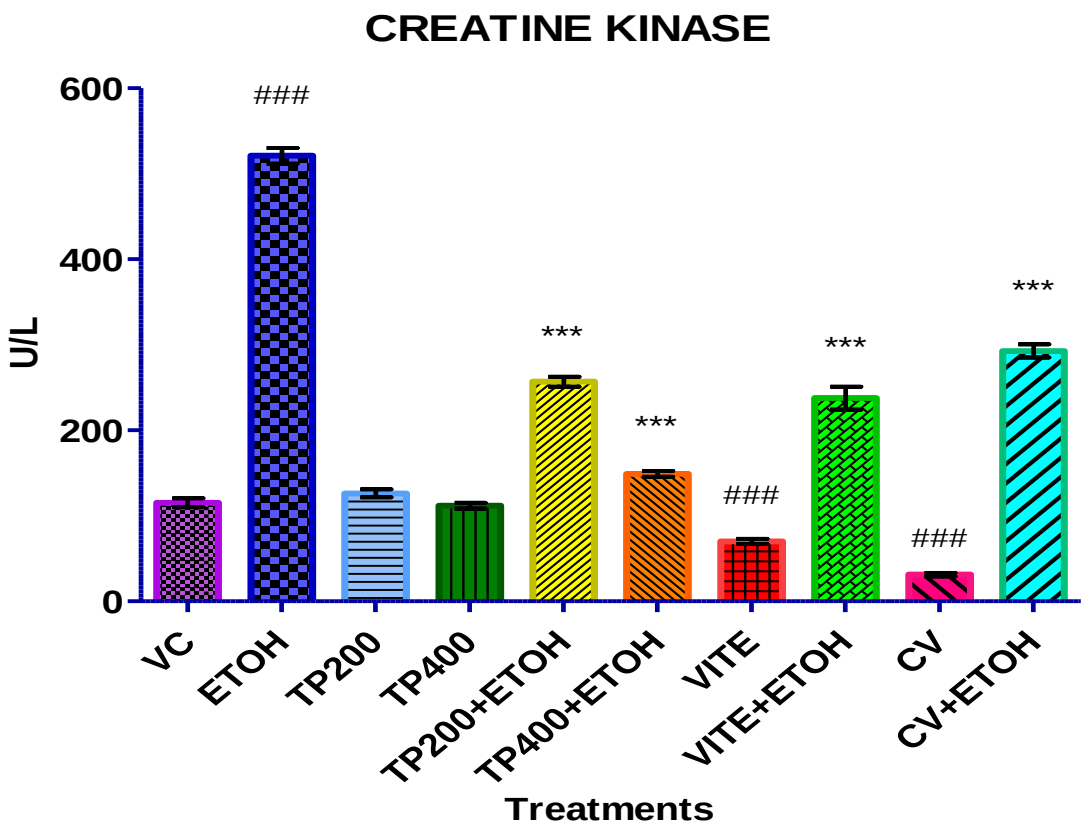

Fig. 59: Histograms representing the changes in serum creatine kinase (CK) activity in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

***:** Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

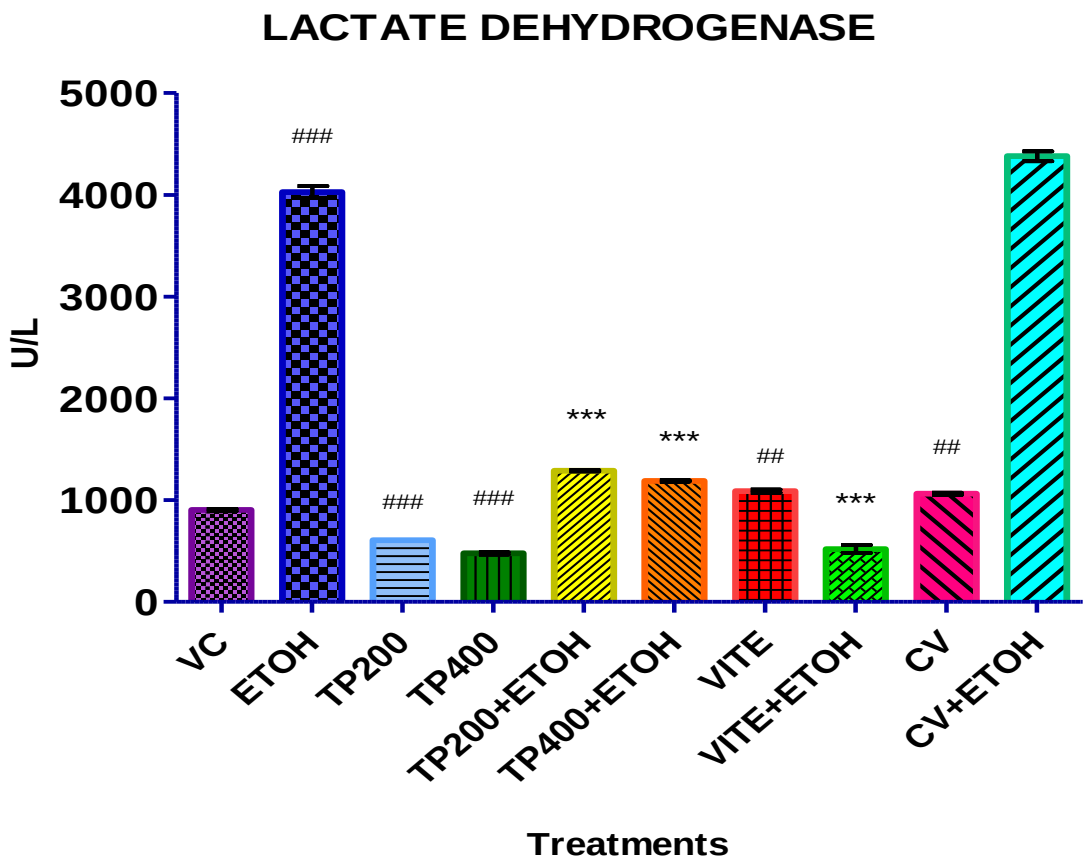

Fig. 60: Histograms representing the changes in lactate dehydrogenase (LDH) activity in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

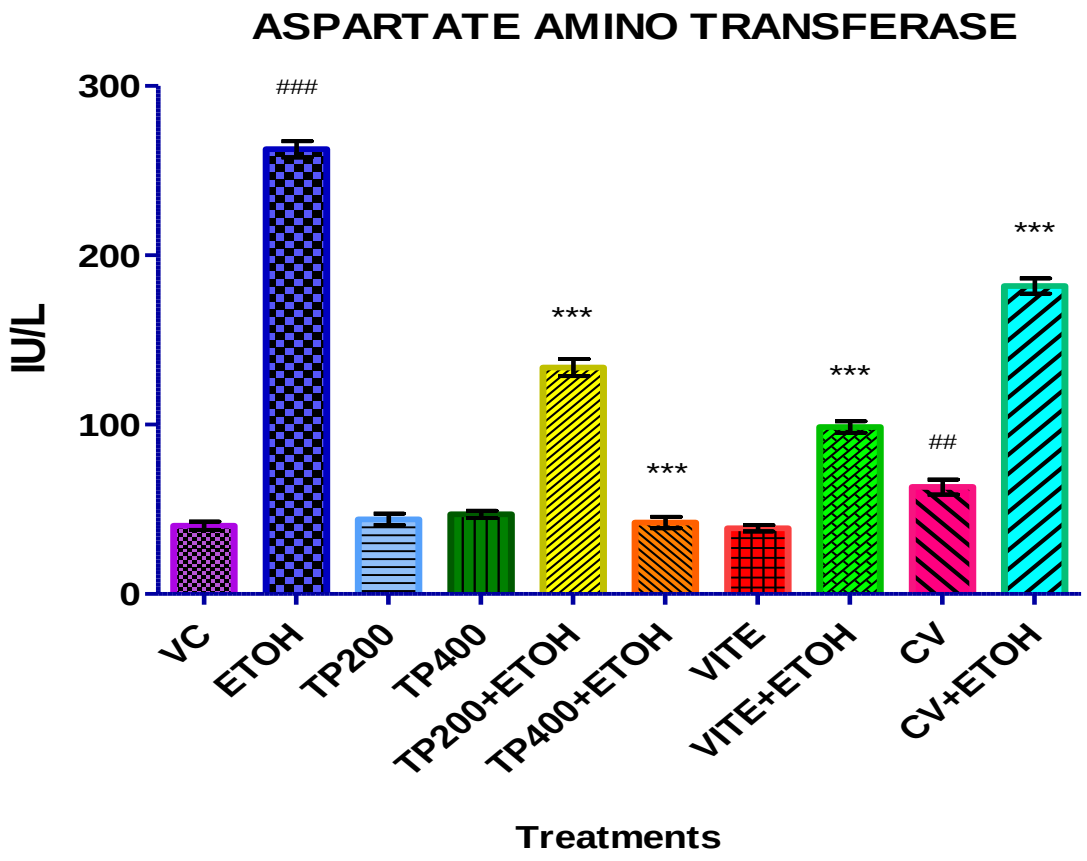

Fig. 61: Histograms representing the changes in serum aspartate aminotransferase (AST) activity in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

***:** Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

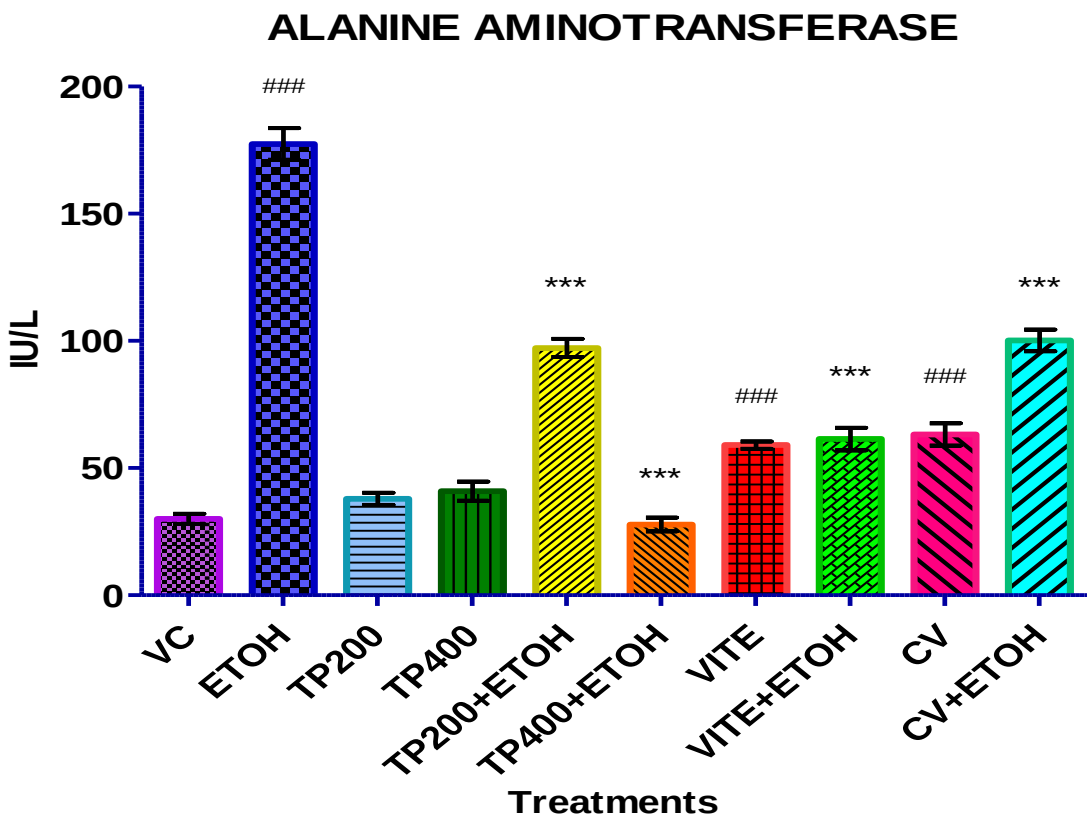

Fig. 62: Histograms representing the changes in serum alanine aminotransferase (ALT) activity in control rats and those receiving different treatments after a treatment period of 6 weeks.

Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt p.o); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **vit E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

4.20 Effect of *T. populnea* leaf extract on alterations in serum lipid profile parameters in ethanol-induced cardiotoxicity:

The effects of ethanol, *Thespesia* leaf extracts, vitamin E and carvedilol, both individually and in combination, on serum lipid profile parameters after the treatment period of 6 weeks are illustrated in Table 22 and Figures 63 to 67**.** The parameters examined were total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL).

4.20.1 Total cholesterol (TC):

Administration of ethanol (disease control) caused a significant increase ($P<0.001$) in TC from the vehicle control. Individual treatments with TP200, TP400, vitamin E and carvedilol caused decreases in TC level to different degrees from the vehicle control, with TP400 causing greater decrease than the other three individual treatments. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol caused a reduction in total cholesterol levels as compared the level in disease control. The reduction in total cholesterol in these groups was nearer to the vehicle control. Of the four prior treatments, TP200, TP400 and vitamin E brought down the TC content to below the vehicle control level, whereas carvedilol was the least effective in preventing the rise in cholesterol levels, with the TC content staying well above the vehicle control level after its treatment. Maximal decrease compared to the disease control was effected by vitamin E and TP400 (Table 22; Fig. 63).

4.20.2 Triglycerides (TG):

Administration of ethanol caused a significant ($P<0.001$) increase in the TG level over the vehicle control. Individually, administration of TP400, vitamin E or carvedilol caused a decrease in the TG level from the vehicle control, while TP200 caused an increase. Administration of TP400 or vitamin E prior to the administration of ethanol brought down the TG level effectively from the level in disease control to the level in vehicle control, while TP200 and carvedilol caused only partial decreases from the disease control, with the TG content staying above the vehicle control level (Table 22; Fig. 64).

4.20.3 High density lipoprotein (HDL):

Administration of ethanol caused nearly 3-fold decrease in the HDL level in the disease control compared to the vehicle control. Administration of TP200, TP 400, vitamin E or carvedilol individually also caused decreases in the HDL content, but the decreases were much smaller in comparison to that caused by ethanol. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol caused an increase in the HDL content from the level in disease control towards the level in vehicle control. Prior administration of carvedilol was most effective and elevated the HDL content completely to the vehicle control level (Table 22; Fig. 65).

4.20.4 Low density lipoprotein (LDL):

Administration of ethanol caused a significant (nearly 4-fold) increase in the LDL content in the disease control compared to the vehicle control. Administration of TP200, TP400 or vitamin E individually caused decreases in the LDL content of the vehicle control, while individual administration of carvedilol caused no perceptible change. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol prevented the rise in the LDL content effectively from the level in disease control towards the level in vehicle control. Of these, TP200 and TP400 caused decreases to below the vehicle control level, with TP400 causing a more significant decrease. Vitamin E reduced the LDL content to slightly higher than the vehicle control level. However, carvedilol caused only a partial reduction in LDL levels in comparison to the disease control, with the LDL content remaining at a relatively higher level than the vehicle control (Table 22; Fig. 66).

4.20.5 Very low density lipoprotein (VLDL):

Administration of ethanol caused an increase ($P<0.001$) in the VLDL level in the disease control compared to the vehicle control. Individual administration of TP200 did not cause any change in the VLDL level from the vehicle control, while TP400, vitamin E and carvedilol individually caused decreases ($P<0.001$) in the VLDL level. Administration of TP200, TP400, vitamin E or carvedilol prior to the administration of ethanol brought down the VLDL content effectively from the level in disease control towards the level in vehicle control. Of these, with TP200, TP400 and vitamin E the VLDL content was brought down to below the vehicle control level from the level of disease control, while with carvedilol the VLDL content remained slightly elevated above the vehicle control level (Table 22; Fig. 67).

Table 22. Effect of *Thespesia populnea* leaf extract, vitamin E and carvedilol on changes in the serum lipid profile parameters comprising levels of serum total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) during ethanol-induced cardiotoxicity in rats.

Experimental Group	**TC (mg/dl)**	**TG (mg/dl)**	**HDL (mg/dl)**	**LDL (mg/dl)**	**VLDL (mg/dl)**
Vehicle Control (5% Gum acacia)	166.40 ± 5.84	126.65 ± 3.69	78.93 ± 4.21	53.33 ± 4.23	33.06 ± 0.86
Disease Control (Ethanol 20%)	274.43 ± 7.36$^{###}$	202.89 ± 10.30$^{###}$	29.08 ± 1.61$^{###}$	204.70 ± 6.11$^{###}$	40.57 ± 2.07$^{###}$
Thespesia Leaf Extract (200 mg/kg)	146.45 ± 6.60 NS	168.92 ± 17.09 NS	69.34 ± 5.07 NS	43.32 ± 8.19 NS	33.70 ± 3.41 NS
Thespesia Leaf Extract (400 mg/kg)	111.40 ± 9.95$^{###}$	101.44 ± 7.69 NS	54.36 ± 6.92$^{#}$	36.74 ± 7.51$^{###}$	20.28 ± 1.53$^{###}$
Thespesia Leaf Extract (200 mg/kg) + Ethanol (20%)	144.20 ± 6.07 ***	157.64 ± 10.24*	68. 47 ± 3.71***	44.20 ± 8.88***	31.52 ± 2.04 NS
Thespesia Leaf Extract (400 mg/kg) + Ethanol (20%)	132.51 ± 4.15***	127.30 ± 8.8***	70.50 ± 9.48***	35.82 ± 8.98***	26.18 ± 1.89***
Standard (Vitamin E, 25 mg/kg, p.o.)	126.05 ± 9.31$^{##}$	112.31 ± 8.51NS	61.14 ± 3.82 NS	42.44 ± 9.50 NS	22.46 ± 1.70$^{###}$
Vitamin E (25 mg/kg, p.o.) + Ethanol (20%)	130.80± 5.63***	123.42 ± 11.86***	46.25 ± 3.30 NS	59.87 ± 5.66***	24.68 ± 2.17***
(Carvedilol, 1mg/kg, p.o.)	143.87 ± 3.46 NS	121.12 ± 6.73 NS	60.78 ± 5.00 NS	58.80 ± 5.79 NS	24.22 ± 1.34 NS
Carvedilol (1mg/kg, p.o.) + Ethanol (20%)	186.05 ± 4.08***	189.50 ± 5.56 NS	30.20 ± 1.87 NS	117.95 ± 5.11***	37.90 ± 1.11 NS

Note: The values are expressed as mean ± SEM from 6 rats, followed by significance.
NS – Not significant.
#: Value differs significantly from vehicle control group **$^{#}$P<0.05, $^{##}$P<0.01, $^{###}$P<0.001**
*: Value differs significantly from disease control group ***P< 0.05, **P<0.01, ***P<0.001**

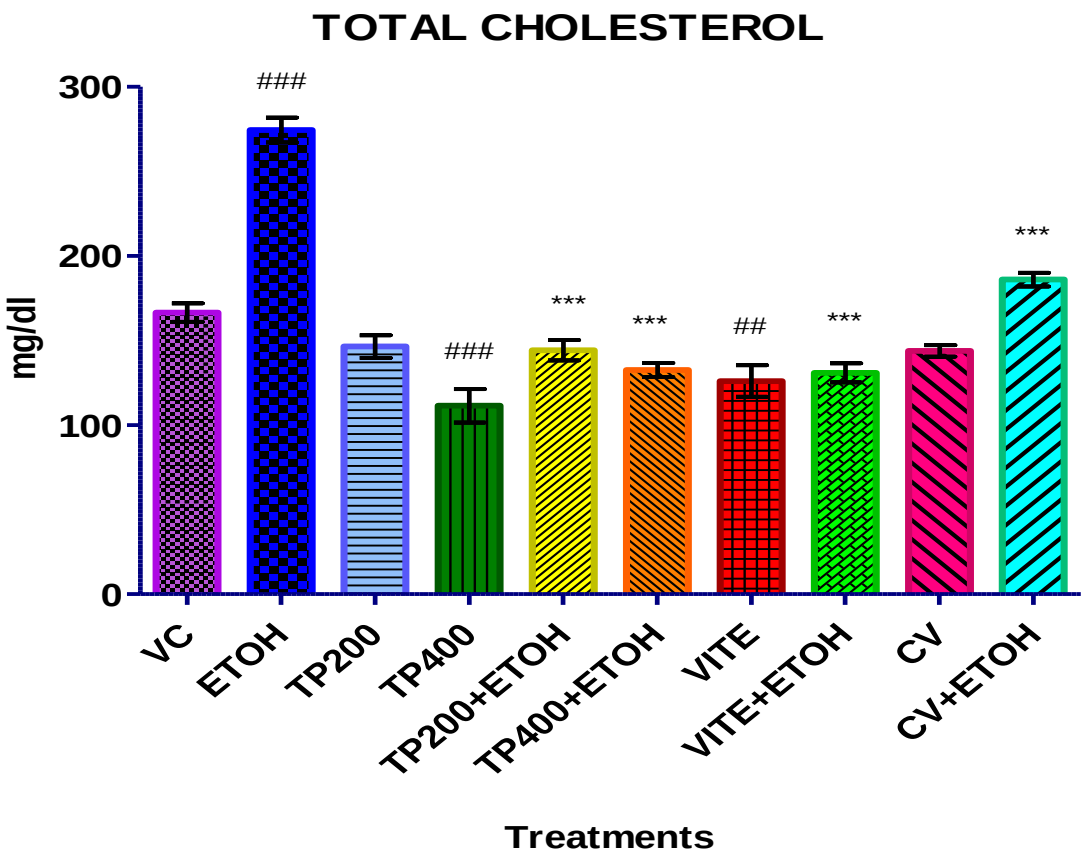

Fig. 63: Histograms representing the changes in serum total cholesterol (TC) levels after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH** - rats treated with carvedilol + ethanol.

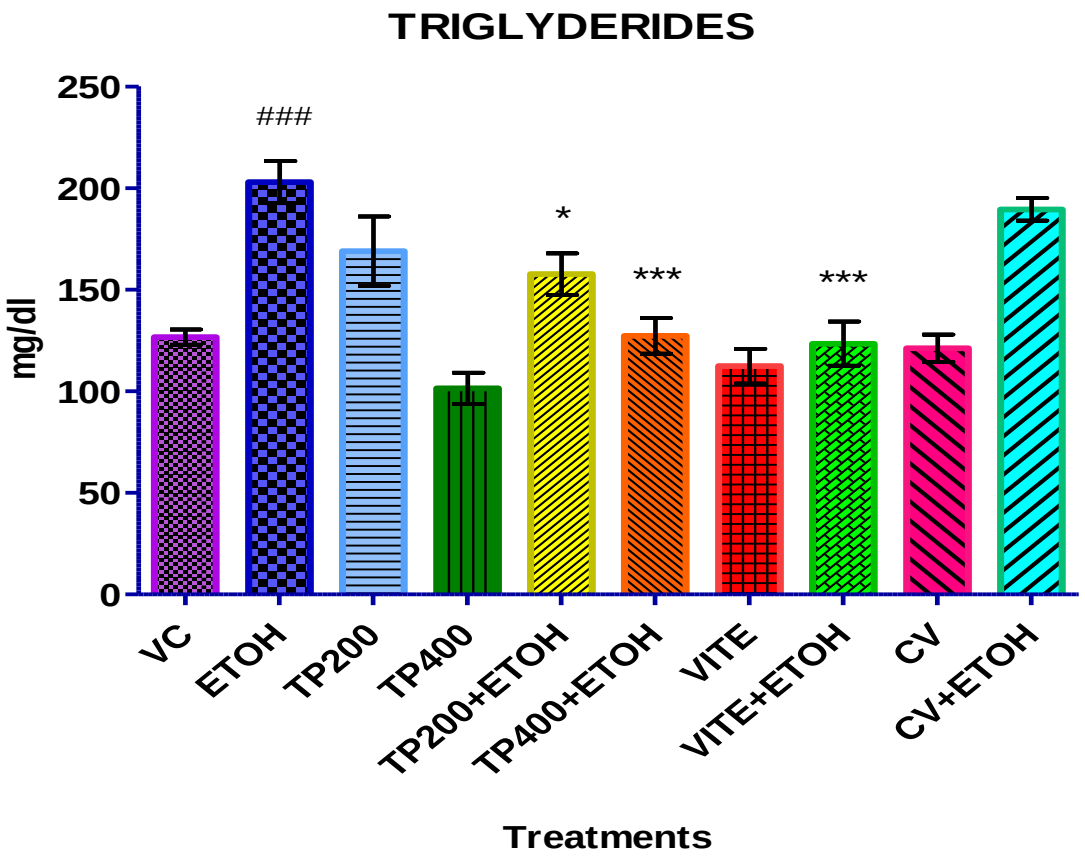

Fig. 64: Histograms representing the changes in serum triglycerides (TG) levels after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

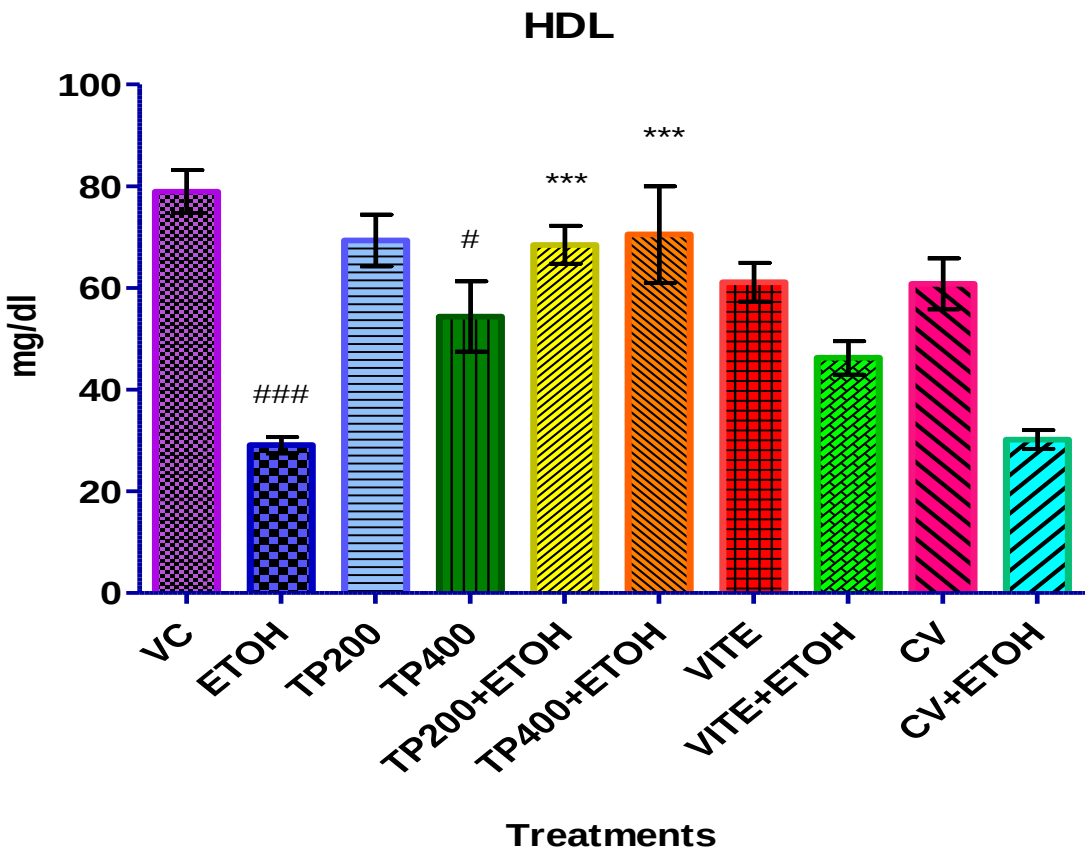

Fig. 65: Histograms representing the changes in serum high density lipoprotein (HDL) levels after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populne*a 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH** - rats treated with carvedilol + ethanol.

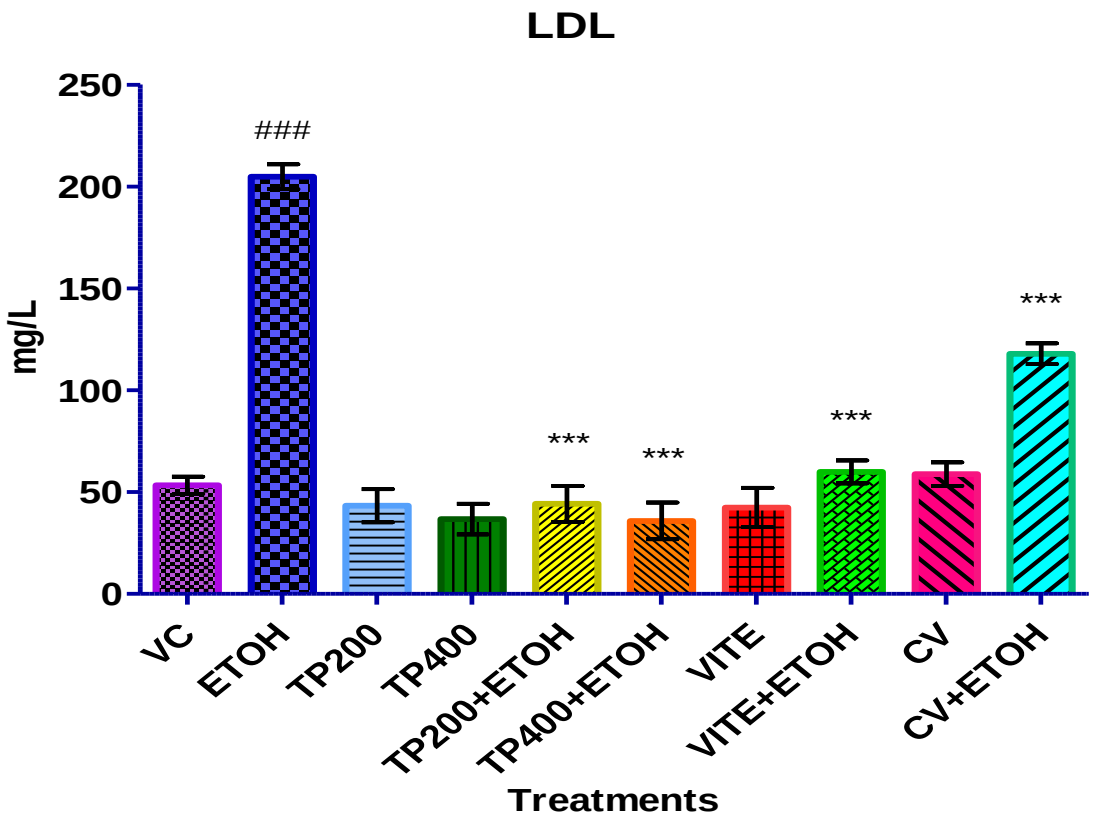

Fig. 66: Histograms representing the changes in serum low density lipoprotein (LDL) levels after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

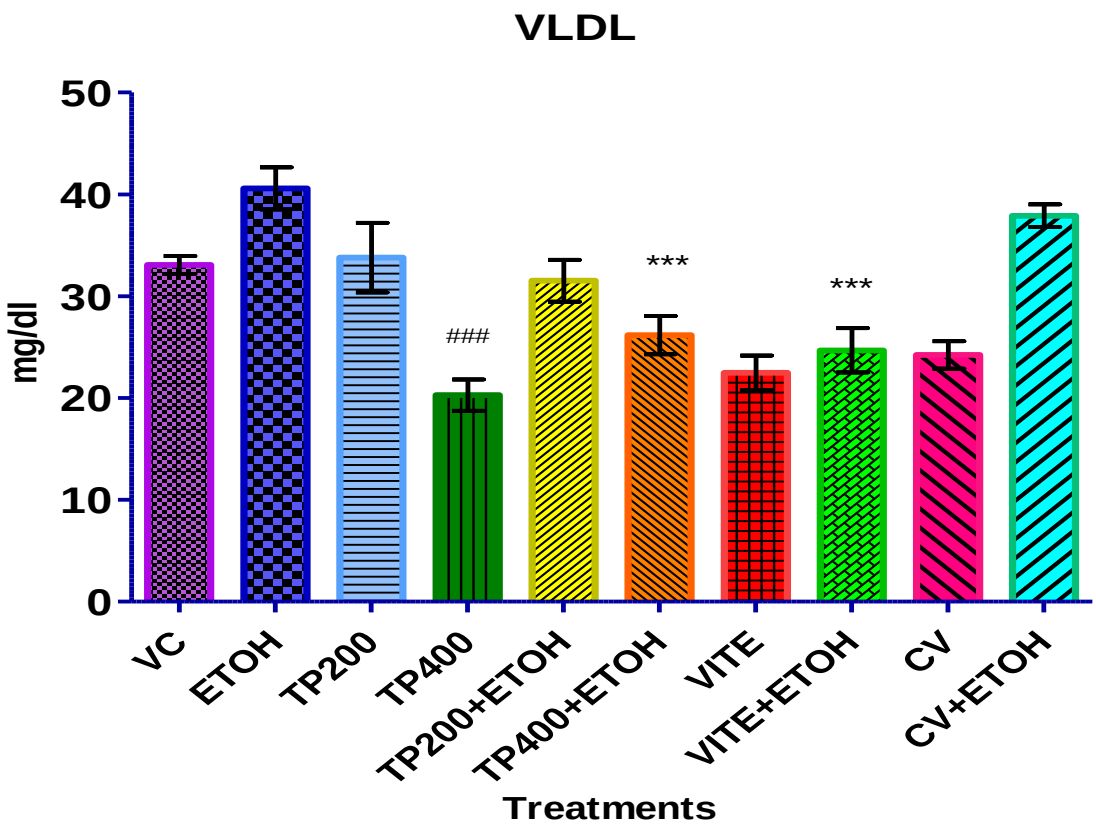

Fig. 67: Histograms representing the changes in serum very low density lipoprotein (VLDL) levels after a treatment period of 6 weeks in control rats and those receiving different treatments.

Data analyzed by one way ANOVA, followed by Tukey's multiple comparisons test, with each point representing mean ± SEM from 6 rats.

#: Value differs significantly from vehicle control group **$^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001**

*: Value differs significantly from ethanol group ***P< 0.05, **P<0.01, ***P<0.001**

Treatments include: **VC -** Vehicle control (rats receiving 5% gum acacia p.o.); **EtOH** - rats administered with ethanol (20%, 2g/kg body wt, p.o); **TP200** - rats receiving *T. populnea* 200mg/kg dose (p.o.); **TP400** - rats receiving *T. populnea* 400mg/kg dose (p.o.); **TP200 + EtOH** - rats treated with TP200 + ethanol; **TP400 + EtOH** - rats treated with TP400 + ethanol; **VIT E** - rats receiving vitamin E (25mg/kg, p.o); **CV** - rats receiving carvedilol (1mg/kg, p.o); **VIT E + EtOH** - rats treated with vit E + ethanol; **CV+ EtOH -** rats treated with carvedilol + ethanol.

4.21 Effect of *Thespesia populnea* leaf extract on histopathological changes in ethanol-induced cardiotoxicity in rats.

Histopathology of cardiac tissue in sections demonstrated a normal architecture of the heart in the vehicle control group (Fig. 68a). Sections of cardiac tissue from ethanol- treatment showed frequent breaks in the muscle strands, with loss of cardiac muscle fibers accompanied by edema indicative of dilated cardiomyopathy (Fig.68b). Cardiac sections of treatment groups receiving TP leaf extracts (200mg/kg and 400 mg/kg respectively), vitamin E and carvedilol (Fig. 68c, 68e, 68g, 68i) showed no notable abnormal histopathological changes compared to ethanol treatment alone. TP200 + ethanol (Fig.68d) exhibited reduced damage to the cardiac architecture with fewer areas of congestion along with vacuolization. TP400 + ethanol (Fig.68f) demonstrated a marked improvement in tissue architecture with preservation of structural integrity of the cardiac muscle fibers in section, absence of edema and congestion with very mild vacuolization.

Vitamin E alone did not affect the appearance of the cardiac tissue and displayed normal, intact myofibrils. With vitamin E plus ethanol treatment (Fig. 68h) decreased myofibrillar vacuolization with mild areas of congestion along with preservation of cardiac tissue structure and absence of congestion and breaks in the muscle strands were observed.

Treatment with carvedilol alone showed no significant alterations in the cardiac tissue as indicated by the absence of edema and presence of intact myofibrillar structure. With carvedilol plus ethanol treatment there were fewer areas of congestion and vacuolization along with partial preservation of myofibrils, marked by decreased breaks in the muscle fibers, myofibrillar vacuolization and marked cellular infiltration was observed.

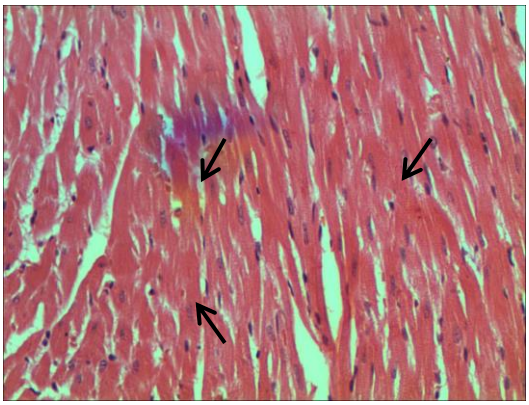

Fig. 68a. Vehicle Control

-Normal cardiac myofibrils
- intact tissue architecture

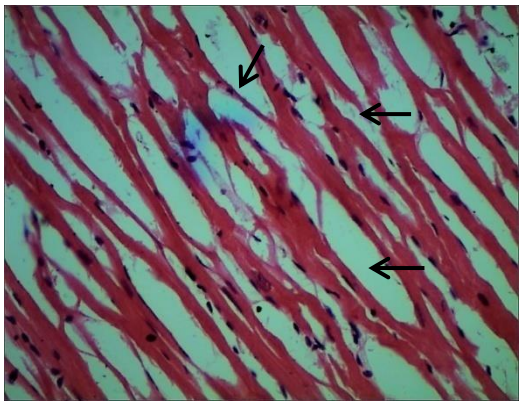

Fig.68b. Ethanol 20% (EtOH)

-Extensive myofibril loss and separation of muscle fibers indicative of dilated cardiomyopathy
-Damage to tissue architecture with breaks in the muscle and large spaces in-between

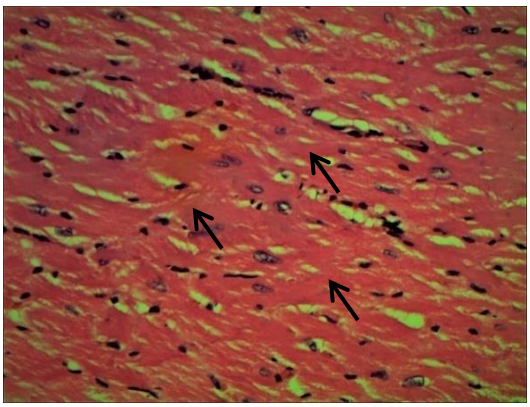

Fig.68c. TP200

-Normal cardiac architecture
-No disruption of muscle fibers

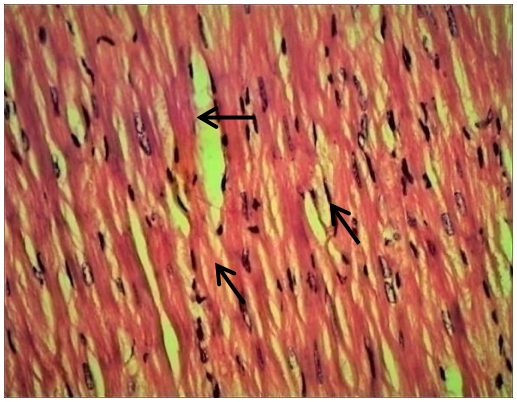

Fig. 68d. TP200+ EtOH

-Myofibrillar vacuolization
-Mild areas of congestion and edema

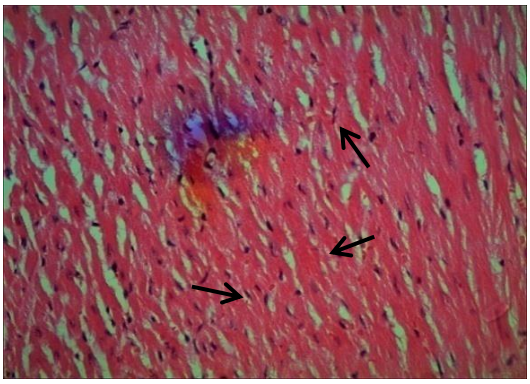

Fig. 68e. TP400

Preserved myocardial architecture

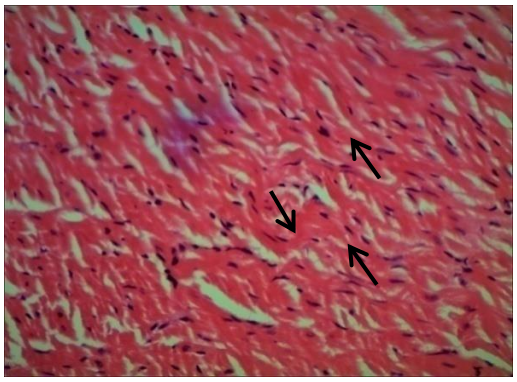

Fig. 68f. TP400+ EtOH

- Minimal Myofibrillar vacuolization
- Absence of necrosis/ Mild areas of congestion and edema

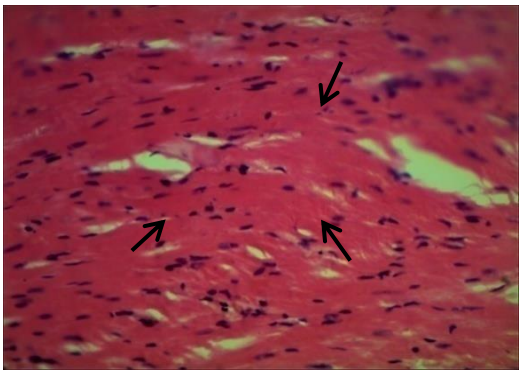

Fig. 68g.Vitamin E

-Normal architecture of cardiac myofibrils
-Absence of congestion and edema

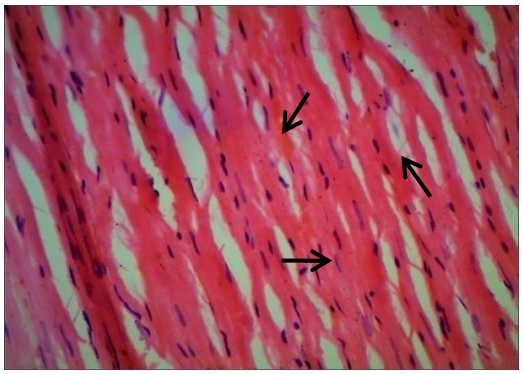

Fig. 68h.Vitamin E + EtOH

-Mild Myofibrillar vacuolization
-Decreased damage to muscle fibers with few areas of mild congestion

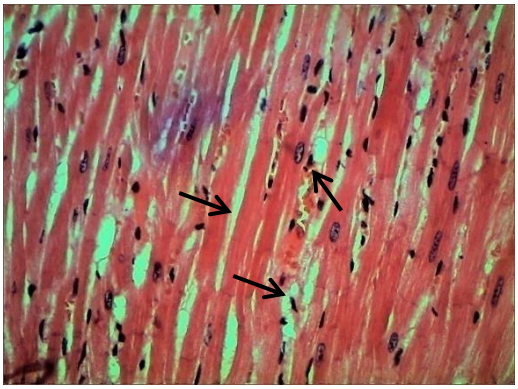

Fig. 68i Carvedilol

-Mild appearance of cytoplasmic organelles and congestion
-absence of fibrillar changes

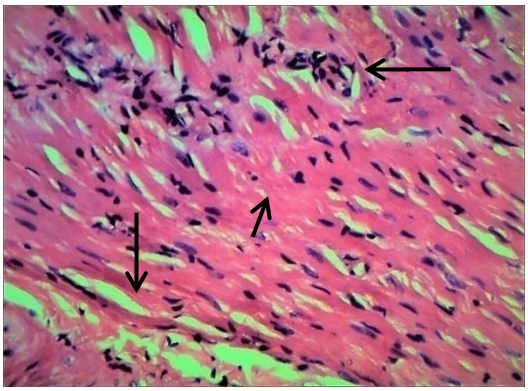

Fig.68j Carvedilol+ EtOH

- frequent damage to myofibril with slight vacuolization and disruption of muscle strands
- Partial preservation of myofibrils
-Accompanied by inflammatory cell infiltration

Fig. 68a-68j: Electrocardiographic recordings representing the changes in QT interval, QRS complex and RR interval durations after a treatment period of 6 weeks in control rats and those receiving different treatments.

CHAPTER-5
DISCUSSION

CHAPTER 5. DISCUSSION

By definition, cardiotoxicity is a condition when there is damage to the heart muscle. As a result of this condition, the heart may not be able to pump blood throughout the body. This may be due to chemotherapy drugs or other medications taken to control disease. Oxidative stress caused by such agents is one of the major factors for the development of cardiotoxicity. This is followed by a subsequent increase in aberrations of cardiac contractility, ultimately resulting in cardiomyopathy and cardiac failure. Cardiotoxic insults resulting due to drug therapy have been reported [267] widely and have been one of the major concerns of cancer chemotherapy. Evidence pointing to several other agents playing an insidious and hazardous role in the development of cardiotoxicity does not rule out the effects of substance abuse such as the use of alcohol or anabolic steroids or cocaine, to name a few [96]. Cardiotoxicity as such has been one of the most devastating conditions of the heart apart from the commonly prevalent cardiovascular disorders such as hypertension, myocardial infarction, congestive cardiac failure, coronary artery disease, etc. Where the insults are cardiotoxic, with drugs or chemicals being the central factors to the conditions, there have been few successful treatments available to check the progress or development of cardiotoxicity.

Herbal drugs or plants possessing phytoconstituents have been one of the principal contrivances of exploring alternatives to conventional treatments for target organ toxicity over the past few decades [269]. Phytoconstituents such as flavonoids, saponins, phenolic compounds, glycosides, plant steroids etc have been researched extensively for their therapeutic benefits. It is also widely acknowledged that such phytochemicals from plant sources are capable of treating a number of disorders [270].

This study encompasses the effects of *Thespesia populnea* on rat heart against adriamycin and ethanol-induced cardiotoxicity, respectively. *Thespesia populnea* (TP) has been reported to contain flavonoids, glycosides, phenolic compounds and saponins which, by virtue of their influence on mitochondrial ion transport and antioxidant effects, confer protection from oxidative damage [52, 271]. The present study was aimed at investigating the cardiac changes *vis-à-vis* serum biochemical changes that develop during chronic administration of adriamycin or ethanol leading to cardiac injury and the possible counteraction of these effects by TP leaf extract. Chronic administration of adriamycin or ethanol to rats was found to effect changes in food-intake and consequently in the body weight apart from the general changes, as reported in the results section. A considerable decrease in the body weight of adriamycin- and ethanol-treated rats was observed during the treatment period. This was accompanied by a reduction in food intake. The decrease in food-intake and body-weight with adriamycin and ethanol as reported earlier [272, 273] could have been possibly because of the decrease in appetite due to these agents. In the present study these changes were effectively countered and the parameters were restored back to normal by TP extract and vitamin E. The striking increase in body weight that was observed with carvedilol treatment in the present study falls in line with the clinical data that suggest an increase in weight gain with carvedilol [274].

A significant increase in heart weight was observed with adriamycin and ethanol treatments, along with a significant decrease in the thickness of the left ventricle wall, followed by a reduction in the percentage cardiac ejection fraction. Changes in the volume of the cardiac tissue with prolonged adriamycin or ethanol administration would be partially reflected by ventricular remodelling that could have occurred as a compensatory protective effect to prevent cardiac damage due to chemical insults. Such increase in heart weight due to either adriamycin or ethanol has been reported in literature [272]. This increase was successfully reduced and brought to near normal with TP leaf extract in the higher dose (TP400).Increased weight gain and heart weight were observed in the carvedilol-treated group compared to the vehicle control. It is reported in literature that carvedilol has a regressive effect on cardiac hypertrophy. Results of the present study are contrary to the reported effects of carvedilol. The reason for this result is not known. The present study just reports the

observed effects of carvedilol and leaf extract, and further exploration on the observed effects is necessary.

A comparison of left ventricular wall (LV) thickness revealed a significantly reduced thickness of LV wall in the disease control (adriamycin/ethanol) groups while it was the thickest in carvedilol-treated group. The thinning of the LV wall in disease control group may be due to an increased after-load, resulting in limiting the cardiac output, while the increased LV wall thickness in carvedilol plus adriamycin or ethanol treatments could be attributed to adaptive cardiac hypertrophy that occurs in the setting of increased after-load. Carvedilol is a non-selective β-blocker without intrinsic sympathomimetic activity. Clinical studies on carvedilol demonstrate a decrease in pre-load and after-load, resulting in an increase in stroke index and cardiac output [275]. The cardio-protective effects of carvedilol are attributed to its antioxidant effects as opposed to its negative inotropic effects [276]. Therefore, the increase in heart weight observed with carvedilol treatment might be due to a decreased wall stress and consequent reduced after-load.TP400 treatment countered such changes resulting due to adriamycin or ethanol treatment, thereby leading to a situation comparable to that of vehicle control. However, further animal studies with *T. populnea* are warranted to elucidate the hypertensive effects that may have led to alterations in the LV wall thickness.

Reduced left ventricular ejection fraction is an important indicator of cardiac failure. Our study is the first to explore the effect of *T. populnea* on cardiac ejection fraction as also in the context of ensuing cardiotoxicity due to adriamycin or ethanol. In the present study Adriamycin or ethanol-treated rats exhibited a significant reduction in ejection fraction, which was effectively countered by TP400, vitamin E and carvedilol treatments respectively. *T. populnea* has been reported to contain flavonoids, glycosides, phenolic compounds, saponins etc. These phytoconstitutents can affect mitochondrial ion transport and oxidative stress [52, 271], leading to amelioration of alteration in left ventricular ejection fraction. Phytoconstituents such as flavonoids, phenolic compounds and saponins in particular may play a role in alleviating the oxidative stress, and probably induce restoration of cardiac function by preserving the myocardial tissue. Presumably, due to the presence of these phytoconstituents in TP leaf extract, an improvement in left ventricular ejection

fraction would have resulted. Improvement in cardiac ejection fraction upon carvedilol and vitamin E treatment in the present study is in confirmation with earlier reports that have suggested an improvement in left ventricular ejection fraction with these drugs [277-279].

The ECG is an invaluable tool to interpret the specific changes that occur in the rhythm of the heart and is a frequently used diagnostic tool by clinicians to characterize conditions of various pathologies of the heart. The ECG obtained in the present study with adriamycin/ ethanol-treatments may be interpreted based on the common premise of a potential cause of toxicity due to oxidative stress. This is consistent with the studies on the rodent ECG [280, 281]. Altered QRS duration and morphology may indicate myocardial fibrosis and block of bundle branch. QT interval prolongation may indicate altered K^+ or Ca^+ ion channel function during repolarization, myocardial infarction and/or heart failure [282, 283].

The ECG findings in adriamycin-treated rats showed a prolongation of QT interval, widening of QRS complex and RR interval, suggestive of ventricular arrhythmias. The abnormal alterations obtained with respect to these intervals are indicative of defective conduction and a decreased ventricular function. Abnormality of ECG changes can also be correlated with a decrease in cardiac function as was evident from the results on percent ejection fraction in the rats receiving adriamycin. These changes could also be the cause of a defective conduction of the myocardium due to alteration of the membrane-bound enzymes. Previous reports have indicated lengthening of QT interval and QRS duration interval following doxorubicin administration to rodents [284, 285], suggesting an increase in the action potential duration. Prolongation of the QT interval and RR interval is suggestive of delayed repolarization and may be indicative of a negative chronotropic effect [286]. These changes in the ECG caused by adriamycin are consistent with similar studies reported by other investigators [287, 288].Effect of TP extract on ethanol-induced changes in the ECG demonstrated a prolongation of QT interval, lengthening of QRS complex and RR interval, indicative of the direct toxic effect of ethanol on the myocardial function. A corresponding decrease in percent ejection fraction with ethanol administration in rats also confirms the aberrations of the heart that might have progressed into alcoholic cardiomyopathy and subsequently cardiac failure due to

ethanol. Alcohols have been studied since many decades for their effects on the myocardial contractility. The abnormal ECG changes are in line with the earlier reports on ethanol-effects on the myocardium [289]. However in contrast to the observations by Ibrahim and co-workers, our observations of the ECG of rats subjected to ethanol intoxication demonstrated a prolongation of QRS complex and RR interval. This could also be related to the duration and dose of ethanol exposure. *T. populnea* in both the doses and vitamin E reduced these abnormal changes caused by adriamycin significantly.

ATPases are membrane-bound enzymes involved in energy mediated translocation of Na^+, Ca^{2+} and Mg^{2+} ions. Studies have shown a reduction in the activity of these enzymes upon damage to the myocardium [290, 291]. Calcium-overload in the myocardial cells during ischemia activates the Ca^{2+}ATPase, depleting high energy phosphate stores and thereby indirectly inhibiting Na^+ and K^+ transport and inactivating Na^+/K^+ ATPase [291]. Reduced activity of Na^+/K^+ATPase and Mg^{2+}ATPase has also been attributed to the loss of sulfhydryl (SH) groups [292] and lipid peroxidation [293]. In the present study, adriamycin and ethanol respectively inhibited Na^+/K^+ ATPase and Ca^{2+} ATPase similar to earlier reports [294]. Ethanol on the other hand interacts with the cellular constituents [295] causing profound changes in their structure, organization and functions. Alteration in lipid components and ion-channels by ethanol can cause changes in membrane function by altering its fluidity [296] and thereby induce inhibition of Na^+/K^+ATPase and Ca^{2+}ATPase activities [297, 298]. *T. populnea* leaf extract also consists of cardiac glycosides and anthraquinone glycosides as its phytoconstituents, as observed from the results of the present study on preliminary phytochemical screening of TP leaf. Cardiac glycosides are basically cardiotonic agents used in the treatment of the failing heart. Due to the presence of cardiac glycosides, *T. populnea* is expected to show inhibitory effects on Na^+/K^+ ATPase. Contrary to this, *T. populnea* brought about an elevation in Na^+/K^+ ATPase. However, this contradictory effect has been observed against adriamycin/ethanol-induced inhibition of Na^+/K^+ ATPase, thus supporting the protective effect of TP extract in chronic cardiomyopathy and ensuing cardiac failure. Similar reports have shown such effects, thus confirming the role of cardiac glycosides in conferring protective effects in drug-induced cardiotoxicity.

Thus, restoration of ATPase activity to normal by TP leaf extract in adriamycin/ethanol-treated rats is attributable to restoration or elevation of the endogenous antioxidant enzyme activities to normal. This may be credited to the antioxidant potential of *Thespesia*, protecting the ATPase enzymes from oxidative degradation [293].

Oxidative stress and free radical damage have been central to all the adverse effects brought about by both adriamycin and ethanol. Both the agents inflict cardiotoxic insult as evidenced from the results of the present study. Although the underlying mechanism for the effects of both these agents (generation of free radicals) is seemingly similar, the toxic effects that could develop to different degrees, in both the settings, have not been completely studied.

Oxidant balance in the heart has a very important role in protecting the heart and in allowing normal cardiac contractile performance. The level of low molecular weight antioxidants and the activity of antioxidant enzymes are lower in myocardium in comparison to other organs. In general, the amount of antioxidants in the heart is sufficient to protect it from any oxidant production that might occur under normal circumstances [299]. Several studies have indicated that adriamycin-induced cardiotoxicity is associated with various complications concerning the heart. One of the most common mechanisms predisposing to adriamycin-mediated cardiac stress is the generation of free radicals such as reactive oxygen species (ROS) [300] and the peroxidation of membrane lipids [126, 301,302]. Morphological changes and reduction of anti-oxidative capacity of the heart have also been observed in animals chronically intoxicated with ethanol, and studies have reported the protective effect of antioxidants against myocardial lipid peroxidation in rats after chronic alcohol ingestion [303].

TP leaf extract was assessed for its antioxidant potential by determining its free radical scavenging activity *in vitro*. This parameter is a noteworthy marker for directly evaluating the antioxidant properties of the leaf extract. A detailed study of *in vitro* antioxidant activity of the plant extract confirmed the free radical scavenging potential of *Thespesia populnea* (TP) which could be attributed to the presence of phytoconstituents such as flavonoids, phenolic compounds, saponins and glycosides in the extract. A concentration-dependent increase was observed in all the radical scavenging assays including the assay of reducing power, superoxide anion, DPPH

and ABTS radicals, indicating that the plant extract might probably confer antioxidant protection *in vivo* as well. The ability to scavenge hydroxyl radicals (TP leaf extract in the concentration of 182.5 μg/ml) and hydrogen peroxide (TP leaf extract in the concentration of 181.98μg/ml) was observed to be higher than all other radical scavenging effects examined in this study. However, the *in vitro* scavenging effects were observed to be, less than those of the standard compounds BHT and ascorbic acid. *In vitro* assessment of the free radical scavenging assays for the extract provided an uncomplicated, quick and expedient method to assess the antioxidant potential and radical scavenging effects of the extract.

Malondialdehyde (MDA) is a major lipid peroxidation end-product, and increased MDA content may contribute to increased generation of free radicals and/or decreased activities of the antioxidant system [304]. In the present study adriamycin-treatment significantly promoted lipid peroxidation which is indicated by an elevation in the MDA levels in the heart tissue of the disease control group. The potential mechanism for increased lipid peroxidation in cardiac tissue may be increased lipid substrate within the myocardium which can serve as a larger target for oxidation by free radicals. *T. populnea* treatment in both the doses (TP200 and TP400), vitamin E and carvedilol effectively suppressed the elevated MDA levels induced by adriamycin in the heart, conferring the desired antioxidant effect for cardio-protection. Acetaldehyde and acetate, produced from the oxidative metabolism of alcohol, contribute to cell and tissue damage in various ways. Most of the acetate resulting from alcohol metabolism escapes the liver to the blood and is eventually metabolized to CO_2 in the heart, skeletal muscle, and brain cells [305]. Acetate increases blood-flow into the liver and depresses the central nervous system, as well as affects various metabolic processes [305]. In patients with alcoholic liver disease the serum markers of lipid peroxidation such as conjugated dienes, MDA, 4-hydroxynonenal and F2-isoprostanes are increased [306]**.** The levels of hydroxyl radicals, which exert their cytotoxic effects by causing peroxidation of membrane phospholipids, are also increased, leading to increased membrane permeability and impairing membrane function [307]**.**

Attenuation of the abnormal changes brought about by adriamycin, with respect to the cardiac antioxidant status was observed with all the treatment groups to different degrees. It has been reported that plant flavonoids such as quercetin, kaempferol,

phenolic compounds and saponins are responsible for protecting tissues from oxidative damage. *T. populnea* has been reported earlier for its antioxidant properties, and its effects on lipid peroxidation, GSH and other antioxidant enzymes have been well documented in various conditions of organ damage [54, 207, 308]**.** In the present study TP leaf extract in both the doses (TP200, TP400) ameliorated adriamycin- and ethanol-induced oxidative stress respectively. This effect could be attributed to the phytoconstitutents such as flavonoids, phenolic compounds and saponins present in the leaf extract. Flavonoids belong to compounds with promising essential features as cardio-protectants. One of the modes of antioxidant action of flavonoids is the chelation of transition metal ions and blocking of their catalytic action in the Fenton and Haber-Weiss reactions, which provide protection to cells from the generation of oxygen oxidants [108]**.** One of the major flavonoids present in TP is quercetin. Quercetin is considered to be a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions. These properties of quercetin allow it to inhibit lipid peroxidation [309, 310]. Chopra and co-workers suggested the lowering of LDL-cholesterol by quercetin in hyperlipidemic patients [311]. Quercetin has been found to reduce the level of ROS by possible suppression of activation of pro-inflammatory nuclear factor-KB (NF-KB)-dependent pathway [312].

The levels of reduced glutathione (GSH) and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidise (GPX), and glutathione-S-transferase (GST) substantially decreased in the heart tissue of the rats receiving adriamycin/ethanol treatment alone. TP400 elevated the enzyme activity and prevented these decrements in antioxidant enzymes by bringing them to normal levels as compared to the ethanol-treated animals.

Cardiomyocytes are more susceptible to adriamycin-induced free radical-mediated damage because these cells have relatively low levels of antioxidant enzymes such as SOD and CAT [32, 313], which provide protection by converting hydrogen peroxide into water and oxygen. There are also several reports that superoxide radicals damage endothelial function, and SOD acts protectively for the endothelial cells [314]. In the present study, SOD activity was significantly inhibited in adriamycin-treated rats and ethanol-treated rats respectively, as reported by earlier studies [315]. Catalysis of dismutation of superoxide radicals to H_2O_2 by SOD thereby reduces the likelihood of superoxide anions interacting with NO to form reactive peroxynitrite [316]. Decrease

in SOD activity due to adriamycin/ethanol indicates reduced efficiency in scavenging of ROS, which might be implicated to oxidative inactivation of the enzyme [317]. Experimental studies on *T. populnea* have shown that the leaf extract exhibits free radical scavenging properties *in vitro* on hydroxyl radicals, peroxyl radicals, and superoxide free radicals [52]. This protection may be credited to the presence of antioxidant principles such as flavonoids, phenolic compounds and saponins, which have proven antioxidant effects [318]. The decrease in activity levels of cardiac SOD and CAT was effectively elevated to normalcy by TP400 leaf extract.

CAT is a heme protein, which decomposes H_2O_2 into a nontoxic product and protects the tissues from highly reactive hydroxyl radicals. CAT is one of the most competent enzymes, and it cannot be saturated by H_2O_2 at any concentration [319]. Ethanol administration reduces CAT activity in cardiac, liver, kidney, and other tissues [320]. In consonance with the earlier findings, a similar trend was noticed in rats treated with alcohol in the present study. The decrease in the CAT activity may be attributed to the inhibition of SOD and ethanol-related loss of NADPH, or excess of H_2O_2 production, or enhanced lipid peroxidation, or a combination of these factors [321-323]. The above findings are in support of our results. The CAT activity was restored to normalcy after treatment with TP extract in the dose of 400mg/kg, which shows the antioxidant property of the leaf extract against ROS. Thus, the analysis of antioxidant status in our study indicates that enzymatic antioxidants decreased due to adriamycin- and alcohol-induced toxicities respectively.

Superoxide ions are removed by SOD in mitochondria [324] and the resultant H_2O_2 is removed by CAT, GPX [325] and peroxiredoxin. Unlike CAT activity, GPX activity depends on the balance between the levels of glutathione and glutathione disulfide [326]. GPX activity was considerably reduced after adriamycin/ethanol treatment when compared to the vehicle control. The observed decrease in GPX activity may also be due to reduced availability of GSH. Thus, decrease in GPX activity may be implicated in both free radical-dependent inactivation of the enzyme [317] and depletion of its co-substrates, that is, GSH and NADPH [327]. In the current study the TP extract in the dose of 400mg/kg showed greater efficacy compared to the TP200 and carvedilol treatments.

Chronic alcohol-intake lowers the mitochondrial GSH [328], which makes these organelles more susceptible to oxidative damage, and precedes the development of mitochondrial dysfunctions such as lipid peroxidation [328] and the impairment of ATP synthesis [329]. The damage accumulated in biomolecules triggered by acetaldehyde exerts its toxic effects by inhibiting the mitochondrial reactions and functions and may injure the function of electron transport chain (ETC). This would lead to the production of ROS, which can oxidize the subunits of ETC complexes, resulting in injury of electron transport and oxidative phosphorylation [330, 331]**,** thus decreasing the ATP levels. In addition, ROS may lead to oxidative stress over lipids, causing lipid peroxidation that affects the permeability of the outer and/or inner mitochondrial membranes.

GSH plays an important role in the maintenance of the intracellular redox state. Perturbations in the redox status and the main protective role of glutathione against oxidative stress are well elucidated by previous reports [332]. Among the endogenous antioxidant systems, reduced GSH plays multiple roles in the detoxification of toxic chemicals [333]. The heart tissue would be more susceptible to oxidative damage compared to other tissues. The observed decrease in GSH levels in the adriamycin-treated rats may be due to diminished activity of GR, which is a crucial enzyme for maintaining GSH/GSSG ratio in the cell. GR is responsible for the regeneration of GSH, and the decrease in GR activity in heart tissue may be due to the decline in the production and availability of GSH to overcome H_2O_2 [334]**.** In consonance with these possibilities, results of the present study demonstrated diminished GR activity accompanied by a decrease in GSH content after adriamycin/ethanol treatment, which was reverted back to normal upon treatment with *T. populnea* extract in the higher dose (TP400). Anthracycline-induced cardiotoxicity is believed to cause a direct damage to mitochondria, mitochondria-dependent apoptosis in the heart and cardiomyocytes, and lipid peroxidation of the cardiac myocyte membrane [335]. Heart tissue is rich in poly-unsaturated fatty acids and is known for its high oxygen consumption. Therefore, it is more susceptible to oxidative stress than other tissues [336].

GST is a detoxifying enzyme that catalyzes the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing less toxic forms [337]. The detoxification of 4-hydroxynonenal, a toxic aldehyde and a product of lipid

peroxidation upon ethanol treatment is compromised when GST activity is reduced. This effect of ethanol has been reported by Alin and co-workers [338]. Thus, ethanol or its metabolic products might specifically target GST isoenzymes and the reduction in enzyme activity. Similar cases of a reduction in GST upon treatment with adriamycin have been reported [339]. In this study, both adriamycin administration and chronic ethanol consumption resulted in a significantly lowered activity of myocardial GST. These reductions in enzyme activities were effectively countered by TP400 and vitamin E respectively against adriamycin or ethanol-induced oxidative stress.

In concurrence with already established reports, cardiotoxicity caused by adriamycin and ethanol, led to the elevation of cardiac biomarkers that included CRP, CKMB, CK, LDH, AST and ALT. This elevation is indicative of their leakage from the subcellular organelles of the heart such as mitochondria [288, 340]. Cardiac biomarkers have also been suggested to increase in conditions of oxidative stress, thereby suggesting a role for free radical generation predisposing to myocardial apoptosis, membrane peroxidation and disruption of cardiomyocytes [341]. Rats treated with TP leaf extract prevented the elevation of these enzymes comparable to near normal values, indicating the protective effect of the leaf extract on the cardiac tissue. TP400 and vitamin E were highly effective in lowering the elevation of these enzyme activities that were significantly increased due to adriamycin or ethanol treatments respectively.

C-reactive protein (CRP) is primarily reckoned as a marker of inflammation and has emerged as an important predictor of cardiovascular risk in both men and women [342-344]]. Studies report the negative effects of elevated levels of CRP on the vascular endothelium. This effect of the cytokine is suggested to be due to reduction of release of nitric oxide along with an increase in endothelin-1 and an induction of expression of endothelial adhesion molecule [345,346]. Therefore, raised CRP levels could also play a role in vascular disease and lead to further cardiac complications such as LV dysfunction, hepatic or renal injury induced by a low cardiac output, hypoperfusion, hypoxia and venous congestion [347]. In line with earlier reports, the present study recorded significantly increased levels of CRP in adriamycin- and ethanol-treated rats, indicating inflammation. Treatment with TP200, TP400, vitamin E or carvedilol prior to adriamycin-/ethanol-administration caused a significant

decrease in the CRP levels, indicating a reduction of the harmful effect of adriamycin or ethanol.

Significant increases in creatine kinases (CK-MB and CK) were observed in Adriamycin- or ethanol-treated rats in the present study which is indicative of myocardial injury. Creatine kinases are cytosolic or mitochondrial enzymes expressed predominantly in the brain, muscle and heart [350]. Myocardial CK plays an important role in the metabolism of phosphate compounds that are closely related to muscle contraction process [351]. Alterations in the serum levels of these enzymes have been suggested to be indicative of cardiac damage. Reports strongly emphasize the role of free radicals as a major predisposing factor for alteration of functioning of CK, leading to damage of heart cells. Cardiotoxicity due to Adriamycin could be due to the inactivation of CK resulting from the generation of oxygen free radicals [352]. This enzyme has been observed to be inactivated by superoxide (O_2^-) radicals [353] that are likely to be elevated after the administration of ethanol. Chronic administration of ethanol leading to raised CK levels in the serum and a low myocardial CK activity have been documented in earlier studies in rats fed for 6 weeks on ethanol-enriched diet [354]. The results of our study are in agreement with these reports. Prior treatment with TP400 and vitamin E with adriamycin or ethanol significantly prevented the abnormal elevations in these enzyme activities, and this could be attributed to their ant oxidative effects, leading to protection of the myocardium.

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of lactate and NAD to pyruvate and NADH [355]. It is predominantly found in skeletal muscles, heart and liver. Decreased oxygen supply to the heart causes hypoxic injury to the myocardium, resulting in cell damage, rupture of the cell membrane and leakage of the enzyme into the blood. The effect of cumulative doses of adriamycin leading to myocardial damage was examined by earlier studies that confirmed the elevation in LDH enzyme activity, suggesting cardiac damage [356]. A similar increase in LDH activity has been observed with adriamycin and ethanol treatments respectively in our study. The protective effects of TP400 in the lowering of the serum levels of this enzyme against otherwise elevated levels of LDH due to adriamycin and ethanol may be primarily due to the cell membrane-stabilizing effects of the plant extract in a dose-dependent manner. Vitamin E was observed to be

conferring a better ameliorative effect on LDH levels, probably due to its superior ant oxidative effects in drug-induced cardiotoxicity as reported earlier [279].

Aspartate (AST) and alanine (ALT) are enzymes found predominantly in liver cells, but they also serve as markers of myocardial damage due to their occurrence in cardiomyocytes. The activities of both these serum transaminases were significantly elevated in our study with adriamycin and ethanol treatments respectively. Elevations in these enzyme activities in the serum is indicative of the myocardial membrane damage, and this negative effect of adriamycin and ethanol respectively was effectively countered by TP200, TP400, vitamin E and carvedilol. Of these, TP400 was found to be the most effective in preventing the elevations of these enzymes and brought their levels to normalcy. These results are in line with the studies carried out on plants, as they are rich sources of antioxidants [357]. Such studies exploring the effects of plant extracts with known phytochemical principles have suggested a beneficial role for flavonoids, phenolic compounds and saponins in countering and reversing the abnormal changes caused by chemicals and drugs at the cellular level. *T. populnea* is rich in flavonoids, phenolic compounds and saponins, and hence the lowering of serum transaminase activities leading to an effective recovery to normal state can be attributed to its phytochemical constituents.

Significant elevations of serum cholesterol, triglycerides, LDL and VLDL were recorded in our study with adriamycin and ethanol treatments respectively. Studies with adriamycin have shown that the drug reduces the rate of lipolysis [358] along with a reduction of HDL cholesterol. The increase in cholesterol, triglycerides, LDL and VLDL was significantly high for ADR-treated rats. This could be attributed to the administration of cumulative dose [359] of adriamycin**.** These results are in line with earlier studies reporting the changes brought about by adriamycin on serum lipids [360, 361]. The results from our study document the adverse effect of adriamycin on serum lipids, inducing a condition of hyperlipidemia. Hyperlipidemia, a condition indicating elevated circulating levels of lipids can cause atherosclerosis and subsequently affect cardiac function. In such a setting of elevated cardiac biomarkers and already established oxidative stress due to adriamycin, it is likely that the drug might be predisposing to cardiac failure. This is in confirmation with the conclusions made from similar studies on adriamycin on serum lipids. High circulating levels of cholesterol, triglycerides and LDL as reported [362] could further the accumulation of these lipids in

the blood vessels and heart tissue. Such developments are well associated with cardiovascular damage [363]. Alterations in the metabolism of lipids can also affect cardiac function through infliction of changes in the properties of cardiac cell membrane. Such changes can also significantly contribute to the reduction in contractility of the myocardium, arrhythmias and cell death, which may ensue with coronary artery occlusion [364]. Similar changes in serum lipids were recorded with ethanol-treatment in the present study. Although alcohol consumption in moderation has been reported to have a beneficial effect on the heart, cardiovascular mortality has been reported in cases of heavy, chronic consumption of alcohol [365].Adverse consequences of chronic heavy consumption of alcohol on the myocardium, such as stroke and sudden cardiac death, can be correlated to increased clotting, rise in low density lipoprotein (LDL) cholesterol concentration and lower threshold for ventricular fibrillation [361]. Reports suggest that alcohol abuse may be associated with hypertriglyceridemia; and that nearly 1 in 5 hospitalized alcoholics have triglyceride levels exceeding 250 mg/dL [366].

Results of the present study derive substantial support from the earlier reports on lipid changes due to adriamycin/ethanol treatment. As suggested in literature, the lipidemia induced by ethanol could be due to the inhibition of lipoprotein lipase (LPL) mediated hydrolysis of chylomicrons [367-369].

The abnormal changes brought about by adriamycin and ethanol respectively were prevented effectively to vehicle control levels by TP400 and vitamin E, while TP200 and carvedilol were less effective in modulating the changes in serum lipids towards the vehicle control. This is suggestive of the lipid lowering properties of *T. populnea* leaf extract that have not been reported till date.

The histopathological changes brought about by adriamycin and ethanol in the rats receiving either of these treatments displayed characteristic deviations from the normal cardiac histo-morphological features. Adriamycin and ethanol respectively caused extensive damage to the cardiac musculature defined by separation of myofibrils and extensive vacuolization with extensive muscle damage. The extent of damage with adriamycin/ethanol administration respectively was countered effectively by prior treatment with TP200, TP400, vitamin E and carvedilol. The histopathological changes observed with these treatments varied to different degrees,

but were milder, with lesser vacuolization of cardiac myocytes, reduced breaks in the muscle fibre stands and preserved integrity of the muscle architecture. The results of histopathological changes explain the cardiac muscle preservative effects and hence cardioprotection conferred by *T. populnea* extract and vitamin E on adriamycin- or ethanol-induced cardiac damage. The amelioration of biochemical changes is also supported by histopathological studies which exhibit the adverse effects of adriamycin on the myocardium. Manifestations like myofibrillar breaks and detachment of muscle strands, accompanied by degeneration of muscle fibers is confirmed by similar studies evaluating adriamycin effects [288].

On an overview, it may be stated that in spite of extensive research on the therapeutic potential of *T. populnea*, till date there are no studies demonstrating its effects on cardiac function. The study demonstrated that the aqueous extract of *Thespesia populnea* leaf possesses antioxidant activity owing to its radical scavenging effects, and this attribute could be of considerable benefit in preventing or reducing the progression of diseases that may occur due to oxidative stress. This study has made an attempt to evaluate the cardio-protective effects of *T. populnea* leaf extract in terms of biochemical, cardiac antioxidant, electrocardiographic and histological parameters. Further investigations of this study can be directed towards elucidating the mechanisms underlying the protection offered by the plant.

CHAPTER -6

SUMMARY AND CONCLUSIONS

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The present study was aimed at evaluating the cardioprotective potential of the plant *Thespesia populnea* in two models of cardiotoxicity, induced by 1) the anti-cancer anthracycline adriamycin and 2) ethanol.

Although the cardiotoxic effects produced by adriamycin and ethanol were observed to be similar, there were notable differences between each, which were significant of their cardiotoxic potential. The percent cardiac ejection fraction upon adriamycin treatment was strikingly low. Adriamycin treatment raised CK levels significantly while a significantly higher serum LDH levels were obtained with ethanol treatment. An abnormal alteration of lipids and lowering of antioxidant enzymes was greater with ADR treatment. Finally, histopathological changes showed that the myocardial damage inflicted by adriamycin was greater. These changes were comparatively less severe in ethanol treated rats; nevertheless, both the agents displayed a derailment of myocardial parameters confirming their cardiotoxic potential.

Under both the protocols the following aspects were observed:

1) Adriamycin (ADR)/ethanol induced a decrease in food intake and body weight. Treatment with *Thespesia populnea* (TP) in the dose of 400mg/kg, vitamin E (25mg/kg) and carvedilol (1mg/kg) along with adriamycin prevented the abnormal changes in both these physical parameters comparable to normal values.

2) Heart weight was increased and thickness of left ventricle wall and percent cardiac ejection fraction were decreased upon adriamycin/ethanol treatment. Treatment with

TP400, vitamin E and carvedilol significantly decreased the heart weight to near normal, while TP200 did not elicit any significant improvement. Thickness of the left ventricle wall and cardiac ejection fraction were comparable to near normal levels with TP400 and vitamin E, while TP200 and carvedilol (CV) did not prevent the abnormal changes in the cardiac function parameters, although they improved the left ventricle wall thickness and cardiac ejection fraction.

3) QT interval, QRS complex duration and RR intervals were prolonged with ADR/ethanol treatment, indicating abnormal ventricular depolarization and repolarization. Treatment with TP200, TP400, vitamin E and carvedilol improved the ECG intervals by reducing their durations to near normal values. Treatment with carvedilol alone did not show any significant changes in ECG intervals compared to ADR/ethanol treatment alone, while carvedilol + ADR exhibited a highly significant increase in RR interval compared to ADR treatment suggesting prolonged repolarization and increased time taken between each heartbeat.

4) The activities of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase were significantly diminished in the heart tissue of adriamycin/ethanol-treated rats. The enzyme activities were elevated upon pre-treatment with TP400 and vitamin E to near control values. TP200 and carvedilol could partly effect an increase in enzyme activities. All the three ATPase activities were comparable to normal values by TP400, vitamin E and carvedilol pre-treatments.

5) Lipid peroxidation (indicated by malondialdehyde, MDA levels) was significantly elevated, and reduced glutathione (GSH) levels were decreased by ADR/ethanol. The activities of superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidise (GP_X), glutathione reductase (GR) and glutathione S-transferase (GST) were significantly lowered by ADR/ethanol treatment. Pre-treatment with TP400, Vitamin E and carvedilol significantly reduced the MDA levels and elevated the GSH levels accompanied by the elevation of SOD, CAT, GP_X, GR, and GST. Although the effects of TP200 and carvedilol were observed to elevate the antioxidant enzymes, the values were not significant in comparison to the normal.

6) Serum biomarkers of cardiac injury represented by C- reactive protein (CRP), activities of creatine kinase-MB (CK MB), creatine kinase (CK), lactate dehydrogenase (LDH),

aspartate aminotransferase (AST), and alanine aminotransferase (ALT) showed a rise in levels with ADR/ethanol treatment. TP in the dose of 400 mg, vitamin E and carvedilol effectively lowered the abnormal elevation in the biomarkers in serum, while TP200 did not exhibit a significant decrease in the serum biomarker levels, although the reduction was significant compared to ADR treatment.

7) Total serum cholesterol (TC), triglycerides (TG), LDL-C and VLDL-C were increased by ADR/ethanol, while HDL-C levels were lowered. The effects of TP200, TP400, vitamin E and carvedilol were significant in that all the groups receiving pre-treatment with these drugs showed lowering of cholesterol, TG, LDL-C, VLDL-C and a rise in HDL-C levels to near normal values. Pre-treatment with TP400, vitamin E and carvedilol were effective in reducing the lipid changes comparable to the control values.

8) Treatment with ADR/ethanol showed extensive loss of myofibrils and separation of muscle fibres, along with damage to tissue architecture with breaks in the muscle fibres and large vacuolated spaces in between. These changes are suggestive of dilated cardiomyopathy. Individual treatments with TP200 and TP400 did showed normal cardiac architecture with intact myofibrils. Prior treatments with TP200 and TP400 followed by adriamycin treatment showed minimal myofibrillar vacuolization and mild areas of congestion and edema. Vitamin E + ADR showed mild myofibrillar vacuolization and decreased damage to muscle fibres with few areas of mild congestion. Carvedilol + ADR treatment group exhibited frequent damage to myofibrils with breaks in the muscle strand length and partial preservation of myofibrils.

In conclusion, the present study attempts to explain the effects of *Thespesia populnea* leaf extract on the abnormalities induced by adriamycin and ethanol, and presents a possibility of cardio-protection by the plant. Further studies to evaluate the several effects produced by adriamycin and ethanol respectively and the potential of *T. populnea* in countering them may pave way for understanding the mechanisms by which cardioprotection is conferred in general and by this plant in particular. For most of the parameters in the present study, one common mode of ameliorative effect by *T. populnea* is presumably through its antioxidative potential.

CHAPTER -7
BIBLIOGRAPHY

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भारत सरकार / Government of India

पर्यावरण एवं वन मंत्रालय / Ministry of Environment & Forest
भारतीय वनस्पति सर्वेक्षण / Botanical Survey of India
शुष्क अंचल क्षेत्रीय केंद्र / Arid Zone Regional Centre
खेमे का कुआँ के पास, पाल–बासनी केनाल लिंक रोड / Near Kheme Ka Kuan, Pal-Basni Canal Link Road
सुभाष नगर–II पी.ओ.–नंदनवन / Subhash Nagar-II, P.O.-Nandan Van
जोधपुर–342008 (राजस्थान) / Jodhpur-342008 (Raj.)

No.: BSI / AZRC / I. 1202/Tech./2012-13 (Pl. Id.)/719 Date: 23/ 01/ 2013

CERTIFICATE

This is to certify that the plant specimen sent to this Regional Centre by **Ms. Sangeeta L.A. Rajbanshi** is identified as follows:

Thespesia populnea (L.) Soland. ex Corr. [Family-Malvaceae]

(Vinod Maina)
Scientist 'C' & HOO

Tel. / दूरभाष: 0291-2740415, 2747163, Fax No. : 91-291-2741736 E-mail ID: bsiazc@yahoo.com

BABARIA INSTITUTE OF PHARMACY

CERTIFICATE

This is to certify that the experimental protocol titled **"EVALUATION OF CARDIOPROTECTIVE EFFECT OF *THESPESIA POPULNEA* WITH SPECIAL REFERENCE TO ANTIOXIDANT ACTIVITY"** and bearing the proposal number **BIP/IAEC/2015/07** has been approved by the IAEC vide its meeting held on **3rd July 2015.**

Name of Chairman, IAEC:
Dr. Vandana B. Patel

03/7/15

Signature with date

Name of CPCSEA nominee
Dr. Ramtej Jayram Verma

3/7/2015

Signature with date

LIST OF PUBLICATIONS

i) Poster presentation:

1. *Thespesia populnea* leaf extract counteracts the effects of adriamycin on ATPase activity in rat heart. Sangeetha L.A.R*, Archana P. & Vasu Appanna, at the Indian Pharmaceutical Congress (IPC), Vishakhapatnam.16-18 December, 2016.

ii) Oral Paper presentation:

1. Oral paper presentation "Ameliorative Effect of *Thespesia populnea* Leaf Extract on Adriamycin-Induced Cardio-Toxicity in Rats". Sangeetha Rajbanshi*, Archana Paranjape[2] & Vasu Appanna[3] at Golden Jubilee International Conference of Indian Pharmacological Society Southern Region 2017, MGMCRI, Puducherry. 4-5th July 2017 and was awarded best paper presentation in the Govind Achari Prize Session.

iii) Papers published:

1. Rajbanshi SLA, Paranjape AN, Appanna V. Counteraction of adriamycin-induced alterations in cardiac enzymes by *Thespesia populnea* leaf extract. *J App Pharm Sci*, 2017; 7 (07): 150-157.

2. Sangeetha LA Rajbanshi, Archana N Paranjape, Vasu Appanna. Ethanol-induced alterations in cardiac enzymes–ameliorative effect of *Thespesia populnea* leaf extract. *Int J Pharm Pharm Sci* 2017; 9(8):161-167.

iv) Grant Received: Received a Minor Research Project Grant of Rs. 3, 00,000/- (Sanction Letter No. GUJCOST/MRP/2014-15/378 dated 30th June 2014) for the project titled " Evaluation of Cardio protective effect of *Thespesia populnea*, with special reference to antioxidant activity" from Gujarat Council on Science and Technology (GUJCOST), Gandhinagar, Gujarat for the period 2014 – 2016.