2. Evaluation of protective role of Fermented Papaya Preparation on multi organ toxicity induced by Doxorubicin.

2.1. INTRODUCTION

2.1.1. CHEMOTHERAPY, from rarely used cancer treatment since centuries, has evolved as one of the most commonly used therapy today. Due to the advances in chemotherapy there is extensive progress in treating cancers. Studies suggest the potential use of cytotoxic chemotherapy as a definitive treatment or as an adjuvant therapy in asymptomatic patients with the aim of improving survival. However, the early progression in a few tumour sites, have not been seen in the more common cancers (Kearsley *et al*., 1986; Braverman *et al*., 1991; Weissman, O'Donnell and Brady *et al*., 1993; Tannock 1998). For most patients, the use of cytotoxic chemotherapy doesn't prolong the survival but the palliation of symptoms is more important outcome, to improve quality of life (Slater *et al*., 2001). Sometimes, however, the quality of life of cancer patients may be seriously compromised, due to the side effects of these chemicals. Chemotherapy is a systemic treatment that affects not only cancer cells but virtually every other cell in the body, especially those that are undergoing constant growth.

2.1.2. DOXORUBICIN, a quinone-containing anti-cancer antibiotic, is one of such chemotherapeutic agent which is very effective against both solid and soft tumours. It is now widely used as an antineoplastic agent for a wide range of cancers including leukaemia (Gelber *et al*., 2003) and solid tumours, i.e. breast cancer (Minotti *et al*., 2004).The clinical efficacy of this drug is greatly restricted because of the development of a severe form of cardiotoxicity as side effect in cancer patients, after cessation of doxorubicin chemotherapy (Carter *et al*., 1975).

2.1.2.1. Side Effects: The side effects of anthracyclines, like any other chemotherapeutic agent, are linked to cytotoxicity of non-differentiated, proliferating normal cells. These side effects include nausea, vomiting, and alopecia. However, the major toxicities of anthracyclines include cardiotoxicity and myelosuppression and these are the major limitations of these drugs. Doxorubicin can also cause severe local tissue necrosis and affect organs like liver and kidney. Cardiomyopathy and congestive heart failure, however, are the two major side effects of anthracyclines.

2.1.3. CARDIOTOXICITY: Cardiotoxicity is defined by the National Cancer Institute as the 'toxicity that affects the heart'. It is a medical emergency that occurs when blood supply to the part of heart is interrupted. Heart becomes weaker and its efficiency in pumping and therefore circulating blood is hampered. This results in ischemia, which causes damage and death of heart tissue. It may also cause arrhythmias or can develop into heart failure. Reperfusion of coronary blood flow following ischemia, has deleterious consequences on cardiac function. This may lead to an extension of myocardial tissue injury due to accumulation of free radicals after reperfusion (Gupta *et al*., 2004).

Cardiotoxicity can also be responsible for long term side effects that may cause severe morbidity in surviving cancer patients. It includes a wide range of cardiac effects from small changes in blood pressure and arrhythmias to cardiomyopathy. In more serious cases it results in congestive heart failure (CHF), heart attack, or death (Marieb *et al*., 2003). Congestive heart failure is a cumulative dose-limiting adverse effect of anthracyclines (Van dalen 1977).

Cardiotoxicity caused by doxorubicin may be divided into acute, sub acute and late forms. The acute form is a myocarditis–pericarditis syndrome with side effects like

nausea, vomiting and arrhythmia. It is rare with current treatment protocols, and is often reversible. The sub-acute form occurs several weeks or months after the end of doxorubicin treatment, and has about 60% mortality. The late forms may be seen as late as 4-20 years after the end of treatment (Lafark *et al*., 1973).

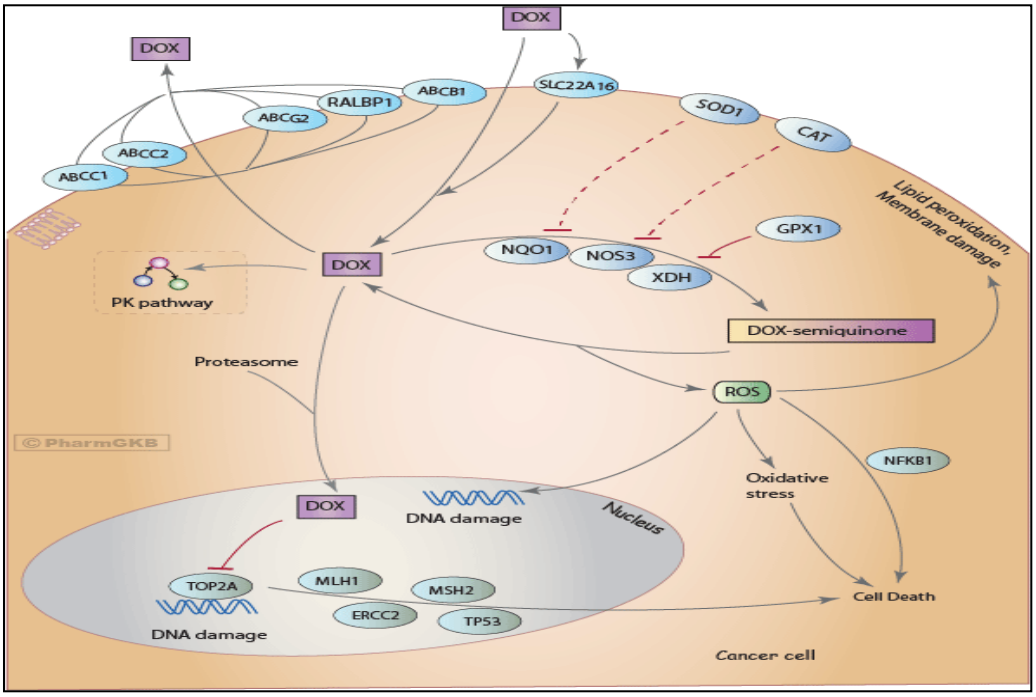
2.1.3.1. Mechanism of action: The antitumor activity of Doxorubicin appears to be due to initiation of DNA damage via the inhibition of topoisomerase II. This leads to the final cellular response like generation of free radicals and peroxidation of lipids. Doxorubicin induced cardiotoxicity, however, has long been stated to involve other mechanisms as well, including **oxidative stress,** cell necrosis, and induction of **apoptoti**c pathways. The intrinsic and extrinsic apoptotic pathways lead to **mitochondrial** impairment (Singal and Iliskovic 1998; Minotti *et al*., 2004).

2.1.3.1.1 Accumulation of anthracyclines in the nucleus of neoplastic and proliferating cells:

Doxorubicin enters the cell through passive diffusion (Skovsgaard 1982). Kiyomiya and colleagues have proposed a mechanism of the selective transport of doxorubicin to the nuclei of neoplastic as well as proliferating cells (Kiyomiya *et al*., 2001; Kiyomiya 2002). It has been demonstrated that, once doxorubicin enters the cells, it binds the proteasomes in the cytoplasm for which it has high affinity. The drug-proteasome complex is then translocated into the nucleus. There is relatively higher transport of doxorubicin into the nucleus of the neoplastic and non-differentiated, proliferative normal cells (Amsterdam *et al*., 1993; Kumatori 1990). Once the doxorubin reaches the nucleus, it dissociates from the proteasomes and bind with DNA due to its higher affinity for DNA. This brings about the DNA mediated effects of doxorubicin. Moreover, binding of doxorubicin to

proteasomes inhibits the protease activity leading to inhibition of degradation of proteins involved in cell growth and metabolism, thus inducing apoptosis of these cells. (Skovsgaard 1982; Kumatori 1990; Palmer 1994)

Fig:2.1.1. Accumulation of anthracyclines in the nucleus of neoplastic and proliferating cells:



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2.1.3.1.2. Free radical generation in mitochondria:

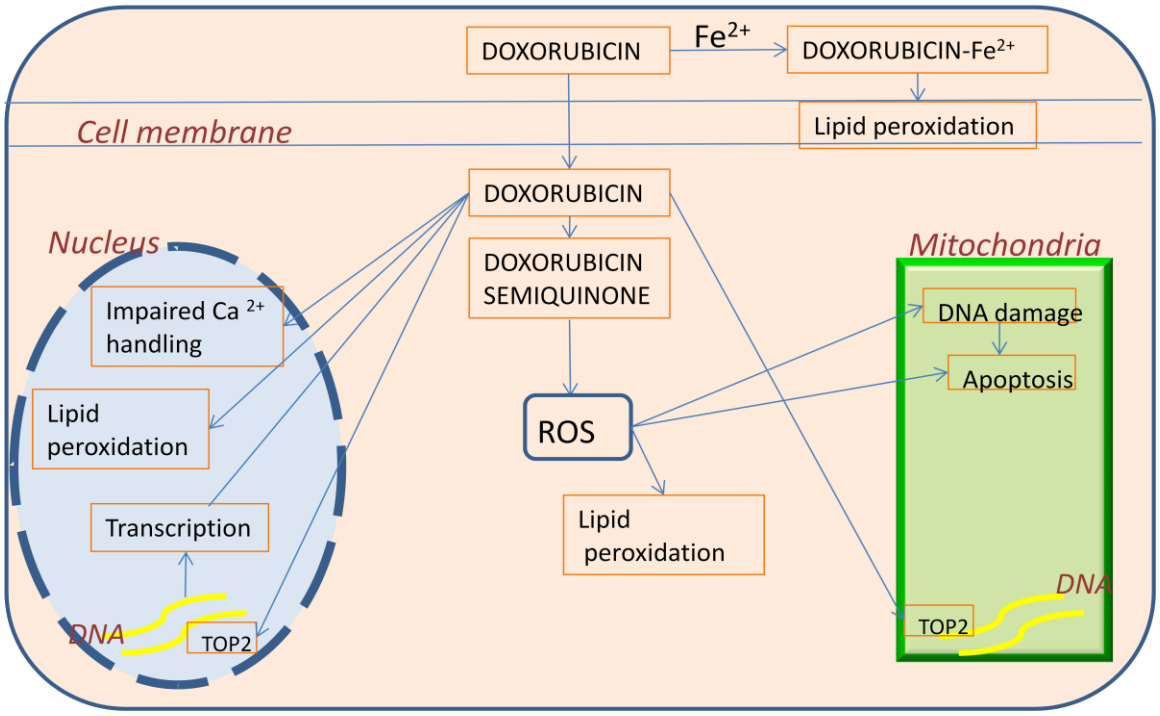
Heart tissue that is rich in mitochondria, heavily relies on oxidative metabolism, and thus produces significant amounts of free radicals. In mitochondria, the affinity of doxorubicin to cardiolipin, an anionic phospholipid specific for the inner mitochondrial membrane, is high. It has been recognized as an essential phospholipid in eukaryotic energy metabolism. Cardiolipin with its particular ability to interact more or less specifically with many proteins is very important not only for mitochondrial structure and function but also for overall cardiac energy metabolism as well as for cell survival. Accumulation of redox active doxorubicin in mitochondria enhance the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Philip and Pharm 2003) .Cardiolipin-bound doxorubicin also induces dissociation of cardiolipin-associated peripheral proteins from the inner mitochondrial membrane, like cytochrome C and Mt.CK. This may affect electron transport chain and energy channeling, as well as favour initiation of programmed cell death (Angelo *et al*., 2005; Kondlepu *et al*., 2014).

2.1.3.1.3 Oxidative stress:

Increased oxidative stress, release of free radicals, including superoxide anion (O_2^-) and other reactive oxygen intermediates as well as endogenous antioxidant deficits have been suggested to play a major role in doxorubicin induced myocardial toxicity and heart failure (Hanaa *et al*., 2005). The reaction starts with a one-electron reduction of doxorubicin to form a doxorubicin semiquinone radical by a reduced flavoenzyme such as NADPH-cytochrome P_{450} reductase. Semiquinone radical forms a complex with iron leading to an anthracycline-iron (Fe^{2+}) free radical complex. This complex reduces oxygen to produce superoxide and to regenerate doxorubicin. The superoxide is

dismutated into hydrogen peroxide and oxygen. Doxorubicin binds to the reductase domain of endothelial nitric oxide synthase. This causes an increase in. superoxide and a decrease in nitric oxide formation. The consequent formation of peroxynitrite may also play a role in the cardiotoxicity (Vasquez-Vivar *et al*., 1997). From the combination of superoxide, hydrogen peroxide and free iron, lipid peroxidation may be initiated. The specific susceptibility of the cardiac cells to the oxidative stress is attributed to relatively low levels of antioxidant enzymes in the heart (Doroshow *et al*., 1980).

Fig: 2.1.2. Oxidative stress:



Several reports suggest that doxorubicin induced apoptosis plays an important role in its cardiotoxicity. This is linked to formation of reactive oxygen species (ROS) derived from redox activation of doxorubicin (Kalyanaraman *et al*., 1980; Sawyer *et al*., 1999;

Kotamraju *et al*., 2000). Apoptosis at low concentrations (1 μM) of doxorubicin and necrosis at high concentrations (>10 μM) was shown by Guchelaar *et al*., 1998.

2.1.3.1.4. Anthracyclines, p53 and apoptosis:

The transcription factor p53 has been reported to play a very important role in apoptosis (Agarwal *et al*., 1998; Ko and Prives 1996). As a tumor suppressor, p53 is responsible for protecting cells from tumorogenic alterations (Ko and Prives 1996; Levine 1997). Mutational inactivation of p53 is frequently observed in various human cancers (Sun and Oberley 1997). Activation of p53, which in turn promotes apoptosis of tumor cells, is considered to be a key mechanism of action of antitumor drugs, including doxorubin (Sawyer *et al*., 1999).

2.1.3.2. Risk Factors:

2.1.3.2.1. Cumulative Dose: The incidence of Congestive Heart Failure (CHF) secondary to doxorubicin-induced cardiomyopathy depends on the cumulative dose of the drug (VonHoff *et al*., 1979). At a cumulative dose of doxorubicin 400mg/m^2, the incidence of CHF, occurring 0 to 231 days after therapy was 0.14%. This increased to 7% at a dose of 55mg/m^2 and to 18% at a dose of 700mg/m^2. Nonetheless, there is a great individual variability in the tolerable anthracycline dose, both in adults and children (VonHoff *et al*., 1979).

2.1.3.2.2. Rate of Administration: Anthracycline-induced cardiotoxicity has been associated with peak plasma drug concentration. There is evidence that doxorubicin becomes less cardiotoxic when administered as a prolonged, continuous intravenous

infusion over more than 48-96 h (Legha *et al*., 1982). Beyond 50mg/m2 dose per day, there appears to be 2.81 times greater risk.

2.1.3.2.3. Gender: Female patients appear to be more vulnerable to the cardiotoxic effects of anthracyclines.

Age: Children at the age of 4 years at the time of exposure appear to be at higher risk of developing anthracycline induced cardiotoxicity (Lipshultz *et al*., 1991, Krischer *et al*., 1997). It was mainly predictive of increased afterload due to reduced ventricular wall thickness (Lipshultz *et al*., 1991). Anthracyclines have been shown to alter transcription of myocellular proteins. The inappropriate reduction in the left ventricular wall thickness found in children, previously treated with anthracycline, could be the result of such an effect. It has been suggested that, although unalterable, the time interval since anthracycline chemotherapy should also be considered as a risk factor particularly in those patients, who received the drug in their childhood. In adults, an increasing risk of doxorubicin-induced CHF with increasing patient age has been observed (VonHoff *et al*., 1979).

2.1.4. HEPATOTOXICITY: One of the major functions of liver in our body is detoxification of various metabolites, along with alcohol and many different medications, such as chemotherapy drugs. It plays a vital role in protein synthesis, and the production of bio chemicals necessary for digestion. It is an accessory digestive gland and produces bile, an alkaline compound which aids in digestion via the emulsification of lipids (Kalender *et al*., 2005). Liver is a specialized tissue; mostly consist of hepatocytes that regulate a wide variety of high-volume biochemical reactions,

as well as the formation and breakdown of small and complex molecules, essential for normal vital functions. The most important is filtration of toxic substances from the blood (Tortora *et al*., 2008).

The liver has a crucial function in the metabolism and elimination of endogenous and exogenous substances including most drugs. Intravenous drugs reach the liver via the hepatic artery, while oral drugs go through the portal blood from the gut (Ganong 1989). Thus, it determines the disposition of many drugs. Hepatotoxicity or Drug-induced liver injury is a cause of acute and chronic liver disease. It leads the liver to under function, or to function irregularly. The liver eliminates toxins and chemicals from the blood stream and changes them into products that can be readily removed through the bile or urine. However, if the accumulation of toxins in the body is faster than the liver can process them, damage can occur leading to hepatotoxicity, which can be a very serious condition. (Zakim and Boyer 2002)

Symptoms that may indicate liver damage after chemotherapy regime include: Jaundice, fatigue, pain in the upper-right abdomen, loss of appetite and enlarged liver. Generally the damage is mild and temporary because the liver recovers in few weeks after the drug is stopped. However, damage can result in cirrhosis of the liver, a long-term problem that causes to formation of scar tissue and interfere with normal liver function. Liver may metabolise a toxic agent to an inactive or non-toxic agent, preventing liver damage. However, the reverse may also occur, transforming an inactive compound to a hepatotoxic compound, subsequently affecting almost every other organ in the body (Benichou 1990).

2.1.4.1. Drug induced Hepatotoxicity: Chemicals that cause liver injury are called hepatotoxins (Friedman *et al*., 2003). Drug-induced liver disease can occur as idiosyncratic reactions, allergic hepatitis, toxic hepatitis, chronic active hepatitis, toxic cirrhosis, and liver vascular disorders (Kirchain and Gil 2005). This can lead to necrosis, steatosis, fibrosis, cholestasis, and vascular injury in the liver tissue (Ishak and Zimmerman 1995).

Chemotherapy drugs may cause liver damage because at specific high doses they behave as toxins and place added stress on the liver's filtering function. Hepatotoxicity is one of the severe side effects of chemotherapy. Hepatotoxic changes from chemotherapy involve a variety of responses, including parenchymal cell injury with fatty change, hepatocellular necrosis or fibrosis, ductal injury with cholestasis, vascular lesions or veno-occlusive disease, and hepatic neoplasms. However, if the patient has multiple pre-existing conditions that interfere with liver function, it becomes difficult to assign the resulting toxicity to chemo-drugs. Also, immunosuppression due to chemotherapy may predispose the patient to infections affecting the liver. For example, chemotherapy has been reported to reactivate chronic hepatitis B viral infection, possibly as a result of an increase in viral synthesis during immunosuppression, followed by a rebounce in the host's immune responses when therapy is discontinued (Perry 1982).

2.1.4.2.Chemotherapy induced hepatotoxicity:

Asparaginase (Kidrolase) has a high potential for causing liver damage.

Alkylating agents can cause liver damage, especially when high doses are used.

Nitrosureas, such as carmustine, cyclophosphamide, cisplatin and antitumor antibiotics like **Doxorubicin,** Vinca alkaloids, such as vinblastine or vincristine, cause liver damage in rare cases.

2.1.4.2.1. Mechanism: The doxorubicin redox-cycle begins with one-electron reduction leading to the formation of doxorubicin radical (doxorubicin*) (Bachur *et al*.,1978). Many NADPH and NADH-dependent enzymes catalyze the reaction, such as, NADPH cytochrome P450 reductase (Doroshow *et al*., 1983), NOS (Vasquez-Vivar *et al*., 1997; Mansour, *et al*., 2003; Nithipongvanitch, *et al*., 2007), NADPH oxidase (Deng *et al*., 2007; Zhao *et al*., 2010) and catalase (Yee and Pritsos 1997). Subsequently, doxorubicin* are reoxygenated to the nonradical parent compound while at the same time the superoxide anion radical is formed (O_2^{-*}). This cycle of reactions can repeat many times leading to overproduction of (O_2^{-*}), which is the source of hydrogen peroxide and much more toxic hydroxyl radical (Minotti *et al*., 2004). These reactive oxygen species (ROS) are responsible for oxidative stress. These enzymes, involved in doxorubicin* production, are abundant in hepatocytes (Crib *et al*., 2005), suggesting that liver may be especially involved in doxorubicin* generation.

2.1.5. NEPHROTOXICITY: Major function of kidneys is to filter out waste products and regulate electrolytes and water levels. Nephrotoxicity is a malfunction of the kidneys in which their vital functioning is hampered, causing difficulty in filtering out waste. Chemotherapy may cause damage to the kidneys that can result in acute kidney failure. Kidney damage is reversible, if it is carefully managed to control the severe complications. Once the administration of drug or combination of drugs that are affecting kidney are stopped, treatment focuses on preventing the excess accumulation of fluid and

waste while allowing the kidneys to heal. Acute renal failure can be caused by decreased blood supply to the kidneys due to drugs or infection, damage to the kidneys, or by blockage in the urinary system. The most common cause of acute renal failure in cancer patients is damage to the cells in the kidney (Michael *et al*., 2007).

When proteins from the diet are catabolized for energy or building tissues, urea is produced. Urea circulates in the blood until it is filtered out by the kidneys and excreted in the urine. Any disturbance in the kidney functioning results in reduced filtration and urea builds up in the blood. In addition, due to the malfunctioning in kidneys, the balance of electrolytes and water cannot be adequately regulated, resulting in a built up of potassium, sodium and fluids too (Kintzel 2001). Kidney damage can also result in increased excretion of proteins in the urine. Under normal conditions, blood proteins do not pass through the kidneys into the urine because of its bigger molecular size. In damaged kidney, protein may pass into urine. Protein in the urine may be a sign of permanent kidney damage, or failure. Some common symptoms of kidney failure are decreased urine output, dark urine, urgency, fatigue, muscle weakness, swelling of hands/feet, nausea and confusion (Heidenreich *et al*.,1999). Many cancers involve the kidneys either directly or indirectly—heightening the risk for kidney injury with exposure to a potential nephrotoxin.

Anti-cancer agents with potential nephrotoxic renal injury have reinforced the need for vigilance amongst all clinicians treating cancer patients. In general, nephrotoxic drugs cause renal injury by inducing a varying combination of intrarenal vasoconstriction, direct tubular toxicity and intratubular obstruction. The vulnerability of the kidney to

various potentially nephrotoxic agents can be attributed to several functional properties of the kidney including a rich blood supply (25% of cardiac output), ensuring high levels of toxicant delivery, a high tubular reabsorptive capacity (via specific transporters) leading to high intracellular tubular cell concentrations, and an ability to concentrate toxins to high levels within the medullary interstitium via the renal counter current mechanisms. In addition, the kidneys are an important site for xenobiotic metabolism and may transform relatively harmless parent compounds into toxic metabolites. As they also have a high metabolic rate, the workload to renal cells results in increased sensitivity to toxicants and a high sensitivity to vasoactive agents (Cummings and Schnellmann 2013). Lastly, the kidneys are a major elimination pathway for many antineoplastic drugs and their metabolites. Thus renal impairment can result in delayed drug excretion and metabolism of chemotherapeutic agents, resulting in increased systemic toxicity. Hence, many drugs require thus dose adjustment when administered in the setting of renal insufficiency (Perazella and Moeckel 2010).

In spite of its high antitumor efficacy, doxorubicin's use in chemotherapy has been largely limited due to its cardiac, renal, pulmonary, testicular, and hematological toxicities (Fadillioglu *et al*., 2003). Doxorubicin causes an imbalance between free oxygen radicals and antioxidants. The disturbance in oxidant-antioxidant systems which has been demonstrated with lipid peroxidation (LPO) and protein oxidation results with tissue injury (Karaman *et al*., 2006).

2.1.5.1. Mechanism: Although the exact mechanism of doxorubicin induced nephrotoxicity remains unknown, it is believed that the toxicity may be mediated through free radical formation, iron-dependent oxidative damage of biological macromolecules,

membrane LPO, and protein oxidation (Liu *et al*., 2007). Doxorubicin induced changes in the kidneys of rats include increased glomerular capillary permeability and tubular atrophy (Wapstra *et al*.,1999). Nephrotoxic action of doxorubicin is also considered to be via drug-induced free radical generation (Shah 1989; Deman *et al*., 2001). The formation of free radicals as well as an increase in response to doxorubicin treatment has already been documented. The disturbance in oxidant-antioxidant system resulting in tissue injury is demonstrated with protein oxidation in renal tissue. This is recognized as one of the possible biochemical mechanisms of doxorubicin-induced nephrotoxicity (Karaman *et al*., 2006). Doxorubicin induces injury by direct toxic damage to the glomerulus with subsequent tubulointerstitial injury. It causes changes in the glomerular filtration barrier, including the glomerular endothelial cells (including glycocalyx), glomerular basement membrane and podocytes. Glycocalyx thickness is reduced, glomerular endothelial cell pore size is increased, glomerular charge selectivity is reduced and podocyte cell foot processes are fused. These changes are associated with reductions in glomerular cell production of proteoglycans and glycosaminoglycans contained within the glycocalyx produced by the glomerular endothelial cells (Jeansson *et al*., 2009). Further evidence for a direct effect of doxorubicin on the kidney comes from a study in which clipping of the renal artery of one kidney protects it from injury (De Boer *et al*.,1999). Additional studies have examined the molecular mechanisms for doxorubicin induced renal injury. Increased free radical production has been proposed as a pathogenetic mechanism. This is supported by isolation perfusion studies of hagfish (*Myxine glutinosa*) glomeruli in which doxorubicin was found to reduce glomerular ATPase activity while glutathione elevated

the levels of lipid peroxide, in association with a reduction in water permeability (Barbey *et al*.,1989).

2.1.6. Chemoprevention of Organ toxicity Induced by Doxorubicin Treatments:

Apart from cumulative dose limitations, several attempts have been made to develop chemoprotectants to prevent the toxicity of anthracyclines without attenuating their anti-tumor effect. In one clinical study, administration of melatonin together with different chemotherapeutic regimens was associated with reduced overall toxicity including cardiotoxicity. The effect was ascribed to its antioxidant capacities (Lissoni 1999).

2.1.7. NON HERBAL TREATMENT:

2.1.7.1. Dexrazoxane : Dexrazoxane hydrochloride is a cardioprotective agent. As a derivative of EDTA it was found to be the most promising agent to prevents free radical formation by binding to intracellular iron and removing the iron from the anthracycline-iron complex (Links and Lewis 1999). Several animals (Herman and Ferrans 1981), and clinical trials have shown that it is efficient in reducing doxorubicin induced cardiotoxicity. But, however, in most of the studies it could not achieve complete protection (Swain *et al*.,1997; Speyer *et al*.,1988). Moreover, it is not known if dexrazoxane provides any protection against late cardiovascular effect (Wiseman and Spencer 1998). Indeed, the American Society of Clinical Oncology concluded that there is insufficient evidence to recommend the use of dexrazoxane in the treatment of paediatric malignancies (Hensley *et al*.,1999).

2.1.7.2. Lipid Lowering Agents: Lipid lowering agents lower the cardiotoxic effects of anthracyclines (Iliskovic and Singal 1997). According to the oxidative stress theory, these

agents improve the antioxidant state of the heart, possibly leading to a better myocardial structure and function (Liu *et al*.,2000; Siveski-lliskovic *et al*.,1995). Recently, a potentiation of anti-tumor activity and a cardioprotective effect by the cholesterol lowering HMG coenzyme-A reductase inhibitor lovastatin, in mice treated with doxorubicin was shown by Feleszko *et al*., (2000).

2.1.7.3. Changes in Formulation: Development of liposomal drug formulations of the anthracyclines has succeeded to achieve reduced cardiotoxicity. Liposomes are preferentially taken up by tissues enriched in phagocytic reticuloendothelial cells and with a sinusoidal capillary system like found in the liver and spleen. The continuous capillaries containing tissues like skeletal and cardiac muscles, therefore hardly take up liposomes. One study suggests that doxorubicin induced cardiotoxicity can be reduced upon using liposomal formulations of the drugs (Safra *et al*., 2000). However, Daemen *et al*., 1995 have shown that doxorubicin entrapped within conventional liposomes (200 nm in diameter; lip-Dox) has major toxic effects on liver macrophages of the rat for a considerable period of time following i.v. administration, with respect to both specific phagocytic capacity and cell numbers.

2.1.8. Herbal Treatment: Considerable interest has been generated in compounds that function as antioxidants and in herbs that contain endogenous antioxidants. It is experimentally revealed that, *Salvia miltiorrhiza, Coprinus comatus, Terminalia chebula, Solanum torvum,* vitamin A, virgin olive oil and selenium as dietary supplements provide anti oxidant properties against doxorubicin (Granados *et al*., 2010). Further, scientists have also revealed that Caffeic acid, phenethyl ester, lipolic acid as well as melatonin

ameliorate oxidative damage caused by the anthracycline (Agapito 2001; Kumaravel 2003; Yagmurca *et al*., 2004).

Several herbs which ameliorates the doxorubicin induced cardiotoxicity have been extensively studied. Grape seed proanthocyanidin extract and *Scutellaria baicalensis* extract, are seen to prevent Dox-induced cardiotoxicity (Arya and Gupta 2011). Prior administration of either turmeric extracts or lipoic acid has been shown not only to modulate the levels of antioxidants such as catalase, GSH and ascorbic acid, but also elevate antioxidant capacity of cardiac cells (El-Sayed *et al*., 2011). As reported by Singh *et al* butanolic fraction of *Terminalia arjuna* bark have protective effects against Dox-induced cardiotoxicity and potential as a cardioprotective agent by increasing the cardiac antioxidant enzymes (Singh *et al*., 2008). The hydroethanol extract of the leaves of the edible plant *Urtica parviflora* is effective against doxorubicin-induced cardiotoxicity in Wistar rats, by virtue of its lipid lowering property and augmenting endogenous antioxidant mechanisms (Barman *et al*., 2013). Catechins, flavonols and flavandiols present in green tea extract are reported to play a major role against doxorubicin-induced cardiotoxicity (Ahmad *et al*., 2011). *Aloe barbadences* having photochemical chromones and methoxy 2 methyl chromone, alleviate doxorubicin induced oxidative stress and calcium overloading (Gurav *et al*., 2011). Reports have suggested that, sitosterol, daucosterol, syringic acid, vanillic acid and feruloyl tyramine present in aqueous ethanol extract of *Aerva lanata* (Linn.) are effective against doxorubicin induced cardiomyopathy in rats (Paramasivam *et al*., 2012). Flavonoids from *Malus hupehensis* show their cardioprotective effects against doxorubicin-induced toxicity in H9c2 cells (Wang *et al*.,

2013). *Phyllanthus niruri* extract has also been reported to play protective role against doxorubicin-induced myocardial toxicity (Thippeswamy *et al*., 2011).

Many studies have shown that several herbs are effective in lowering the extent of hepatotoxicity caused by doxorubicin. Studies have shown that Silymarin is used as supportive treatment for liver diseases of different etiology where it is hepatoprotective through its antioxidant activity. It stimulates protein synthesis, influences lipid metabolism and stabilizes membrane phospholipids (Aleksandar *et al*., 2011). Celery and parsley juices have shown antioxidant activities in doxorubicin treated rats (Kolarovic *et al*., 2010). It is reported that Vitamin E and Catechin alliviate Doxorubicin hepatotoxicity and hepatic free radical metabolism in rats (Kalender *et al*., 2005). Berberine has been proved to be protective against doxorubicin-induced hepatotoxicity, such as reduction in lethality in mice, alleviation of liver dysfunction and attenuation of liver tissue injury (Xiaoyan *et al*., 2012). Studies have shown that, pretreatment with rutin and hesperidin may protect the liver from the hepatotoxic effect caused by doxorubicin (Walaa *et al*., 2014). As reported by Ahmed and Abdella 2010, aqueous rosemary leaves extract ameliorated doxorubicin induced histological lesions, apoptosis and oxidative stress in male mice tissues. Chrysin (5,7-dihydroxyflavone), a natural flavonoid present in several plant extracts, attenuated nephro and hepatic damage induced by doxorubicin (Summya *et al*., 2013).

The implication of oxidative stress in the etiology of anti cancer drug induced nephrotoxicity has widely led to the suggestion that incorporation of antioxidant-rich foods into the diet can potentially target oxidative stress pathways and reverse or reduce the complications. A study revealed that *Luffa acutangula* prevents oxidative

stress by neutralizing the free radicals, augmenting enzymatic and non-enzymatic antioxidants, and stabilizing cell membrane ultimately leading to cardio- and nephro-protection (Jadhav 2013). Nicotinamide (NAD), a derivative of Vitamin B3, has been reported to show its effects on lipid peroxidation, antioxidant status, and iNOS activity in DOX-induced nephrotoxicity in rats (Sule *et al*., 2011). Studies showed that *Lepidium sativum* offer protection against the deleterious renal side-effects of doxorubicin (Shinde *et al*., 2010). It is proved by Ajith and Aswathy 2007 that *Zingiber officinale* roscoe show protective effect against doxorubicin-induced acute nephrotoxicity. Scientists have also revealed that Caffeic acid, phenethyl ester, lipolic acid and melatonin curtail the oxidative damage caused by the anthracycline (Yagmurca *et al*., 2004). Lycopene, *Ginko biloba* leaf extract and multiple antioxidants in Chinese herbal medicine render protection against doxorubicin induced nephrotoxicity (Yilmaz *et al*., 2006; Abd El-Aziz *et al*., 2001; Quin *et al*., 2007). A study showed that A 43 kD protein (CI protein) from the leaves of the herb *Cajanus indicus L*. modulates doxorubicin induced nephrotoxicity via MAPKs and both mitochondria dependent and independent pathways (Pal and Sil 2012). Several studies have shown that Fermented Papaya Preparation can also modulate oxidative injury as well as injury due to inflammation and improve the immune function. It exhibits anti-inflammatory, antioxidant, immunostimulatory (at the level of the mucus membrane) activities and induction of antioxidant enzymes, and have beneficial prophylactic potential in the management of chronic diseases with overt inflammation.

2.1.8.1. Neutraceuticals: There is now a growing interest in the utilization of plant extracts as dietary food supplements. A wide spectrum of beneficial activity for the human health has been advocated for such supplements, at least in part, due to their

antioxidant activity (Rice-Evans 1996). More recently, the ability of antioxidant nutrients to affect cell response and gene expression has been reported *in vitro*, providing a novel and different mechanistic prospective, underlying the biological activity of plant derived nutraceuticals (Virgili *et al*., 1998, Prajda *et al*., 1995, Csokay *et al*., 1997).

2.1.8.2. Fermented Papaya Preparation (FPP) is one such nutraceutical. Made by yeast fermentation of *Carica Papaya Linn*., FPP is used as natural food health supplement in different parts of the world. FPP has been used in Japan for decades. It is also an extremely popular supplement in France and other parts of Europe. FPP, a granular substance available over the counter as **"Immun'Age"**, is known to possess antioxidant properties.

The process of long fermentation, by means of yeasts, is the unique process, which not only preserves the papaya anti-oxidant properties but also offers new and important immune-modulating features. Many new classes of oligosaccharides are present at a different polymerisation as well as monomers similar to the basic structure of ß 1-3 D-glucan in the fermented product but not in the fresh fruit. Such oligosaccharides, mainly those exhibiting a low molecular weight, exhibit a wide spectrum immune-modulating activity (Francesco *et al*., 2012).

It is well known for a long time that the natural anti-oxidant properties of the papaya, mainly depending on vitamins (A and C) and amino acids, were consistent both in the fruit and papain enzyme (Arginine among all). Papain has digestive property, but in the FPP, such property is not witnessed. FPP stimulate superoxide dismutase (SOD) and glutathione peroxidase (GPX) to eliminate free-radicals from the system. FPP is more than an antioxidant as it doesn't turn into a pro-oxidant (Santiago *et al*., 1991). FPP has

been shown to up-regulate phorbol ester and zymosan induced superoxide production in rat peritoneal macrophages (Osato *et al*., 1995), natural killer cell activity (Okuda *et al*., 1993) and the level of interferon (IFN-γ) in human blood (Santiago *et al*., 1991). Studies have demonstrated that FPP affects NO and hydrogen peroxide production as well as tumor necrosis factor alpha secretion in RAW 264.7 macrophages (Kobuchi and Packer 1997). Such evidence suggests a role of FPP as an immunomodulator.

It has also been reported that FPP protects the brain of aged rodents *in vivo*, challenged either by oxidative stress or by ischemia reperfusion injury (Santiago *et al*., 1993). Furthermore, accumulation of thiobarbituric acid reactive substances were found to be lower in heart homogenates from FPP supplemented rats exposed to peroxyl radicals as compared to non-supplemented controls (Marcocci *et al*., 1996).

Studies on the alcoholic liver disease have proved how FPP allows reducing the alcoholic oxidative stress and also the chronic alcoholic abuse. The alcoholic oxidative stress reduces plasma and erythrocyte level of malonyldialdehyde as well as plasma lipoperoxides while during the initial phases of withdrawal it is possible to observe a persistence of the microsomal system activation. Later leads to the ethanol oxidation, with a consequent maintenance of the pro-oxidative state (Marotta *et al*., 1997). In chronic liver disease, unrelated to alcohol, it was shown that a significant improvement of redox status was obtained by alpha-tocopherol present in FPP regime. However, only FPP significantly decreased 8-OHdG and the improvement of cytokine balance with FPP was significantly better than with vitamin E treatment. It was also found that patients with liver cirrhosis showed a significantly time-dependent upregulated TNF-α production from *ex vivo* LPS-stimulated monocyte and this effect was more pronounced in more

advanced stages of the disease together with higher serum level of thioredoxin (Trx) (Marotta *et al*., 2007). Again, FPP has shown to reach a normalization of Trx and partial but significant down regulation of TNF-α mRNA (Marotta *et al*., 2011).

Scientists, studying FPP efficacy have found that the genes for antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase as well as the gene for the DNA repair enzyme *hOGG1* were up-regulated by FPP in humans (Marotta *et al*., 2010). FPP appeared to exert protective effects on leukocyte DNA adducts formation, irrespective of genotype profile, while enhancing DNA repair mechanisms against the highly mutagenic base modification, but only in GSTM1-null genotype participants (Aruoma *et al*., 2010). Tumor necrosis factor-α and interleukin-lβ are involved in the induction of iNOS gene as well as the immune system. FPP may not only be directly involved in the expression of iNOS, but shows synergistic interaction with IFN-γ to induce NO synthesis (Kobuchi and Packer 1997). Nitrilotriacetic acid (NTA), a synthetic amino-tricarboxilic acid, forms water-soluble complexes with iron at neutral pH. This complex (a Fenton reaction catalyst), is nephrotoxic and induces renal proximal tubular damage associated with oxidative damage after repeated administration. These eventually lead to a high incidence of renal cell carcinoma in rodents (Toyokuni 1996). Fe–NTA in the presence of H_2O_2 *in vitro* is also known to cause increased oxidative DNA damage. Fe–NTA depletes cellular GSH levels in lymphocytes both of which are counteracted by FPP. Electronic Paramagnetic Resonance spin trapping studies demonstrated that antioxidant properties of FPP are related to both hydroxyl scavenging as well as iron chelating properties (Rimbach *et al*., 2000). Thus, FPP when translated to an *in vivo* environment would be expected to exert antioxidant protection.

From the above survey of literature it is evident that, Fermented Papaya Preparation (FFP) has shown its immense anti oxidant property in curing several ROS generated disturbances in various organs of the body. Therefore, the present study is designed to test the efficacy of FFP to act as protective agent against doxorubicin induced toxicity.

2.2. MATERIALS AND METHODS

2.2.1. CHEMICALS

Doxorubicin was purchased from Sigma Aldrich (St Louis, MO, USA). Fermented Papaya Preparation was gifted by Venkatesh Food Products, Indore (Prepared by fermenting *Carica papaya* with glucose, yeast and lactic acid bacterium). All other biochemical reagents and chemical were of analytical grade.

2.2.2. ANIMALS

Female Wistar rats (180-220g) were housed and maintained in a clean propylene cages under controlled room temperature. Food (commercially available rat chow, standard laboratory diet: M/s Pranav Agro Ltd Baroda, India) and water was provided ad libitum. Experiments were performed in accordance with guidelines of Institutional Animal Ethical Committee (Approval no.827/ac/04), a Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

2.2.3. EXPERIMENTAL PROTOCOL

i. **Induction of Experimental organ toxicity in Rats**

Doxorubicin was dissolved in normal saline and injected to rats (10mg/kg, i.p.) at an interval of 24 h for 2 days to induce experimental organ toxicity. Animals were sacrificed 48 hr after the last dose

ii. **Experiment design:** After acclimatization, the animals were randomly divided into the following groups consisting of 5 rats each. They received standard laboratory diet and drinking water ad libitum.

Table : 2.2.1. Experiment design

Experiment design	**SALINE**	**FERMENTED PAPAYA PREPARATION**	**DOXORUBICIN**
Group 1 (Control)	Animals received normal saline orally for 28 days and through intraperitoneal injection on 29th and 30th day	----------	----------
Group 2 (Dox Control)	Animals received normal saline orally for 28 days	----------	Animals received DOX treatment (10 mg/kg bw, i.p) for 2 days (29th and 30th day)
Group 3 (FPP Control)	Animals received normal saline (i.p) on 29th and 30th day	Animals received FPP treatment (250 mg/kg bw, orally) for 28days	----------
Group 4 (FPP+DOX)	----------	Animals received FPP treatment (100 mg/kg bw, orally) for 28 days.	Animals received DOX treatment (10mg/kg bw i.p) for the following 2 days. (29th and 30th day)
Group 5 (FPP+DOX)	----------	Animals received FPP treatment (250 mg/kg bw, orally) for 28 days.	Animals received DOX treatment (10mg/kg bw i.p) for the following 2 days. (29th and 30th day)

iii. **Biochemical analysis in serum and tissue**

Twenty-four hours after the treatment period, animals were sacrificed and blood samples were collected. Serum was separated from each blood sample and was used for the biochemical analysis. Immediately after sacrifice, tissues was excised and blotted free of blood as well as tissue fluid, weighed and stored at -80^{o} C till further use for analysis.

Biochemical parameters in serum

1. Assessment of cardiac function markers

The serum was used for the estimation of the cardiac marker enzyme Creatinine Kinase MB (CK MB) and Lactate Dehydrogenase (LDH) using commercially available kits (Reckon Diagnostics kits Pvt. Ltd. India) following manufacturers instruction.

CK MB

CK MB (NAC act.) Kit used for the determination of CK-MB Activity in Serum is from Reckon diagnostics.

PRINCIPLE

CK-M fractions of the CK-MM and the CK-MB in the sample are completely inhibited by an anti CK-M antibody present in the reagent. Then the activity of the CK-B fraction is measured by the CK (NAC act.) method. The CK-MB activity is obtained by multiplying the CK-B activity by two.

WORKING REAGENT PREPARATION

For sample start assays a single reagent is required. Pour the contents of 1 bottle of L_2 (Starter Reagent) into 1 bottle of L_1 (Enzyme Reagent). This working reagent is stable for at least 10 days when stored at 2-8°C.

PROCEDURE

Pipette into a clean dry test tube labeled as Test (T):

PIPETTE INTO TEST TUBES	**TEST**
• WORKING REAGENT (ml)	1.0
Incubate at the assay temperature for 1 minute and add	
• SAMPLE (ml)	0.05

Mix well and read the initial absorbance A after 5 minutes and repeat the 0 absorbance reading after every 1, 2, & 3 minutes. Calculate the mean absorbance change per minute (Δ/ min).

CALCULATIONS

CK - MB activity in U/L = **Δ**A/ min. x 3333x2

LDH (UV-Kinetic)

Reagent kit for quantitative estimation of Lactate Dehydrogenase activity in serum was from Reckon diagnostic.

PRINCIPLE: LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD.

Lactate + NAD $\xrightarrow{LDH}$ Pyruvate + NADH

WORKING REAGENT PREPARATION

For 20 x 1.1 ml: Reconstitute one tablet of 1 LDH with 1.1 ml of 2 LDH. Mix gently to dissolve the contents. Use after 5 minutes.

PROCEDURE

PIPETTE INTO TEST TUBES	**TEST**
• WORKING REAGENT (ml)	1.0
• SAMPLE (ml)	0.05

Mix and read first absorbance of the test exactly at one minute & thereafter at 30, 60 & 90 seconds at 340 nm. Determine the mean change in absorbance per minute and calculate test results.

CALCULATIONS

LDH activity = Δ A/min X F

Where F = $\frac{1}{6.22} \times \frac{T.V.}{S.V} \times 1000 = 3376$

T.V. = Total Volume = 1.05 ml.

S.V. = Sample Volume = 0.05 ml.

6.22 = Millimolar Extinction Coefficient of NADH at 340 nm.

Assessment of lipid profile

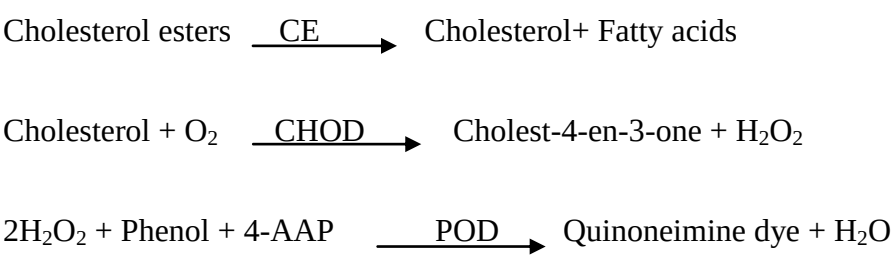
Lipid profile including total Cholesterol and Triglycerides was estimated using commercially available standard enzymatic kits following manufacturer instruction. (Reckon diagnostics Pvt. Ltd, India)

***TOTAL CHOLESTEROL** (TC)*:

Quantitative estimation of TC in serum was performed according to manufacturer's instructions provided in commercially available standard diagnostic kit (Span Diagnostics Pvt. Ltd., India) using Perkin Elmer Lambda UV-Vis Spectrophotometer.

PRINCIPLE

Cholesterol esters are hydrolysed by Cholesterol Esterase (CE) to give free Cholesterol and Fatty acids. In subsequent reaction, Cholesterol Oxidase (CHOD) oxidizes the 3-OH group of free Cholesterol to liberate Cholest-4-en-3-one and Hydrogen Peroxide. In presence of Peroxidase (POD), Hydrogen Peroxide couples with 4 Aminoantipyrine (4-AAP) and Phenol to produce Red Quinoneimine dye. Absorbance of coloured dye is measured at 505 nm and is proportional to amount of Total Cholesterol concentration in the sample.



PROCEDURE:

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Test (T) as shown below

	Blank	Standard	Test
Serum/plasma	--	--	10µL
Reagent 2	--	10µL	--
Regent 1	1000µL	1000µL	1000µL

Mix well. Incubate at 37^{o} C for 10 minutes or at Room Temperature (15-30 ^{o} C) for 30 minutes. Take absorbance of standard and test against blank at 505 nm and calculate results as per following formula.

CALCULATIONS

Cholesterol concentration (mg/dl) = Absorbance of Test / Absorbance of Standard × 200

***TRIGLYCERIDES** (TG):*

Quantitative estimation of TG in serum was performed according to manufacturer's instructions provided in commercially available standard diagnostic kit (Reckon Diagnostics Pvt. Ltd., India) using Shimadzu 1800 UV-Vis Spectrophotometer.

Principle: Triglycerides in the sample are hydrolyzed by microbial lipase to glycerol and free fatty acid (FFA). Glycerol is phosphorylated by adenosine 5-triphosphate (ATP) to glycerol 3-phosphate (G-3-P) in reaction catalyzed by glycerol kinase (GK). G-3-P is oxidized to dihydroxy acetone phosphate in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO). In this reaction hydrogen peroxide (H_2O_2) is produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacts

with 4-aminoantipyrine and ADPS in the reaction catalyzed by peroxidases (POD). The result of this oxidative coupling is a quinoneimine purple colored dye. The absorbance of this dye in solution is proportional to the concentration of triglycerides in sample.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty Acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-Phosphate} + \text{ADP}$$

$$\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone Phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + H_2$$

PROCEDURE

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Test (T) as shown below:

	Blank	Standard	Test
Working Reagent (ml)	1.0	1.0	1.0
Standard (ml)	--	0.02	--
Sample (ml)	--	--	0.02

Mix & incubate at 37˚C for 10 mins. & read absorbance of test & standard against reagent blank at 520 nm (500-550nm or GREEN filter).

CALCULATIONS

Triglyceride (mg/dl) = Absorbance of Test/ Absorbance of Standard ×200

2.2.3.3.1.2. Assessment of hepatic function markers:

Serum was estimated for SGPT, SGOT and ALP using commercially available kits (Reckon Diagnostics kits Pvt. Ltd. India), following manufacturers instruction.

SGPT

Kit used for the estimation of glutamate pyruvate transaminase activity in serum or plasma is from Reckon diagnostics.

PRINCIPLE

This method is for estimating glutamate pyruvate transaminase activity (also called alanine transminase, ALT). The primary transaminase reaction was coupled with lactate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH).

1) In this reaction, L-Alanine and alpha-ketoglutarate react in the presence of GPT in the sample to yield pyruvate and L-glutamate.

L-Alanine + α-Ketoglutarate $\xrightarrow{\text{GPT}}$ Pyruvate + L-Glutamate

2) Pyruvate is reduced by lactate dehydrogenase to yield lactate with the oxidation of NADH to NAD. The reaction is monitored by measurement of the decrease in absorbance of NADH at 340 nm. The rate of reduction in absorbance is proportional to GPT activity in sample.

Pyruvate + NADH $\xrightarrow{\text{LDH}}$ Lactate + NAD

The rate of reduction in absorbance is proportional to GPT activity in sample.

WORKING REAGENT PREPARATION

Add 1.1 ml. of 2 SGPT to one tablet of 1 SGPT. Mix well to dissolve and wait for 15 minutes prior to use. The working reagent is stable for 30 days at 2-8° C

PROCEDURE

PIPETTE INTO TEST TUBES	**TEST**
• WORKING REAGENT (ml)	1.0
• SAMPLE (ml)	0.05

Mix and after incubation at 37°C for 60 seconds, measure the absorbance at an interval of 30 seconds for 2 minutes at 340 nm.

CALCULATION

Serum GPT activity (IU/L) = Δ A/min. x F

Where F = 3376 (based on the Milli molar extinction Coefficient of NADH at 340 nm)

SGOT

Estimation of Glutamate Oxaloacetate Transaminase activity in serum was done from Reckon diagnostic kit.

PRINCIPLE

In 1955, Karmen published a method for the determination of glutamate oxaloacetate transaminase activity (also called aspartate transaminase AST.) The primary transaminase reaction was coupled with malate dehydrogenase (MDH) and reduced nicotinamide adenine dinucleotide (NADH).

1) In this reaction L-Aspartate and Alpha-Ketoglutarate react in the presence of GOT in the sample to yield oxaloacetate and L-glutamate.

L-Aspartate + Alpha-Ketoglutarate $\xrightarrow{\text{SGOT}}$ Oxaloacetate + L-Glutamate

2) The Oxaloacetate is reduced by malate dehydrogenase (MDH) to yield L-malate with the oxidation of NADH to NAD. The reaction is monitored by measurement of the decrease in absorbance of NADH at 340 nm

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

The rate of reduction in absorbance is proportional to GOT activity in sample.

WORKING REAGENT PREPARATION

Add 1.1 ml. of 2 SGOT to one tablet of 1 SGOT. Mix well to dissolve and wait for 15 minutes prior to use. The working reagent is stable for 30 days at 2-8° C.

PROCEDURE

PIPETTE INTO TEST TUBES	TEST
• WORKING REAGENT (ml)	1.0
• SAMPLE (ml)	0.05

Mix and after incubation at 37° C for 60 seconds, measure the absorbance at an interval of 30 seconds for 2 minutes at 340 nm.

CALCULATIONS

Serum GOT activity (IU/L) = Δ A/min. x F

Where F = 3376 (based on the Milli molar extinction Coefficient of NADH at 340 nm)

ALP

Kit used for ALP estimation (p-NPP method) is procured from Reckon diagnostics Pvt Ltd.

PRINCIPLE

The increase in absorbance due to formation of 4 - nitroophenolate israte of is measured photo metrically and is directly proportional to the ALP (Alkaline Phosphatase) activity in the sample.

P - Nitrophenylphosphate + H2O $\xrightarrow{ALP}$ Phosphate + p – Nitrophenol

PROCEDURE

Take 20 µl of sample of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 1 minute. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 405 nm.

2.2.3.3.1.3. Assessment of renal function markers:

Serum was estimated for Urea and Creatinine, using commercially available kits (Reckon Diagnostics kits Pvt. Ltd. India), following manufacturers instruction.

UREA

Urea (DAM Method) Kit used for the determination of urea in serum is from Reckon diagnostics.

PRINCIPLE

Urea in an acidic medium condenses with Diacetyl monoxime at 100°C to form a red coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Urea + Diacetyl monoxime → Red Coloured Complex

PROCEDURE

Contents :L1: Urea Reagent , L2: Acid Reagent , L3: DAM Reagent , S: Urea Standard

Addition sequence	**Blank**	**Standard**	**Test**
L1	1.0 ml	1.0 ml	1.0 ml
L2	1.0 ml	1.0 ml	1.0 ml
L3	1.0 ml	1.0 ml	1.0 ml
Distilled water	0.01 ml	---	---
S	---	0.01 ml	---
Sample	---	---	0.01 ml

Mix well and keep the test tubes in boiling water (100°C) for 10 minutes. Cool under running tap water and measure the absorbance of the Standard (Abs. S), and Test Sample (Abs. T) against blank.

CALCULATIONS

$$\text{Urea in mg/dl} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 40$$

CREATININE

Creatinine (Alkaline Picrate Method) kit used for the determination of creatinine in serum is from Reckon diagnostics.

PRINCIPLE

Creatinine reacts with alkaline picrate to produce a red colored complex; the rate of red colored complex formation is directly proportional to the Creatinine concentration.

Creatinine + Alkaline Phosphate ⟶ Orange Coloured Complex

WORKING REAGENT PREPARATION

Prepare working reagent by combining one volume of 1 CREATININE with one volume of 2 CREATININE.

PROCEDURE

PIPETTE INTO TEST TUBE	**STANDARD (ST)**	**TEST (TS)**
WORKING REAGENT (ml)	1.0	1.0
STANDARD (ml)	0.1	-
SAMPLE (ml)	-	0.1

Mix and aspirate. Record the absorbance of Standard (ST) and Test (TS) at 20 seconds (ST_1, TS_1) and again at 80 seconds (ST_2, TS_2) at 510 nm, against distilled water.

CALCULATION

$$\text{Creatinine concentration} = \frac{(TS_2-TS_1) \times 2}{(ST_2-ST_1)}$$

To convert (mg/dL) to micromol/liter, use the following equation; micromol/liter = (mg/dL) x 88.5

2.2.3.3.2.Biochemical parameters in tissue

The excised heart, liver and kidney tissues were thawed and homogenized in chilled PBS buffer (0.1M, pH 7.4). The 10% tissue homogenate was centrifuged at 3000g at 10^{o}C using the Plastografitis Super Spin R centrifuge. The clear supernatant obtained was used for the assay of endogenous antiperoxidative enzymes.

CATALASE

METHOD: (Hugo *et al*., 1987)

PRINCIPAL- Catalase (CAT) is a heme protein contains four ferriprotoporphyrin groups per molecules. This enzyme is also found in all aerobic organisms and is important in removal of H2O2 generated in peroxisomes (microbodies). Highest CAT activity is found in liver and kidney and lowest in connective tissue. In tissue it is mainly present bound to the membranes of mitochondria and peroxisomes, whereas it exists in soluble state in erythrocyte (Hugo *et al.,* 1987).

PRINCIPLE: Catalase activity was estimated by the method of Hugo *et al*., (1987) where in, Hydrogen Peroxide is decomposed by Catalase. Catalase is a heme containing enzyme which catalyzes dismutation of hydrogen peroxide into water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240 nm, since hydrogen peroxide absorbs UV light maximally at this wavelength. The values expressed as nm of H_2O_2 decomposed /min/mg tissue.

$$H_2O_2 \xrightarrow{CAT} H_2O + O_2$$

REAGENTS

1. Phosphate Buffer: (50 mM, pH 7.0)

a. 36 g KH2PO4 in 250 ml D/W.

b. 13 g Na2HP04 in 300 ml D/W. Mix both in the ratio 1:1.5 and adjust pH to 7.0 2.

2. Hydrogen peroxide (30 mM)

3. Absolute alcohol (ethanol)

4. Triton X-100 (10%)

5. PBS (0.1M, pH 7.4)

SAMPLE PREPARATION

Tissue sample was prepared as 10% tissue homogenate in PBS, centrifuged at 1000 rpm to remove cell debris. Supernatant was used for enzyme analysis. 10 µl ethanol was added to 1.0 ml supernatant and these tubes incubated in ice water bath for 30 minutes. Just before the assay 10 l of Triton X-100 and 9 ml of phosphate buffer were added.

Reagents	Blank	Tissue
Sample	0.2ml	0.2ml
Phosphate buffer	2.8ml	1.8ml
H_2O_2	------	1.0ml

Immediately after adding H_2O_2 decrease in the absorbance was recorded at every 5 seconds interval for 15 seconds at 240nm.

CALCULATION - CAT activity = $\frac{2.303}{\Delta t} \times (\log E1 \div \log E2) \times$ dilution factor

Unit - mmoles ofH_2O_2 decomposed /sec/ g tissue

GLUTATHIONE reduced

METHOD: (Beutler *et al*., 1963)

Glutathione (g-glutamylcysteinylglycine, GSH) is highly concentrated intracellular antioxidants, accounts for 90% intracellular non-protein thiol content. Highest concentration of GSH is present in liver. Glutathione exists in two forms: reduced glutathione (GSH) and the oxidized form glutathione disulfide (GSSG). The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Parris 1997). Glutathione status is homeostatically controlled both inside the cell and outside, being continually self-adjusting with respect to the balance between GSH synthesis (by GSH synthetase enzymes), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases, and transpeptidases).

PRINCIPLE: Red cell contains GSH as a non - protein sulfhydryl compounds. 5-5' Dithiobis (2 nitrobenzoic) acid (DTNB) is a disulfide compound, which is readily reduced by sulfhydryl compounds forming a highly colored yellow anion, which can be read at 412 nm.

REAGENTS:

1. Precipitating (ppting) reagent: Glacial metaphosphoric acid (1.67g), EDTA (0.20g), NaCl (30g) and total volume was made up to 100 ml with distilled water (D/W.)

2. Na2HP04. (0.3M)

3. DTNB: 40 mg DTNB dissolved in 100 ml 1% sodium citrate.

4. PBS (0.1M, pH 7.4).

5. Standard GSH solution (2mM GSH). (Standard range 10-100p.g)

Sample preparation - Anticoagulated whole blood or 10% tissue homogenate in Phosphate buffered saline (PBS) (0.1M, pH 7.4).

PROCEDURE:

Reagents	blank	Tissue
Sample	----	1.0ml
D/W	1.0ml	----
Ppting reagent	1.5ml	1.5ml

Keep the tubes for five minutes then centrifuge at 3000rpm for 15min.

Reagents	blank	Tissue
Supernatant	0.5ml	0.5ml
Na2HP04 solution	2.0ml	2.0ml
DTNB	2.5ml	2.5ml

Read absorbance at 412 nm within a minute after adding DTNB

CALCULATIONS- Calculation was done according to the slope calculated from the standard graph.

Unit -GSH mg/g of tissue

SUPEROXIDE DISMUTASE (SOD)

METHOD- (Kakkar *et al*., 1984)

PRINCIPLE- SOD is present in all the aerobic organisms. It provides an essential defense against the potential toxicity of molecular oxygen. SOD helps to prevent tissue damage

by superoxide radicals (O_2^-). It is a metalloenzyme, which catalyzes dismutation of superoxide radicals to hydrogen peroxide (H_20_2) and oxygen (0_2).

$$2\ O_2^- + 2H^+ \xrightarrow{SOD} H_20_2 + 02$$

Two isoenzymes i.e. Cu-Zn SOD (cytosol) and Mn-SOD (mitochondria) are present in eukaryotic animals, which are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments, but how it is communicated at molecular level is unknown.

REAGENTS:

1. 0.89%KC1
2. PBS (0.1M, pH 7.4)
3. Sodium pyrophosphate (pH 8.3) - 0.052 Mm
4. PMS -186 pM
5. NBT - 300 pM
6. NADH - 780 pM
7. D/W

SAMPLE PREPARATION -.4% tissue homogenate in 0.89% KC1 was prepared and centrifuged at 3000 rpm for 15 min. Supernatant was used for SOD estimation. Principle: Mixtures of NADH and phenazine methosulfate (PMS) generate to superoxide under non - acidic conditions via the univalent oxidation of reduced PMS. NBT serve as a detector molecule for superoxide through reduction in to a stable, blue coloured formazone product, which can be measured at 560nm.

PROCEDURE

Reagents	Test	Control
Sodium pyrophosphate	1.2 ml	1.2 ml
PMS	0.1 ml	0.1 ml
NBT	0.3 ml	0.3 ml
Diluted enzyme	0.01ml/0.02ml	
D/W	1.2 ml/1.18ml	1.2 ml
NADH	0,2ml	0.2 ml

All tubes were incubated for 90 seconds at 37°C then reaction was terminated by adding 1.0 ml glacial acetic acid and shaken vigorously. Reduced NBT was extracted in 4 ml of n-Butanol. Tubes were centrifuged and absorbance was read at 560 nm against butanol blank.

CALCULATIONS-

$$\text{SOD (U/g)} = \frac{OD^{Control} - OD^{test}}{OD^{Control}} \times \frac{100}{0.01} \times \frac{60}{90} \times \frac{1}{\text{tissue wt(g/dl)}}$$

Unit - One unit of SOD is defined as the amount of enzyme required to inhibit NBT reduction by 50% as compared to control.

GPx

METHOD: (Rotruck *et al*., 1973)

PRINCIPLE: Glutathione peroxidase catalyse the reduction of hydrogen peroxide by reduced glutathione resulting in H_20 and oxidised glutathione which is then instantly and continuously converted in to GSH by excess of GR use with NADPH providing for a constant level of GSH. Reduced glutathione acts as a reductant. The estimation is based

on the oxidation of GSH by 5,5' dithio bis 2, nitro benzoic acid (DTNB) to measure the total glutathione content of biological samples.

GPx

ROOH+2GSH -> ROH +H_20 + GSSG

H_20_2+2GSH ⟶ $2H_20$ + GSSG

REAGENTS:

1. Tris-HCL buffer: 0.1M, pH 7.4,

2. GSH for reaction mixture: 2 mM,

3. Sodium azide: 10 mM,

4. Dibasic (Na2HPO4): 0.3M, pH 8.0,

5. DTNB: 0.04%,

6. TCA: 10% and

7. H2O2: 10 mM

PROCEDURE: The assay mixture containing 0.4 ml of phosphate buffer (0.4 M,PH 7.0), 0.1 ml sodium azide (10 mM), 0.2 ml of reduced glutathione (4mM) and 0.2ml of tissue homogenate was mixed and 0.1 ml of H_20_2 (30 mM) was added and made up to 2.0 ml with water. The tubes were incubated at 37 °C for 10 min along with control tubes containing ail reagents except the enzyme. The reaction was terminated by addition of 0.5 ml of 10% TCA which was then centrifuged at 4000rpm for 10 min at 4°C. 1ml of this supernatant was taken and added to 3.0 ml of disodium hydrogen phosphate and 1.0 ml DTNB solution (40mg/100ml of 1% sodium citrate). The color formed was measured at 412 nm. The blank contained disodium hydrogen phosphate and 1.0 ml of DTNB solution.

Calculations (U/min/mg tissue):

$$\frac{\Delta OD/min \times GSH\ Std. \times Total\ reaction\ Vol.}{Std\ OD \times 307.32 \times Vol.\ of\ enzyme\ source \times tissue}$$

307.32 = Molecular weight of GSH

LPO

METHOD: (Buege *et al*., 1978)

PRINCIPLE: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric acid reactive substance (TBARS). TBARS gives a characteristic pink color that can be measured caiorimetrically at 535 nm. The degree of lipid peroxidation was estimated by the rate of Malondialdehyde (MDA) production using the Thiobarbuteric Acid (TBA) method as previously described by Beuge and Aust (1978). The absorbance was recorded at 535.

PROCEDURE: The test system contained tissue extract of 1ml, 1ml TBA reagent solution, was kept in water bath (90-100° C) for 20 minutes, allowed to cool and centrifuged at 3000 rpm for 15 minutes. Absorbance was recorded against a blank recorded at 535nm against reagent blank and the values expressed as MDA nmol/g of heart tissue.

iv. **Histopathological examination of heart, liver and kidney section**

After sacrifice the heart was harvested and washed immediately with saline and fixed in 4% buffered paraformaldehyde. Briefly, after 1-2 days the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 6μ thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinised in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The haematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted in DPX. Each section was stained with hematoxylin and eosin. The sections were examined under the light microscope (Lieca) for any histopathological changes and were photographed with canon S70.

v. **Statistical analysis**

Result of all the above estimations were expressed in terms of Mean ± SE. Difference between the groups was statistically determined by ANOVA followed by Tukey's Multiple Comparison test with the level of significance set at $P<0.05$.

2.3. RESULTS

2.3.1. Body weight, Tissue weight and Ratio of Tissue weight to Body weight:

The changes in the body weight, heart, liver and kidney weight and ratio of their weights to body weight are shown in Table 1. In Doxorubicin treated rats, body weight decreased and heart, liver and kidney weight increased as compared to that of control rats. It is evident from the significant change in body weight ($p<0.05$) and tissue weights [heart ($p<0.01$) ,liver ($p<0.05$) and kidney($p<0.05$)] as compared to the respective weights of control animals that treatment of 250 mg/kg bw of Fermented Papaya Preparation (FPP) averted such adverse effects of doxorubicin

Table 2.3.1. Tissue weight and Body weight ratio.

TREATMENT	Body wt	Heart wt	Heart wt/body wt ratio	Liver wt	Liver wt/body wt ratio	Kidney wt	Kidney wt/body wt ratio
CONTROL	223.2±2.70	0.77±0.02	0.34	5.63±0.36	2.52	1.23±0.096	0.51
FPP CONT	220.2±3.33	0.76±0.03	0.34	4.7±0.42	2.13	0.94±0.066	0.42
DOX CONT	200.6±2.78^{aa}	0.93±0.03^{aaa}	0.46	7.65±0.44^{aaa}	3.81	1.9±0.085^{aa}	0.94
100mg/kgbw FPP+DOX	208.6±3.44^{a}	0.88±0.02^{aa}	0.42	6.65±0.38^{a}	3.19	1.5±0.079^{a}	0.71
250mg/kgbw FPP+DOX	215.2± 3.02^{b}	0.79±0.03^{bb}	0.37	6.32±0.46^{b}	2.90	1.3±0.091^{b}	0.60

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

2.3.2. Serum function markers

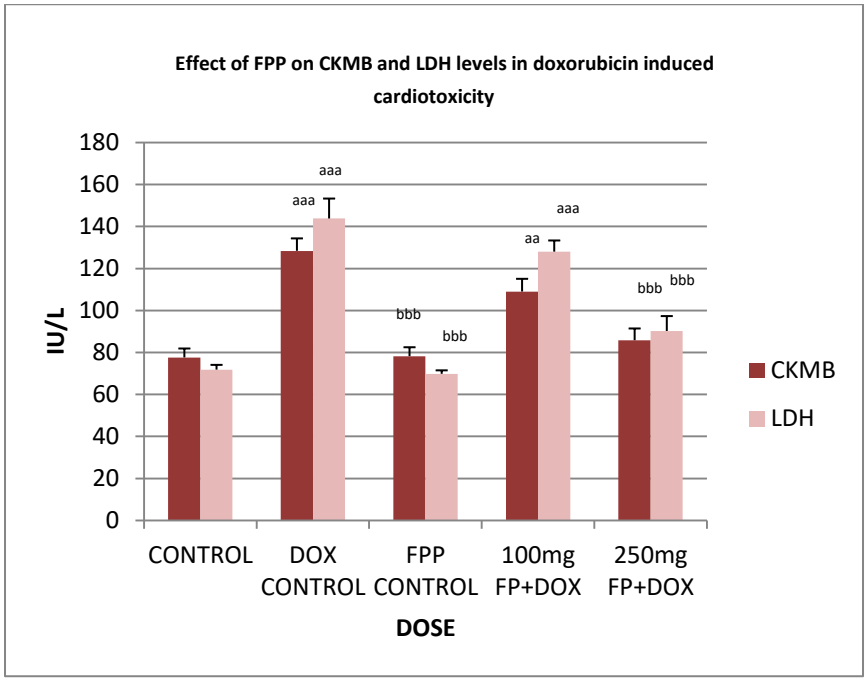
Cardiac function markers: Notable increase ($p<0.001$) in CK-MB and LDH activity was observed after the treatment of Doxorubicin, which was significantly ($p<0.001$) attenuated by FPP pretreatment with 250mg/kg bw (Group 5) but not by FPP pretreatment with 100mg/kg bw (Group 4)(Table 2.3.2).

Table-2.3.2. Effect of FPP on CK MB and LDH in doxorubicin induced cardiotoxicity in rats

TREATMENT	CK_MB	LDH
CONTROL	77.6 ± 4.26	71.8 ± 2.26
DOX CONTROL	128.4 ± 5.91^{aaa}	143.8 ± 9.48^{aaa}
FPP CONTROL	78.2 ± 4.25^{bbb}	69.8 ± 1.65^{bbb}
100mg/kg bw FPP+DOX	109 ± 6.05^{aa}	128 ± 5.31^{aaa}
250mg/kg bw FPP+DOX	85.8 ± 5.59^{bbb}	90.2 ± 7.1^{bbb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG:2.3.1.



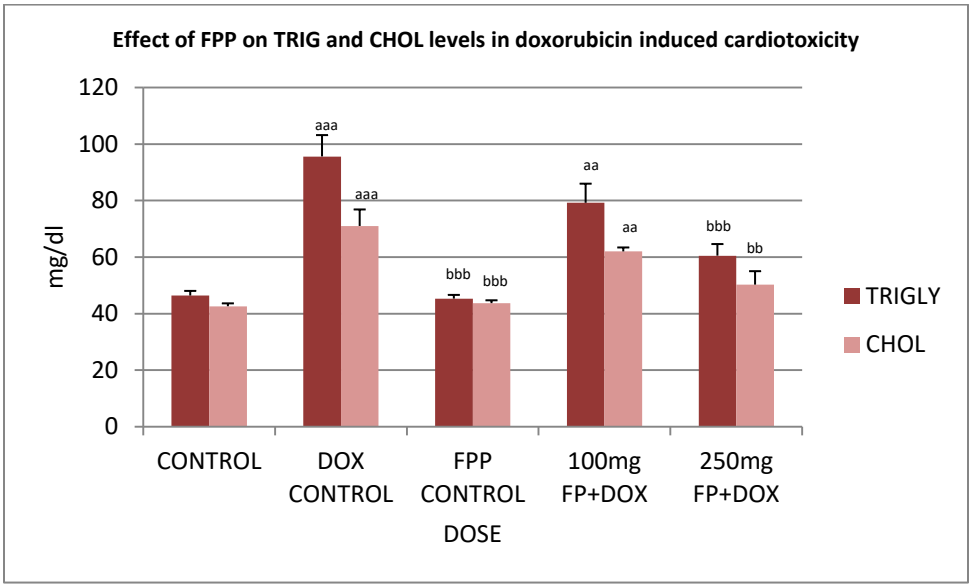
Lipid profile: Doxorubicin treated animals showed a marked increase in cholesterol and triglyceride levels. FPP 100mg/kg bw pretreatment (Group 4) showed insignificant decrease in both cholesterol as well as triglyceride levels. FPP 250 mg/kg bw (Group 5) however, showed a significant correction ($p<0.01$) in the levels of cholesterol and triglycerides. (Table 2.3.3)

Table-2.3.3. Effect of FPP on Cholesterol and Triglycerides in doxorubicin induced cardiotoxicity in rats

TREATMENT	TRIGLYCERIDE	CHOLESTEROL
CONTROL	46.4 ± 1.63	42.6 ± 1.02
DOX CONTROL	95.6 ± 7.55^{aaa}	71 ± 5.85^{aaa}
FPP CONTROL	45.2±1.31^{bbb}	43.6±1.02^{bbb}
100mg/kg bw FPP+DOX	79.2 ± 6.76^{aa}	62 ± 1.40^{aa}
250mg/kg bw FPP+DOX	60.54± 4.04^{bbb}	50.2 ± 4.76^{bb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{a}P<0.05, ^{aa}P<0.01, ^{aaa}P<0.001
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{b}P<0.05, ^{bb}P<0.01, ^{bbb}P<0.001

FIG: 2.3.2.



Hepatic function markers: Doxorubicin treated rat showed a significant ($p<0.001$) elevation in levels of hepatic serum markers compared to control rats . FPP supplementation of dose 250mg/kg Bw to rats resulted in significant decrement in hepatic function marker SGPT ($p<0.001$), SGOT ($p<0.01$) and ALP ($p<0.01$) compared to doxorubicin treated rats. FPP 100mg/kg bw pretreatment (Group 4) showed insignificant decrease in both SGOT and ALP levels but SGPT levels raised with minimum significance ($p<0.05$)

Table-2.3.4. Effect of FPP on SGPT, SGOT and ALP in doxorubicin induced hepatotoxicity in rats

TREATMENTS	SGPT	SGOT	ALP
CONTROL	30±4.11	59.2±5.0	167.2±8.413
DOX CONTROL	80±4.43^{aaa}	115±8.06^{aaa}	257.8±6.763^{aaa}
FPP CONTROL	31.36±3.11^{bbb}	67.1±4.6^{bb}	156.3±4.234^{bbb}
100mg FP+DOX	57±5.21^{aab}	92.8±10.8^{a}	226±11.815^{aa}
250mg FP+DOX	39±7.273^{bbb}	69.4±7.47^{bb}	202.25±13.664^{bb}

Values are expressed as Mean ± SE.(n=5)

Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{a}P<0.05, ^{aa}P<0.01, ^{aaa}P<0.001

Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{b}P<0.05, ^{bb}P<0.01, ^{bbb}P<0.001

FIG: 2.3.3.

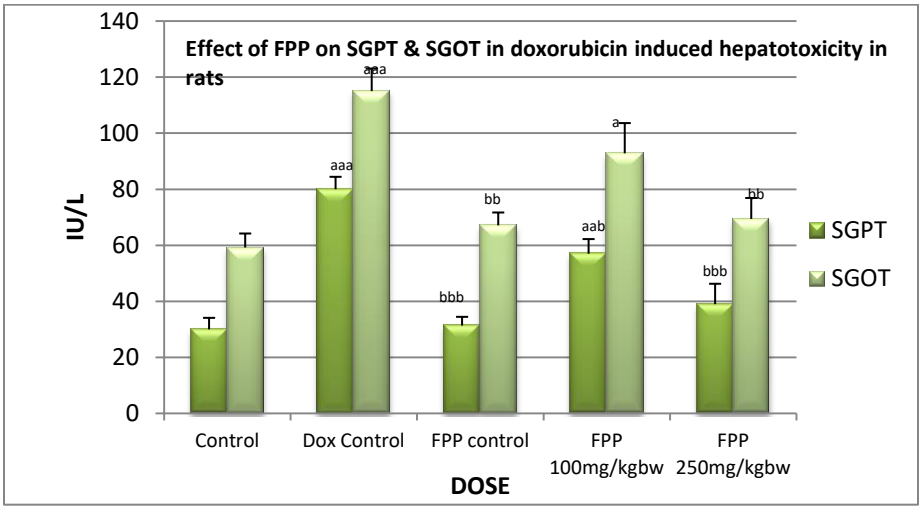
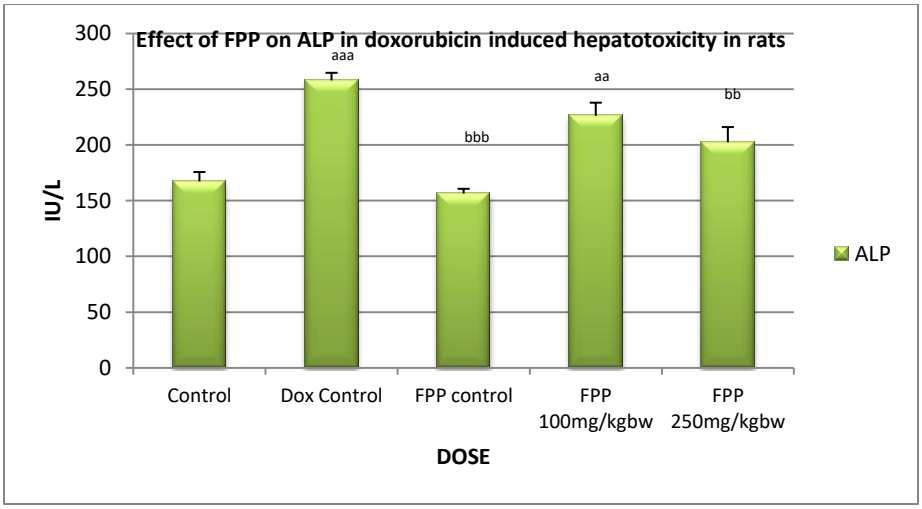


FIG: 2.3.4.



Renal function markers: A significant increase in ($p<0.001$) in the levels of Urea and Creatinine were observed after the treatment of Doxorubicin. But 100mg/kg bw dose of FPP helped to decrease the levels of urea insignificantly and Creatinine significantly ($p<0.01$).These levels were significantly ($p<0.001$) attenuated by FPP pretreatment with 250mg/kg bw (Group 5)

Table- 2.3.5. Effect of FPP on Urea and Creatinine in doxorubicin induced hepatotoxicity in rats

TREATMENTS	UREA	CREATININE
CONTROL	35±1.517	0.86±0.0143
DOX CONTROL	60.2±3.861^{aaa}	1.38±0.089^{aaa}
FPP CONTROL	31.1±1.35^{bbb}	0.841±0.013^{bbb}
100mg FP+DOX	50.6±3.20^{aa}	1.038±0.019^{aabb}
250mg FP+DOX	43±1.476^{bbb}	0.905±0.035^{bbb}

Values are expressed as Mean ± SE.(n=5)

Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$

Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG:2.3.5.

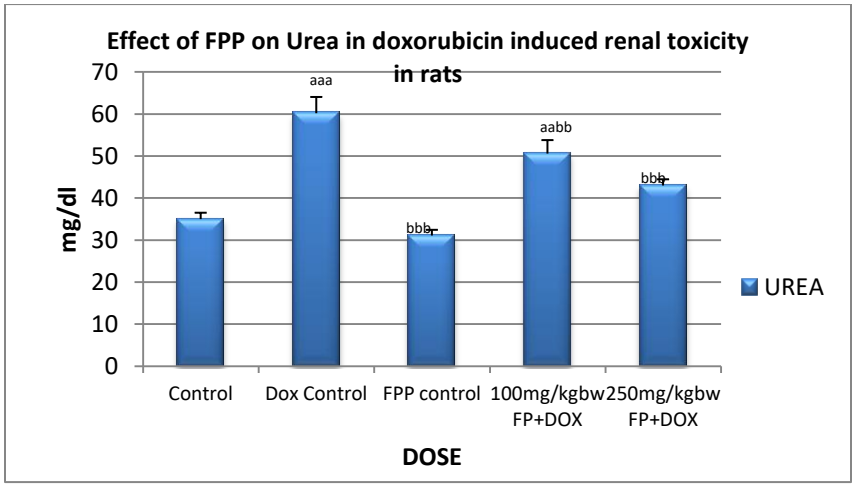
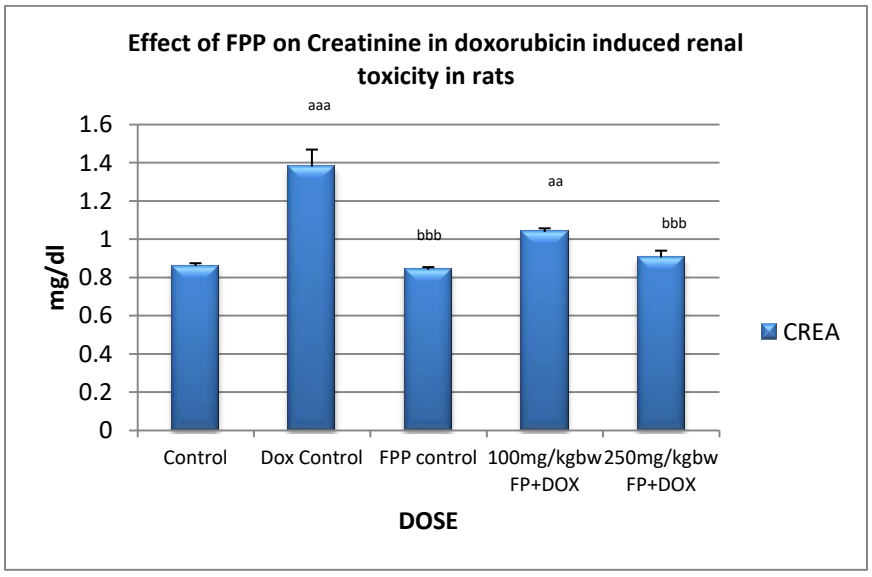


FIG: 2.3.6.



2.3.3. Antioxidant Status and Lipid Peroxidation in Tissues.

Glutathione, Glutathione Peroxidase, Superoxide dismutase, Catalase and LPO in heart:

GSH- Group 2 rats showed a significant decrease ($p<0.001$) in GSH levels when compared to control (Group 1). Group 4 rats showed insignificant increase in GSH levels but pretreatment with 250 mg/kg bw FPP was helpful in negating the adverse effect induced by Dox. ($P<0.05$). (Table 2.3.6)

Dox treatment reduced the activity of all the three enzymes viz. GPx, SOD and Catalase significantly ($p<0.001$) in the heart tissue. Pretreatment with FPP 250 mg/kg bw restored the activities of these enzyme to near normal however pretreatment with FPP 100 mg/kg bw did not show any significant attenuation of Dox rendered effects. (Table 2.3.7)

Doxorubicin significantly($p<0.01$) increased the levels of LPO in group 2 rats but dose of 250mg FPP significantly ($p<0.05$) attenuated the adverse effect of doxorubicin. (Table 2.3.8)

Table-**2.3.6. Effect of FPP on Glutathione peroxidise & Glutathione in doxorubicin treated heart of rats.**

TREATMENT	GPX	GSH
CONTROL	4.2 ± 0.21	5.08 ± 0.4
DOX CONTROL	1.12 ± 0.13^{aaa}	1.3 ± 0.4^{aaa}
FPP CONTROL	3.56 ± 0.14^{bbb}	5.32 ± 0.53^{bbb}
100mg/kgbw FPP+DOX	1.92 ± 0.18^{aaab}	2.68 ± 0.71^{aa}
250mg/kgbw FPP+DOX	3.8 ± 0.16^{bbb}	3.82 ± 0.49^{b}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG: 2.3.7.

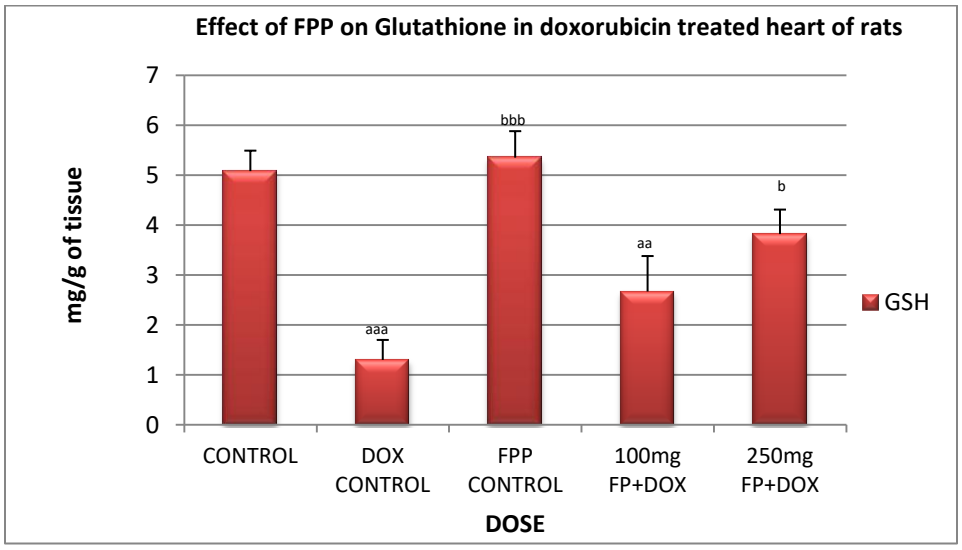


FIG: 2.3.8.

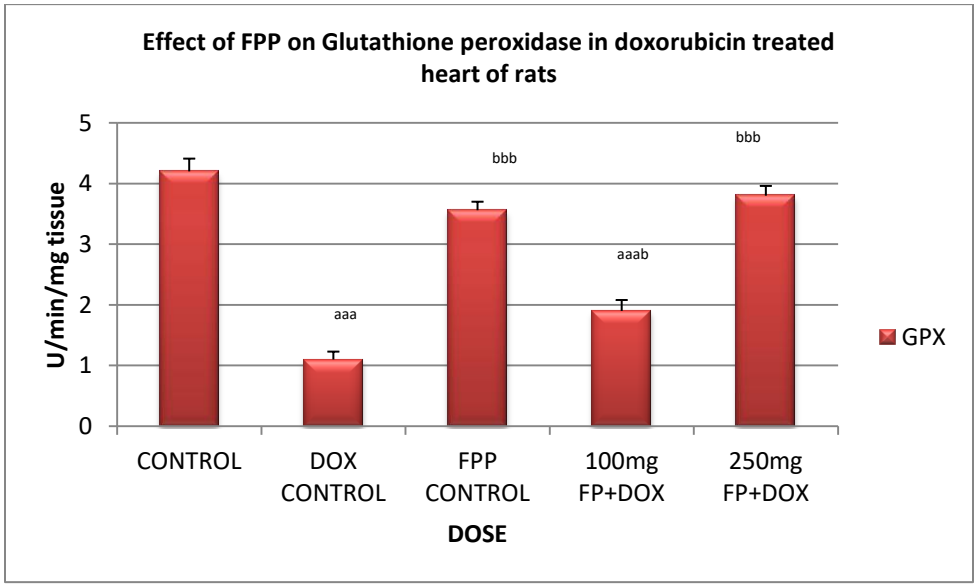


Table- 2.3.7. Effect of FPP on Superoxide dismutase & Catalase in doxorubicin treated heart of rats.

TREATMENT	CATALASE	SOD
CONTROL	8.08 ± 0.88	6.19 ± 0.52
DOX CONTROL	3.34 ± 0.72^{aa}	2.03 ± 0.46^{aaa}
FPP CONTROL	8.02±0.76^{bb}	5.89±0.47^{bbb}
100mg/kgbw FPP+DOX	3.85 ± 0.7^{aa}	3.3 ± 0.62^{a}
250mg/kgbw FPP+DOX	6.54± 0.64^{b}	5.04 ± 0.71^{bb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{a}P<0.05, ^{aa}P<0.01, ^{aaa}P<0.001
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{b}P<0.05, ^{bb}P<0.01, ^{bbb}P<0.001

FIG: 2.3.9.

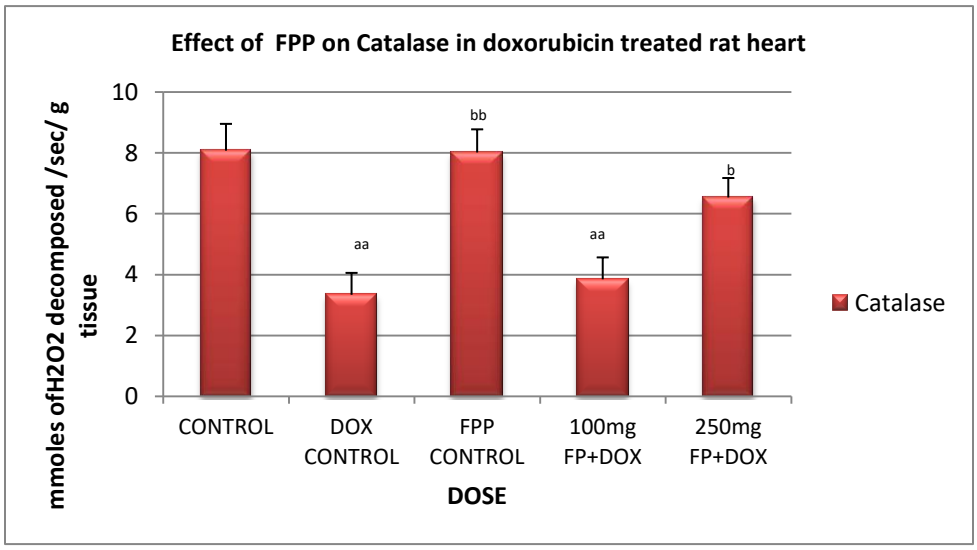


FIG:2.3.10.

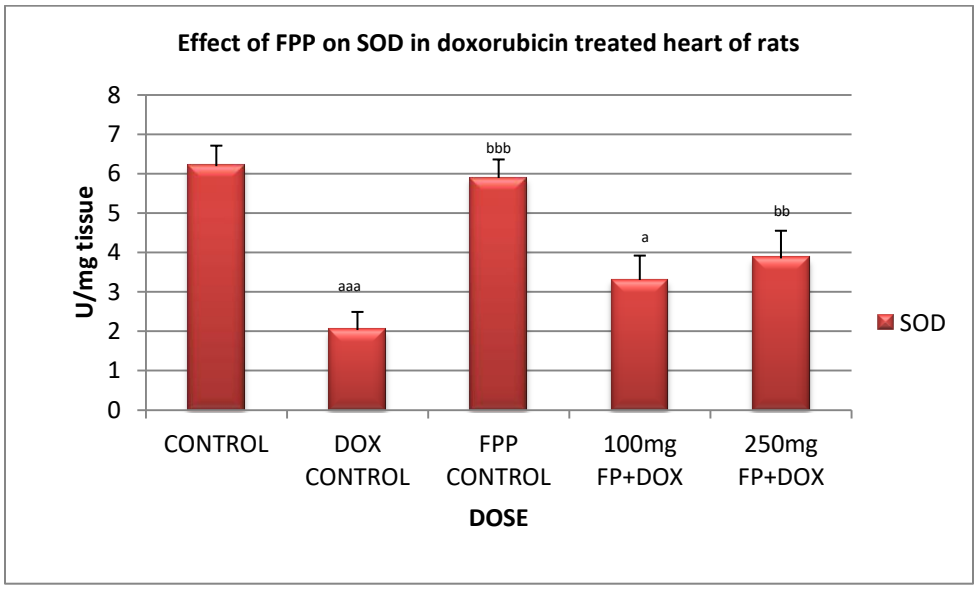
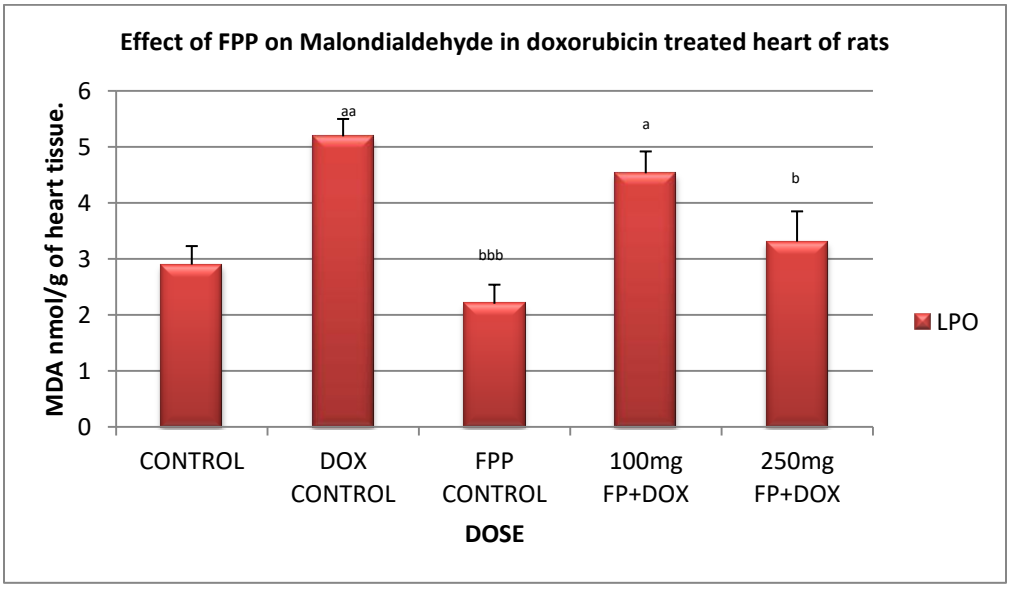


Table-2.3.8. Effect of FPP on Malondialdehyde, in doxorubicin treated heart of rats.

TREATMENT	LPO
CONTROL	2.9 ± 0.33
DOX CONTROL	5.19 ± 0.3^{aa}
FPP CONTROL	2.2 ± 0.34^{bbb}
100mg/kgbw FPP+DOX	4.53 ± 0.39^{a}
250mg/kgbw FPP+DOX	3.31 ± 0.54^{b}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG: 2.3.11.



Glutathione, Glutathione Peroxidase, Superoxide dismutase, Catalase and LPO in liver:

GSH- Doxorubicin control (Group 2) rats showed a significant decrease ($p<0.001$) in GSH levels when compared to Control (Group 1). Group 4 rats, treated with low dose of FPP showed significant increase in GSH levels ($P<0.05$) but pretreatment with 250 mg/kg bw FPP was helpful in negating the adverse effect induced by Dox more efficiently ($P<0.001$)(Fig: 2.3.12)

Doxorubicin treatment reduced the activity of all the three enzymes viz. GPx, SOD and Catalase significantly ($p<0.001$) in the liver tissue. Pretreatment with FPP 250 mg/kg bw restored the activities of these enzyme to near normal however pretreatment with FPP 100 mg/kg bw did not show any significant attenuation of Dox rendered effects. (TABLE-2.3.10, Fig: 2.3.12)

Levels of LPO in liver increased in doxorubicin control ($p<0.001$) rats but significantly decreased ($p<0.01$) with higher dose of FPP. (Fig: 2.3.16**)**

TABLE-2.3.9. Effect of FPP on Glutathione peroxidise and Glutathione in doxorubicin treated liver of rats

Treatments	GSH	GPX
CONTROL	7.26±0.21	8.56±0.56
DOX CONTROL	3.3 ± 0.43^{aaa}	3.28 ± 0.19^{aaa}
FPP CONTROL	7.65 ± 0.63^{bbb}	8.12 ± 1.67^{bb}
100mg FPP+DOX	5.06 ± 0.43^{a}	4.76 ± 0.35^{a}
250mg FP+DOX	6.733 ± 0.32^{bbb}	6.94 ± 0.93^{b}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG: **2.3.12.**

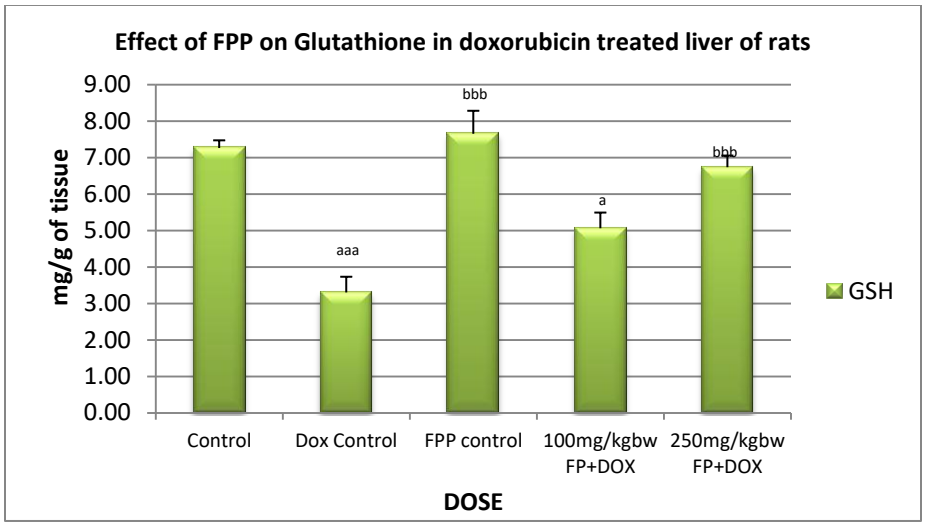


FIG: **2.3.13.**

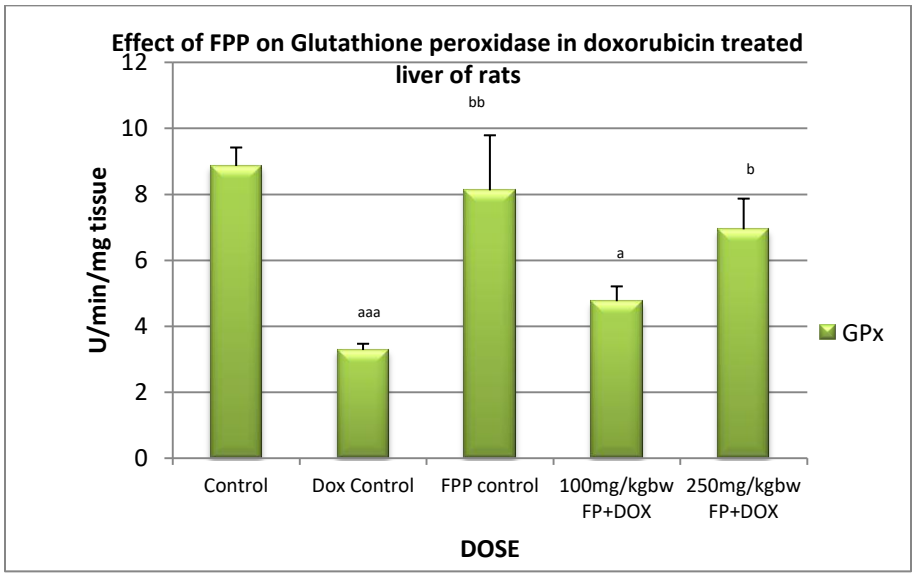


TABLE-2.3.10. Effect of FPP on Catalase & Superoxide dismutase in doxorubicin treated liver of rats

Treatments	Catalase	SOD
CONTROL	32.98±2.67	10.28±0.07
DOX CONTROL	14.56±1.79^{aaa}	3.67±0.37^{aaa}
FPP CONTROL	34.02±1.45^{bbb}	9.833±1.75^{bbb}
100mg FPP+DOX	21.96±1.82^{a}	5.98±0.63^{a}
250mg FP+DOX	27.38±3.568^{bb}	7.86±0.94^{b}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{a}P<0.05, ^{aa}P<0.01, ^{aaa}P<0.001
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. ^{b}P<0.05, ^{bb}P<0.01, ^{bbb}P<0.001

FIG: **2.3.14**

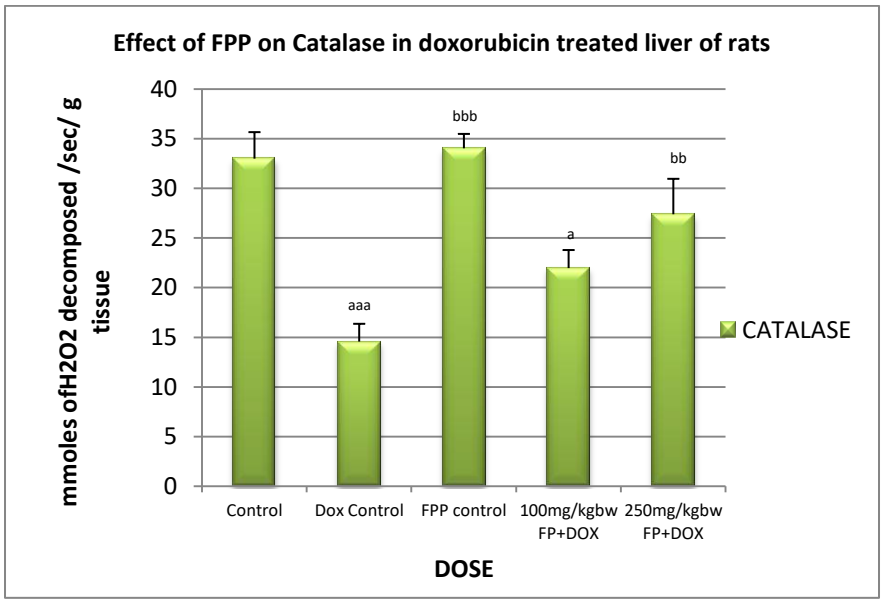


FIG: **2.3.15**

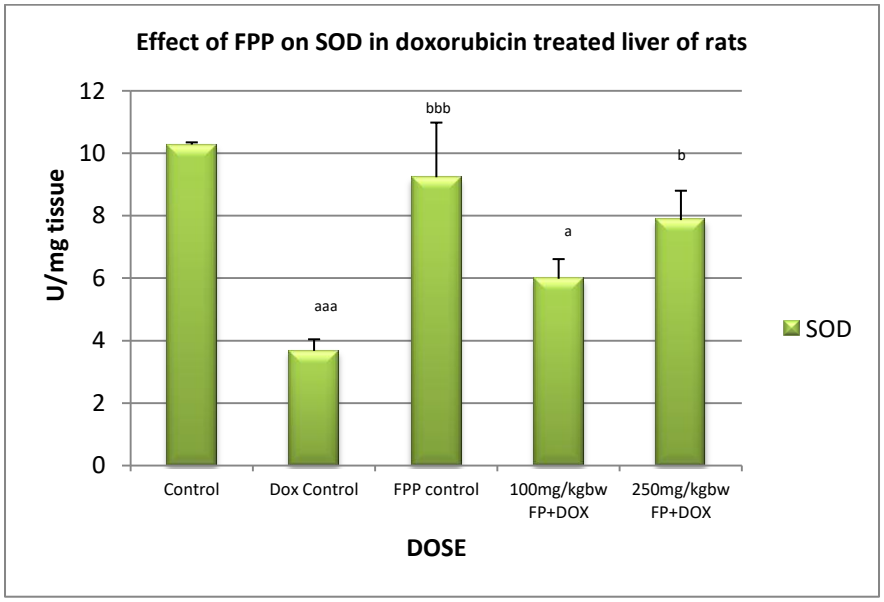
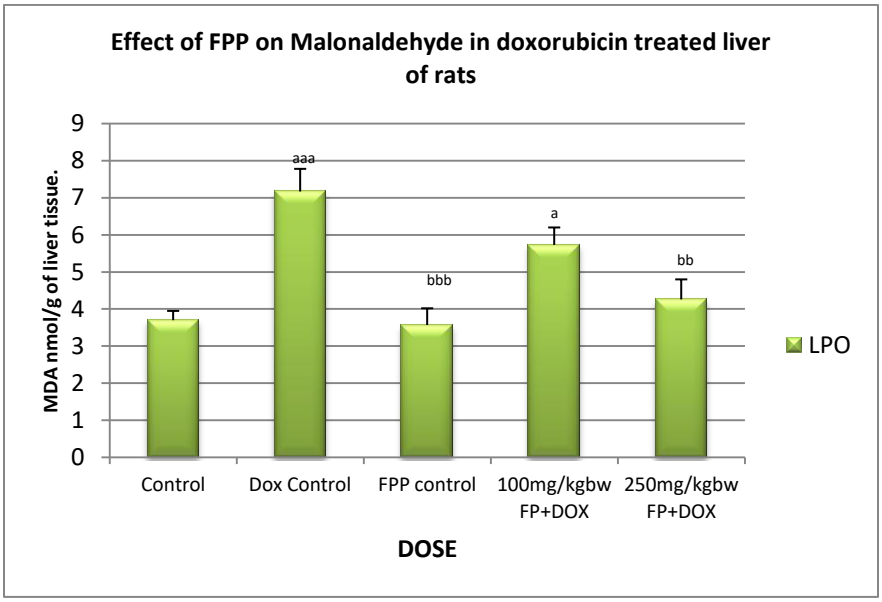


TABLE-2.3.11. Effect of FPP on Malondialdehyde, in doxorubicin treated liver of rats

Treatments	LPO
CONTROL	3.7±0.25
DOX CONTROL	7.17 ± 0.61^{aaa}
FPP CONTROL	3.57 ± 0.45^{bbb}
100mg FPP+DOX	5.73 ± 0.47^{a}
250mg FP+DOX	4.26 ± 0.54^{bb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG: **2.3.16**



Glutathione, Glutathione Peroxidase, Superoxide dismutase, Catalase and LPO in kidney:

GSH and GPX levels in kidney, significantly decreased in the rats treated with Doxorubicin ($p<0.001$) but GSH and GPX level increased more significantly in rats treated with 250 mg/kg bw of FPP as compared to lower dose of FPP.

There is an insignificant increase in the levels of Catalase and SOD in rats treated with 100mg/kg bw FPP which was lowered due to doxorubicin treatment. But FPP 250mg/kg bw dose significantly ($p<0.01$) and ($p<0.05$) respectively, increased the levels of Catalase and SOD.

Doxorubicin significantly increased the levels of LPO in group 2 rats but dose of 250mg FPP significantly ($p<0.001$) attenuated the adverse effect of doxorubicin. (Table-2.3.12, Table-2.3.13, Table-2.3.14)

TABLE-2.3.12. Effect of FPP on Glutathione peroxidise Glutathione in doxorubicin treated kidney of rats

Treatments	GSH	GPX
CONTROL	5.75±0.35	4.83±0.45
DOX CONTROL	1.25 ± 0.07^{aaa}	1.5 ± 0.19^{aaa}
FPP CONTROL	5.13 ± 0.23^{bb}	4.58 ± 0.29^{bbb}
100mg FPP+DOX	2.89 ± 0.65^{a}	2.6 ± 0.6^{aa}
250mg FP+DOX	4.8 ± 1.28^{bb}	3.64 ± 0.21^{bb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG: **2.3.17**

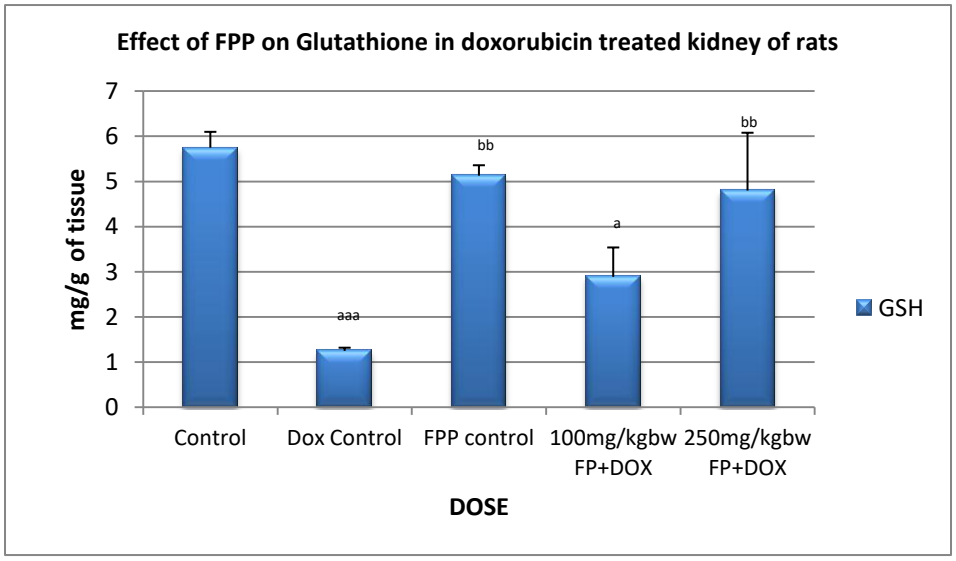


FIG: **2.3.18**

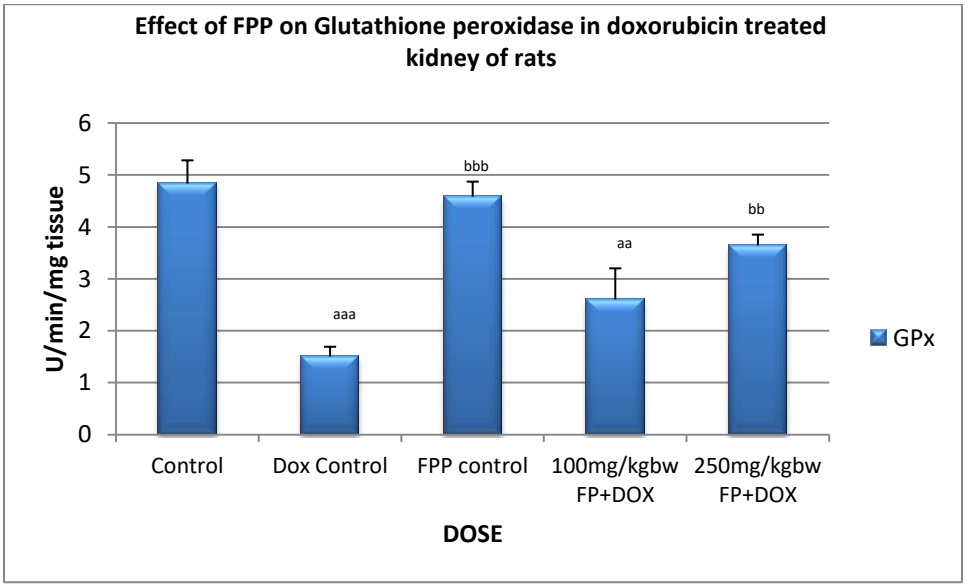


TABLE-2.3.13. Effect of FPP on Catalase & Superoxide dismutase in doxorubicin treated kidney of rats

Treatments	Catalase	SOD
CONTROL	39.35±1.56	4.85±0.96
DOX CONTROL	18.85 ± 2.7^{aaa}	0.95 ± 0.1^{aaa}
FPP CONTROL	35.68 ± 1.75^{bbb}	4.57 ± 0.2^{bbb}
100mg FPP+DOX	24.2 ± 1.56^{aaa}	1.68 ± 0.63^{aa}
250mg FP+DOX	29.35 ± 1.43^{bb}	3.55 ± 0.2^{b}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

FIG: **2.3.19**

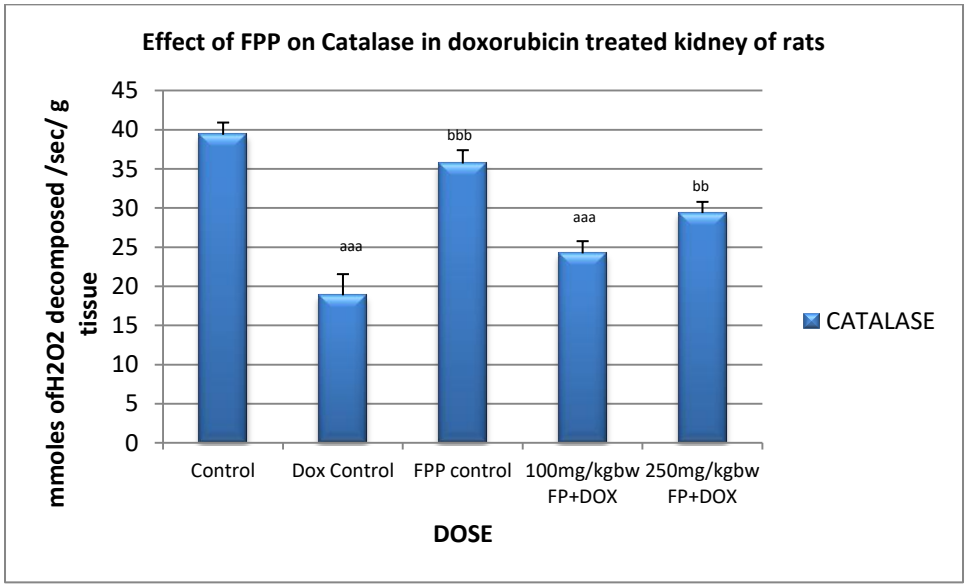


FIG: **2.3.20**

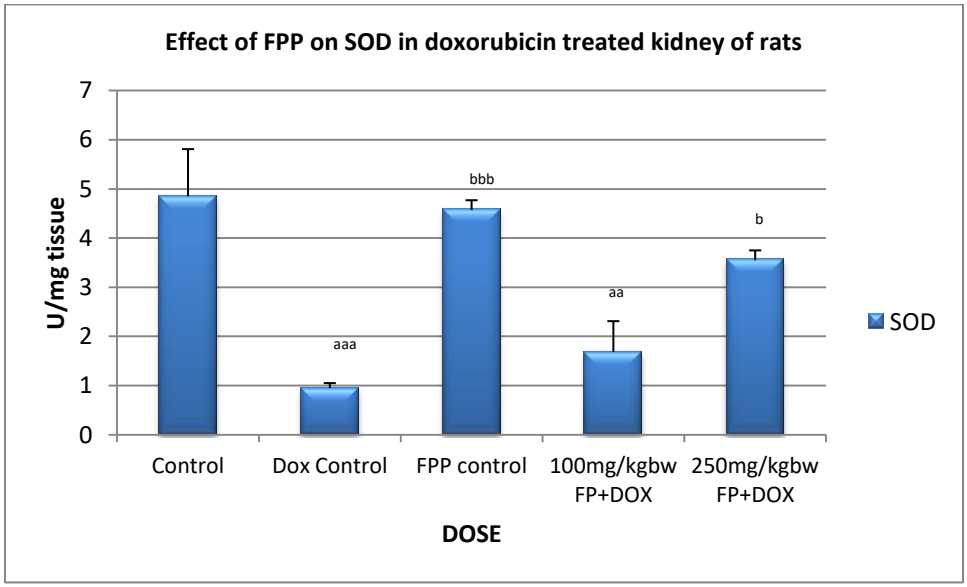
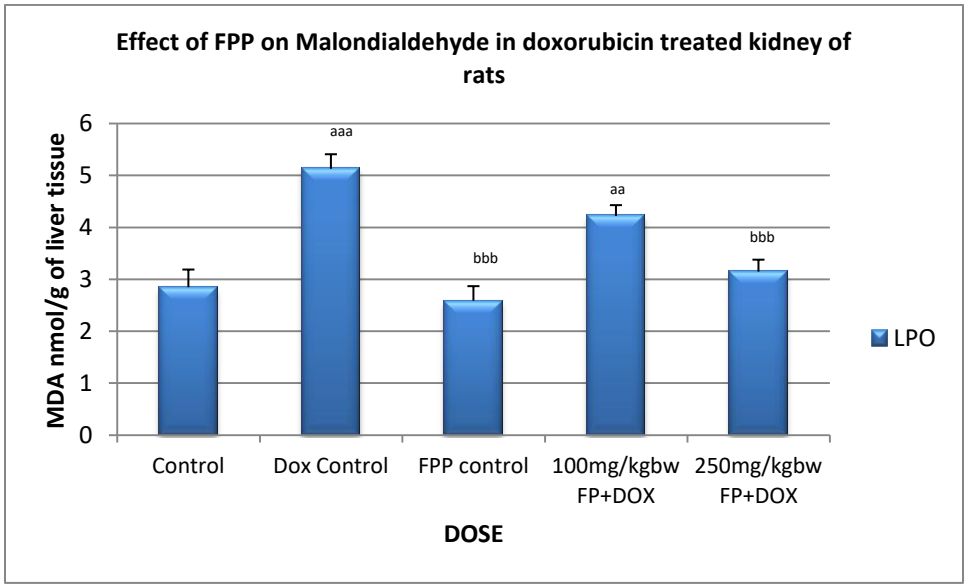


TABLE-2.3.14. Effect of FPP on Malondialdehyde in doxorubicin treated kidney of rats

Treatments	LPO
CONTROL	2.85±0.34
DOX CONTROL	5.13 ± 0.28^{aaa}
FPP CONTROL	2.59 ± 0.12^{bbb}
100mg FPP+DOX	4.22 ± 0.21^{aa}
250mg FP+DOX	3.15 ± 0.23^{bbb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{a}P<0.05$, $^{aa}P<0.01$, $^{aaa}P<0.001$
Dox control is compared with 100mg FPP+DOX & 250mg FPP+DOX resp. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

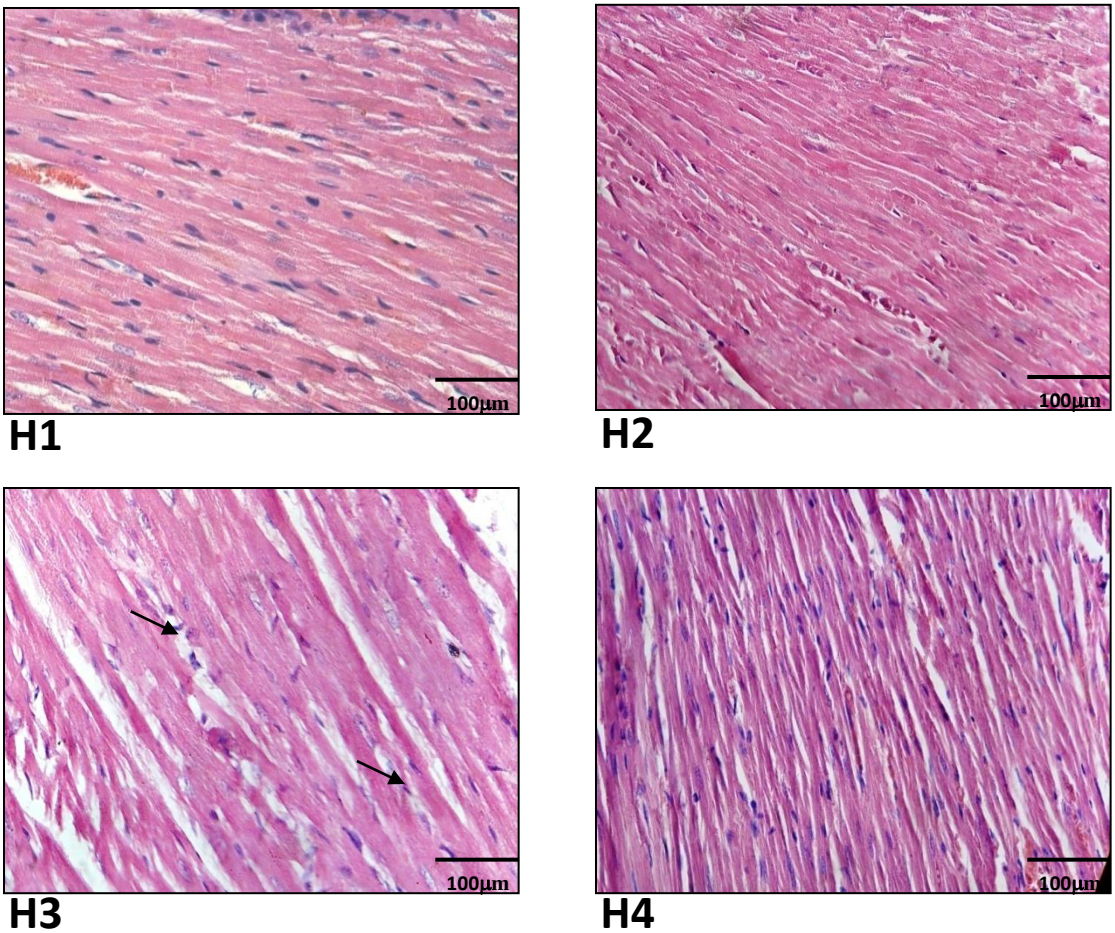
FIG: **2.3.21**



Histopathological examination of tissue section

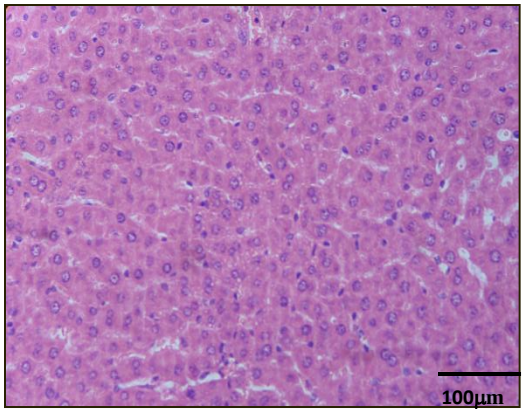
In histopathological examination it was observed that in doxorubicin treated rat heart, loss of striations and myofibrillar loss is seen but pre treatment of 250mg/kg bw dose FPP reduced the cardiac damage and showed minimal loss of striations and myofibrillar loss. The kidney sections of the doxorubicin treated animals showed thickening of the glomerular basement membrane and destructive changes in the renal tubule which is reduced to minimal in the animals pretreated with 250 mg/kg bw dose of FPP. In the liver sections, loss of tissue structural pattern and vacuolization is seen in doxorubicin treated animals but pre-treatment of 250 mg/kg bw dose of FPP protected the hepatic tissue.

PLATE 1 : Histopathological examination of rat heart(H&Ex40)

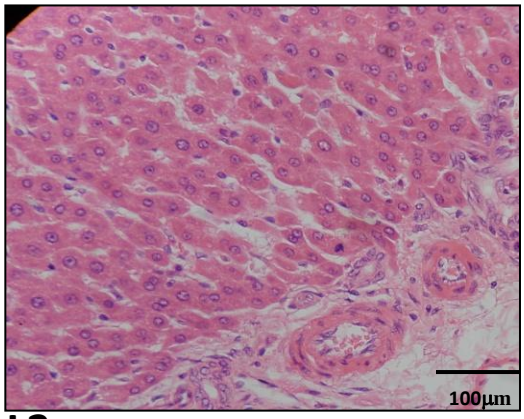


H1:- Control rat showing normal morphological appearance.
H2:- FPP treated rat showing normal morphological appearance.
H3:-DOX treated rat heart showing loss of striations and myofibrillar loss (arrow).
H4:- DOX + FPP treated heart rat showing minimal loss of striations and myofibrillarloss

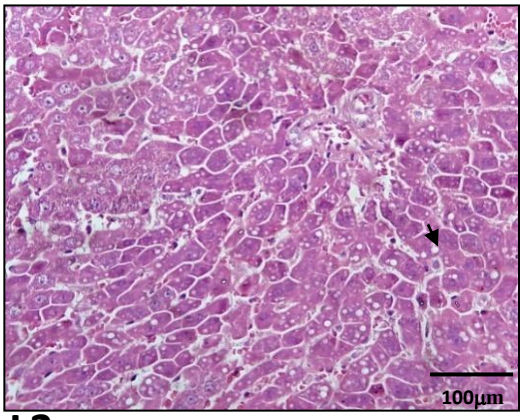
PLATE 2 : Histopathological examination of rat Liver (H&Ex40)



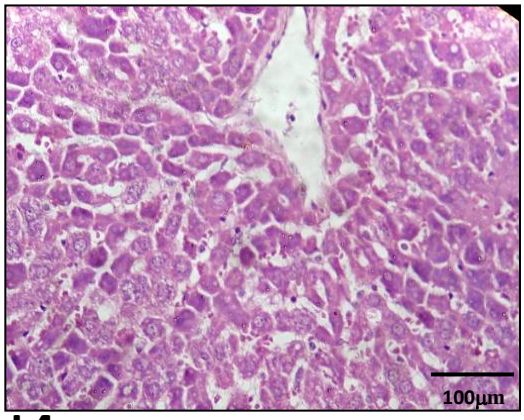
L1



L2



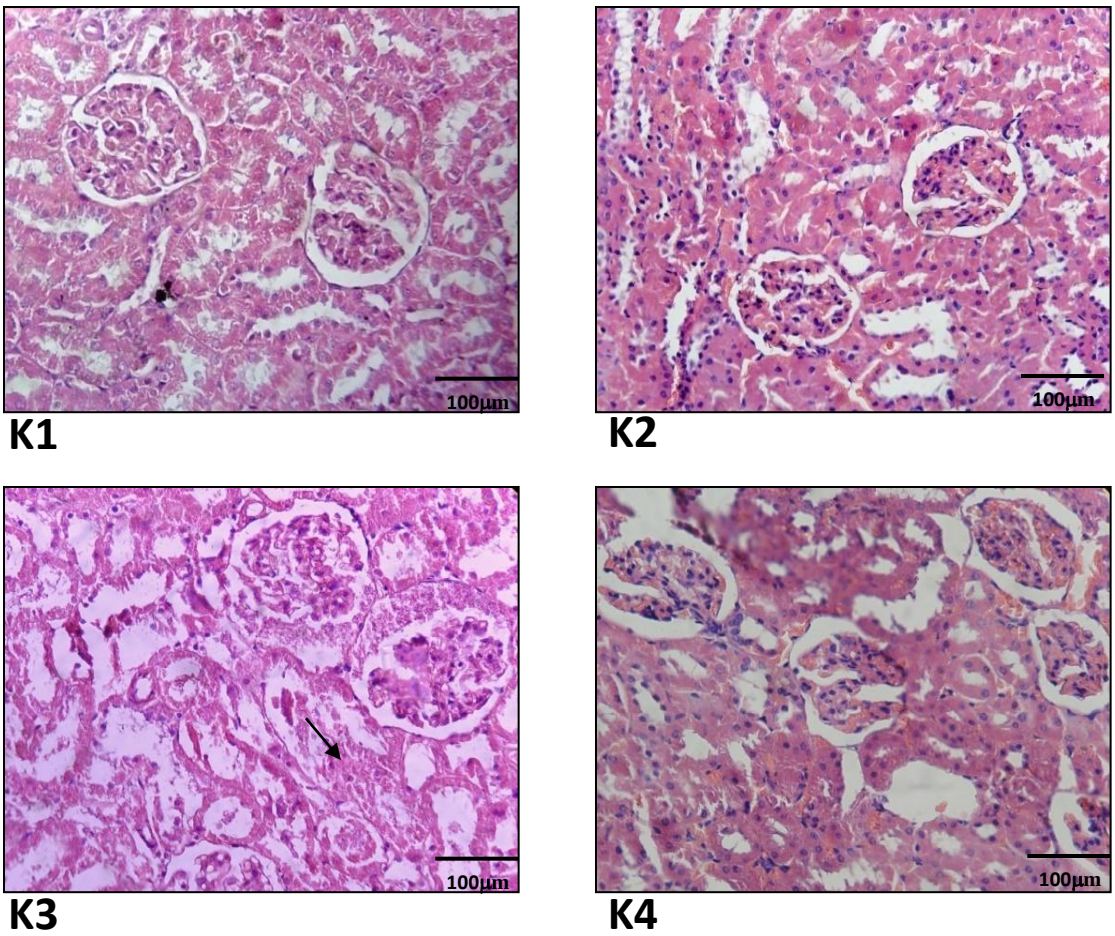
L3



L4

L1:- Control rat showing normal morphological appearance.
L2:- FPP treated rat showing normal morphological appearance.
L3:- DOX treated rat showing vacuolization and loss of hepatic tissue structural pattern.
L4:- DOX + FPP treated rat showing minimal vacuolization and near normal tissue structural pattern.

PLATE 3 : Histopathological examination of rat kidney (H&Ex40)



K1:- Control rat showing normal morphological appearance.
K2:- FPP treated rat showing normal morphological appearance.
K3:- DOX treated rat showing thickening of basement membrane *(GBM) and destructive changes in renal tubules (arrow)
K4:- DOX + FPP treated rat showing minimal lesions in renal tubules and normal GBM.

2.4. DISCUSSION

Doxorubicin is a very potent antitumor antibiotic. Its use is severely limited for its toxicity, which have been documented in a variety of animal models (Kolarovic *et al.,* 2010; Thomas *et al.,* 2002). Anticancer therapy usually demolishes the physiological homoeostasis and affects multiple organs during treatment process. Effective anticancer therapy with anthracyclines is limited because of its toxicity to various organs including heart, kidney and liver (Psotova *et al.,* 2002).

Doxorubicin induced cardio toxicity has been attributed as the basic mechanisms responsible for ROS generation and lipid per oxidation (Thomas *et al.,* 2002; Psotova *et al.,* 2002). Tissues with less developed antioxidants such as the heart are particularly susceptible to injury by doxorubicin induced free radical generation (Olson and Mushlin 1990). Doxorubicin has been demonstrated to induce cardiac toxicity in cultured cells (Chan *et al.,* 1996), isolated heart preparations (Repine 1991) animal models (Singal and Iliskovic 1998) and in humans (Lafark *et al.,* 1973). It is shown in previous studies that doxorubicin in the form of semiquinone plays the main role in nephrotoxicity and hepatotoxicity. The administration of doxorubicin leads to production of hydroxyl radicals, hydrogen peroxide and superoxide anions (Oz *et al.,* 2006). Reactive oxygen species are produced by the reaction of molecular oxygen with the semiquinone, which results in the tissue damage (El-Shitany *et al.,* 2008; Liu *et al.,* 2007; Mohan *et al.,* 2010). The objective of this study therefore was to investigate the protective effect of Fermented Papaya Preparation on doxorubicin induced hepatic and renal toxicity.

Several antioxidants have showed promising effect in reducing the Doxorubicin induced cardiotoxicity (Herman and Ferrans 1981; Speyer *et al.,* 1985; Speyer *et al.,* 1988; Iliskovic and Singal 1997; Liu *et al.,* 2002) , hepatotoxicity and nephrotoxicity, however FPP does this by affecting superoxide dismutase (SOD) and glutathione peroxidase (GPx), the very genetic pathway that eliminate free radicals from the system. Importantly, FPP does not turn into a pro-oxidant if taken in large doses, the way standard antioxidant turn out to be (Santiago *et al.,* 1991, Osato *et al.,* 1995).

In the present study, pretreatment of FPP was able to reduce the doxorubicin induced cardiotoxic, hepatotoxic and renal toxic manifestation in multiple ways. Glutathione (GSH) is a tripeptide which has many biological roles including protection against reactive oxygen species. It participates not only in antioxidant defense system but also in many metabolic processes, and conjugation and excretion of toxic compounds (Wani A 2011). Reduced levels of GSH, hamper the cellular defense mechanism against ROS. In the present study, both liver and kidney tissues from Wistar rats treated with Doxorubicin showed decreased levels of GSH. This led to decrease in the level of GPx as GSH act as a substrate for GPx (Wu *et al.,* 2004). However, pre-treatment of Fermented Papaya Preparation significantly restored the levels of GSH and GPx.

Several studies have shown that doxorubicin produce free radicals such as superoxide, hydroxyl and hydrogen peroxide which extensively react with lipids that causes lipid per oxidation (LPO) (Oz *et al.,* 2006).Lipid per oxidation is known to cause cellular damage and is primarily responsible for reactive oxygen species induced organ damage. LPO is measured in the terms of the extent of Malondialdehyde formation (Guéraud *et al.,* 2010). In the present study doxorubicin increased the Malondialdehyde formation in heart, liver

and kidney, which was successfully reduced in the animals pre-treated with FPP. (Fig 2.3.11, Fig 2.3.16 & Fig 2.3.21)

Superoxide dismutase is an important anti-oxidant defense in nearly all the cells exposed to oxygen which protect the cells from superoxide toxicity. SOD transforms superoxide ion ($O2^-$) to H_2O_2 which is later acted upon by Catalase. Catalase is a tetramer, which have four porphyrin heme groups that allow enzyme to react with H_2O_2. In the present study, it is noted that decreased amount of catalase and SOD in treated animals, led to decrease in superoxide ion and hydroxyl scavenging, which in turn, increased free radicals in the tissue and affected the normal functioning of cells. Similar results were observed by (Damodara *et al.,* 2007; Tu *et al.,* 2010). SOD and Catalase activity in the cardiac, hepatic and renal tissues increased with pre treatment of higher dose of FPP and helped the cells to retain their near normal functioning. (Table.2.3.7, Table 2.3.10, Table 2.3.13)

FPP pretreatment restricted the doxorubicin induced cardio toxic manifestation like increase in the levels of triglycerides and total cholesterol in serum significantly (Table.2.3.2). Elevated levels of alkaline phoshatase was noted in the serum of doxorubicin treated rats which may be due to obstruction of the biliary tract in liver and glomular malfunctioning in kidney but higher dose of FPP protected the tissues from this negative effect of doxorubicin (Table.2.3.4).

Urea and creatinine are very sensitive renal function markers and their infiltration from cell to serum is the sign of cellular damage. Similarly SGPT and SGOT are liver transaminases which act as potent biomarkers of liver injury. Doxorubicin increased the levels of SGPT, SGOT, Urea and Creatinine in serum significantly, which indicated the

dysfunctioning of liver and kidney cells. In the present study FPP pretreatment showed successful amelioration of this effect induced by doxorubicin. (Table.2.3.4 &Table.2.3.5)

The biochemical findings in the present study are seconded by the histological studies of tissues. Here, doxorubicin treatment caused significant histological changes in the myocardium, including marked myofribillar loss and loss of striations in heart (Plate 1) These changes in the doxorubicin treated group were in agreement to those observed by (Danese *et al.,* 2006). The myocardial tissue of rats pretreated with FPP showed minimal changes, suggesting protection from cellular damage induced by Doxorubicin treatment. The heart tissue of animals treated with FPP alone (Group 3) did not show any histological abnormalities (Plate 1). Doxorubicin treatment caused significant histological changes in the renal tissue cells which led to thickening of glomerular basement membrane. However, the FPP pretreatment helped to restore the normal architecture of the cells (Plate 3). In the liver tissue too, distortion of cells and vacuolization were clearly observed in the rats treated with doxorubicin but FPP successfully prevented the damage by the anticancer drug (Plate 2)