3. Assessment of preventive and therapeutic role of FPP in Doxorubicin induced organ toxicity

3.1. INTRODUCTION

Doxorubicin is one of the effective and useful antineoplastic agents commonly used for the treatment of a variety of tumors including solid and haemopoietic malignancies (Pointon *et al.,* 2010). However, its practical therapeutic use is limited by late-onset and acute and chronic cardiotoxicities (Lafark *et al.,*1973). The chronic cardiotoxicity is dose-dependent and causes irreversible myocardial damage, resulting in dilated cardiomyopathy with fatal congestive heart failure (VonHoff *et al.,*1979). The mechanism of doxorubicin induced cardiomyopathy is not completely understood, but several hypothesis have been postulated which include inhibition of nucleic acid (Algria *et al.,* 1990), protein synthesis, release of vasoactive amines (Bristow *et al.,* 1978) alterations in sarcolemmal Ca^{2+} transport (Singal and Pierce 1986), alterations in membrane bound enzymes (Boelsterli 2003), abnormalities in mitochondria and lysosomal alterations (Singal *et al.,* 1987) and an imbalance of myocardial electrolytes (Olson and Mushlin 1990). The most comprehensively evaluated cardiotoxicity of doxorubicin is cumulative and dose-related progressive myocardial damage leading to clinical events, ranging from an asymptomatic reduction in left ventricular ejection fraction (LVEF) to irreversible life-threatening CHF (Barrett-Lee *et al.,* 2009).

Discontinuation of doxorubicin therapy at a total dose of 550 mg/m2 has been generally advised to reduce the incidence of the cardiotoxicity. Unfortunately, this attitude prevents administration of doxorubicin to patients who might further benefit from the antitumor effect of this drug (Salouege 2014). The liposome-encapsulated anthracyclines was designed to reduce the toxicity of doxorubicin while preserving its antitumor efficacy by altering its tissue distribution and pharmacokinetics. It is found that the liposomal formulations have low incidence of CHF, alopecia, neutropenia and

thrombocytopenia. Pegylated (polyethylene glycol coated) liposome-encapsulated (PLD) form of doxorubicin, Doxil has preferential concentration in the skin because of the polyethylene glycol coating. The main dose limiting side effects associated with Doxil is the palmar plantar erythrodysesthesia (PPE), otherwise known as hand-foot syndrome. Following administration of Doxil, small amounts of the drug can leak from capillaries in the palms of the hands and soles of the feet. The result of this leakage is redness, tenderness, and peeling of the skin that can be uncomfortable and even painful. Myocet and DaunoXome are non-pegylated liposomal daunorubicin which is indicated as the first line treatment of cancers (Rafiyath and Rasul 2012). The extents of side effects are lowered in liposomal doxorubicin but not the toxicity. A study revealed that doxorubicin treatment *in vivo* causes cumulative and irreversible decrease in mitochondrial calcium loading capacity (Zhou *et al.,* 2001). Congestive heart failure, cardiomyopathy and electrocardiographic changes were demonstrated after cumulative Doxorubicin administration by Lenaz and Page 1976. Frequent administration of doxorubicin has been shown to cause cardiomyopathic changes in patients (Lafark *et al.,*1973) as well as in a variety of animal models (Singal *et al.,*1987).

Doxorubicin induced Hepatotoxicity and Renaltoxicity although rare, never the less, is an equally important toxicity effect and needs dose adjustments as well.

Some studies have been carried out showing positive effect of herbal treatments which subsides the side effects of Doxorubicin in cumulative dose regime. Jing Sun et al. has revealed that isorhamnetin can be used as an adjuvant therapy for the long-term clinical use of Doxorubicin as it protect against doxorubicin induced cardiotoxicity (Sun *et al.,* 2013). A study suggested that administration of CardiPro, with an antioxidant property, protected the Doxorubicin induced cardiotoxicity in

mice (Mohan *et al.,* 2006). The traditional Chinese medicine "Sheng-Mai-San (SMS)" which has been used for treating patients with coronary heart disease for a long time and was found to have antioxidative effects showed partial protection against Adriamycin induced cardiomyopathy (You *et al.,* 2008). A study had shown that *A. sinensis* a Chinese traditional medicine, elicited a typical protective effect on doxorubicin-related oxidative stress and Chronic Cardiotoxicity (Yan *et al.,* 2007).A study revealed that Lipistat is also cardioprotective against doxorubicin induced myocardial toxicity in albino rats (Koti *et al.,* 2009).

In the present study, acute dose of Doxorubicin (20mg/Kg) encountered significant toxicity in all the organs studied which was evident from both Biochemical parameters as well as Histopathological studies (Chapter I). Generally, chemotherapy regime is spread over a span of time (several cycles) with low dose of Dox per cycle rather than acute high dose. Several studies have reported, primarily, cardiotoxic effect of higher cumulative dose of Dox.

The aim of our study therefore was to (i) assess the effect of same total concentration of Doxorubicin in cumulative dose regime and compare its effect with that of the acute study done earlier. (ii) study the protective as well as therapeutic effect of FPP.

3.2. MATERIALS AND METHODS

3.2.1.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.

3.2.2. Experiment design

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. CPCSEA 827/ac/04), a Committee for the Purpose of Control and Supervision on Experiments on Animals.

After acclimatization, the animals were randomly divided into the following groups consist of 5 rats each.

Table 3.2.1 Experimental design

Experiment design	**SALINE**	**FERMENTED PAPAYA PREPARATION**	**DOXORUBICIN**
Group I (Control)	Animals received 1 ml normal saline orally for 5weeks	----------	----------
Group II (FPP Control)	----------	Animals received daily 250 mg/kg bw FPP orally for 5 weeks.	----------
Group III (DOX Control)	----------	----------	Animals received cumulative dose of 20mg/kg bw doxorubicin intraperitoneally twice a week for 5 weeks. (Each dose 2mg/kg bw)
Group IV (Pre-FPP + Dox-FPP + Post-FPP)	----------	Animals received 250 mg/kg bw FPP daily, starting 7 days prior to doxorubicin administration (20mg/kg bw, cumulative dose). FPP administration was further extended for 7 days as post treatment.	Animals received doxorubicin (20mg/kg bw, cumulative dose) for 5 weeks.
Group V (Pre-FPP + Dox-FPP)	----------	Animals received 250 mg/kg bw FPP daily through oral gavage for starting 7 days prior to doxorubicin administration (20mg/kg bw, cumulative dose) over a period of 5 weeks.	Animals received doxorubicin (20mg/kg bw, cumulative dose) for 5 weeks.
Group VI (Dox-FPP + Post-FPP)	----------	Animals received 250 mg FPP orally for 5 weeks along with doxorubicin (20mg/kg bw, i.p). FPP treatment was further extended for 7 days as post treatment	Animals received doxorubicin (20mg/kg bw, i.p) for 5 weeks.

Twenty-four hours after the treatment periods, overnight fasted animals were sacrificed, blood samples were collected and serum was used for the biochemical

analysis. Immediately after sacrifice heart, liver and kidney were excised and blotted free of blood as well as tissue fluid, weighed and stored at -80^{o} C till further use for analysis.

3.2.3. Observations

In the present study, FPP was given for different time periods ie. prior, post and with the treatment of doxorubicin. Serum markers of cardiac, hepatic and renal damage and lipid levels were assessed with kit methods. Among biochemical parameters Glutathione (Beutler *et al*., 1963), Glutathione peroxidase (Rotruck *et al*., 1973), Catalase (Hugo *et al*., 1987), Superoxide dismutase (Kakkar *et al*., 1984) and lipid per oxidation (Buege *et al*., 1978) were assessed. Histopathological changes in tissues were also assessed in different groups.

(Materials and Methods are described in Chapter 1)

3.3. RESULT

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

As seen in the table Table 3.3.1 the body weight of the animals significantly decreased in GROUP III. But FPP supplementation significantly raised the weight of rats in GROUP IV ($P<0.001$) ,GROUP V($P<0.001$) and GROUP VI($P<0.01$). When body weight and tissue weight ratio were calculated, it was observed that in GROUP V due to FPP supplementation heart weight/ body weight ratio and kidney weight/ body weight ratio was highly reduced as compared to that in GROUP III. Liver weight/ body weight ratio was minimum in GROUP VI.

Table 3.3.1.Body weight, Tissue weight and Ratio of Tissue weight to Body weight:

	Body weight	Heart weight	Heart wt/body wt ratio	Liver weight	Liver wt/body wt ratio	Kidney weight	Kidney wt/body wt ratio
CONTROL (I)	243.2±2.6	0.73±0.02	0.3	6.63±0.02	2.73	1.23±0.029	0.51
FPP CONTROL (II)	230.2±2.34	0.76±0.03	0.33	5.72±0.03	2.48	1.35±0.046	0.59
DOX CONTROL (III)	195.6±2.78^{aaa}	0.95±0.03^{aaa}	0.49	7.45±0.05^{aa}	3.81	1.8±0.017^{aaa}	0.92
PRE-FPP+DOX-FPP+POST FPP (IV)	218.6±3.4^{bbb}	0.98±0.02^{bb}	0.36	6.61±0.04^{bb}	3.02	1.4±0.045^{bb}	0.41
PRE-FPP+DOX (V)	215.2±3.02^{bbb}	0.85±0.03^{b}	0.41	6.32±0.03^{bb}	2.94	0.9±0.031	0.65
DOX-FPP+FPP (VI)	207±2.14^{bb}	0.79±0.02 ^{bb}	0.38	6.9±0.04 ^{b}	3.33	1.5±0.035 ^{bb}	0.63

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. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

3.3.2. Serum function markers

In FPP treated animals **cardiac** function marker CKMB showed significant restoration in GROUP IV ($P<0.001$), GROUP V ($P<0.001$) and GROUP VI ($P<0.05$) as compared to (GROUP III). Similarly LDH levels were restored significantly ($P<0.001$) in all the three FPP treated groups. (Table 3.3.2)

Triglyceride levels significantly decreased near to normal in GROUP IV, GROUP V and GROUP VI as compared to doxorubicin control animals. Cholesterol levels drop is significantly seen in GROUP IV but insignificant in GROUP V and GROUP VI as compared to doxorubicin control animals. (Table 3.3.3)

The results of the **hepatic** function test revealed that the altered SGPT levels restored significantly with the prophylactic oral administration of FPP at a dose of 250 mg/kg bw in GROUP IV($P<0.001$), GROUP V($P<0.001$) and GROUP VI($P<0.05$). SGOT levels significantly restored in GROUP IV($P<0.01$) and GROUP V($P<0.001$) but the change was insignificant in GROUP VI. (Table 3.3.3)

The **renal** function markers levels showed a significant decrease in the rats treated with 250mg FPP. Urea levels significantly decreased in GROUP IV ($P<0.001$), GROUP V ($P<0.01$) and insignificantly in GROUP VI when compared to doxorubicin control (GROUP III). ALP and Creatinine levels significantly decreased($P<0.001$) in GROUP IV, GROUP V and GROUP VI as compared to doxorubicin control animals. (Table 3.3.4)

Table 3.3.2-Effect of FPP on CK MB and LDH in doxorubicin induced cardiotoxicity in rats.

CONTROL (I)	CKMB	LDH
FPP CONTROL (II)	76.16±2.86	66.5±2.32
DOX CONTROL (III)	71.67±3.7	62±2.89
PRE-FPP+DOX-FPP+POST FPP (IV)	119.16±4.71^{aaa}	137.67±3.2^{aaa}
PRE-FPP+DOX (V)	69.34±3.83^{bbb}	91±6.6^{aabbb}
DOX-FPP+FPP (VI)	87.5±4.17^{bbb}	97.67±6.93^{aaabbb}

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:3.3.1

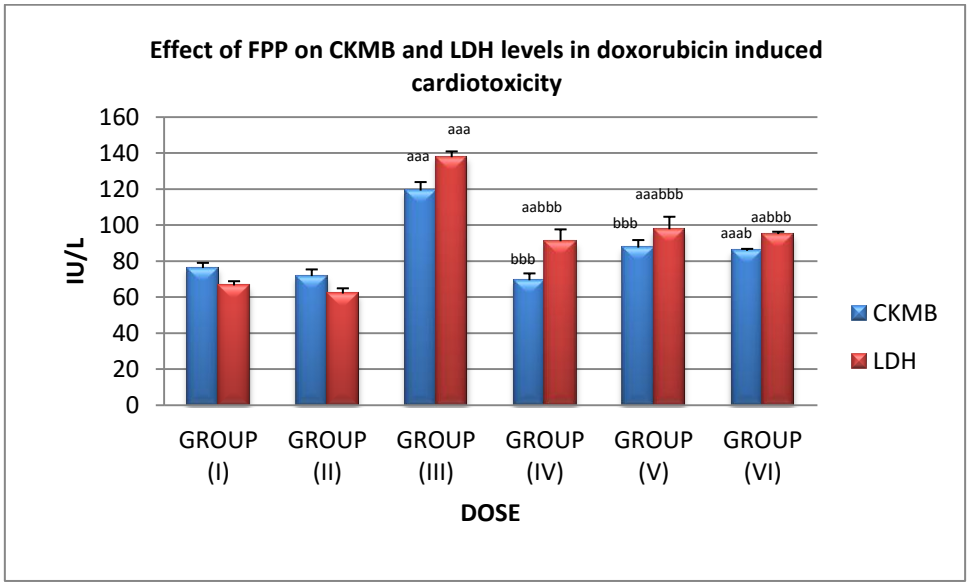


Table 3.3.3 Effect of FPP on Cholesterol and Triglycerides in doxorubicin induced cardiotoxicity in rats

CONTROL (I)	TRIG	CHOL
FPP CONTROL (II)	54.67±3.62	53.16±3.85
DOX CONTROL (III)	52.17±3.44	51.83±4.43
PRE-FPP+DOX-FPP+POST FPP (IV)	95.17±2.92^{aaa}	64.5±2.96^{aa}
PRE-FPP+DOX (V)	58±5.23^{bbb}	47.67±1.55^{bbb}
DOX-FPP+FPP (VI)	59.17±2.91^{bbb}	58.83±4.16

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:3.3.2

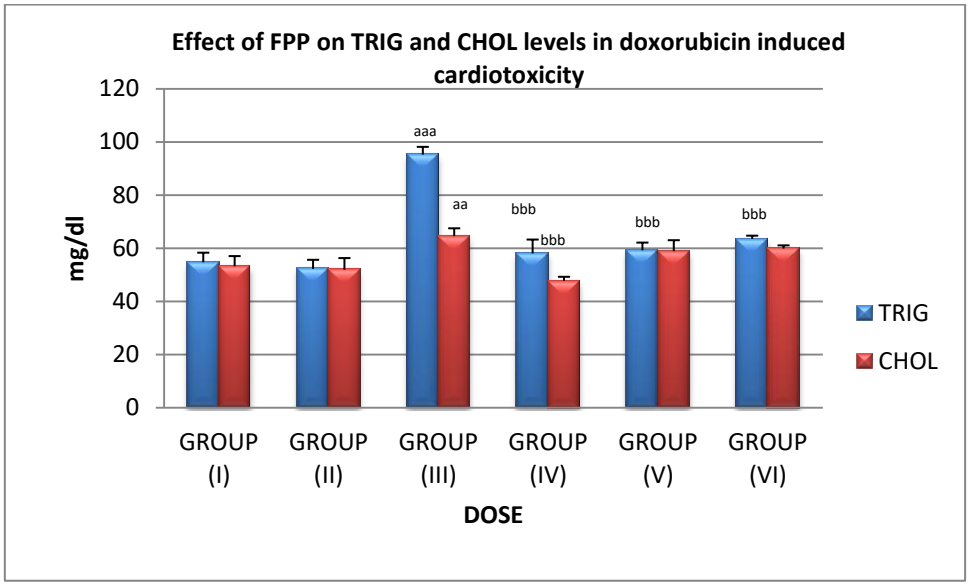


Table 3.3.4 -Effect of FPP on SGPT, SGOT and ALP in doxorubicin induced hepatotoxicity in rats

CONTROL (I)	SGPT	SGOT	ALP
FPP CONTROL (II)	31.5±2.52	53.83±4.02	149.5±3.2
DOX CONTROL (III)	31±3.58	44±3.67	154.34±5.78
PRE-FPP+DOX-FPP+POST FPP (IV)	70.5±4.39 ^{aaa}	101.67±5.89^{aaa}	283±6.61 ^{aaa}
PRE-FPP+DOX (V)	40.34±3.93 ^{bbb}	78.84±6.29^{aabb}	163.67±5.3^{bbb}
DOX-FPP+FPP (VI)	43.5±2.46 ^{bbb}	84.17±4.67^{aaa}	192.67±224^{aaabbb}.

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:3.3.3

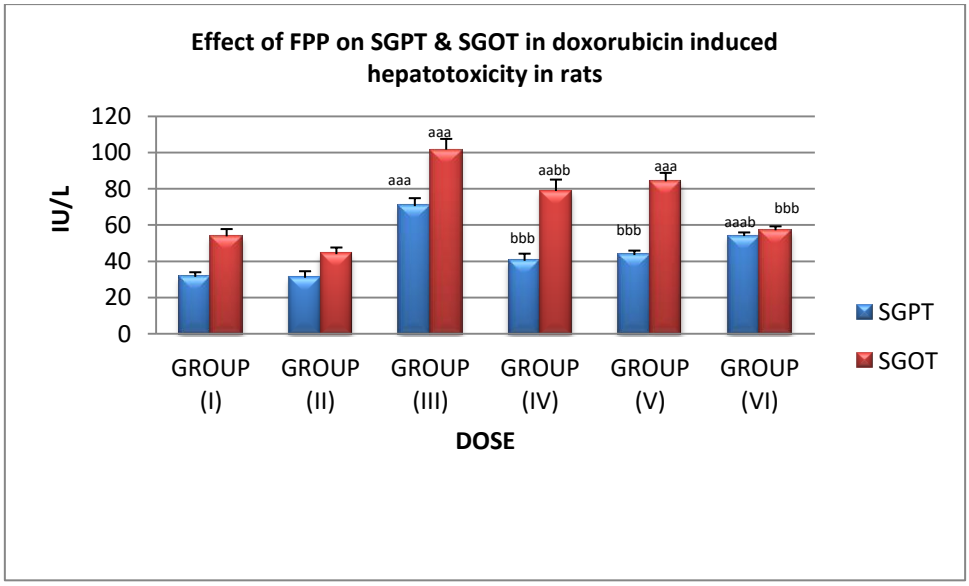


FIG:3.3.4

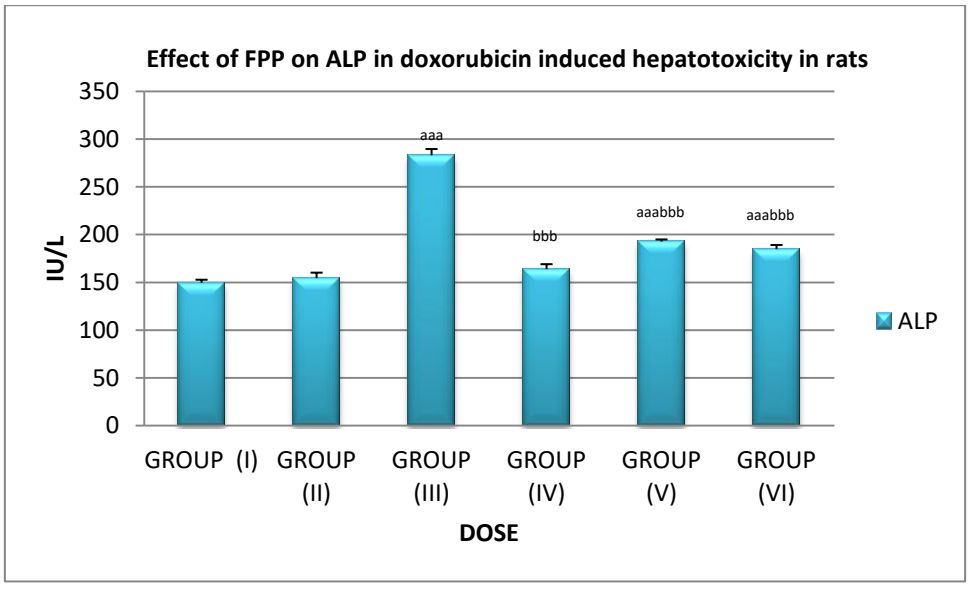


Table 3.3.5. Effect of FPP on Urea and Creatinine in doxorubicin induced hepatotoxicity in rats

CONTROL (I)	UREA	CREA
FPP CONTROL (II)	30.33±3.82	0.46±0.03
DOX CONTROL (III)	32.34±2.7	0.49±0.04
PRE-FPP+DOX-FPP+POST FPP (IV)	65.34±2.49 ^{aaa}	1.087±0.035^{aaa}
PRE-FPP+DOX (V)	41.67±1.41 ^{bbb}	0.55±0.035^{bb}
DOX-FPP+FPP (VI)	48.34±2.26^{aabb}	0.54±0.046^{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:3.3.5

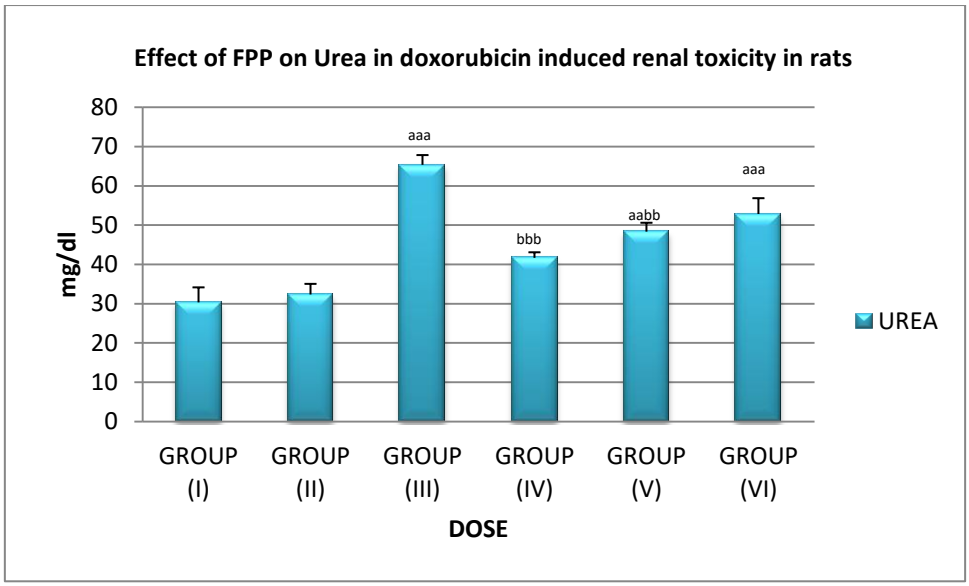
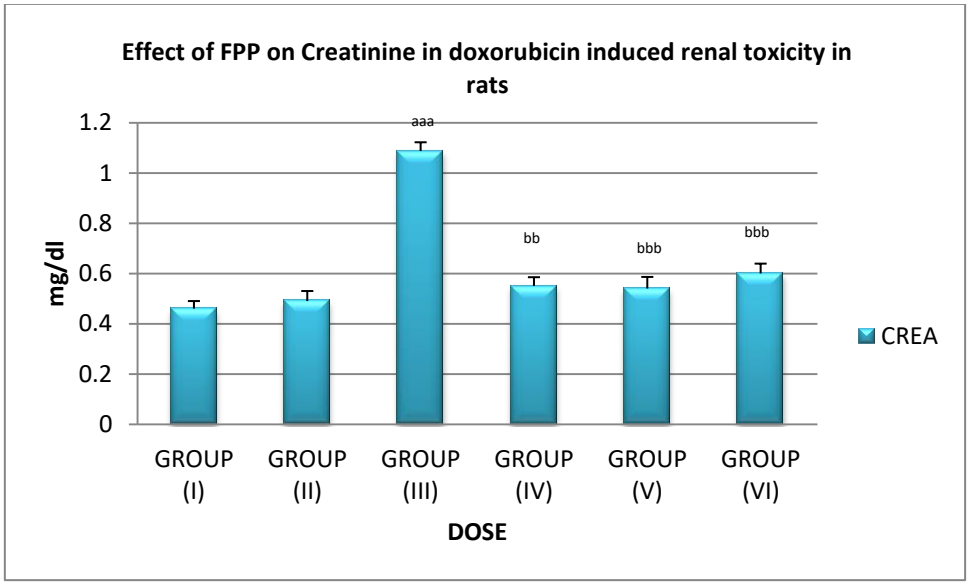


FIG:3.3.6



3.3.3. Antioxidant status and lipid peroxidation in heart.

In heart, the cumulative dose of doxorubicin decreased the levels of SOD, Catalase, GSH and GPx significantly ($P<0.001$) and increased the LPO levels less significantly ($P<0.05$) as compared to that of Control animals. In GROUP IV, there is a high significant ($P<0.001$) restoration of the levels of GSH,GPx,Catalase and LPO compared to Dox Control group. SOD levels were increased but with moderate significance as compared to GROUP III. In GROUP V, SOD ($P<0.01$), Catalase, GSH, GPx and LPO($P<0.001$ resp.) restored the effect doxorubicin treatment. In GROUP VI, SOD ($P<0.001$), Catalase($P<0.001$),GSH($P<0.01$), GPx ($P<0.05$) levels increased and LPO($P<0.001$) decreased as compared to GROUP III. (Table 3.3.6, Table 3.3.7& Table 3.3.8)

Table 3.3.6. Glutathione & Glutathione Peroxidase in heart

CONTROL (I)	GSH	GPX
FPP CONTROL (II)	5.65±0.53	3.83±0.27
DOX CONTROL (III)	5.67±0.71	3.88±0.33
PRE-FPP+DOX-FPP+POST FPP (IV)	1.15 ± 0.3^{aaa}	1.5 ± 0.13^{aaa}
PRE-FPP+DOX (V)	4.54 ± 0.34^{bbb}	4 ± 0.27^{bbb}
DOX-FPP+FPP (VI)	3.97 ± 0.45^{abb}	3.7 ± 0.36^{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:3.3.7

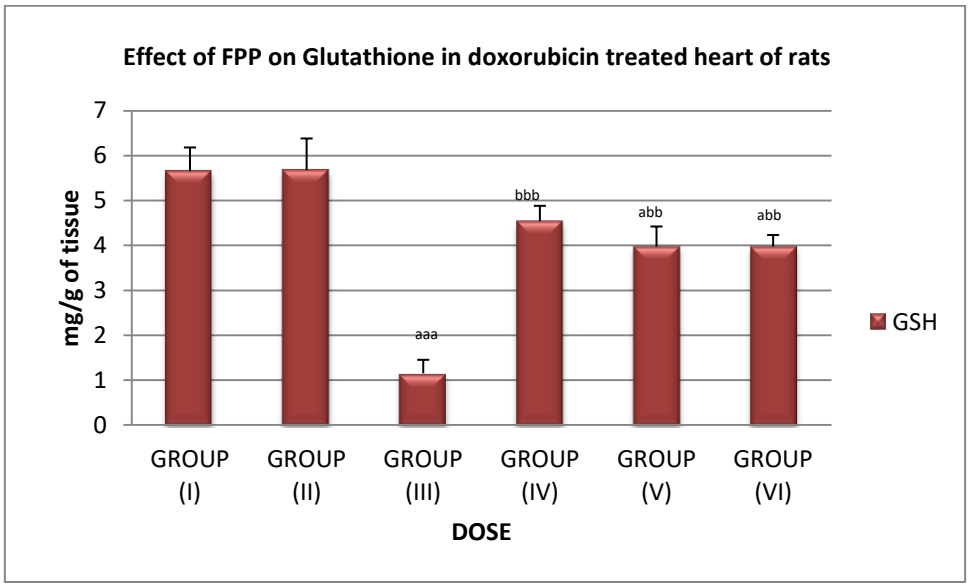


FIG:3.3.8

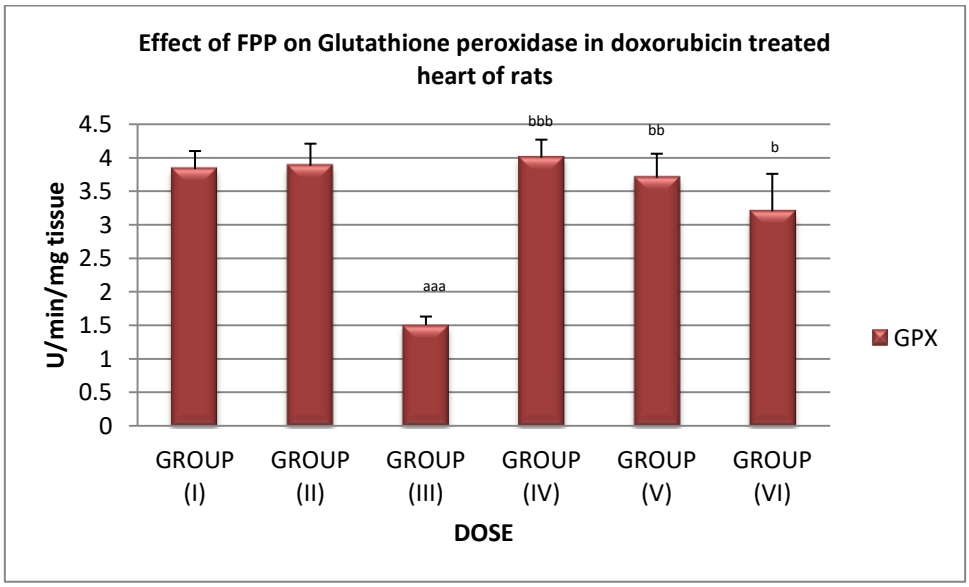


Table 3.3.7. Effect of FPP on Superoxide dismutase & Catalase in doxorubicin treated heart of rats.

CONTROL (I)	CATALASE	SOD
FPP CONTROL (II)	7.56±0.24	6.25±0.34
DOX CONTROL (III)	6.23±0.33	7.6±0.33
PRE-FPP+DOX-FPP+POST FPP (IV)	3.97 ± 0.14^{aaa}	2.25 ± 0.18^{aaa}
PRE-FPP+DOX (V)	6.9 ± 0.57^{bbb}	4.8 ± 0.3^{abb}
DOX-FPP+FPP (VI)	6.14 ± 0.21^{bb}	4.87 ± 0.27^{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:3.3.9

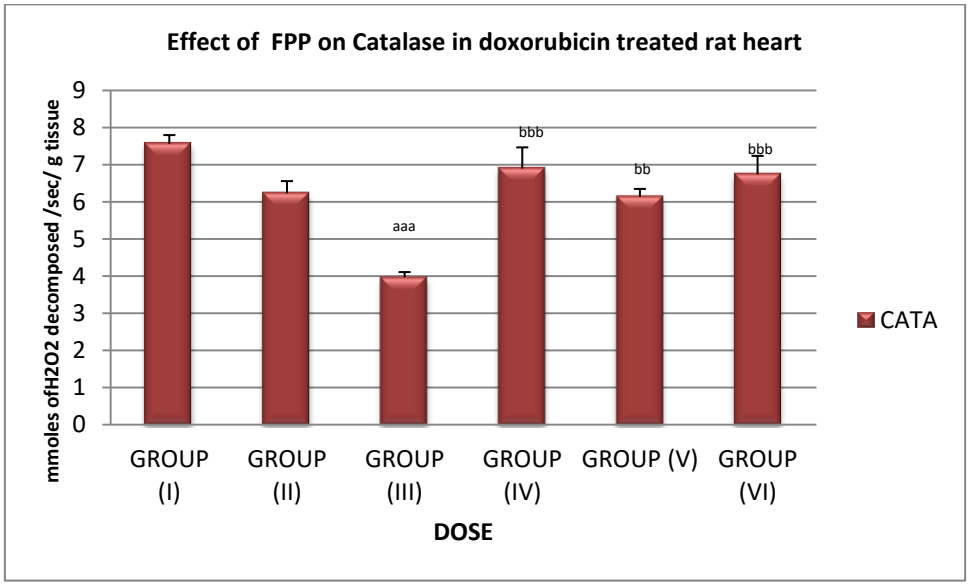


FIG:3.3.10

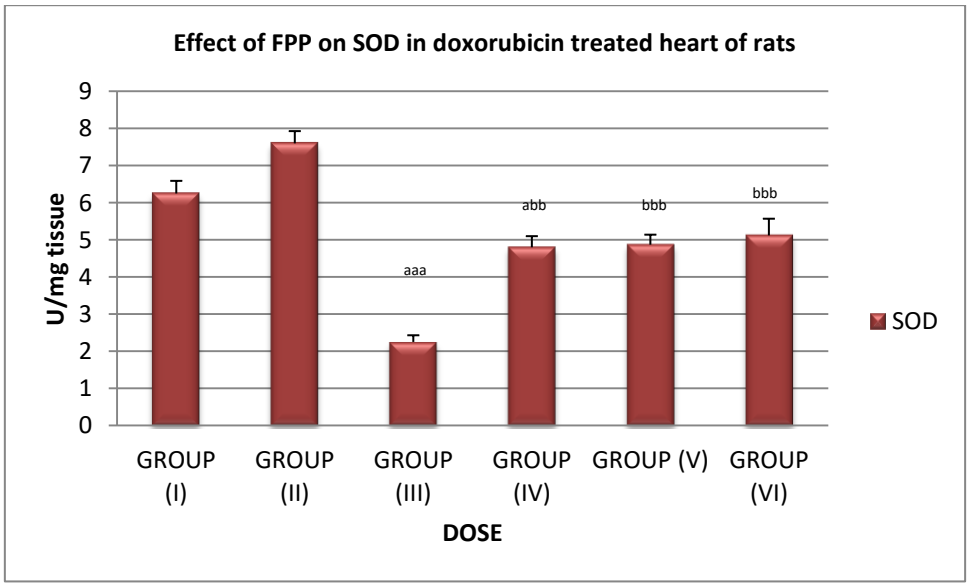


Table 3.3.8. Effect of FPP on Malondialdehyde, in doxorubicin treated heart of rats.

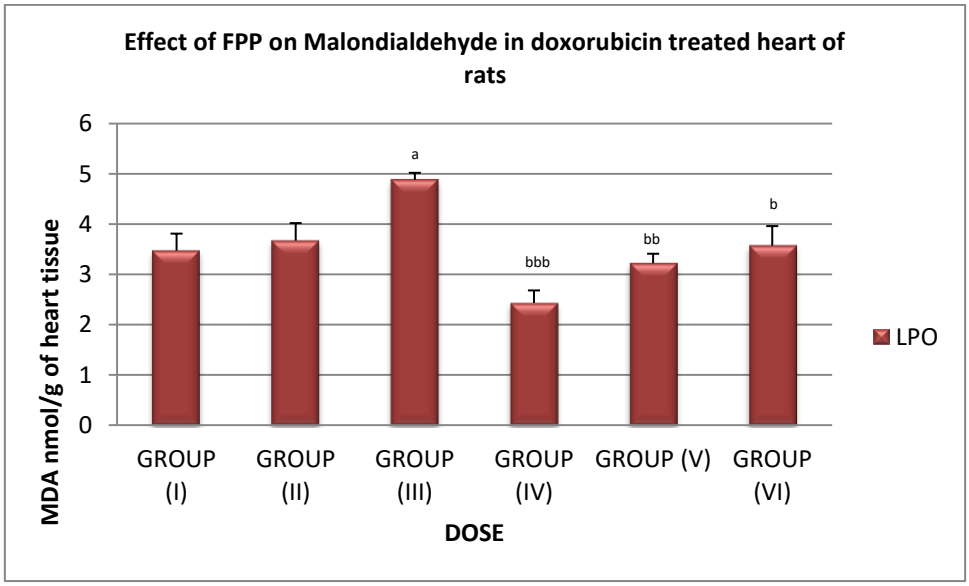
CONTROL (I)	LPO
FPP CONTROL (II)	3.47±0.34
DOX CONTROL (III)	3.67±0.35
PRE-FPP+DOX-FPP+POST FPP (IV)	4.88 ± 0.14^{a}
PRE-FPP+DOX (V)	2.43 ± 0.25^{bbb}
DOX-FPP+FPP (VI)	3.22 ± 0.19^{bb}

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:3.3.11



3.3.4.Antioxidant status and lipid peroxidation in liver

In **liver**, doxorubicin treated (GROUP III) significantly showed marked decreased in levels of SOD, CAT, GPx and GSH and increase in LPO. GROUP IV restored the levels of SOD($P<0.001$), Catalase ($P<0.001$), and GPx($P<0.05$), GSH($P<0.05$) and LPO($P<0.05$) significantly as compared to(GROUP III). In GROUP V, GSH and SOD showed a significant increase in their levels due to FPP treatment. But, changes in the levels of GPX, LPO and Catalase were insignificant as compared to GROUP III. In GROUP VI SOD and Catalase restoration levels are moderately significant ($P<0.05$and $P<0.01$ resp.) when compared to GROUP III.changes in GSH, GPX and LPO are insignificant. (Table 3.3.9, Table 3.3.10 & Table 3.3.11)

Table 3.3.9. Effect of FPP on Glutathione peroxidise and Glutathione in doxorubicin treated liver of rats

CONTROL (I)	GSH	GPX
FPP CONTROL (II)	4.75±0.3	7.23±0.46
DOX CONTROL (III)	4.2±0.34	7.37±0.45
PRE-FPP+DOX-FPP+POST FPP (IV)	2.73 ± 0.28^{aa}	3.78 ± 0.2^{aaa}
PRE-FPP+DOX (V)	4.78 ± 0.24^{b}	5.15 ± 0.31^{aab}
DOX-FPP+FPP (VI)	4.17 ± 0.33^{bb}	4.47 ± 0.18^{aaa}

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 3.3.12

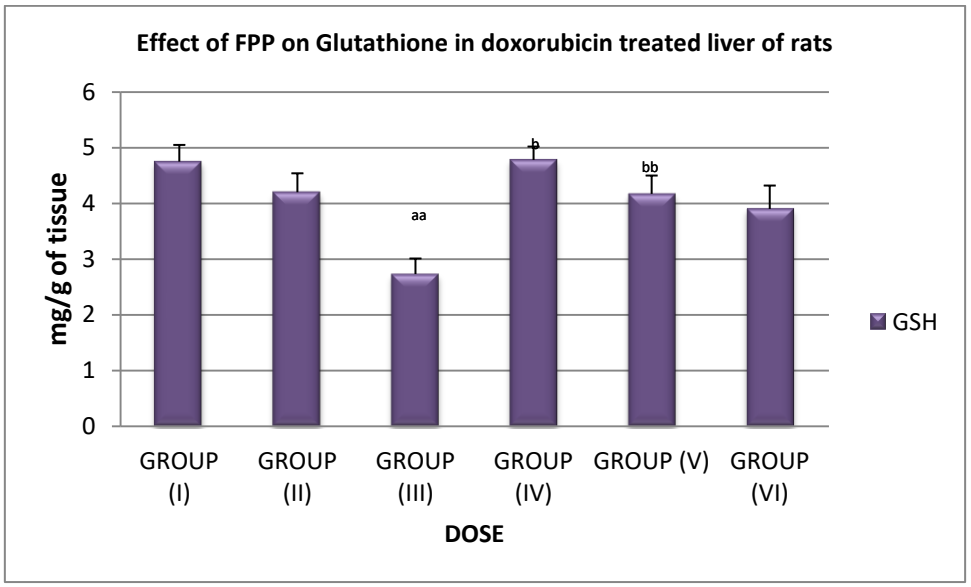


FIG 3.3.13

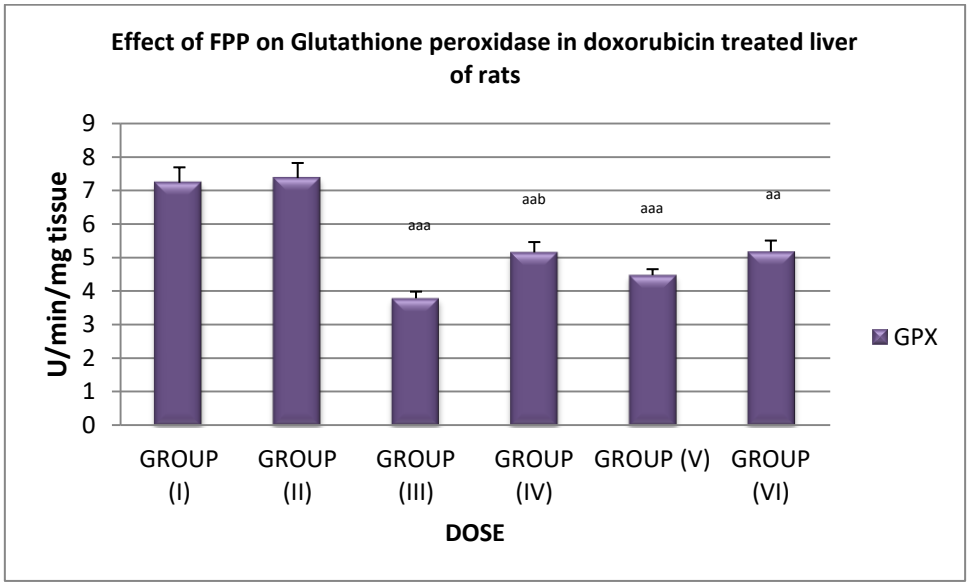


TABLE-3.3.10. Effect of FPP on Catalase & Superoxide dismutase in doxorubicin treated liver of rats

CONTROL (I)	CATA	SOD
FPP CONTROL (II)	8.43±0.51	8.65±0.33
DOX CONTROL (III)	8.45±0.45	9.17±0.33
PRE-FPP+DOX-FPP+POST FPP (IV)	4.22 ± 0.37^{aaa}	5.27 ± 0.11^{aaa}
PRE-FPP+DOX (V)	7.97 ± 0.61^{bbb}	8.83 ± 0.24^{bbb}
DOX-FPP+FPP (VI)	5.28 ± 0.34^{aaa}	6.83 ± 0.425^{aab}

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:3.3.14

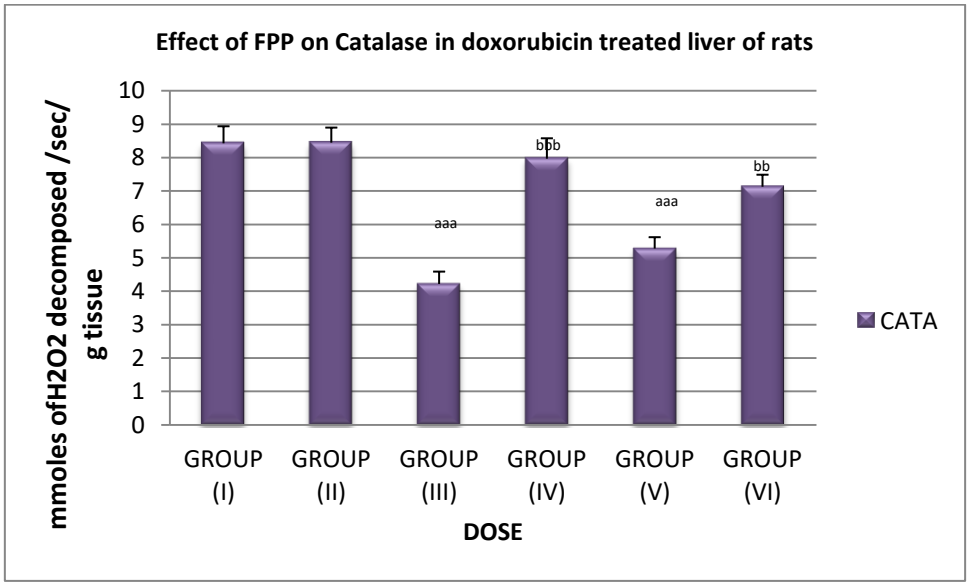


FIG:3.3.15

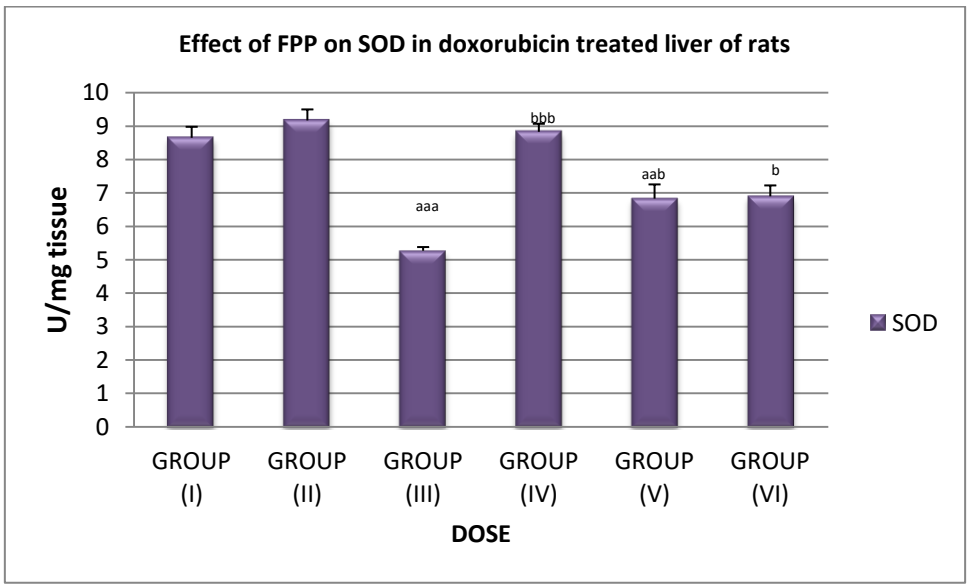


TABLE-3.3.11. Effect of FPP on Malondialdehyde in doxorubicin treated liver of rats

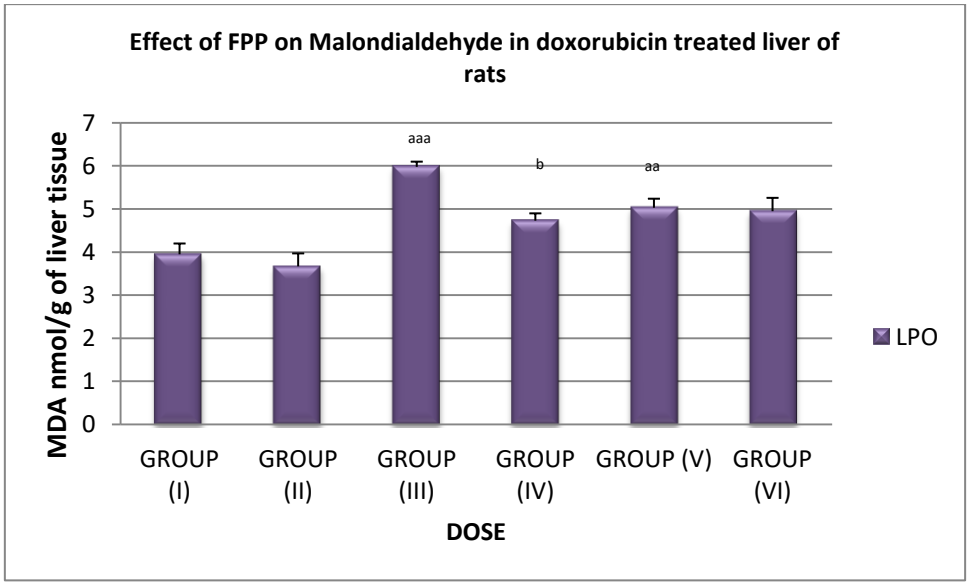
CONTROL (I)	LPO
FPP CONTROL (II)	3.95±0.25
DOX CONTROL (III)	3.67±0.3
PRE-FPP+DOX-FPP+POST FPP (IV)	5.98 ± 0.12^{aaa}
PRE-FPP+DOX (V)	4.73 ± 0.17^{b}
DOX-FPP+FPP (VI)	5.03 ± 0.21^{aa}

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:3.3.16



3.3.5. Antioxidant status and lipid peroxidation in kidney

The antioxidant status of **kidney** was lowered in the DOX alone treated animals(GROUP III), Therefore the concentration of MDA equivalents, as a result of lipid peroxidation (LPO) ($P<0.01$), increased along with decreased SOD, CAT, GPx activities and GSH level significantly($P<0.001$). SOD and Catalase levels increased to near normal significantly in GROUP IV($P<0.001$), GROUP V($P<0.01$) and GROUP VI($P<0.001$).The levels of GSH increased significantly in GROUP IV($P<0.001$), GROUP V($P<0.01$) and GROUP VI ($P<0.001$) as compared to (GROUP III). GPX level change was significant in GROUP IV($P<0.01$) but insignificant in GROUP V and GROUP VI. LPO levels were restored in GROUP VI significantly ($P<0.05$). (Table 3.3.12, Table 3.3.13& Table 3.3.14)

TABLE-3.3.12. Effect of FPP on Glutathione peroxidise Glutathione in doxorubicin treated kidney of rats

CONTROL (I)	GSH	GPX
FPP CONTROL (II)	5.57±0.31	4.58±0.2
DOX CONTROL (III)	5.55±0.56	4.77±0.38
PRE-FPP+DOX-FPP+POST FPP (IV)	1.42 ± 0.23^{aaa}	1.92 ± 0.21^{aaa}
PRE-FPP+DOX (V)	4.16 ± 0.25^{bbb}	3.67 ± 0.2^{bb}
DOX-FPP+FPP (VI)	3.32 ± 0.29^{aabb}	2.68 ± 0.13^{aa}

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:3.3.17

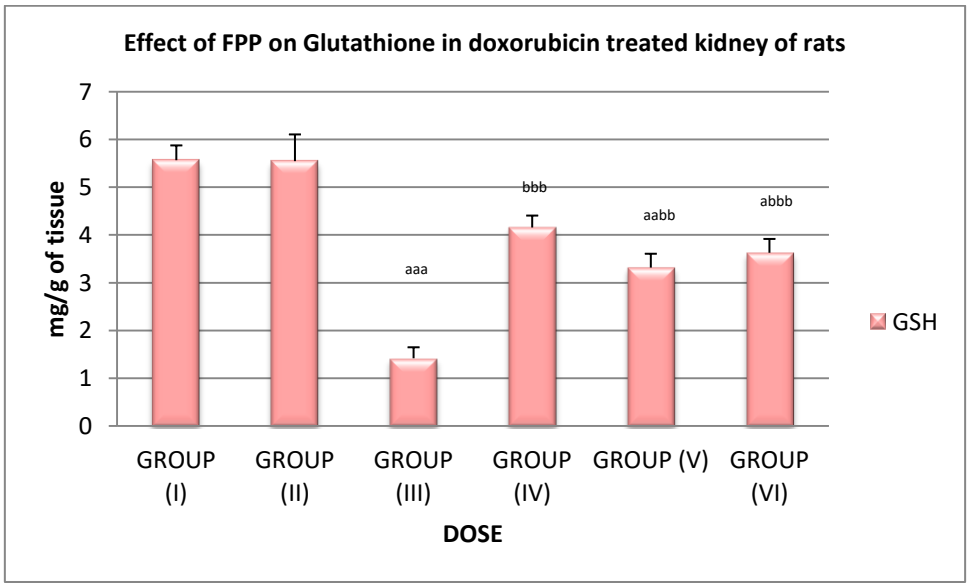


FIG:3.3.18

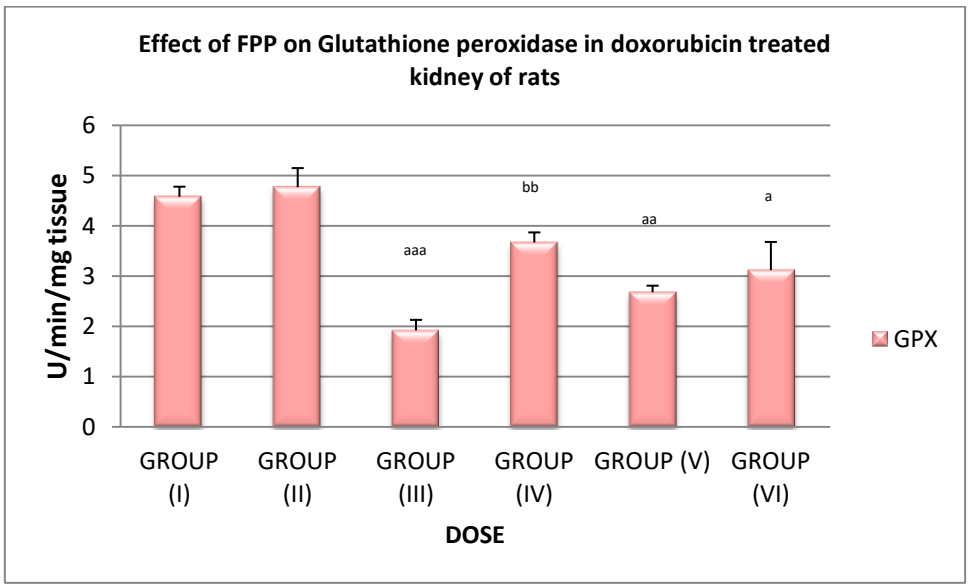


TABLE-3.3.13. Effect of FPP on Catalase & Superoxide dismutase in doxorubicin treated kidney of rats

CONTROL (I)	CATA	SOD
FPP CONTROL (II)	9.74±0.5	6.4±0.22
DOX CONTROL (III)	8.25±0.27	7.63±0.31
PRE-FPP+DOX-FPP+POST FPP (IV)	3.92 ± 1.27^{aaa}	1.95 ± 0.3^{aaa}
PRE-FPP+DOX (V)	8.2 ± 0.3^{bbb}	5.65 ± 0.22^{bbb}
DOX-FPP+FPP (VI)	7.017 ± 0.23^{ab}	5.88 ± 0.02^{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:3.3.19

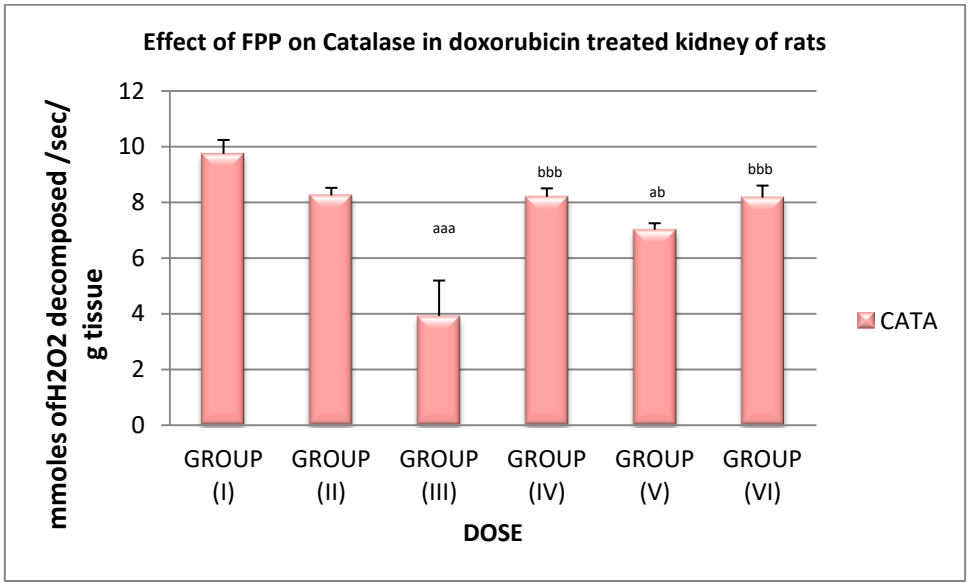


FIG:3.3.20

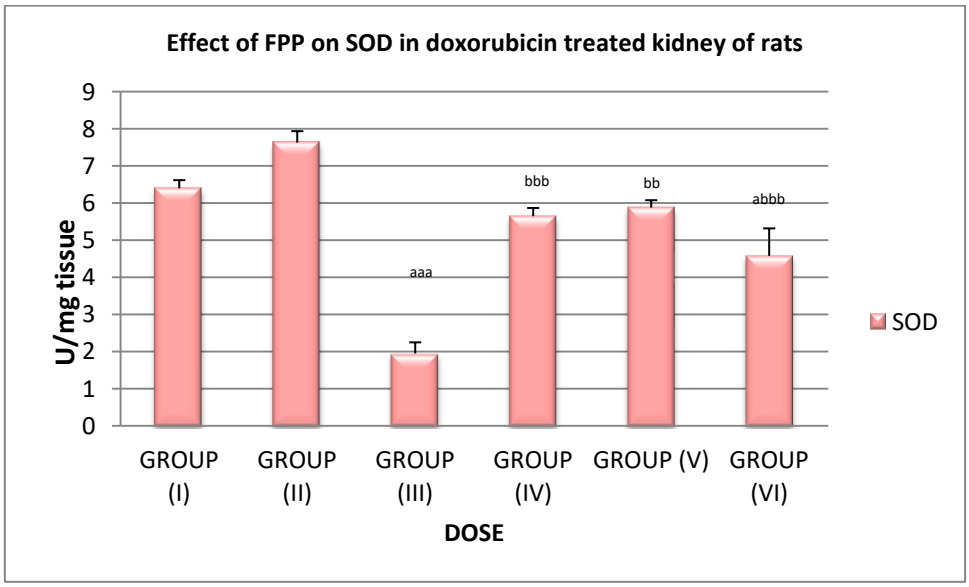


TABLE-3.3.14. Effect of FPP on Malondialdehyde in doxorubicin treated kidney of rats

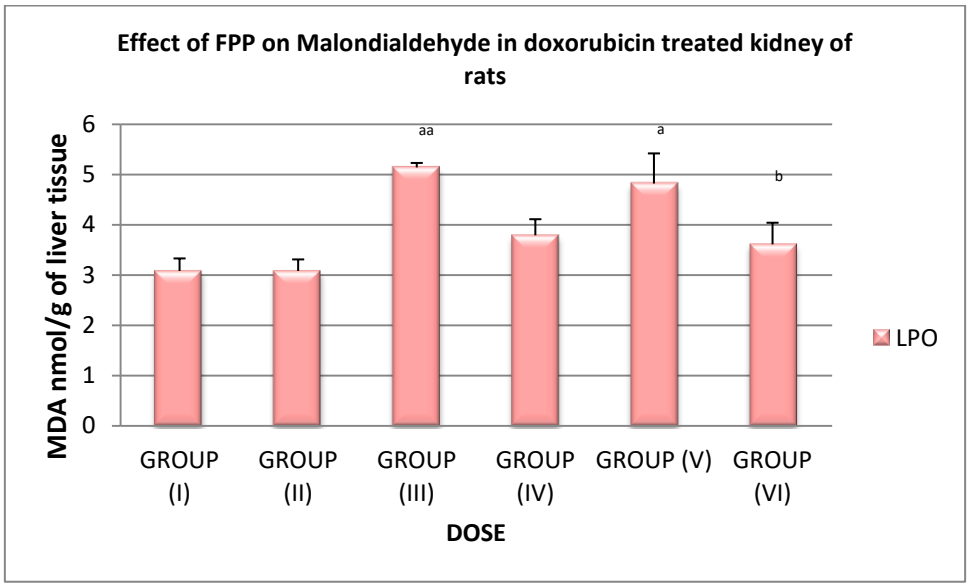
CONTROL (I)	LPO
FPP CONTROL (II)	3.08±0.25
DOX CONTROL (III)	3.08±0.23
PRE-FPP+DOX-FPP+POST FPP (IV)	5.14±0.09^{aa}
PRE-FPP+DOX (V)	3.79±0.32
DOX-FPP+FPP (VI)	4.82±0.6^{a}

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:3.3.21



3.3.6. Comparison between pretreatment of FPP in Acute dose and Cumulative dose in rats

Group V (Pre FPP + DOX) biochemical results were compared with that of acute toxicity study (Chapter I) in which FPP pretreatment was given with the same dose concentration to wistar rats. It showed a non-significant variation between the serum parameters and biochemical parameters.

TABLE- 3.3.15 : Comparison between pretreatment of FPP in Acute dose and Cumulative dose in rats(serum)

	PRE-FPP+DOX		Dox control	
	Acute	Cummulative	Acute	Cummulative
SGPT	130.00	138.10	266.67	223.81
SGOT	117.23	156.36	194.26	188.87
ALP	120.96	128.88	154.19	189.30

FIG:3.3.22

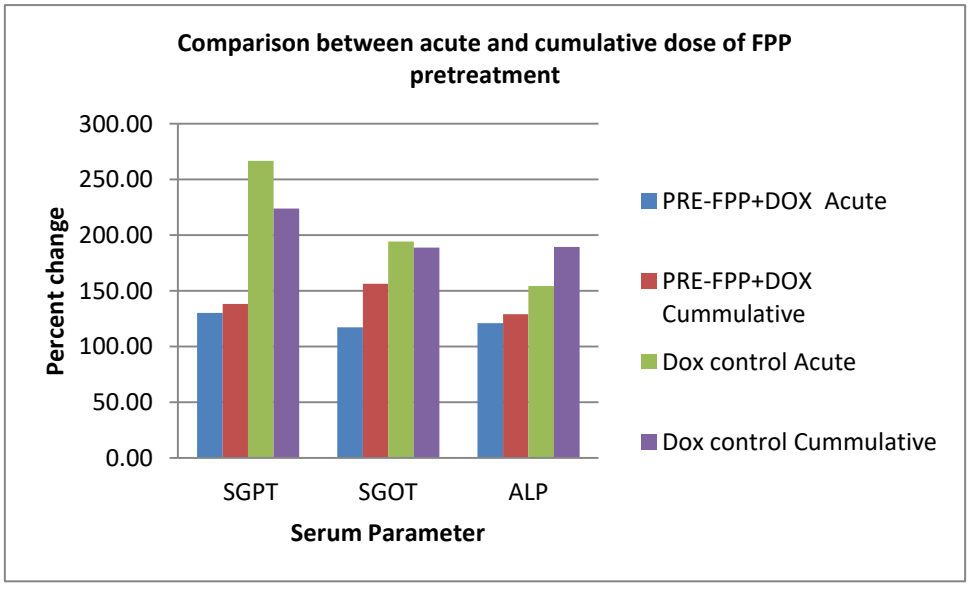


TABLE- 3.3.16 - Comparison between pretreatment of FPP in Acute dose and Cumulative dose in rats.(serum)

	PRE-FPP+DOX		Dox control	
	Acute	Cummulative	Acute	Cummulative
UREA	122.86	159.38	172.00	215.43
CREA	105.23	117.39	160.47	236.30
CKMB	110.57	114.89	165.46	156.46
LDH	125.63	146.87	200.28	207.02
TRIG	130.47	108.23	206.03	174.08
CHOL	117.84	110.67	166.67	121.33

FIG:3.3.23

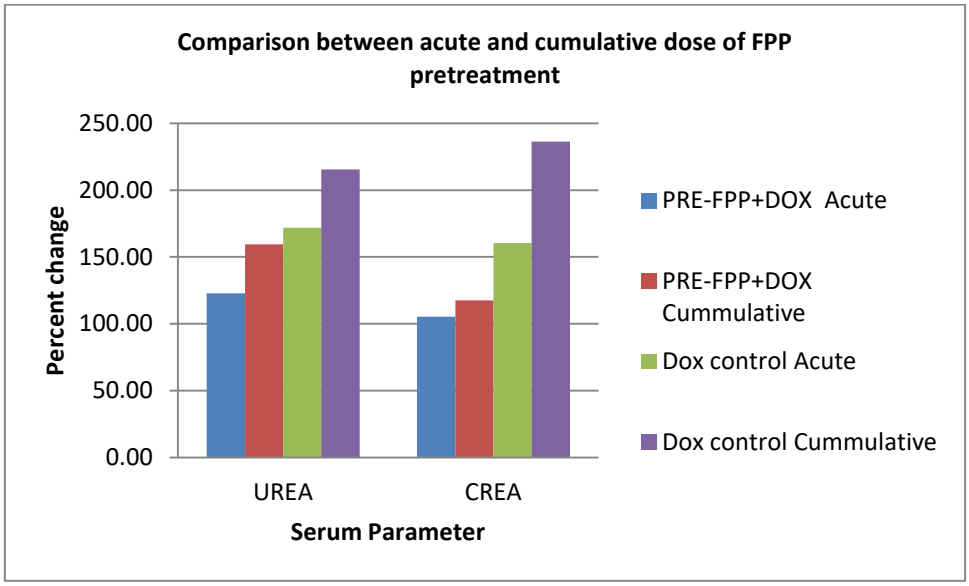


FIG:3.3.25

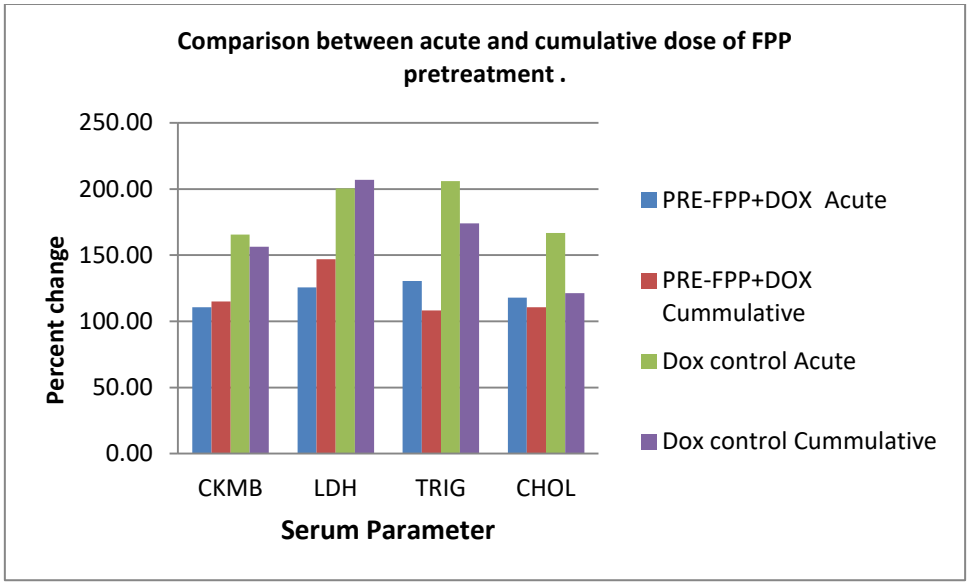


TABLE- 3.3.17: Comparison between acute and cumulative dose of FPP pretreatment in rats.(Heart)

	PRE-FPP+DOX		Dox control	
	Acute	Cummulative	Acute	Cummulative
GSH	75.20	70.27	25.59	20.35
GPX	90.48	96.61	26.67	39.16
LPO	114.14	92.80	178.97	140.63
CATA	80.94	81.22	41.34	52.51
SOD	81.42	77.92	32.79	36.00

FIG:3.3.25

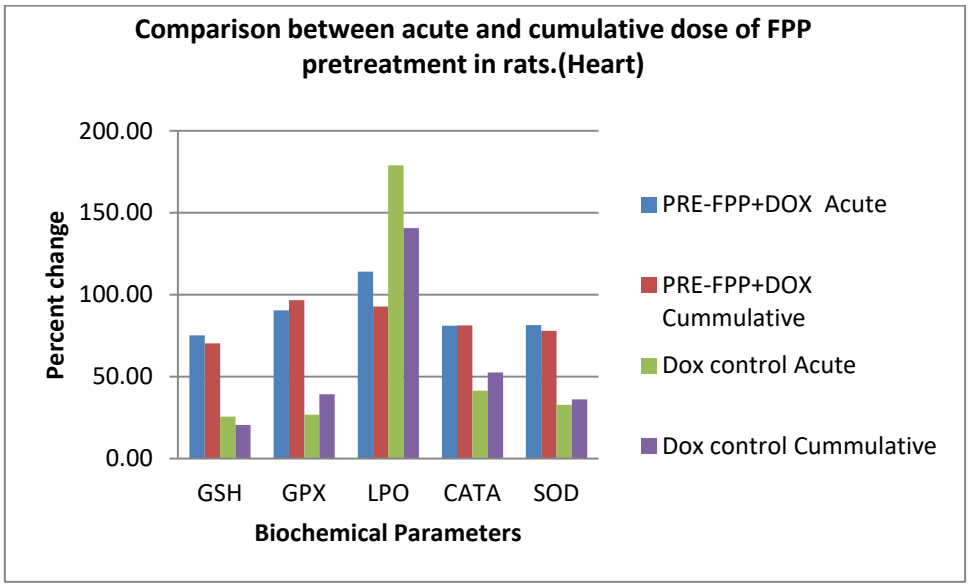


TABLE-3.3.18. Comparison between acute and cumulative dose of FPP pretreatment in rats.(Liver)

	PRE-FPP+DOX		Dox control	
	Acute	Cummulative	Acute	Cummulative
GSH	92.74	87.79	45.45	57.47
GPX	81.07	61.83	38.32	52.28
LPO	115.14	127.34	193.78	151.39
CATA	83.02	62.63	44.15	50.06
SOD	76.46	78.96	35.70	60.92

FIG 3.3.26

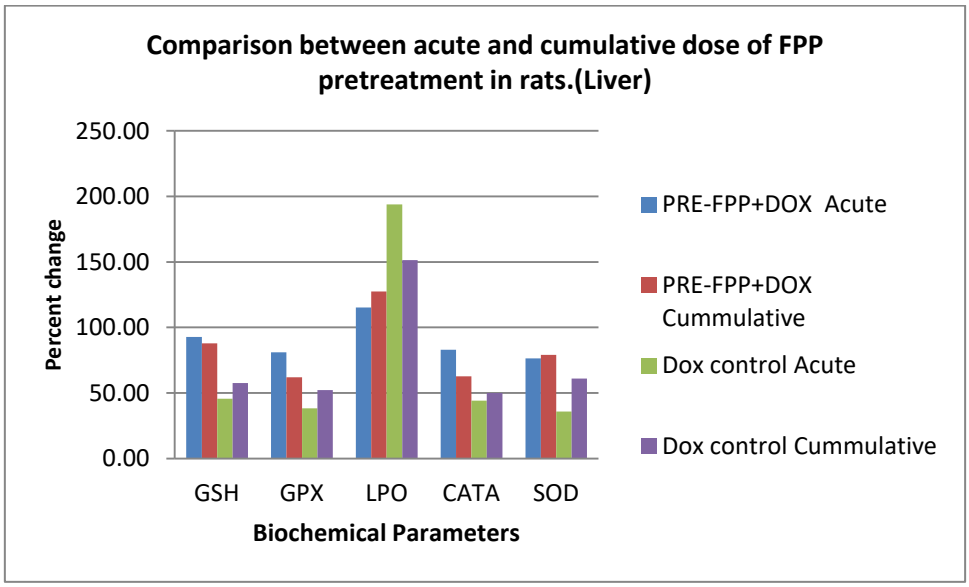
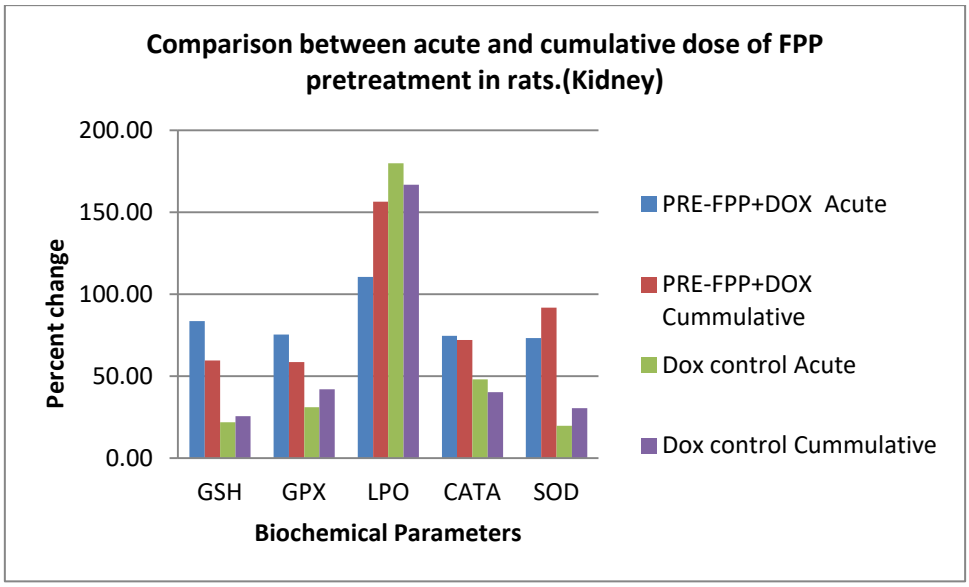


TABLE- 3.3.19. Comparison between acute and cumulative dose of FPP pretreatment in rats.(Kidney)

	PRE-FPP+DOX		Dox control	
	Acute	Cummulative	Acute	Cummulative
GSH	83.48	59.61	21.74	25.49
GPX	75.36	58.52	31.06	41.92
LPO	110.53	156.49	180.00	166.88
CATA	74.59	72.04	47.90	40.25
SOD	73.20	91.88	19.59	30.47

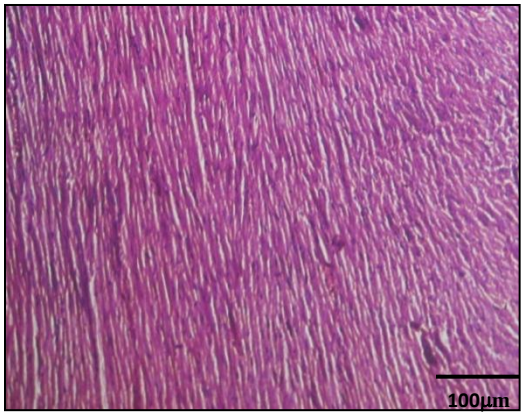
FIG 3.3.27



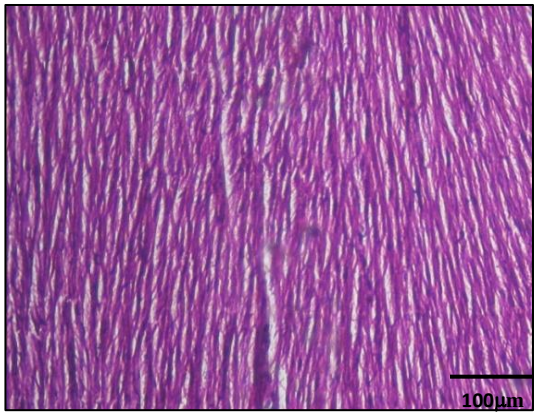
3.3.7 Histopathological examination of tissue section

The histological sections of heart showed interstitial hemorrhage, sarcoplasmic edema and RBC & WBC infiltration. The Liver histopathology revealed central vein congestion, bile duct hyperplasia and RBC infiltration, while the kidney showed vacuolated glomeruli and tubular degeneration. The size of few glomeruli was found to be reduced. Exudative lesions were visible at few locations.

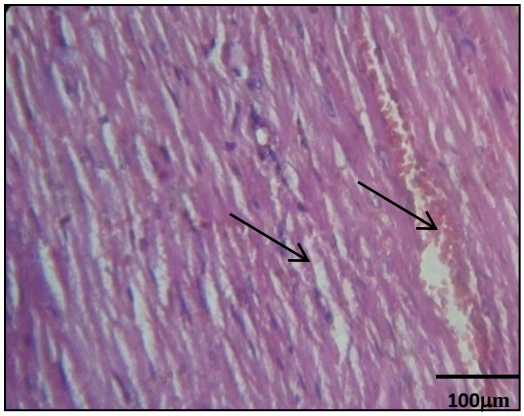
PLATE 4 : Histopathological examination of rat heart (H&Ex40)



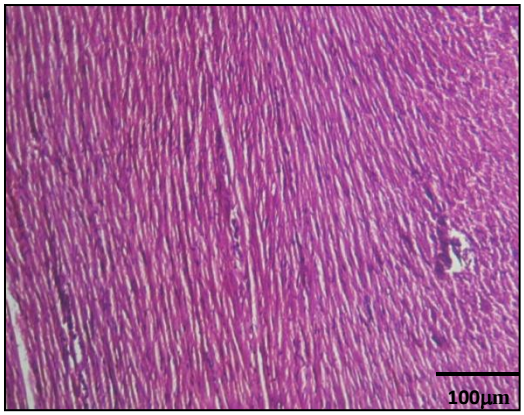
H1



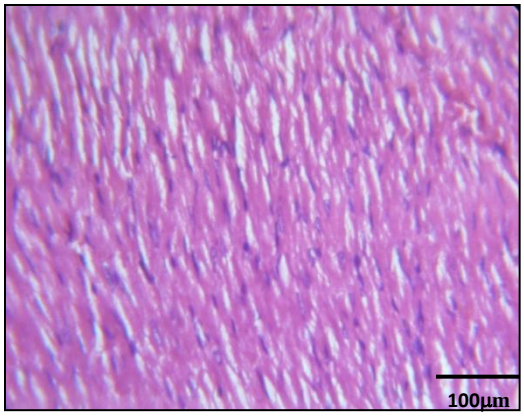
H2



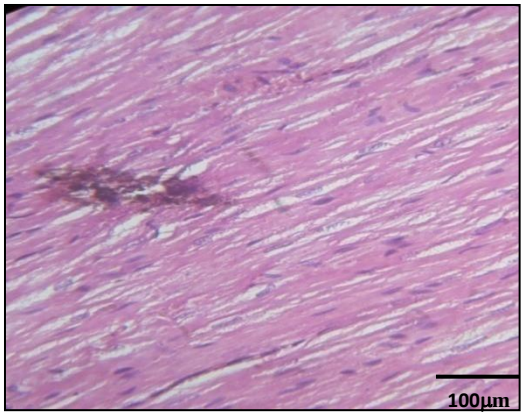
H3



H4



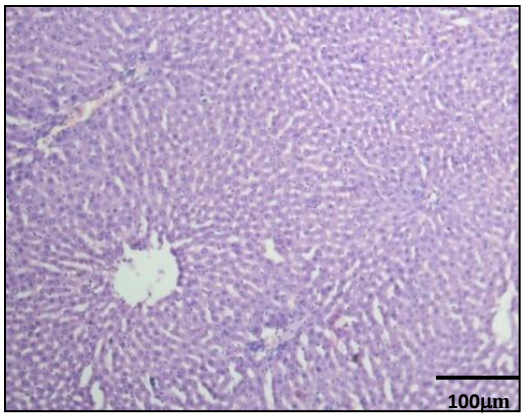
H5



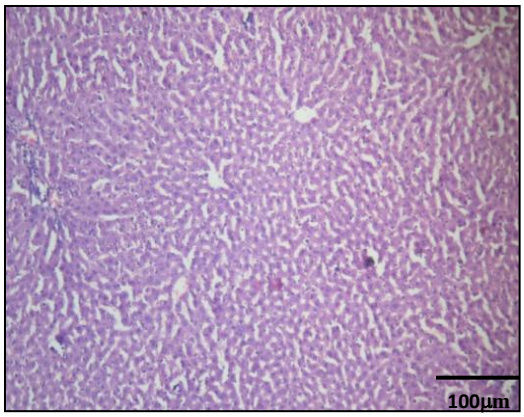
H6

H1:- Control rat showing normal morphological appearance.
H2:- FPP treated rat showing normal morphological appearance.
H3:- DOX showing necrosis and infilteration
H4:-Pre FPP + FPP-DOX +Post FPP
H5:-Pre FPP + DOX
H6:- DOX +Post FPP

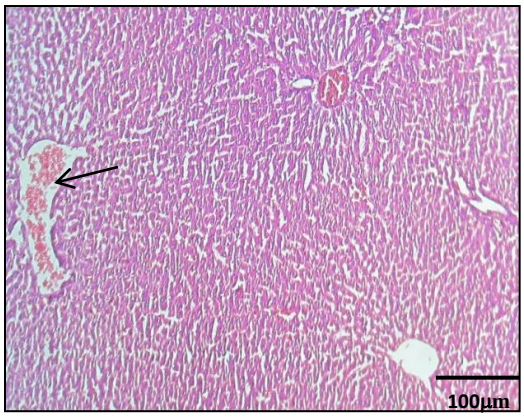
PLATE 5 : Histopathological examination of rat liver (H&Ex40)



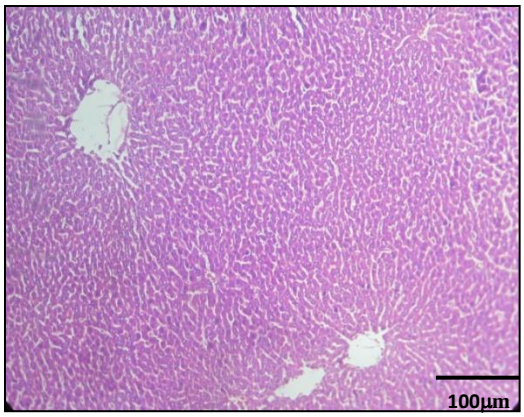
L1



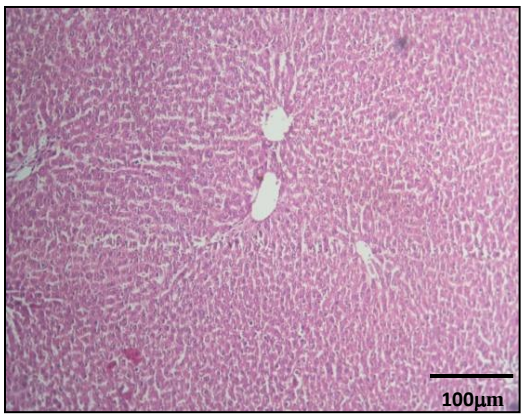
L2



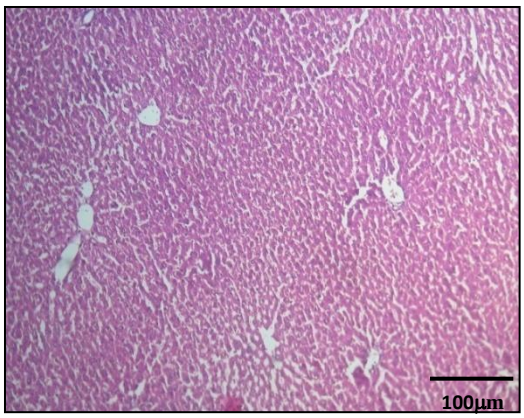
L3



L4



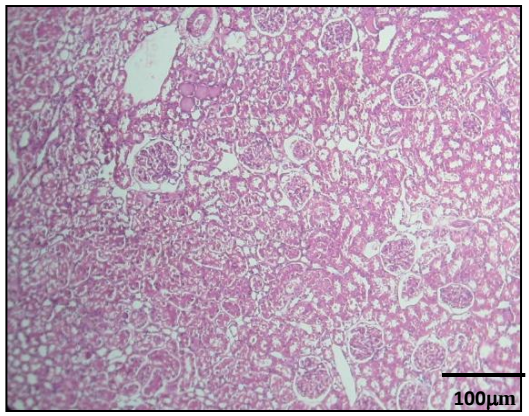
L5



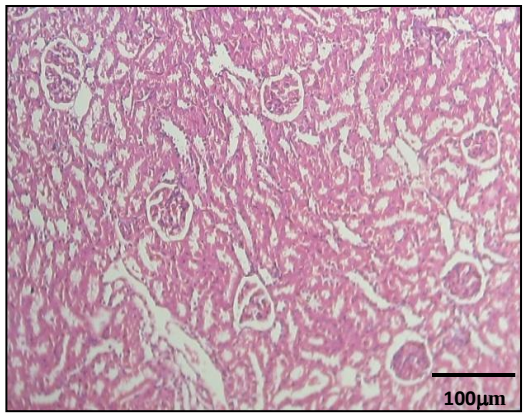
L6

L1:- Control rat showing normal morphological appearance.
L2:- FPP treated rat showing normal morphological appearance.
L3:- DOX treated rats showing RBC infilteration.
L4:-Pre FPP + FPP-DOX +Post FPP
L5:-Pre FPP + DOX
L6:- DOX +Post FPP

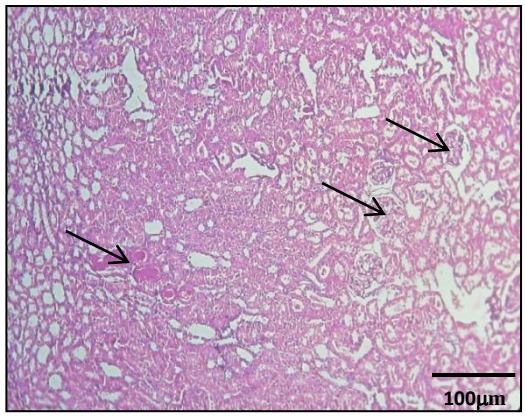
PLATE 6 : Histopathological examination of rat kidney (H&Ex40)



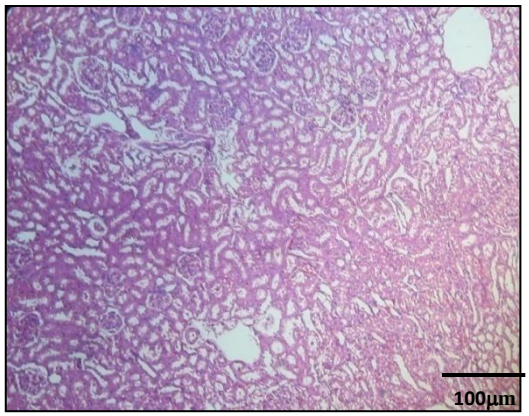
K1



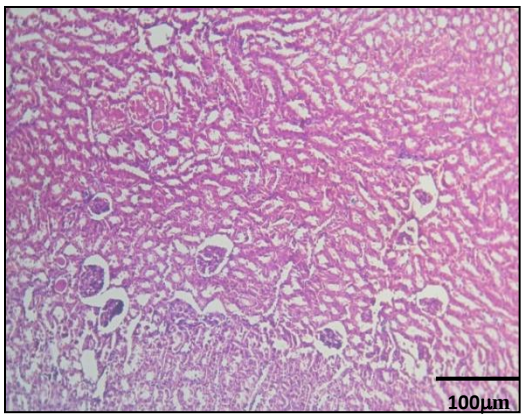
K2



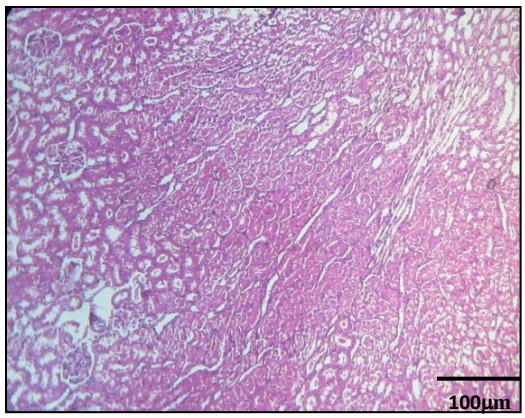
K3



K4



K5



K6

K1:- Control rat showing normal morphological appearance.
K2:- FPP treated rat showing normal morphological appearance.
K3:- DOX treated rat showing hematoma and shrunken glomeruli.
K4:-PreFPP + FPP-DOX +Post FPP
K5:-Pre FPP + DOX
K6:- DOX +Post FPP

3.4. DISCUSSION

Doxorubicin (Dox) was introduced in cancer therapy in the late 1960s. It has emerged as one of the most potent broad-spectrum antitumor anthracycline antibiotics. Dox can be administered as a single agent or in combination with other chemotherapeutic agents. It is widely used to treat a variety of cancers, including leukemias, lymphomas, soft-tissue sarcomas, and solid tumors. Its cytotoxic effects on malignant cells, however, are complicated by an increase in the risk of organ toxicity especially cardiotoxicity (Lafark *et al.,* 1973; Singal and Illosik 1998).

Although mechanisms responsible for Doxorubicin-induced toxicity on organs are not clearly known, oxidative damage to cellular components is believed to be a major factor in the Doxorubicin cardiotoxicity (Elberry *et al.,* 2010; Boghdady 2013). An increase in the formation of reactive oxygen species (ROS) and alteration in prooxidant-antioxidant balance in favor of prooxidation have been observed in cardiac muscular tissues of dox-treated animals (Andreadou *et al.,* 2007; Patel *et al.,* 2010). Some investigators have also detected increases in hepatic (Dudka *et al.,* 2012; Espinosa *et al.,* 2012) and renal (Abo-Salem 2012; Kavimani 2014) oxidative stress parameters associated with tissue damage in dox -treated animals. Therefore, many investigators have tested the preventive effects of several antioxidants against dox-induced organ toxicity (Andreadou *et al.,* 2007; Dudka *et al.,* 2012).

Several reports suggest that Doxorubicin induced apoptosis plays an important role in its cytotoxicity that 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). Most hypotheses believed that the primary pathogenic mechanisms of doxorubicin induced cytotoxicity are mediated via its ability to generate reactive oxygen species (ROS) including lipid peroxides, super oxide anions,

hydroxyl radicals and hydrogen peroxides (Abd El-Aziz *et al.,* 2001; Kalender *et al.,* 2005; Yagmurca *et al.,* 2007)

Considerable interest has been generated in compounds that function as antioxidants and in herbs that have endogenous antioxidants. Based on the complex nature of antioxidants and ROS, it would be extremely unlikely that a treatment with a high dose of one or a few particular antioxidants such as vitamin C, vitamin E, or β-carotene would protect all parts of the cells, organs, and tissues against oxidative damage and oxidative stress. Indeed, supplementation with antioxidants has often resulted in no effect or even adverse disease outcomes. The beneficial effect for supplemental vitamin C, vitamin E, or β-carotene has been challenged recently by many researchers (Bjelakovic *et al.,* 2007; Bjelakovic *et al.,* 2008).

Plant products contain a large combination of different antioxidants. These antioxidants were selected, in the course of evolution, to protect every part of the plant cells against oxidative damage. Therefore, an alternative antioxidant strategy is established to test protection against oxidative stress and related diseases using the potential beneficial effects of such antioxidant-rich plants. (Ingvild *et al.,* 2011; Eidelman *et al.,* 2004; Bjelakovic *et al.,* 2007)

Some of the natural products find their use not as pharmaceuticals (real medicine) but as a novel class of dietary supplements or nutraceuticals that fall well into the concept of functional foods. Nutraceuticals can be defined as food product consumed or administered under medical supervision based on medical evaluation of specific dietary management of a disease (Hardy 2000). Fermented papaya preparation (FPP) is a specific product, derived from the technologically advanced and controlled bio-fermentation process of *Carica papaya* Linn., in the absence of genetic manipulation.

A long fermentation, by means of yeasts, is the unique process, supporting the preservation of papaya anti-oxidant properties while offering important new immune-modulating features. In the final fermented product many new class of oligosaccharides are present, at a different polymerization, as well as monomers similar to the basic structure of ß 1-3 D-glucan. Such oligosaccharides, mainly oligosaccharides exhibiting a low molecular weight, exhibit a wide spectrum immune-modulating activity (Marotta *et al.*, 2012).

Scientists at Pasteur Institute in Kyoto evidenced a positive effect of FPP on the Natural Killer population of a sarcoma in experimental animals. They further proved its capacity on human beings and showed that FPP affected the γ-interferon production. Such data was further proved by studies supporting the positive activity of FPP on the macrophage function on rats (Marcocci *et al.*, 1996) and human beings too. Simultaneously it was proved that FPP had consistent protection effect on oxidizing stress on isolated rat hearts (Haramaki *et al.*, 1995). Such data have been recently confirmed and gained further insights from Aruoma *et al.*, (2006) that have showed the ability of FPP to modulate oxidative DNA damage due to H_2O_2 in rat pheochromocytoma (PC12) cells and protection of brain oxidative damage in hypertensive rats.

We therefore hypothesized that antioxidant-rich FPP may be beneficial and provide a balanced combination of a variety of antioxidants in appropriate doses that would protect against excessive oxidative stress and oxidative damage caused by Doxorubicin, without disturbing the normal role of ROS.

In our previous study, the efficacy of FPP 250 mg/kgbw was identified against Doxorubicin induced organ toxicity (20mg/Kg acute dose), indicating its preventive role. Based on these reports, a study was planned in which FPP 250mg/kgbw was co-

administered with doxorubicin (20mg/Kg cumulative dose) along with pre, and post administration in a 5 week regime. The chemotherapy treatment in cancer patients generally follows a cumulative dose regime spanning several weeks. Therefore our study mimicked the human treatment, at the same time looked into the therapeutic potential of FPP. The dose of Doxorubicin used in this study was slightly higher than the dose that is currently being used in clinical practice (Chabner *et al.,* 2001). The corresponding dose in human being (60 Kg) is approximately 98mg/m2. We found that the dose of Doxorubicin used in this study was effective in inducing organ toxicity without any mortality.

As expected, administration of cumulative dose of doxorubicin (GROUP III) animals significantly showed impaired function in all the organs, which was evident from the marked increase in hepatic function markers (SGPT, SGOT), renal function markers (Urea, Creatinine), and cardiac function markers (CK MB, LDH) along with Triglycerides and Cholesterol when compared to control animals ($p<0.001$). FPP administration showed ameliorating effects in all groups of animals. However group IV animals where the FPP treatment extended from 7 days prior to 7 days post doxorubicin regime, showed the maximum amelioration.(Table 3.3.2,Table 3.3.3 & Table 3.3.2)

Cumulative administration of doxorubicin in GROUP III animals resulted in a significant decrease in the levels of cardiac, hepatic and renal enzymes viz. SOD and Catalase as well as decrease in levels of GSH and GPx as compared to the control group (GROUP I). Significant increase in Lipid peroxidation, in all the organs, was also observed, indicating induction of organ toxicity in these animals. All groups treated with FPP showed much less toxic effect of doxorubicin treatment (cumulative

dose) with minimum damage seen in GROUP VI animals. (Table 3.3.6 to Table 3.3.14).

Doxorubicin has been shown to induce accumulation of inflammatory cells (Saad et al,. 2001), associated with increased activities of tissue aminotransferases, LDH and ALP, indicating hepatic damage (Deepa and Varalakshmi 2003).

It has been investigated by Oz and Ilhan (2006) that the use of Doxorubicin results in an increased production of free radicals such as superoxide, hydroxyl radicals and hydrogen peroxide, which have a great potential to react rapidly with lipids that cause LPO formation. Enhanced LPO is known to be one of the toxic manifestations of Doxorubicin ingestion and is measured in terms of MDA levels. In the present study, Doxorubicin -treated rats showed increased level of MDA compared to control rats in heart, kidney as well as liver. The administration of FPP, to Doxorubicin-treated rats, significantly decreased the level of MDA, in the tissues compared to Doxorubicin-treated rats without FPP supplementation (GROUP III).(FIG.3.3.11,FIG.3.3.16,FIG 3.3.21) The augmented levels of antioxidants on FPP supplementation may be associated with the decrease in LPO which may be due to its free radical scavenging property of the hydroxyl groups. The efficacy of FPP to improve the intensity of antioxidants along with its antilipid-peroxidative action suggests that it might be potentially beneficial in thwarting the free radical-induced damage involved in the progression of organ injury due to Doxorubicin treatment.

SOD is extensively distributed in all cells and has a significant shielding role against oxidative injury induced by ROS. It transforms superoxide ion (O_2^-) to hydrogen peroxide (H_2O_2), which is later acted upon by CAT and GPx. One of the most efficient defence mechanism against various diseases is to scavenge superoxide ions and hydroxyl radicals. In our study, the activities of SOD and CAT were significantly

decreased in all organs in Doxorubicin-treated rats as compared to control rats. The accumulation of highly reactive free radicals lead to reduced activity of SOD and CAT which in turn results in damaging effects in the form of loss of cell membrane integrity and function. However, decrease in the SOD and CAT activity may be related with the increase in the intracellular levels of H_2O_2. CAT has been reported to be responsible for the detoxification of H_2O_2, which is an effective inhibitor of SOD (Damodara *et al.,* 2007; Mahesh *et al.,* 2009; Mohan *et al.,* 2010). FPP supplementation to the Doxorubicin-treated group elevated SOD and CAT activity in the organs under study emphasizing the antioxidant activity of FPP.(TABLE 3.3.7, TABLE 3.3.10, TABLE 3.3.13)

GSH is an antioxidant and plays an efficient role in the detoxification of ROS, conjugation and excretion of toxic compounds (Wu *et al.,* 2004; Wani *et al.,* 2011). Reduction of GSH level in tissues may result in peroxidative injury and impairment of the cellular defense against ROS. Our findings are consistent with previous reports that showed decreased GSH concentration upon Doxorubicin treatment (Mohan et al,. 2010; Ali 2012).GSH acts as a substrate for GPx, besides being a direct free radical scavenger. However, Doxorubicin treatment led to decrease in the activity of GPx, which may be due to unavailability of GSH. All groups of animals treated with FPP showed increased levels of GSH and GPx, indicating the ameliorating effect of FPP.

Treatments with FPP showed reduction in circulating CKMB levels with simultaneous decrease in the extent of lipid peroxidation. CKMB, an enzyme found primarily in the myocardium is used to evaluate the existence and extent of myocyte injury. FPP was found to inhibit the Doxorubicin-induced CKMB release in the serum of rats. It is widely reported that Doxorubicin-induced free-radical generation triggers

membrane peroxidation and disruption of cardiac myocytes, which can lead to increased release of CKMB in the serum (Liu *et al.,* 2002).

We have shown that FPP treatment led to inhibition of CKMB release. There was a near complete inhibition of CKMB release in Doxorubicin-treated animals in Group IV. Cardioprotective activity of FPP was further supported by increased myocardial antioxidant enzyme activity and decreased extent of lipid peroxidation. The most abundant ROS generated in living cells are superoxide anion and its derivatives, particularly highly reactive and damaging hydroxyl radical, which induces peroxidation of cell membrane lipids (Hemnani and Parihar 1998). Lipid peroxidation is known to cause cellular damage and is primarily responsible for ROS-induced organ damage (Halliwell and Gutteridge 1989). Present studies have shown that Doxorubicin-induced considerable increase in lipid peroxidation, which was significantly prevented by FPP treatment in all groups of animals.

Non-enzymatic antioxidants can also play a critical role in the defense against oxidative stress. Thus, the antioxidant property of FPP may facilitate in boosting the other antioxidants such as SOD, CAT, GSH and GPx. This may be the rationale behind the increased level of these enzymic and nonenzymic antioxidants in heart, kidney and liver tissues. The reduced activities of antioxidant enzymes in Doxorubicin-treated group support the participation of oxidative stress in the pathophysiology of Doxorubicin-induced organ toxicity.

Besides variation in antioxidant enzyme levels in the cell, Urea and serum creatinine are the most sensitive marker of nephrotoxicity implicated in the diagnosis of renal injury (Sallie *et al.,* 1991; Khan and Sultana 2005) and SGOT, SGPT, ALP for liver damage, respectively (Mohan *et al.,* 2010). Doxorubicin-induced kidney damage is manifested by an elevation in Creatinine and Urea levels. Our results are in good

agreement with those previously reported (Al-Nasser *et al.*, 1998; Saad *et al.,* 2001; Mohan *et al.,* 2010). In contrast, rats administered with FPP (all Groups) showed lower Urea and creatinine levels as compared to Doxorubicin-treated rats. This is an indicator of the possible nephroprotective efficacy offered by FPP against Doxorubicin toxicity. (TABLE 3.3.4)

Serum transaminases have long been known as sensitive markers of liver damage. Membrane permeability and transport function are altered by injured hepatocytes, which lead to the leakage of enzymes from the cells that cause increase in the levels of SGOT, SGPT and ALP in serum (Mohan *et al.,* 2011). Administration of Doxorubicin to rats significantly increased serum SGOT, SGPT and ALP while all groups treated with FPP restored the activities significantly.(Table 3.3.3,Table 3.3.4)

The protective effect of FPP is splendidly correlated with histopathological studies. In the present study, light microscopic evaluation of rat heart, liver and kidney revealed Doxorubicin induced alteration in the histological architecture. Similar alterations in Doxorubicin-treated mice have been reported earlier (Liu *et al.,* 2002). The histological sections of heart showed interstitial hemorrhage, sarcoplasmic edema and RBC & WBC infiltration. The Liver histopathology revealed central vein congestion, bile duct hyperplasia and RBC infiltration, while the kidney showed vacuolated glomeruli and tubular degeneration. The size of few glomeruli was found to be reduced. Exudative lesions were visible at few locations. (Plate 4,5,6)

The results of the present study concluded that FPP significantly protected the organ toxicity either by enhancing the Doxorubicin-induced declined antioxidant status or by its direct antioxidant activity. Further, the results demonstrate our hypothesis to be true. FPP acts both as a protective/preventive and therapeutic nutraceutical.

Further, when Group V results (Biochemical & Histological) were compared with acute toxicity study (Chapter I) results, it showed a non-significant variation between the serum parameters, organ function tests as well as the histopathological observations. Overall, however, we may conclude that Doxorubicin, when administered in cumulative doses, is slightly better tolerated as compared to acute dose (same concentration). (TABLE.3.3.15 to TABLE 3.3.19)

The increased level of ROS in cancer cells is balanced by an increased defense against ROS so that the cell does not exceed the ROS threshold for cell death. The increase in ROS leads to activation of signaling pathways that favor cell growth, migration, and proliferation. Furthermore, cancer therapies like radiation and chemotherapy induce massive amounts of ROS that exceed the ROS threshold and induce cancer cell death (Trachootham *et al.*, 2009). Thus, although antioxidants may theoretically prevent transformation of normal cells to cancerous cells, they may theoretically also lower the efficacy of cancer treatment.

Therefore, although the results of our studies strongly suggest the possible use of FPP as nutraceutical against oxidative stress induced organ toxicities, the interference of FPP in the antitumor efficacy of Doxorubicin must be evaluated for its possible clinical application.