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Research Article

EFFECT OF FERMENTED PAPAYA PREPARATION ON DOXORUBICIN INDUCED MYOCARDIAL TOXICITY

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ABSTRACT: Many antitumor drugs cause "on treatment" cardio toxicity or introduce a measurable risk of delayed cardiovascular events. The problem of anthracycline-induced cardio toxicity has been around for some 40 years. Doxorubicin (DOX) is an anthracycline derivative used as an anticancer agent. However, its clinical use is limited due to its severe cardio toxic manifestations. The present aim is to evaluate the protective role of Fermented Papaya Preparation (FPP) in combating doxorubicin induced cardio-toxicity/oxidative stress. Female *Wistar* rats were pretreated with FPP (100 mg/kgbw or 250 mg/kgbw) or saline daily for 28 consecutive days followed by doxorubicin (10 mg/kgbw) induction for next 2 days. Results indicated that pretreatment with FPP significantly decreased serum levels of CK-MB and LDH cardiac marker enzymes. Further, FPP supplementation significantly increased SOD, GSH-Px and GSH ($p<0.05$) while decreased Malondialdehyes and Catalase levels in heart. Histological observations demonstrated that FPP pretreatment attenuated DOX induced myofribillar derangement and vascular congestion in heart tissue. Thus our results suggest that FPP exhibits significant preclinical potential in combating DOX induced oxidative stress.

Key Words: Doxorubicin, Fermented Papaya Preparation, Oxidative stress, Cardiotoxicity

INTRODUCTION

For many years, antitumor drugs have been known to induce adverse effects that challenge patient's compliance and quality of life. Some of these effects (such as bone marrow toxicity) are unavoidable consequences of the mechanism(s) of action of cytostatic and/or cytotoxic agent in rapidly dividing normal cells. Appropriate medications or scheduled modifications, introduce minimal long-term sequelae and can spontaneously reverse such effects. Other effects are more sinister and tend to surface in normal tissue, like the heart, that has limited regenerative capability. It is now believed that, anthracyclines show their toxic effects after one or two electron reductive activation, which cause oxidative stress, iron dysregulation and concomitant alterations of cardiac specific gene expression thereby induce cardio toxicity (Minotti G *et al*, 2004). The findings, that the treatment of animals with a variety of antioxidants, protect heart against DOX toxicity, supported the role of ROS (Reactive Oxygen Species) in Doxorubicin (DOX) induced cardio toxicity (Elberry AA *et al*, 2010, Alkreathy H *et al*, 2010, Xin Y *et al*,2011, El-Shitany NA *et al*, 2008). It is known that DOX or EPI (4'-epi-doxorubicin) diffuse from cardiomyocytes back into plasma, thereby cause secondary alcohol metabolites to accumulate in the heart and form a long lived reservoir, which may help to understand how anthracycline introduce a lifelong risk of cardio toxicity(Menna P *et al*, 2012, Salvatorelli E *et al*, 2007). In patients with a clinical history of cumulative anthracycline regimens, antitumor drug induced full blown CHF (congestive heart failure) does not always respond to standard medications. In many cases heart transplant is the only vital option for patients, who have survived cancer, but experienced delayed full blown CHF (Thomas X *et al*, 2002).Therefore the problem of anticancer drug induced cardio toxicity must also be weighed on social and economical ground, such as the loss of human resources and the cost of hospitalization and treatment. 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 CA *et al*, 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 F *et al*,1998, Prajda N *et al*, 1995, Csokay B *et al*, 1997). Fermented Papaya Preparation (FPP), made by yeast fermentation of Carica Papaya Linn. is used as natural food health supplement in different parts of the world. FPP has been shown to up-regulate phorbol ester and zymosan induced superoxide production in rat peritoneal macrophages (Osato JA *et al*, 1995), natural killer cell activity (Okuda D *et al*, 1993) and the level of interferon (IFN-γ) in human blood (Santiago LA *et al*, 1994). Recent 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 H *et al*, 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 (Santiago LA *et al*, 1993) or by ischemia reperfusion injury (Santiago LA *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 L *et al*, 1996). From these reports it has been proposed that besides immune modulating, FPP also possess the antioxidant activities. The hypothesis proposed was that if doxorubicin cardio toxicity is related to free radical formation and oxidative stress, an antioxidant such as FPP may protect against doxorubicin induced cardio toxicity. Therefore the aim of this study was to investigate, evaluate and confirm the potential cardio protective effect of FPP pretreatment in rats after the administration of doxorubicin.

MATERIAL AND METHODS

Chemicals

Doxorubicin was purchased from Sigma Aldrich (St Louis, MO, USA). Fermented Papaya Preparation was procured from 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.

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).

Experimental Protocol.

Induction of Experimental Cardio 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 Cardio toxicity. Animals were sacrificed 48 hr after the last dose.

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.

Group 1 (Control): Animals received normal saline orally for 28 days and through intraperitoneal on 29th and 30th day.

Group 2 (DOX control): Animals received DOX treatment (10 mg/kg bw, i.p) for 2 days.

Group 3 (FPP control): Animals received FPP treatment (250 mg/kg bw, orally) for 28days and normal saline (i.p) on 29th and 30th day

Group 4 (FPP+DOX): Animals received FPP treatment (100 mg/kg bw, orally) for 28 days and DOX treatment (10mg/kg bw,i.p) for the following 2 days.

Group 5 (FPP+DOX): Animals received FPP treatment (250 mg/kg bw, orally) for 28 days and DOX treatment (10mg/kg bw, i.p) for the following 2 days.

Biochemical analysis

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, heart 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

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.

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).

Biochemical parameters in heart tissue

The excised heart tissue was 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 activity was estimated by the method of Hugo *et al*, (1987) where in, Hydrogen Peroxide is decomposed by Catalase and concentration of remainder H_2O_2 was measured spectrophotometrically at 240 nm and the values expressed as nm of H_2O_2 decomposed /min/mg tissue. Glutathione (GSH) contents in the tissue was measured spectrophotometrically using Ellman's reagent with 5,5'-dithiobis 2 nitrobezoic acid (DTNB) as coloring agent, according to the method of Beutlar *et al*, (1963). The absorbance was recorded at 412nm and the values expressed as nmol/mg tissue. Glutathione Peroxidase (GPx) activity in tissue was measured by using Hydrogen Peroxide as a substrate, by applying the method of Rotruck *et al*, (1973) and the values expressed as U/min/mg tissue. For Superoxide dismutase (SOD), the samples were homogenized in 0.89% KCl and were centrifuged at 3000g for 15 minutes at 10^{o}C. SOD was estimated by the method of Kakkar*et al*, (1984). This method is based on the ability of SOD to inhibit oxidation of reduced PMS under specific conditions. Reading was taken at 560 nm and the values expressed as U/mg tissue.

Estimation of lipid peroxide (measured as MDA)

The degree of lipid peroxidation was estimated by the rate of Malonaldehyde (MDA) production using the Thiobarbuteric Acid (TBA) method as previously described by Beuge and Aust (1978).The absorbance was recorded at 535 nm against reagent blank and the values expressed as MDA nmol/g of heart tissue.

Histopathological examination of heart section

After sacrifice the heart was harvested and washed immediately with saline and fixed in 4% buffered paraformaldehyde. The fixed tissues were processed and embedded in paraffin wax and serial sections (6um thick) were cut. Each section was stained with hematoxylin and eosin. The sections were examined under the light microscope (Leica) for any histopathological changes and were photographed with canon S70.

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 $P<0.05$.

RESULTS

Body weight, Heart weight and Ratio of Heart weight to Body weight

The changes in the body weight, heart weight and ratio of heart weight to body weight are shown in Table 1. In Dox treated rats, body weight decreased and heart weight increased as compared to that of control rats. Treatment of 250 mg/kg bw of Fermented Papaya Preparation (FPP) averted such adverse effects of doxorubicin, as evidenced from the insignificant change in body weight and heart weight as compared to the respective weights of control animals.

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). (Fig.2).

Table 1- Heart weight and Body weight ratio.

TREATMENT	Body wt	Heart wt	Heart wt/body wt ratio
CONTROL	223.2±2.70	0.77±0.02	0.34±0.01
FPP CONT	220.2±3.33	0.76±0.03	0.34±0.01
DOX CONT	200.6±2.78^{aa}	0.93±0.03^{aaa}	0.46±0.02
100mg/kgbwFPP+DOX	208.6±3.44^{a}	0.88±0.02^{aa}	0.42±0.01
250mg/kgbwFPP+DOX	215.2± 3.02^{b}	0.79±0.03^{bbb}	0.37±0.02

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

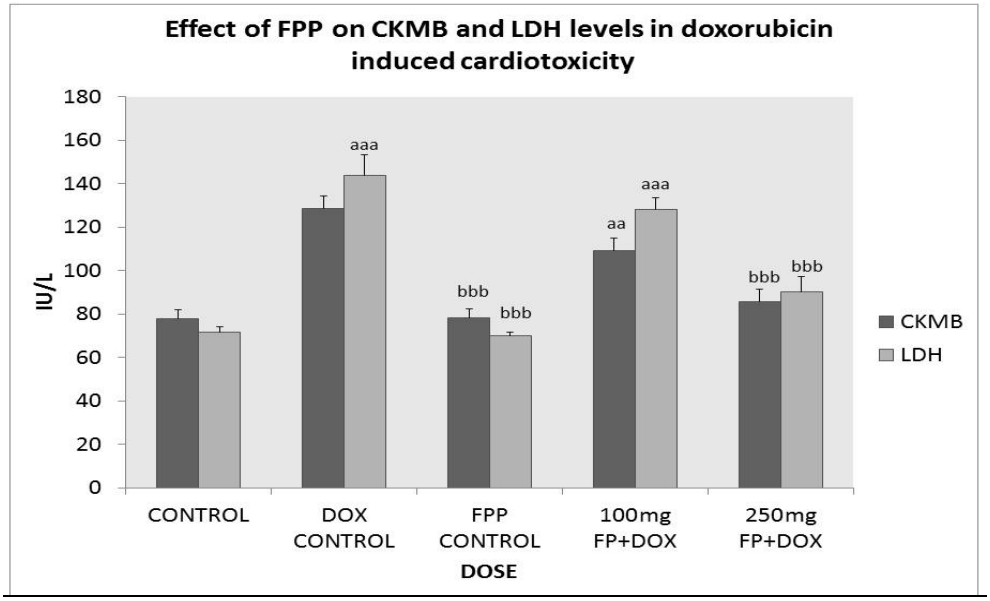

Figure 2: Effect of FPP on CKMB and LDH levels in doxorubicin induced cardio toxicity in rats

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).

Table 2: 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

Cardiac tissue biochemical parameters

LPO-The MDA levels were found to be significantly higher ($p<0.01$) in Group 2 rats as compared to control animals (Group 1). Group 4 rats showed insignificant change in MDA levels, but in Group 5 animals, the MDA levels were significantly ($p<0.05$) decreased in comparison to Dox control (Group 2) rats. (Table 3).

Glutathione, Glutathione Peroxidase, Superoxide dismutase and Catalase:

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-3). 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 3).

Table 3: Effect of FPP on Malonaldehyde, Glutathione peroxidase, Glutathione, Superoxide dismutase & Catalase in doxorubicin treated heart of rats.

TREATMENT	LPO	GPX	GSH	SOD	CATALASE
CONTROL	2.9 ± 0.33	4.2 ± 0.21	5.08 ± 0.4	6.19 ± 0.52	8.08 ± 0.88
DOX CONTROL	5.19 ± 0.3^{aa}	1.12 ± 0.13^{aaa}	1.3 ± 0.4^{aaa}	2.03 ± 0.46^{aaa}	3.34 ± 0.72^{aa}
FPP CONTROL	2.2 ± 0.34^{bbb}	3.56 ± 0.14^{bbb}	5.32 ± 0.53^{bbb}	5.89 ± 0.47^{bbb}	8.02 ± 0.76^{bb}
100mg/kgbwFPP+DOX	4.53 ± 0.39^{a}	1.92 ± 0.18^{aaab}	2.68 ± 0.71^{aa}	3.3 ± 0.62^{a}	3.85 ± 0.7^{aa}
250mg/kgbwFPP+DOX	3.31 ± 0.54^{b}	3.8 ± 0.16^{bbb}	3.82 ± 0.49^{b}	5.04 ± 0.71^{bb}	6.54 ± 0.64^{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. $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$

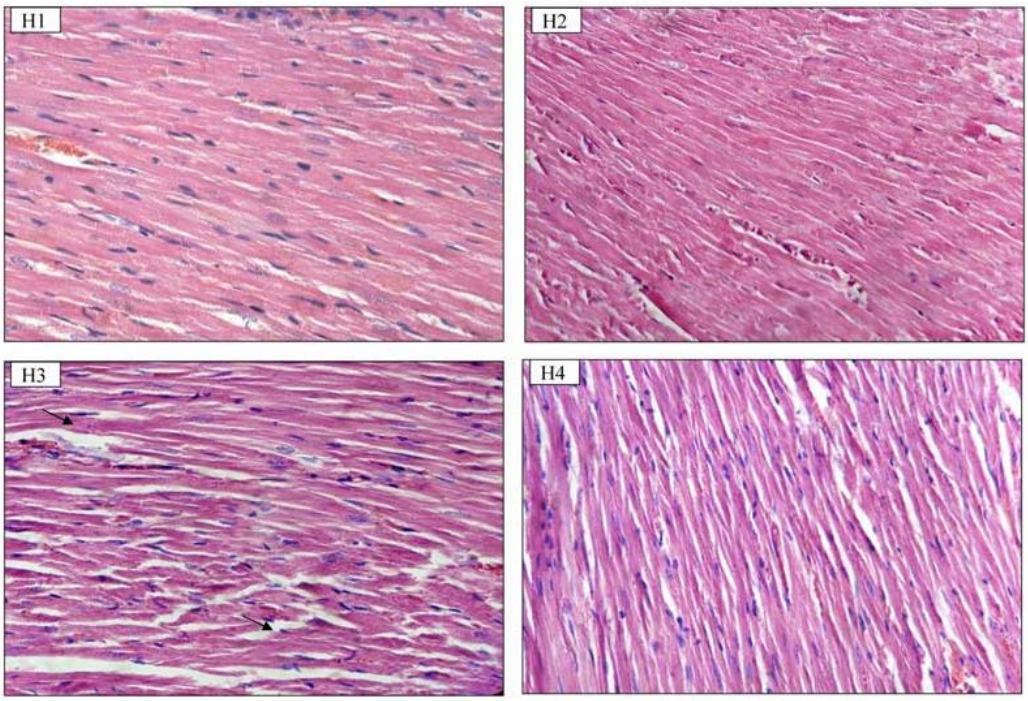

Figure 1: Histopathological examination of rats heart (H&EX40).

H1: Control rat heart showing normal morphological appearance.
H2: FPP treated rat heart 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.

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 (Thomas X*et al*,2002, Psotova J *et al*,2002, Kolarovic J *et al*,2010, Kolarovic J *et al*,2009). Doxorubicin induced cardio toxicity has been attributed as the basic mechanisms responsible for ROS generation and lipid per oxidation (Thomas X*et al*,2002,Psotova J *et al*,2002, Injac R *et al*,2008,Injac R *et al*,2009). Tissues with less developed antioxidants such as the heart are particularly susceptible to injury by Dox induced free radical generation (Olson RD*et al*,1990). Doxorubicin has been demonstrated to induce cardiac toxicity in cultured cells (Chan EM *et al*,1996), isolated heart preparations (Repine JE *et al*,1991), animal models (Singal PK *et al*,1987) and in humans (Lefrak EA *et al*,1973). Several antioxidants have showed promising effect in reducing the Dox induced cardiotoxicty (Herman EH *et al*,1985, Speyer JL *et al*,1985, Speyer JL *et al*,1988, Siveski-Iliskovic N *et al*,1994, Liu X *et al*,2002), 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 L *et al*,1991, Osato JA *et al*,1995).

In the present study, pretreatment of FPP was able to reduce the doxorubicin induced cardio toxic manifestation in multiple ways. FPP pretreatment restricted the Dox induced increase in the levels of triglycerides and total cholesterol significantly (Table 1). Cardio protective activity of FPP was further supported by increased myocardial antioxidant enzyme activity and decreased extent of lipid per oxidation (Table 3). Lipid per oxidation is known to cause cellular damage and is primarily responsible for reactive oxygen species induced organ damage. Increased level of MDA while decreased level of GSH, GPx, SOD and Catalase were observed in heart tissue of doxorubicin treated animals. Pretreatment of FPP counteracted doxorubicin induced cardiac damage by significant decrease in MDA and increase in GSH, GPx, SOD and Catalase activity levels.

As seen in the present study, Dox treatment caused significant histological changes in the myocardium, including marked myofribillar loss and loss of striations (Fig 2). These changes in the Dox treated group were in agreement to those observed by Danesi R *et al*,1992. The myocardial tissue of rats pretreated with FPP showed minimal changes, suggesting protection from cellular damage induced by Dox treatment. The heart tissue of animals treated with FPP alone (Group 3) did not show any histological abnormalities (Fig.2).

CONCLUSION

Although, improvements in cancer treatment strategies have been witnessed in recent decades, doxorubicin toxicity remains a clinical dilemma. In order to reap optimal benefits from this drug, it has become necessary to develop complementary therapies that offsets Dox induced toxicity. In view of our present study, we believe that FPP treatment is a strategy that may provide significant benefits, particularly cardiovascular, for cancer patients undergoing doxorubicin treatment. Patients taking FPP may show better tolerance to doxorubicin treatment or possibly even tolerate higher doses of doxorubicin. Such an improved response to doxorubicin treatment may ultimately increase cure rate, long term survival, and the quality of life for cancer survivors

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