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EFFECT OF FERMENTED PAPAYA PREPARATION ON DOXORUBICIN INDUCED HEPATOTOXICITY AND NEPHROTOXICITY

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ABSTRACT
Doxorubicin (DOX) is an anthracycline derivative used as an anticancer agent. However, its clinical use is limited due to its severe toxic manifestations. In the present study, we aim to evaluate the protective role of Fermented Papaya Preparation (FPP) in combating doxorubicin induced oxidative stress. Wistar rats were pretreated with FPP (100 mg/kg bw or 250 mg/kg bw) or saline daily for 28 consecutive days followed by doxorubicin (10 mg/kg bw) induction for next 2 days. Results indicated that pretreatment with FPP significantly decreased serum levels of hepatic markers Serum Glutamic-Pyruvate Transaminase (SGPT) and Serum Glutamic Oxalicacetic Transaminases (SGOT) along with renal marker Creatinine. Further, FPP supplementation significantly increased Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Reduced Glutathione (GSH) ($p<0.05$) while decreased *Malondialdehyde* and Calatase levels in kidney and liver. Histological observations demonstrated that FPP pretreatment attenuated DOX induced thickening of the glomerular basement membrane and destructive changes in the renal tubule as well as loss of tissue structural pattern and vacuolization in liver. Thus our results suggest that FPP exhibits significant preclinical potential in combating DOX induced oxidative stress.

KEYWORDS: Doxorubicin, Fermented Papaya Preparation, Organ toxicity, Oxidative stress Liver, Kidney.

INTRODUCTION
Doxorubicin is a broad spectrum and potent anticancer characterized under anthracyclines. The limitation in use of Doxorubicin is its lack of selectivity in targeting cancerous cells, leading to adverse side effects which can interfere with treatment outcome and affect subject's quality of life. Doxorubicin is firmly established as a major therapeutic agent in the treatment of a wide variety of tumors. It is used for treatment of lung, breast, ovarian and uterine cancer.[1] It exerts its antitumor effect through arresting tumor angiogenesis[2] and discontinues growth by eliminating the cancer cells from the source of primary tumor. It is thought to be involved in the interference with synthesis of macromolecules, covalent DNA binding and DNA cross-linking, DNA unwinding or DNA strand separation and helicase activity[3], inhibition of topoisomerase II, arresting of tumor cell cycle progression to G_2 phase, induction of apoptosis and generation of reactive oxygen radicals.[4] However, it doesn't discriminate between a cancerous and normal cell and hence act not only on cancer cells but also on healthy growing cells in the body. Its clinical use is limited by its serious side effects including bone marrow depression[5], liver and kidney injury[6,7] and cardiac toxicity.[8] Bone marrow depressions may reverse spontaneously, can be managed through appropriate medications or schedule modifications, and introduce minimal long-term sequel. Other effects are more insidious and tend to surface in normal tissues, like the heart, kidney & liver composed of cells with a limited regenerative capability. Studies have shown that ROS generation and oxidative injury to membrane lipids and other cellular components are the basic factors in doxorubicin toxicity. Recently, apoptosis was also considered to be one of the major processes that lead to the progressive deterioration of tissue functions leading to some tissue pathologies.[9–11]

In liver, doxorubicin causes cell cycle arrest of hepatocytes, oxidative stress and disruption of electron transport.[12] In kidney, doxorubicin causes tubular atrophy and affect glomerular capillary permeability.[13]The exact mechanism of doxorubicin-induce nephrotoxicity is not yet understood, however, it has been suggested by many investigators that cellular damage induced by doxorubicin is mediated by the formation of an iron anthracyclin free radical which in turn causes severe damage to the plasma membrane.[14]

Nowadays herbal treatment is very effectively used to overcome the drug induced toxicity. Stream of studies have confirmed that FPP has a powerful antioxidant effect. It helps in scavenging the ROS by chelating with free iron and decreasing the unbound iron level in blood

and thereby reduce the oxidative stress and free radical damage.[15].Fermented Papaya Preparation (FPP), a natural health food, made by yeast fermentation of Carica Papaya Linn fruit has been reported to possess hydroxyl radical scavenging action resistant to both heat and acid treatment.[16] FPP has been shown to up-regulates phorbol ester and zymosan induced superoxide production in rat peritoneal macrophages [17], natural killer cell activity[18] and the level of interferon (IFN-γ) in human blood.[19] Recent studies have demonstrated that FPP affects NO and hydrogen peroxide production as well as tumour necrosis factor alpha secretion in RAW 264.7 macrophages.[16] 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[20] or by ischemia reperfusion injury.[21] 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.[17] Experimental studies using (FPP) was found to posses highly protective antioxidant properties despite lacking any specific antioxidant vitamin[17,22–24] Such studies have been followed by clinical investigations.[25–29] In particular, recent gastroenterology studies have demonstrated that FPP was able to significantly decrease the oxidative stress in gastric mucosa affected by chronic atrophic gastritis associated with metaplasia and importantly to curb the mucosal concentration of 8-OHdG.[30] From these reports it has been proposed that besides immune modulating, FPP also possess the antioxidant activities.

The aim of this study therefore was to investigate, the potential ameliorating effect of FPP on doxorubicin induced hepatic and renal toxicity.

MATERIAL AND METHODS

2.1. Chemicals

Doxorubicin was purchased from Sigma Aldrich (St Louis, MO, USA). Fermented Papaya Preparation was procured from Venkatesh Naturals, Chhindwara, Madhya Pradesh, India. (Prepared by fermenting Carica papaya with glucose, yeast and lactic acid bacterium). All other biochemical reagents and chemical used were of analytical grade.

2.2. Animals

Female Wistar rats (180-220g) were housed and maintained in 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 (CPCSEA).

2.3. Experimental Protocol.

2.3.1. 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.

2.3.2. Experimental design:

After acclimatization, the animals were randomly divided into the following groups consisting of 6 rats each. They received standard laboratory diet and drinking water ad libitum.

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

Group:2 (DOX control): Animals received normal saline for 28 days orally and DOX treatment (10 mg/kg bw, i.p) on 29th and 30th day.

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.

2.4. Biochemical analysis

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

2.4.1. Biochemical parameters in serum

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

2.4.2 Biochemical parameters in tissues

The excised kidney and liver 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 anti per oxidative enzymes.

Catalase activity was estimated by the method of Hugo *et al* .[31] 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* ., [32]. 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* ., [33] 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* ., [34]. This method is based on the ability of SOD to inhibit oxidation of reduced Phenazine methosulphate 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), as previously described by Buege and Aust., [35]. The absorbance was recorded at 535 nm against reagent blank and the values expressed as MDA nmol/g of tissue.

2.5. Histopathological examination of kidney and liver section

After sacrifice the liver and kidney tissues from the rats of all groups were harvested and washed immediately with saline and fixed in 4% buffered paraformaldehyde for histopathological studies. The fixed tissues were processed and embedded in paraffin wax. 6µm thick sections were cut and stained with hematoxylin-eosin. Sections were examined under the light microscope (Lieca) for any histopathological changes. The sections were photographed with canon S70.

2.6. Statistical Analysis

Results 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

Biochemical parameters in tissues and serum Doxorubicin treatment reduced the activity of all the four enzymes viz. GSH,GPx, SOD and Catalase significantly in the liver and kidney tissue. Pretreatment with FPP 250 mg/kg bw restored the activities of these enzymes to near normal. However pretreatment with FPP 100 mg/kg bw did not show significant attenuation of Dox rendered effects. (Table 1 &2).

Levels of LPO both in liver and kidney increased in doxorubicin treated rats but significantly decreased with higher dose of FPP. (Table 1 &2)

TABLE-1 Biochemical Parameters in liver

Treatments	GSH	GPX	Catalase	SOD	LPO
CONTROL	7.26±0.21	8.56±0.56	32.98±2.67	10.28±0.07	3.7±0.25
DOX CONTROL	3.3±0.43^{aaa}	3.28±0.19^{aaa}	14.56±1.79^{aaa}	3.67±0.37^{aaa}	7.17±0.61^{aaa}
FPP CONTROL	7.65±0.63	8.12±1.67	34.02±1.45	9.833±1.75	3.57±0.45
100mg FPP+DOX	5.06±0.43^{a}	4.76±0.35^{a}	21.96±1.82^{a}	5.98±0.63^{a}	5.73±0.47^{a}
250mg FPP +DOX	6.733±0.32^{bbb}	6.94±0.93^{b}	27.38±3.568^{bb}	7.86±0.94^{b}	4.26±0.54^{bb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with FPP Control, 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

TABLE-2 Biochemical Parameters in Kidney

Treatments	GSH	GPX	Catalase	SOD	LPO
CONTROL	5.75±0.35	4.83±0.45	39.35±1.56	4.85±0.96	2.85±0.34
DOX CONTROL	1.25±0.07^{aaa}	1.5±0.19^{aaa}	18.85±2.7^{aaa}	0.95±0.1^{aaa}	5.13±0.28^{aaa}
FPP CONTROL	5.13±0.23	4.58±0.29	35.68±1.75	4.57±0.2	2.59±0.12
100mg FPP+DOX	2.89±0.65^{a}	2.6±0.6^{aa}	24.2±1.56^{aaa}	1.68±0.63^{aa}	4.22±0.21^{aa}
250mg FPP +DOX	4.8±1.28^{bb}	3.64±0.21^{bb}	29.35±1.43^{bb}	3.55±0.2^{b}	3.15±0.23^{bbb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with FPP Control, 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

Table 3 indicates significant increase in serum parameters SGPT, SGOT, ALP, Urea and Creatine in Doxorubicin treated groups and near normal levels in FPP 250mg/kg bw treated group (Group 5).

TABLE-3 Serum Parameters

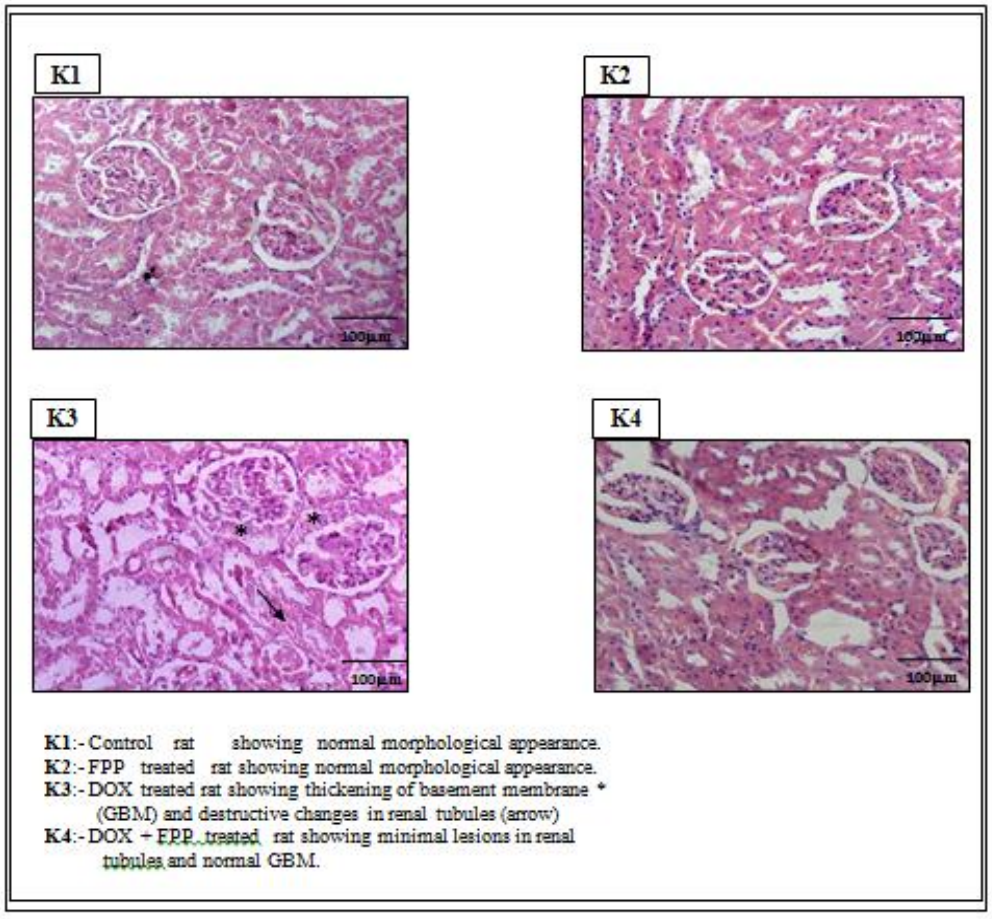
Treatments	SGPT	SGOT	ALP	Urea	CREA	TRIGLY	CHOL
CONTROL	30±4.11	59.2±5.0	167.2±8.413	35±1.517	0.86±0.0143	46.4±1.61	42.6±1.02
DOX CONTROL	80±4.43^{aaa}	115±8.06^{aaa}	257.8±6.763^{aaa}	60.2±3.861^{aaa}	1.38±0.089^{aaa}	75.6±3.51^{aaa}	71±5.84^{aaa}
FPP CONTROL	31.36±3.11	67.1±4.6	156.3±4.234	31.1±1.35	0.841±0.013	49.3±1.24	43.7±1.01
100mg FP+DOX	57±5.21^{aab}	92.8±10.8^{a}	226±11.815^{aa}	50.6±3.20^{aa}	1.038±0.019^{aabb}	69.2±6.726^{a}	62±1.3038^{aa}
250mg FP+DOX	39±7.273^{bbb}	69.4±7.47^{bb}	202.25±13.664^{bb}	43±1.476^{bbb}	0.905±0.035^{bbb}	57.5±4.101^{b}	50.25±4.8^{bb}

Values are expressed as Mean ± SE.(n=5)
Control is compared with FPP Control, 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

Doxorubicin treated animals showed marked increase in cholesterol and triglyceride levels. Animals treated with FPP 250 mg/kg bw (Group 5) however, showed a significant correction in the levels of cholesterol and triglycerides. (Table 3)

Histopathological examination of kidney and liver section. 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. (FIG 1&2)

FIGURE 1: 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.

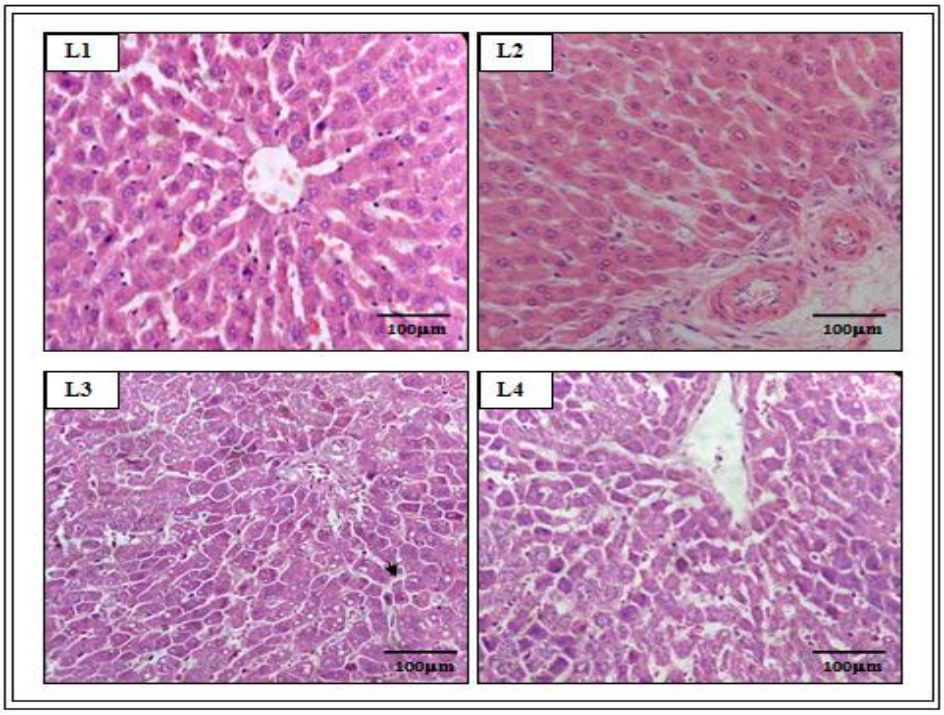


Figure 2: Histopathological examination of rat liver (H&Ex40)

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.

DISCUSSION

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 kidney and liver.[36,37]

Doxorubicin is a potent antitumor drug but causes wide range of side effects. The administration of DOX leads to production of hydroxyl radicals, hydrogen peroxide and superoxide anions.[38] It is shown in previous studies that doxorubicin in the form of semiquinone plays the main role in nephrotoxicity and hepatotoxicity. Reactive oxygen species are produced by the reaction of molecular oxygen with the semiquinone, which results in the tissue damage.[11,39–41] The objective of this study therefore was to investigate the protective effect of Fermented Papaya Preparation on doxorubicin induced liver and kidney toxicity.

Several antioxidants have showed promising effect in reducing the DOX induced nephro and hepatotoxicity [11,42,43,44] however FPP does this by affecting superoxide dismutase (SOD) and glutathione peroxidase (GPx), the very genetic pathway that eliminate free radicals from the system.[16] Importantly, FPP does not turn into a pro-oxidant if taken in large doses, the way standard antioxidant turn out to be.[17,23]

In the present study, pretreatment of FPP was able to reduce the doxorubicin induced 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.[45] 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.[46] 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).[38] LPO is measured in the terms of the extent of Malondialdehyde formation.[47] In the present study doxorubicin increased the Malondialdehyde formation in liver and kidney, which was successfully reduced in the animals pre-treated with FPP.

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(O_2^-) 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* and Tu *et al* .[48,50] SOD and Catalase activity in the hepatic and renal tissue increased with pre treatment of higher dose of FPP and helped the cells to retain their near normal functioning.

FPP pretreatment restricted the DOX induced increase in the levels of triglycerides and total cholesterol in serum significantly (Table 3). 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 in rats.

Urea and creatinine is 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. Previous studies in our lab have shown a similar protection against Doxorubicin induced cardiotoxicity by Fermented Papaya Preparation pre-treartment.[51]

The biochemical findings in the present study were seconded by the histological studies of both liver and kidney. 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 (Fig 2). 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.

CONCLUSION

Nutraceuticals can be defined as food product consumed or administered under medical supervision based on medical evaluation of specific dietary management of a disease.[52] Some of the natural products find their use not as pharmaceuticals (real medicine) but as nutraceuticals that fall well into the concept of functional foods. The results of the present study concluded that FPP significantly protected the nephrotoxicity and hepatotoxicity either by enhancing the DOX-induced declined renal & hepatic antioxidant status or by its direct antioxidant activity. The results can further suggest the possible use of FPP as nutraceutical against oxidative stress induced organ toxicities. However, the interference of FPP in the antitumor efficacy of Doxorubicin needs to be evaluated and these studies are under way.

CONFLICT OF INTEREST

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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