4. Validation of Protective Role of FPP in Doxorubicin Induced Toxicity in vitro System.

4.1. INTRODUCTION

For the last decade or so, in vitro toxicology has been studied as a method for determining human toxicity, as well as hazard identification and characterization. One purpose of in vitro studies is to determine the possibility of developing systems in which the toxicity of chemicals can be quantified, without the dependence on animal experimentation. The other purpose is to validate the findings made in the in vivo system.

Late onset of doxorubicin cardiotoxicity is frequently observed among childhood cancer survivors, which has generated a great interest in the mechanisms involved. By using a juvenile mouse model, Huang *et al.,* (2010) and coworkers demonstrated that hearts from juvenile mice exposed to doxorubicin developed abnormal vasculature and were highly susceptible to myocardial infarction. During doxorubicin treatment decreased number of progenitor cardiac cells was observed, suggesting that these undifferentiated cells were more susceptible and thus were selectively eliminated (Huang *et al.,* 2010). This selective cell toxicity may increase cardiac susceptibility to several stressors. A similar results were observed by De Angelis *et al.,* (2009) who reported different sensitivities of adult and neonatal rat cardiomyocytes to doxorubicin treatment, with the mitochondrial apoptotic pathway being more active in immature cardiac cells than in adult cardiac cells (Konorev *et al.,*2008).

The liver is the metabolic centre of the body (Sadauskas *et al.,* 2009). It has a crucial role in metabolic homeostasis, as it is responsible for the storage, synthesis, metabolism and re-distribution of carbohydrates, fats and vitamins. It also produces large numbers of serum proteins and an array of enzymes and cytokines (Kiemic 2001). The liver receives and accumulates materials at much higher volumes compared to other organs and alongside the kidneys might be responsible for the clearance of toxins from the blood.

There are several studies suggesting the involvement of oxidative stress in the pathogenesis of various disorders and diseases. Reactive oxygen species (ROS) and other free radicals are critical intermediates in the normal physiology and pathophysiology of the liver. Oxygen species are important in the creation of oxidative stimuli required for normal physiologic homeostasis of hepatocytes, as well as playing a role in gene expression (Diesen *et al.*, 2009). Since ROS are ubiquitous in the normal physiology of so many processes, it is not surprising that when excess ROS are produced, some normal functions of healthy cells are affected.

To neutralize the excess ROS, cells utilize antioxidants. If the equilibrium between ROS generation and the antioxidant defence within a cell is disrupted it may result in oxidative stress (Kang 2002). Common antioxidants in hepatocytes include glutathione (GSH), glutathione peroxidase, superoxide dismutase (SOD), hemeoxygenase (HO) and peroxidases (Glantzounis *et al.,* 2005). Glutathione is a ubiquitous tri-peptide which primarily functions to react with hydrogen peroxide utilising glutathione peroxidise to create glutathione disulfide (GSSG). GSH also scavenges other ROS molecules and prevents oxidation of protein sulfhydryl groups (Diesen *et al.*,2009)

The effects of oxidative stress are usually dependent upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, long lasting or severe oxidative stress can cause cell damage and death. Even moderate oxidative stress can trigger apoptosis, while more intense stress may cause necrosis (Martindale *et al.,* 2002). Mild ROS/oxidative stress can activate cells via redox sensitive transcription factors (i.e. NFκβ) leading to elevated gene expression of pro-inflammatory mediators (Brown *et al.,* 2004), while severe ROS insult can lead to genotoxicity (Friedberg 1995; Abrahm 2001). Apoptosis is an energy dependent, natural, genetically-controlled process by which an organism eliminates unnecessary single cells (Mallat *et al.,* 2000). The term "apoptosis" has been coined to explain the morphological processes principal to controlled cellular self-destruction, and was first discovered in a publication by Kerr *et al.,* (1972). Bax belongs to a family of proteins which share homology with Bcl-2 in several highly conserved regions. The Bcl-2 proteins are involved in the regulation of apoptosis, and act to promote or suppress cell death. An overexpression of Bax promotes cell death. Bax can form homodimers and can heterodimerise with other Bcl-2-related proteins. The formation of heterodimers between Bax and Bcl-2 homologues with death repressor function (Bcl-2 and Bcl-Xl) leads to the inhibition of the death-promoting effects of Bax. It has been proposed that the relative expression of the different Bcl-2 families of proteins controls the sensitivity of cells to apoptotic stimuli (Reed 1994). Bcl-2 is a member of the Bcl-2 family of proteins that regulates apoptosis. The gene which encodes Bcl- 2 was first identified at the chromosomal translocation point (14:18) in human B cell follicular lymphoma (Rosse *et al.,* 1998). The overexpression of Bcl-2 enhances cell survival by suppressing apoptosis in a number of cells that are subjected to a wide range of apoptosis-inducing stimuli, including nerve growth factor withdrawal, radiation and chemotherapeutic agents (Rosse *et al.,* 1998)

The earlier experiments in this study established the role of FPP as a protective agent against the organ toxicity induced by Doxorubicin. The present study was designed to validate and prove that FPP indeed show protection against DOX induced toxicity not only in vivo studies but also in in vitro cells, using two cell line viz. H9c2 and BRL-3A. H9c2 cell line is derived from embryonic rat heart and has been used as an in vitro model for both skeletal and cardiac muscle cells. As progenitor embryonic cells isolated from the heart (Kimes *et al.,* 1976), H9c2 cells are able to differentiate from mononucleated myoblasts to myotubes when cultured in a low serum concentration media (Hescheler *et al.,* 1991), acquiring in the process mostly a skeletal muscle phenotype. In addition, chronic treatment with all trans retinoic acid in a low serum concentration media has been reported to generate cells with a cardiac phenotype (Menard *et al.,* 1999). Undifferentiated H9c2 myoblast cells are now accepted as in vitro model to study the effects of ischemia/reperfusion and diabetes on the cardiac muscle (Zhu *et al.,* 2011; Yu *et al.,* 2011)and the cardiac toxicity of several agents such as resveratrol (Gurusamy 2009), tertbutylhydroperoxide (Silva *et al.,* 2010; Sardao *et al.,* 2007), homocysteine (Sipkens *et al.,* 2011), nucleoside analogs (Lund *et al.,* 2007), diazoxide (Comelli *et al.,* 2007), or chemotherapeutics such as doxorubicin (Huelsenbeck *et al.,* 2011, L'Ecuyer *et al.,* 2011)The BRL-3A is a rat liver cell line extensively used for in vitro toxicity studies.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

- Doxorubicin was purchased from Sigma Aldrich (St Louis, MO, USA).
- Fermented Papaya Preparation was procured from Venkatesh Food Products, Indore.
- Fetal bovine serum , Dulbecco's Modified Eagle medium (DMEM) and trypsin and other tissue culture reagents were obtained from_Gibco, invitrogen, USA
- Antibiotic and antimycotic solution (himedia laboratories, India.)
- 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)
- Cell lines: Rat cardiac cells H9C2 and liver cells BRL-3A were procured from National Centre For Cell Science ,Pune, India.

4.2.2. Cell viability

Cytotoxicity was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye-reduction assay, to determine the cytotoxic effect of Doxorubicin and FPP at various concentrations. Briefly, cells were plated onto 96-well flat-bottom culture plates with various concentrations of Doxorubicin (10-100µm) and FPP (5-320µg). All cultures were incubated for 24 hours at 37°C in a humidified incubator. After 24 hours of incubation (37°C, 5% CO_2 in a humid atmosphere), 10 µL of MTT (5 mg/mL in PBS) was added to each well, and the plate was incubated for a further 4 hours at 37°C. The resulting formazan was dissolved in 100 µL of dimethyl sulfoxide with gentle shaking at 37°C, and absorbance was measured at 490 nm with an enzyme-linked immunosorbent assay reader (Biotek Instruments EL800). The results were given as percentage mean of three independent experiments. Concentrations of Doxorubicin & FPP showing a 50% reduction in cell viability (ie, half-maximal inhibitory

concentration [IC_{50}] values) were then calculated. Further studies were carried out with the IC_{50} value for both Doxorubicin and FPP.

4.2.3. Cell culture

Rat cardiac cells H9c2 and liver cells BRL-3A were cultured in DMEM Hg supplemented with 10% fetal bovine serum (Gibco, invitrogen, USA) with antibiotic and antimycotic solution (himedia laboratories, India.) in 5% CO2 at 37 °C. The cells were fed every 2–3 days and subcultured once they reached 70–80% confluence. Cells were plated at an appropriate density according to each experimental design.

4.2.4. RNA extraction and RT-PCR

Total RNA was isolated from H9C2 and BRL-3A cells respectively using Trizol (Invitrogen). First-strand cDNAs were generated by reverse transcription using oligo (dT) from RNA samples. Primer sequences (Integrated DNA Technologies) are shown below.

Gene name	Primer sequence	Tm	Condition for rt-PCR
GAPDH	LP:5′ACTTGGCATCGTGGAAGGG3′, RP:5′ACTTGGCAGGTTTCTCCAGG 3′;	55.4 °C	95°C:3min, 95°C:30 s } 35 cycles Tm:30s, 72°C:30s 72°C:10m 4°C:10m
Bcl-2	LP: 5′TCTCATGCCAAGGGGGAAAC 3′, RP: 5'TATCCCACTCGTAGCCCTC 3′.	55.0°C	
Bax	LP: 5′GCTGGACACTGGACTTCCTC 3′, RP: 5′CTCAGCCCATCTTCTTCCAG 3'.	53.1 °C	

PCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide (EtBr). The gels were visualized on Biorad Gel Doc^{TM} EZ system (BIORAD USA). The relative

expression was quantified densitometrically using the Image J Software and calculated according to the reference bands of GAPDH.

4.2.5. Invitro Assessment of ROS generation

H9C2 (Rat embryonic cardiomyoblast) and BRL 3A (Rat normal hepatocytes) were maintained in DMEM Hg and 10 % FBS. At ~ 90% confluency 0.1 million cells per well in 12 well plate were seeded. Plate was divided in to 4 groups i.e. Control, doxorubicin treated (2ug/ml), FPP treated (250 ug/ml) and FPP + DOX group (250 ug/ml fpp and 2 ug/ml doxorubicin). Cells were pretreated with FPP followed by doxorubicin treatment, plate was incubated for 24 hrs after treatment. Cells were washed twice with PBS (0.1M) after removal of test compounds. Finally cells were incubated with 10 mM H_2DCFDA in PBS for 15 min in dark. Incubated cells were washed to remove residual DCFDA and were observed with Applied biosystems Floid Imaging station at 460x magnification under green filter (530nm) in dark. The amount of fluorescence is directly proportional to ROS present in the cells.

4.2.6. Invitro Mitochondrial Integrity Analysis:

Similar treatment groups were used for the tests. Finally cells were incubated with 1mM Rhodamine 123 solution in treatment groups after removal of treated media in dark for 10 min. After staining procedure the residual Rhodamine was removed by washing 4 times. The stained plate was observed with Applied Biosystems Floid Imaging station at 460 X magnification with green filter (540 nm). Here amount of fluorescence is directly related to Membrane potential of Mitochondria, Healthier mitochondria will yield better fluorescence.

4.2.7. Nuclear Staining:

Cells in each group was fixed with 2% Paraformaldehyde following treatment. Cells were washed twice and stained with DAPI (5mg/ml) for 1 min. Extensive washing was done before observing under microscope under UV filter (emission) at 405 nm.

4.3. RESULTS

4.3.1. Cell viability after exposure to different concentrations of Dox and FPP alone:

The viability of H9c2 cardiomyoblast cells and liver cells BRL-3A was evaluated after 24h of exposure to different concentrations of doxorubicin and FPP by MMT method. As shown in FIG.4.3.2,doxorubicin treatment induced significant cytotoxicity in a dose dependent manner. The IC_{50} value was found to be 5.1µM. The working drug concentration of 2µM was fixed for all further experiments related to drug studies. We examined the effect of different concentrations of FPP on H9c2 and BRL-3A cell viability to select a concentration of FPP which is both non-toxic to cells as well as effective in preventing/improving doxorubicin induced cytotoxicity. The cells were incubated with FPP at different concentrations (10–100µg) for 24h. The percentage of cell viability under different treatment conditions has been shown in Fig.4.3.1 .The results clearly showed that FPP was not cytotoxic towards the relevant cell lines at tested concentrations. Since the cytotoxicity was at a negligibly lower range, the concentration of 50µg was used for further studies in conjunction with doxorubicin.

4.3.2. **FPP reduces Dox induced cytotoxicity:**

Fig.4.3.2 shows the effect of co-administration of FPP concentrations (10–100µg) on doxorubicin induced cytotoxicity in H9c2 and BRL-3A cell lines. Doxorubicin treatment at a concentration of 5µM showed significant cytotoxicity. The cell viability was found to be 48% after 24h. The results shown indicate that co-administration of FPP significantly reduced the cell toxicity induced by doxorubicin and helped to retain almost 100% cell viability at 50 µg concentration.

Graphs showing percentage viability in FPP and Doxorubicin

FIG 4.3.1 Percentage Viability of Dox and FPP in H9c2 cells

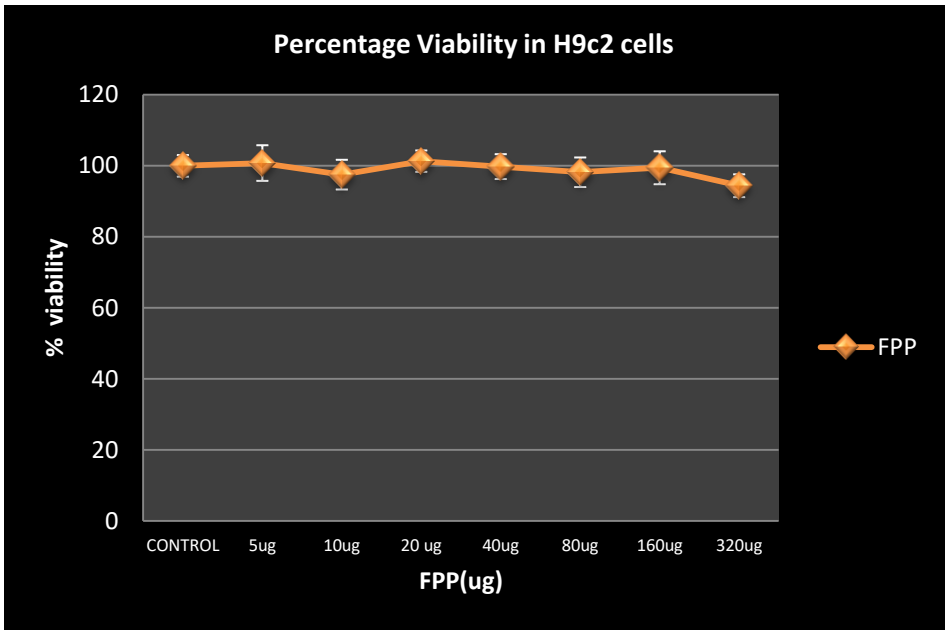


FIG 4.3.2 Percentage Viability of FPP +Dox H9c2 cells

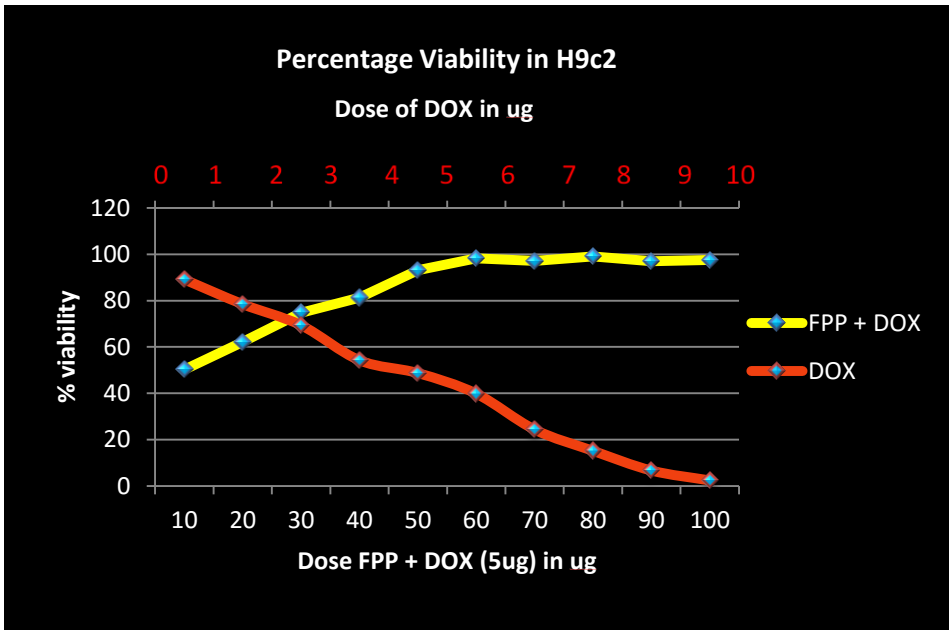


FIG 4.3.3 Percentage Viability of Dox and FPP in BRL-3A cells

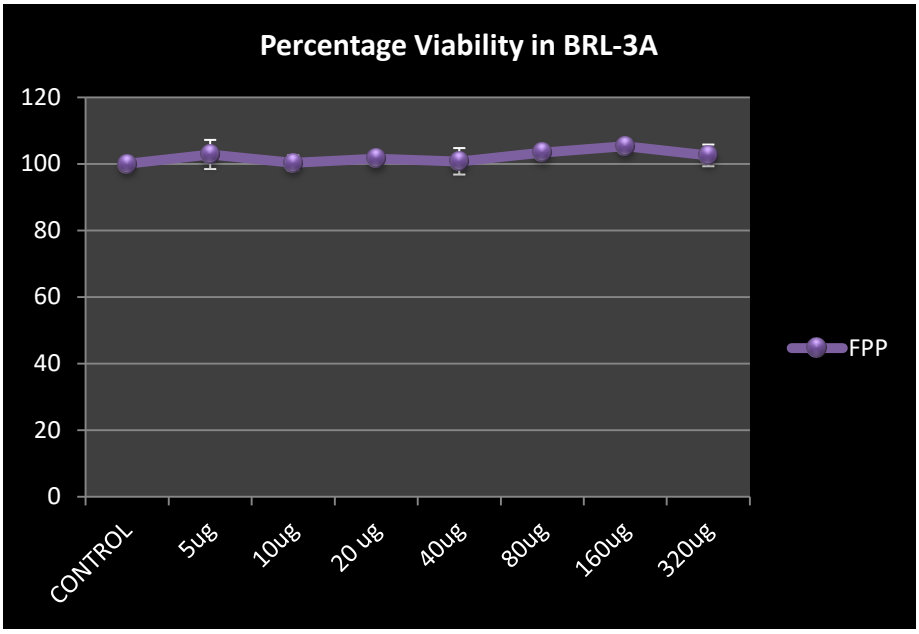
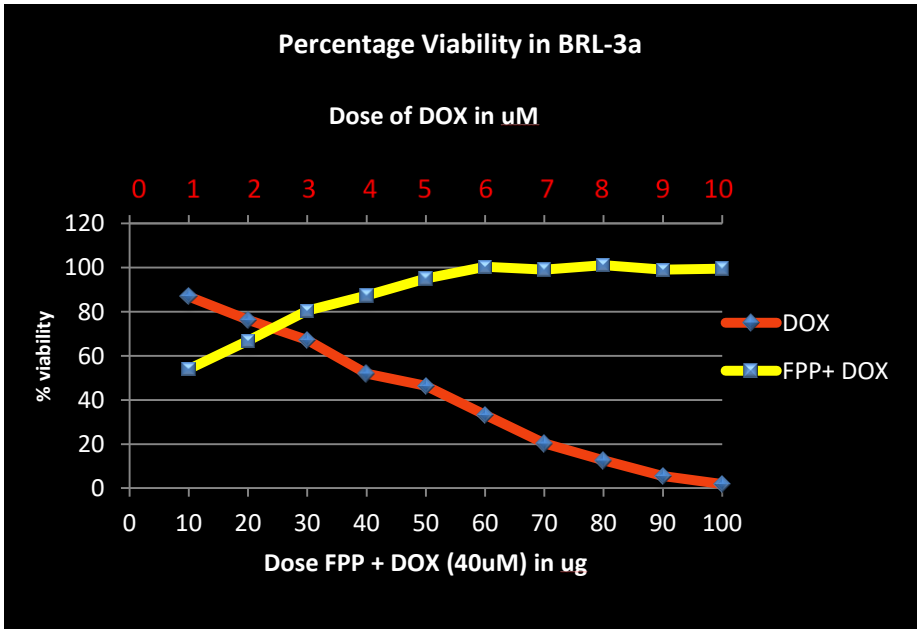


FIG 4.3.4 Percentage Viability of Dox and FPP + Dox in BRL-3A cells



4.3.3. FPP inhibits doxorubicin induced apoptosis in H9c2 and BRL-3A cell lines:

Since doxorubicin decreased cell viability, it was hypothesised that apoptosis might be involved in the reduction of cell viability induced by doxorubicin.

4.3.3.1. FPP retained Bcl-2/Bax Ratio in the doxorubicin treated cells (H9c2 & BRL3A).

The ratio between anti-apoptotic protein (Bcl-2) and pro-apoptotic protein (Bax), namely Bcl-2/Bax ratio is vital to determination of cell survival or death in the apoptosis response. Therefore, gene expression study was conducted to examine Bcl-2 and Bax protein expression in H9c2 & BRL3A cells during doxorubicin treatment. Our results show that Dox mitigated Bcl-2 expression and elevated Bax expression, while treatment with FPP preserved the Bcl-2 protein level and inhibited the elevation of Bax level (plate 7) thus collectively resulting in the maintenance of the Bcl-2/Bax ratio in the doxorubicin treated cells.

When the treated and control cells of H9c2 & BRL3A were stained with the DNA-binding dye DAPI, the cell morphology of treated cells revealed fragmented nuclei, which is a characteristics of apoptotic cells .When the H9c2 and BRL-3A cells were exposed to 2μM doxorubicin for 24h, the total number of viable cells and the size of most cells reduced noticeably. Shrunken/swollen cytoplasm and nuclei pyknosis could also be observed (Plate 9). Co-administration of *FPP* showed significant reduction in these properties induced by Dox. Treatment with FPP *alone* showed cells morphology and viability comparable to that of control, indicating non-cytotoxic nature of the compound. Co-administration *of* FPP showed significant reduction in the numbers of non-viable compared to the cells treated with 2 μM Dox alone (Plate 7)

4.3.3.2. FPP modulates ROS production in H9c2 and BRL-3A cells:

Plate 10 shows the effect of ROS production in doxorubicin and FPP treated cells. Treatment with 2 μM doxorubicin showed significant increase in ROS production as indicated by increase in DCFDA fluorescence.Cells treated with FPP alone showed DCFDA fluorescence at normal levels. Cells cotreated with FPP + Dox showed a significant reduction in ROS production as evident from reduction in DCFDA fluorescence as compared to cells treated with doxorubicin alone (Plate 10)

4.3.3.3. **FPP modulates MI in H9c2 & BRL-3A cells exposed to doxorubicin:**

Plate 8 shows the variations in mitochondrial integrity in doxorubicin and *FPP* treated cells. Treatment with 2 μM doxorubicin showed a significant decrease in MI as indicated by significant reduction in RHO-123 fluorescence as shown in Plate 8. Cells treated with FPP alone showed a normal MMP. Co-administration of FPP showed a significant restoration of MMP as evident from the higher fluorescence in comparison to exclusively Doxorubicin treated cells.

4.4. DISCUSSION

The multi organ toxicity caused by doxorubicin treatment jeopardizes the use of more effective drug dosages in cancer treatment. Chronic doxorubicin cardiotoxicity, which can be latent for many years or decades (Steinherz and Steinherz 1991) is a serious concern for survivors of childhood cancers (Lipshultz 2006). To understand the molecular mechanisms by which doxorubicin causes persistent multi organ toxicity has remained the biggest challenge in recent years. It is known that doxorubicin alters mitochondrial capacity (Oliveira *et al.*,2004; Pereira *et al.*,2011), which may suggest persistent bioenergetic deficits (Berthiaume and Wallace 2007). Other possible mechanisms for cardiotoxicity may involve selective removal of cardiac progenitor cells during doxorubicin childhood treatment or enhanced the activation of the mitochondrial apoptotic pathway (Oliveira *et al.,* 2006)

The mitochondrial membrane potential assay is a sensitive indicator of mitochondrial function in early apoptosis. The disruption of the mitochondrial membrane potential is an early event in apoptosis, which may result in activation of apoptotic cascades (Desagher *et al.*,1999; Cheng *et al.*,1997).

In the present study, we found for the first time that FPP has an anti-apoptotic effect. Rhodamine 123 stained H9c2 rat embryonic cells and BRL-3A rat liver cell showed significant reduction in florescence in Doxorubicin treated cells which was ameliorated appreciably with FPP co-treatment.

We demonstrated that doxorubicin induced excess ROS production in normal cell types is negated by FPP. Doxorubicin treatment reduced viability in both the cell types indicating that Doxorubicin induced apoptosis. Two gene expressions Bcl-2 and BRL-3A were studied. The Bcl-2 family contains both proapoptotic and antiapoptotic proteins that are recognized as key

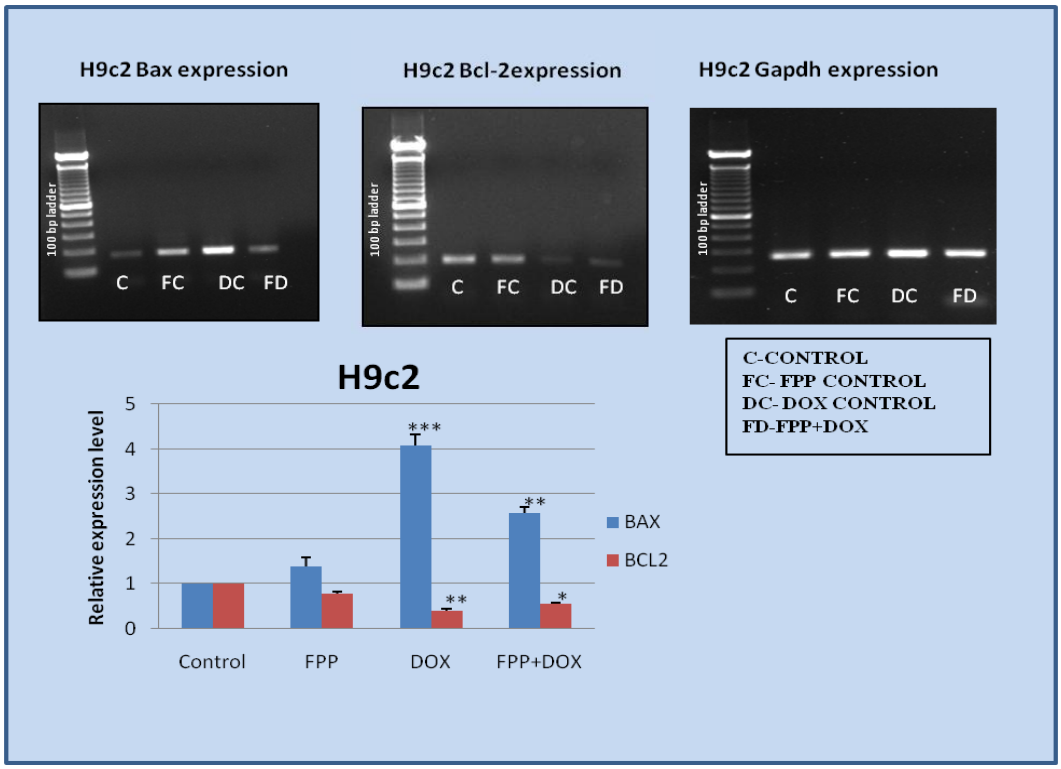
regulatory components of the mitochondrial apoptosis pathway (Gustafsson and Gottlieb 2007). Bcl-2 and Bcl-xl are the principal Bcl-2 family proteins that protect cells from apoptosis. They combine with Bax, a proapoptotic protein, to prevent its oligomerization. The oligomeric form of Bax promotes the loss of mitochondrial membrane integrity and causes the release of cytochrome C (Mikhailov *et al.*,2001). Other proapoptotic proteins, such as Bad, compete for binding to Bcl-2 or Bcl-xL. This causes release of Bax, which induces apoptosis. Thus, the balance between pro- and antiapoptotic Bcl-2 family proteins influences the rate of apoptosis (Valks *et al.*,2003; Markou *et al.,* 2009). Our findings demonstrated that Doxorubicin treatment induces increase in Bax expression and decrease in Bcl-2 expression. FPP reversed these changes and enhanced the Bcl-2-to-Bax ratio in H9c2 cardiomyocytes and BRL-3A rat liver cells exposed to Doxorubicin

Further evidence came from the DNA binding dye DAPI. DAPI staining was done to detect the changes in nuclear morphology and DNA damage during treatment with Doxorubicin, FPP and Doxorubicin in presence of FPP. The results clearly revealed the anti apoptotic nature of FPP.

Cells constantly generate reactive oxygen species (ROS) during aerobic metabolism. The ROS generation plays an important protective and functional role in the immune system. The cell is armed with a powerful antioxidant defense system to combat excessive production of ROS. Oxidative stress occurs in cells when the generation of ROS overwhelms the cells' natural antioxidant defenses. Over the years, ROS has been perceived as a biological hazard causing oxidative damage to the cellular components leading to cancer, neuro-, and cardiovascular degeneration, and disorders related to aging (Molavi and Mehta 2004). More recent studies reveal that ROS also have roles in modulating normal cellular functions (Forman *et al.*,2004)

In the present study cellular ROS levels measured in H9c2 and BRL3A cells by DCFDA technique in both Doxorubicin treated and Doxorubicin co-treated with FPP, once again confirmed the protective effect of FPP against Doxorubicin toxicity. The result also reveals that FPP does not cause any oxidative stress to the cells.

PLATE 7-Bax and Bcl-2 gene expression in H9c2 and BRL-3A



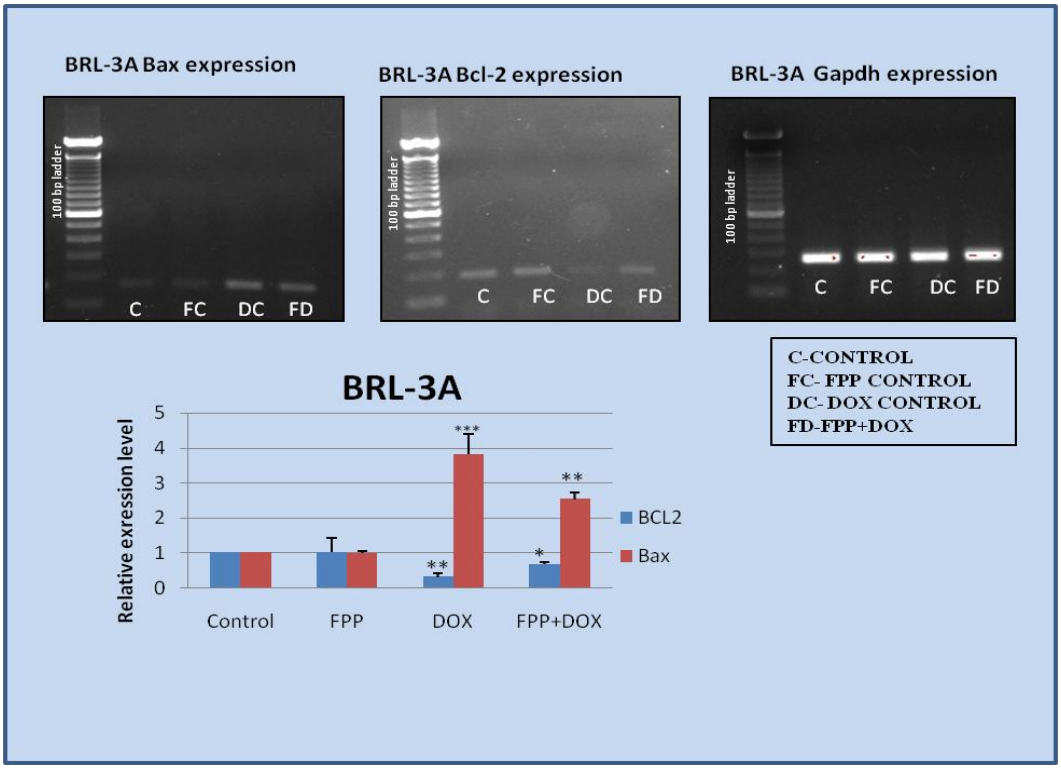
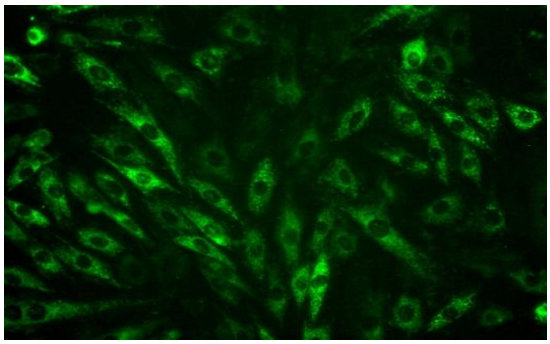


Plate 8-FPP modulates MMP in H9c2 & BRL-3A cells exposed to doxorubicin

H9c2 (Rhodamine)



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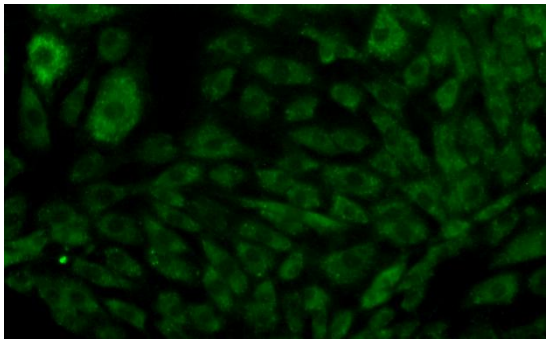
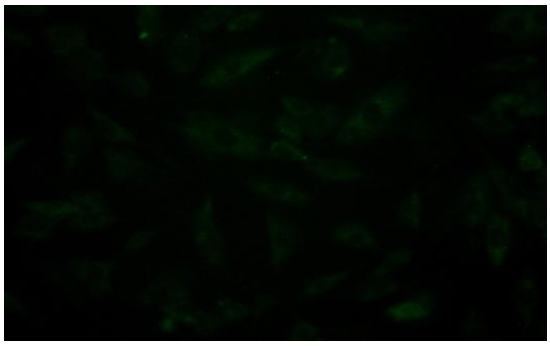
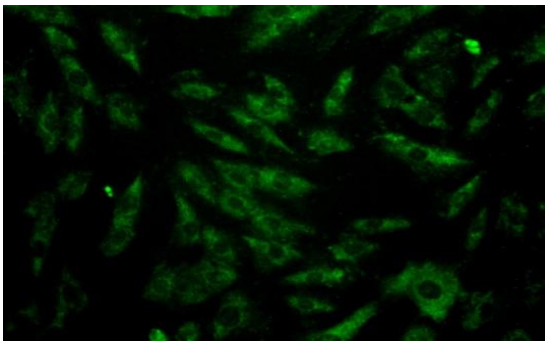


FPP-CON (250 ug/ml)

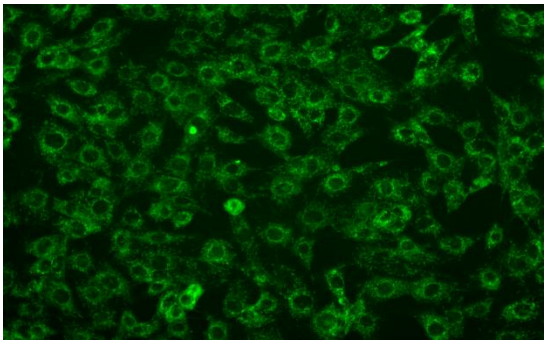


DOX-CON



FPP (250 ug/ml)+DOX (2ug/ml)

BRL-3A (Rhodamine)



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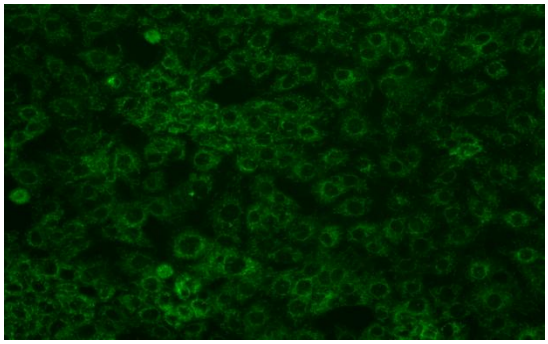
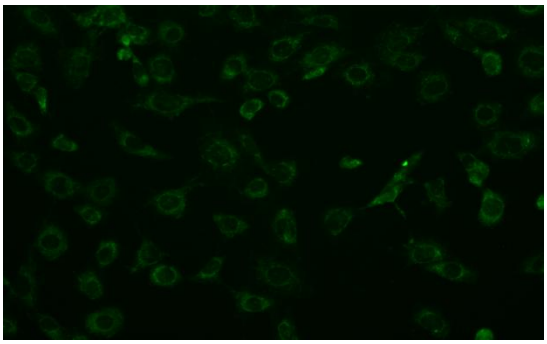
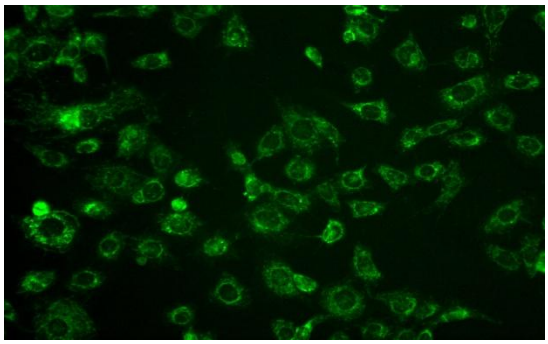


FPP-CON (250 ug/ml)



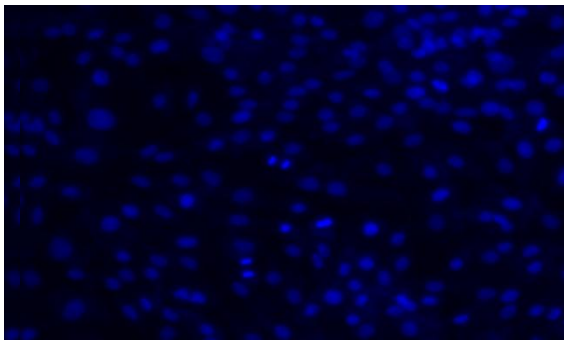
DOX-CON



FPP (250 ug/ml)+DOX (2ug/ml)

Plate 9-FPP modulates Dapi staining in H9c2 & BRL-3A cells exposed to doxorubicin

H9c2 (DAPI)



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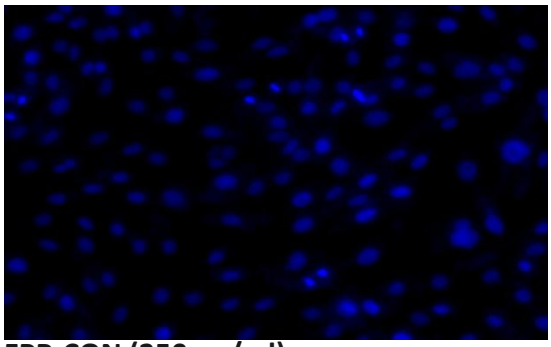
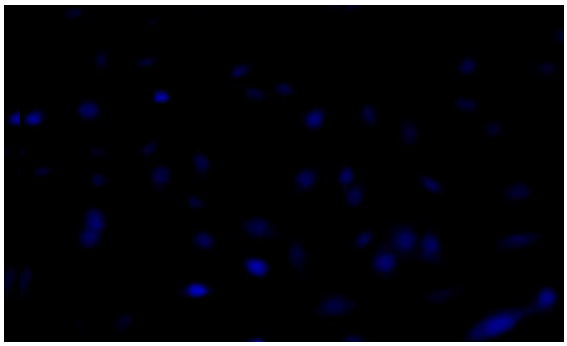
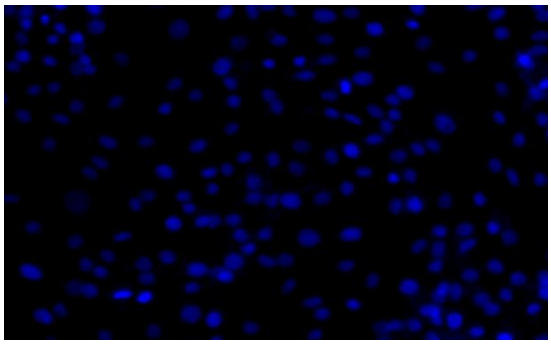


FPP-CON (250 ug/ml)

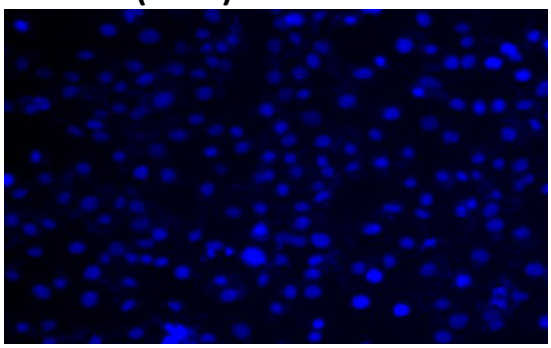


DOX-CON



FPP (250 ug/ml)+DOX (2ug/ml)

BRL-3A (DAPI)



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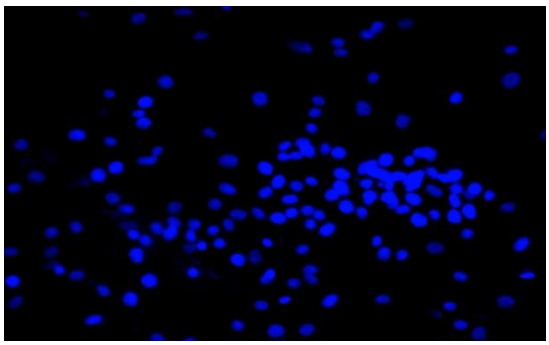
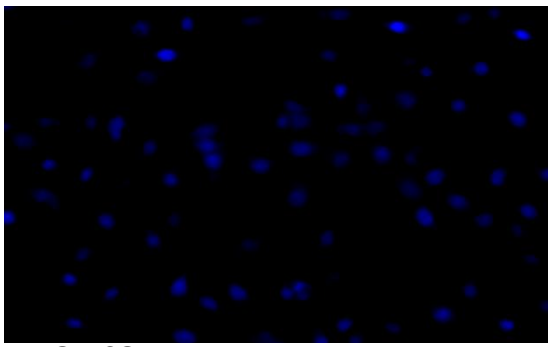
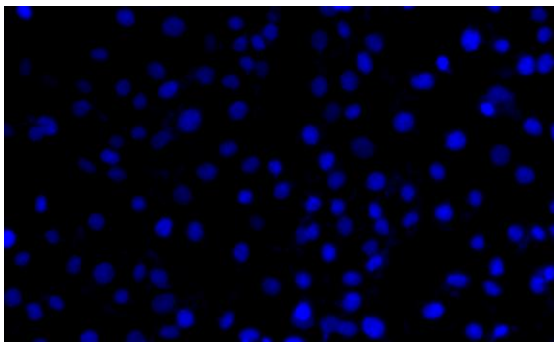


FPP-CON (250 ug/ml)



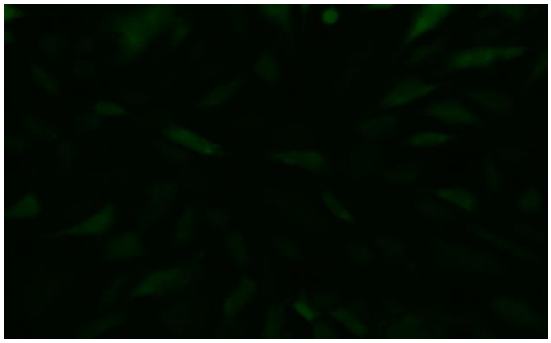
DOX-CON



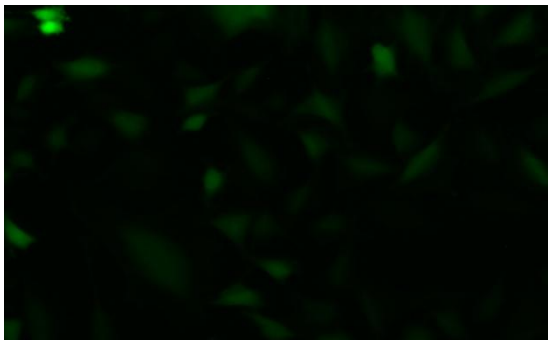
FPP (250 ug/ml)+DOX (2ug/ml)

PLATE 10-FPP modulates ROS production in H9c2 and BRL-3A cells:

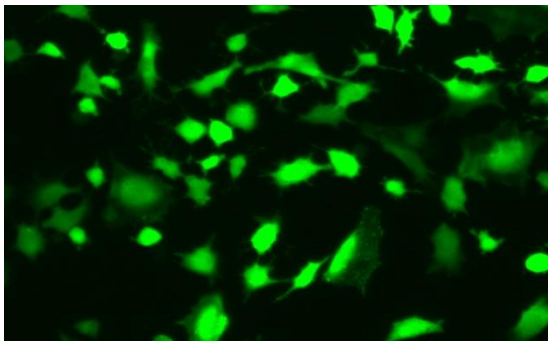
H9c2 (DCFDA)



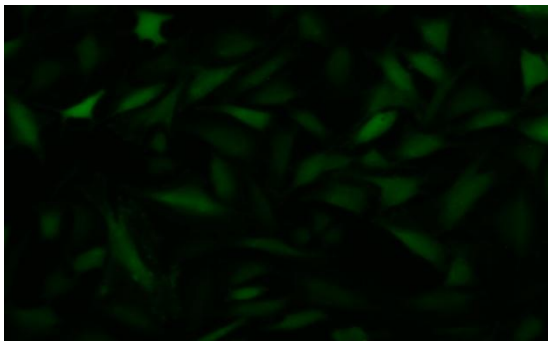
CONTROL



FPP-CON (250 ug/ml)

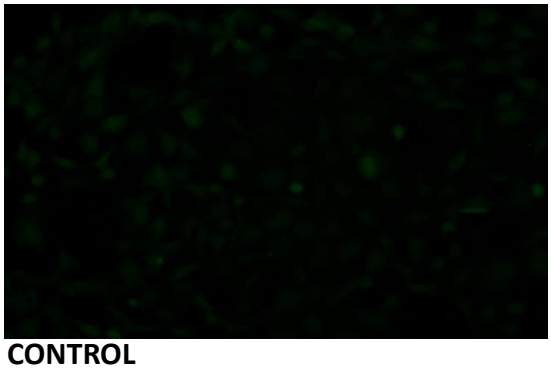


DOX-CON

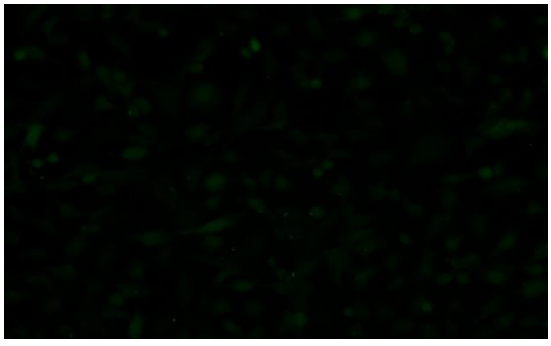


FPP (250 ug/ml)+DOX (2ug/ml)

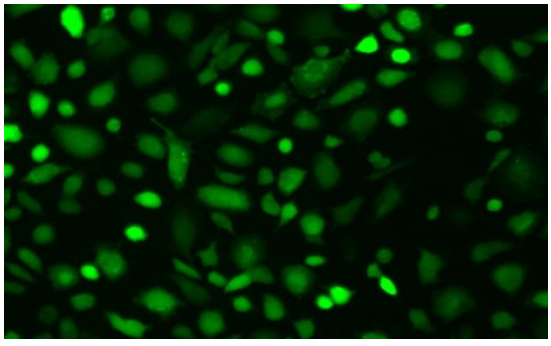
BRL-3A (DCFDA)



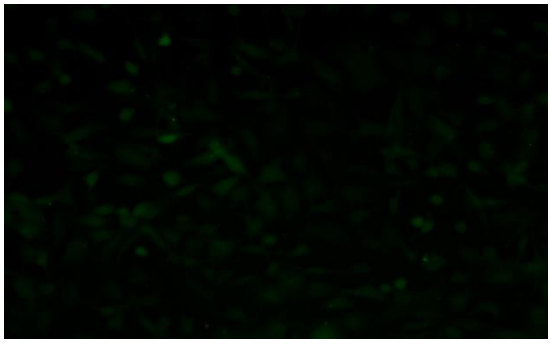
CONTROL



FPP-CON (250 ug/ml)



DOX-CON



FPP (250 ug/ml)+DOX (2ug/ml)

4.4. DISCUSSION

The multi organ toxicity caused by doxorubicin treatment jeopardizes the use of more effective drug dosages in cancer treatment. Chronic doxorubicin cardiotoxicity, which can be latent for many years or decades (Steinherz and Steinherz 1991) is a serious concern for survivors of childhood cancers (Lipshultz 2006). To understand the molecular mechanisms by which doxorubicin causes persistent multi organ toxicity has remained the biggest challenge in recent years. It is known that doxorubicin alters mitochondrial capacity (Oliveira *et al.*,2004; Pereira *et al.*,2011), which may suggest persistent bioenergetic deficits (Berthiaume and Wallace 2007). Other possible mechanisms for cardiotoxicity may involve selective removal of cardiac progenitor cells during doxorubicin childhood treatment or enhanced the activation of the mitochondrial apoptotic pathway (Oliveira *et al.*, 2006)

The mitochondrial membrane potential assay is a sensitive indicator of mitochondrial function in early apoptosis. The disruption of the mitochondrial membrane potential is an early event in apoptosis, which may result in activation of apoptotic cascades (Desagher *et al.*,1999; Cheng *et al.*,1997).

In the present study, we found for the first time that FPP has an anti-apoptotic effect. Rhodamine 123 stained H9c2 rat embryonic cells and BRL-3A rat liver cell showed significant reduction in florescence in Doxorubicin treated cells which was ameliorated appreciably with FPP co-treatment.

We demonstrated that doxorubicin induced excess ROS production in normal cell types is negated by FPP. Doxorubicin treatment reduced viability in both the cell types indicating that Doxorubicin induced apoptosis. Two gene expressions Bcl-2 and BRL-3A were

studied. The Bcl-2 family contains both proapoptotic and antiapoptotic proteins that are recognized as key regulatory components of the mitochondrial apoptosis pathway (Gustafsson and Gottlieb 2007). Bcl-2 and Bcl-xl are the principal Bcl-2 family proteins that protect cells from apoptosis. They combine with Bax, a proapoptotic protein, to prevent its oligomerization. The oligomeric form of Bax promotes the loss of mitochondrial membrane integrity and causes the release of cytochrome C (Mikhailov *et al.*,2001). Other proapoptotic proteins, such as Bad, compete for binding to Bcl-2 or Bcl-xL. This causes release of Bax, which induces apoptosis. Thus, the balance between pro- and antiapoptotic Bcl-2 family proteins influences the rate of apoptosis (Valks *et al.*,2003; Markou *et al.,* 2009). Our findings demonstrated that Doxorubicin treatment induces increase in Bax expression and decrease in Bcl-2 expression. FPP reversed these changes and enhanced the Bcl-2-to-Bax ratio in H9c2 cardiomyocytes and BRL-3A rat liver cells exposed to Doxorubicin

Further evidence came from the DNA binding dye DAPI. DAPI staining was done to detect the changes in nuclear morphology and DNA damage during treatment with Doxorubicin, FPP and Doxorubicin in presence of FPP. The results clearly revealed the anti apoptotic nature of FPP.

Cells constantly generate reactive oxygen species (ROS) during aerobic metabolism. The ROS generation plays an important protective and functional role in the immune system. The cell is armed with a powerful antioxidant defense system to combat excessive production of ROS. Oxidative stress occurs in cells when the generation of ROS overwhelms the cells' natural antioxidant defenses. Over the years, ROS has been perceived as a biological hazard causing oxidative damage to the cellular components

leading to cancer, neuro-, and cardiovascular degeneration, and disorders related to aging (Molavi and Mehta 2004). More recent studies reveal that ROS also have roles in modulating normal cellular functions (Forman *et al.*,2004)

In the present study cellular ROS levels measured in H9c2 and BRL3A cells by DCFDA technique in both Doxorubicin treated and Doxorubicin co-treated with FPP, once again confirmed the protective effect of FPP against Doxorubicin toxicity. The result also reveals that FPP does not cause any oxidative stress to the cells.