Research Article

The grape fruit flavonone naringin protects mice against doxorubicin-induced cardiotoxicity

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Abstract

Doxorubicin (DOX), an anthracycline drug widely used for the treatment of various cancers, causes a cumulative dose-dependent cardiac toxicity that is characterized by an irreversible cardiomyopathy and congestive heart failure. The cardioprotective effect of 2.5, 5, 7.5 and 10 mg/kg naringin (NIN) was studied in mice treated with 15 mg/kg DOX. The animals were killed 30 h after DOX treatment. The latter induced acute cardiotoxicity indicated by a significant elevation in glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), creatine kinase (CK-MB) and lactate dehydrogenase (LDH) in mice serum. Treatment of mice with NIN before DOX administration significantly reduced serum levels of GPT, GOT, CK-MB and LDH indicating that NIN protected against the DOX-induced cardiotoxicity. DOX induced a significant increase in the 8-OHdG DNA adducts and the PARP activity in the heart and liver of mice, whereas NIN treatment of mice before DOX administration significantly reduced 8-OHdG DNA adducts and PARP activity in the heart and liver. Similarly, NIN inhibited the DOX-induced decline in the glutathione concentration, catalase and superoxide dismutase activities and abated DOX-induced lipid peroxidation in the heart and liver of mice. Intraperitoneal administration of 1.25 mg/kg DOX significantly elevated survival of Ehrlich ascites carcinoma (EAC) bearing mice, whereas the combination of 10 mg/kg NIN with DOX did not alter the tumor cell growth, median survival time or average survival time of tumor bearing mice when compared to DOX treatment alone, indicating that NIN does not interfere with the antineoplastic activity of DOX. Our study demonstrates that naringin reduced the doxorubicin-induced cardiotoxicity, without affecting its antineoplastic activity, which may be due to reduction in the DOX-induced 8-OHdG DNA adducts and PARP activity, increase in the antioxidant enzymes and alleviation of lipid peroxidation by naringin.

Introduction

Anthracyclines constitute a major class of cytotoxic agents for the treatment of cancer. Among these, doxorubicin (DOX) a quinone-containing anthracycline antibiotic, is an important anticancer drug used for treating a wide spectrum of human neoplasms including Hodgkin's and non-Hodgkin's disease, soft tissue sarcoma, breast, ovarian, lung, bladder, thyroid, liver and stomach cancer, Wilm's tumor, neuroblastoma as well as acute lymphoid leukemia (Carter 1975, Martin & McNally 1980, Weiss 1992). However, its clinical utility is limited due to dose-dependent cumulative cardiotoxicity that includes electrocardiographic changes, arrhythmias, irreversible degenerative cardiomyopathy and congestive heart failure (Minotti *et al.* 2004, Tacar *et al.* 2013). DOX binds to nucleic acids, presumably by specific intercalation of the planar anthracycline nucleus with the DNA double helix (Gewirtz 1999). The anthracycline ring is lipophilic, but the saturated end of the ring system contains abundant hydroxyl groups adjacent to the amino sugar, producing a hydrophilic center. The molecule is amphoteric, containing acidic functions in the ring phenolic groups and a basic function in the sugar amino group. It binds to cell membranes as well as plasma proteins. Despite extensive research, the mechanism underlying the cardiac damage induced by DOX is still not completely understood. It has been reported that mitochondrial dysfunction and iron catalyzed free-radical production (oxidative stress) leads to cardiotoxicity (DeAtley *et al.* 1999, Gewirtz 1999, Asensio-Lopez *et*

al. 2013), because the heart tissue has a limited defense system and can not cope with the free radical induced toxicity of DOX (Mimić-Oka *et al.* 1999).

DOX has been reported to be metabolically activated to a free radical state and interacts with molecular oxygen to generate superoxide radicals (Powis 1989, Olson & Mushlin 1990), that are generated through redox cycling of DOX in vivo. Superoxide radicals can react with hydrogen peroxide to form highly reactive hydroxyl radicals via the iron catalyzed Haber-Weiss reaction. The secondarily derived hydroxyl radicals can cause protein and DNA damage and initiate lipid peroxidation (Halliwell & Gutteridge 1989). Increased lipid peroxidation, and enhanced free radical generation in the heart have been found to be the major cause of DOX induced cardiotoxicity (Olson & Mushlin, 1990). Iron plays a crucial role in DOX-induced toxicity, as the generation of oxygen free radicals is facilitated by the formation of DOX-iron complexes (Myers 1998).

ROS can cause oxidative damage to both nuclear and mitochondrial DNA. The nature of DNA damage mainly includes base modification, deoxyribose oxidation, strand breaks and DNA-protein crosslinks. Guanine is by far the most prevalent target, although adducts have been reported for all bases (Poirier *et al.* 2000). ROS react with heterocyclic bases in DNA and cause hydroxylation of guanine at the C-8 position. This guanine base hydroxylation leads to the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is a common base modification (Kasai & Nishimura 1983). DOX has been reported to generate 8-OHdG, an indicator of oxidative DNA damage, in HL-60 and MCF-7 cells (Mizutani *et al.* 2003, Swift *et al.* 2006, Coldwel *et al.* 2008). Increased levels of 8-OHdG have been indicated in various pathophysiological processes such as carcinogenesis, aging, cardiovascular and degenerative diseases (Wu *et al.* 2004, Valavanidis *et al.* 2009).

Poly (ADP-ribose) polymerase (PARP), also known as poly-(ADP ribose) transferase (pADPRT) or poly-(ADP ribose) synthetase (PARS), [EC 2.4.2.30] is an abundant nuclear enzyme in eukaryotic cells. It is activated by DNA strand breaks and initiates an energy-consuming cycle by transferring ADP ribose units from NAD+ to nuclear proteins. This process results in rapid depletion of the intracellular NAD+ and ATP pools, slowing the rate of glycolysis and mitochondrial respiration and eventually leading to cellular dysfunction and death (Zingarelli *et al.* 1998, Burkart *et al.* 1999, Szabo *et al.* 2004). Overactivation of PARP leads to tissue damage in various pathological conditions associated with oxidative stress, including myocardial reperfusion injury (Zingarelli *et al.* 1998, Soriano *et al.* 2001a, b, Pacher *et al.* 2002), stroke (Eliasson *et al.* 1997), circulatory shock (Oliver *et al.* 1999, Liaudet *et al.* 2000, Szabo *et al.* 2005), autoimmune cell destruction (Burkart *et al.* 1999) and cardiovascular dysfunction (Soriano *et al.* 2001a, b, Pacher *et al.* 2002, 2006). Doxorubicin induces oxidative and nitrosative stress and, subsequently, overexpression of PARP in the rat or mice heart leading to cardiomyopathy (Szenczi *et al.* 2005, Szántó *et al.* 2011, Lim 2013). The excessive oxidative and nitrosative stress triggers DNA damage, overactivation of PARP, decrease in cellular NAD+ and can impair glycolysis, the Krebs cycle together with mitochondrial electron transport, and will eventually reduce the ATP pool. This eventually leads to cell dysfunction and death by necrosis (Pacher & Sazbo 2007). There is a close relationship between oxidative and nitrosative stress and PARP activation in doxorubicin-treated animals, as well as in humans undergoing doxorubicin anticancer therapy (Bachur *et al.* 2002, Pacher & Szabo 2008, Singal *et al.* 1997). The role of calcium in DOX-induced cardiotoxicity has begun to unfurl. The calcium overload in mitochondria triggers permeability changes in the latter, leading to alteration in its membrane potential, mitochondrial swelling and increased permeability of its outer membrane, causing apoptosis of myocytes (Wallace 2007). PARP activation contributes to the development of the disturbances in cellular calcium handling that develop in the myocardium, in response to doxorubicin treatment (Orsolya *et al.* 2005). PARP inhibitors have been found to provide benefit in clinical trials against BRCA mutant cancers that are unable to repair DNA double strand breaks by homologous recombinant repair (Curtin & Szabo 2013, Do & Chen 2013). Similarly, PARP inhibitors have been reported to increase the effect of ionizing radiation and certain chemotherapeutic drugs (Curtin & Szabo 2013). Recent reports indicate that PARP inhibitors are also active in homologous repair-sufficient tumors (Luo & Kraus 2012).

The use of antioxidants to prevent DOX-induced toxicity has been advocated and strategies have been devised to prevent DOX-induced cardiotoxicity without affecting its antitumor activity by combining it with cardioprotectives and antioxidants (Singal *et al.* 1997). Dexrazoxane, a bisdioxopiperazine compound is the clinically approved drug for the prophylaxis of DOX-induced cardiotoxicity in cancer patients (Weiss 1992). The toxic potential of this agent (especially haemato and hepatotoxicities) encourages further investigation of new cardioprotectants with lower toxicities. Therefore, a drug which can protect against the cardiotoxicity of DOX without compromising its antineoplastic activity is needed.

In this regard, flavonoids are a class of naturally occurring compounds with excellent iron chelating and radical scavenging properties (Kaiserová *et al.* 2007) and are therefore of interest as possible modulators of DOX-induced cardiotoxicity and genotoxicity. Naringin (naringenin 7-rhamnoglucoside), a grapefruit flavanone is present in most of the citrus species and has metal chelating, antioxidant, and free radical scavenging properties (Jagetia & Reddy 2011). It has been reported to offer some protection against mutagenesis and lipid peroxidation (Miyake & Shibamoto 1997, Jagetia *et al.* 2004, Jagetia & Reddy 2005, 2011). Naringin has been reported to inhibit human breast cancer cell proliferation and delay mammary tumorigenesis (So & Guthrie 1996). It has also been reported to reduce constitutive expression of tumor necrosis factor in walker's carcinoma (Camargo *et al.* 2012). Naringin has been reported to protect cells against the radiation-induced chromosome damage as well as iron and radiation-induced oxidative stress (Nagaratnam *et al.* 1999, Jagetia *et al.* 2003, 2004, Jagetia & Reddy 2002, 2005, 2011). Treatment of mice with naringin has also been reported to reduce the tumor burden and tumor incidence in forestomach carcinoma (Jagetia & Reddy 2004). However, a systematic evaluation of the effect of NIN on DOX-induced cardiotoxicity, 8-OHdG DNA adducts and oxidative stress in mice is lacking. Therefore, the present study was undertaken to obtain an insight into the effects of naringin on the doxorubicin-induced cardiotoxicity in Swiss albino mice.

Materials and Methods

Chemicals
Naringin was procured from Acros Organics Ltd, Geel, Belgium, whereas doxorubicin hydrochloride was obtained from Biochem Pharmaceutical Industries, Mumbai, India. The DNA adducts estimation kit (8-OHdG Check ELISA Kit, Catalog # KOG-200SE) and PARP cellular ELISA kit (Catalog # QIA 105) were procured from Gentuar, Brussels, Belgium and Oncogene Research Products, EMD Biosciences, Inc. La Jolla, CA, USA, respectively. All other chemicals were supplied by Merck India Ltd., Mumbai, India.

Animal care and handling
The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and INSA (Indian National Science Academy, New Delhi, India). Ten to twelve weeks old male Swiss albino mice weighing 30 to 36 g were selected from an inbred colony maintained under the controlled conditions of temperature (23±2ºC), humidity (50±5%) and light (12 h of light and dark, respectively). The animals had free access to sterile food and water. Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was approved by the Institutional Animal Ethical Committee of the Kasturba Medical College, Manipal, India, where the study was carried out.

Preparation of drugs & mode of administration
Naringin (NIN) or doxorubicin hydrochloride (DOX) was freshly prepared in sterile double distilled water (DDW), immediately before use. The animals were injected with either 0.01 ml/g per body weight (b.wt.) DDW or various doses of NIN orally (*p.o.*) (Popper & Sons, Inc., New Hyde Park, NY, USA).

Effect of NIN on DOX-induced cardiotoxicity
The animals were divided into the following groups: a) NIN group: the animals of this group were orally administered with 0, 2.5, 5, 7.5 or 10 mg/kg b. wt. of NIN once daily for 5 consecutive days. B) DDW and DOX group: the animals of this group received orally sterile double distilled water, once daily for 5 consecutive days. One hour after the last administration, the animals of this group received a single injection of 15 mg/kg b. wt. of DOX. C) NIN and DOX group: The animals of this group were orally administered with 2.5, 5, 7.5 and 10 mg/kg b. wt. of NIN once daily for 5 consecutive days followed by a single intraperitoneal injection of 15 mg/kg b. wt. of DOX one hour after the last treatment with NIN. A total of four animals (n = 4) were used in each group and a total of 40 animals were used for this experiment

Estimation of serum enzymes
The mice were killed by decapitation 30 h after DOX treatment (Abd *et al.* 2001). The blood was collected by cardiac puncture, the serum was separated by centrifugation at 5000 rpm for 10 minutes and frozen at –70°C for estimation of creatine kinase isoenzyme (CK-MB), lactate dehydrogenase (LDH), glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) (Reitman & Frankel 1957). The enzymes were estimated using standard procedures.

Biochemical estimations
The livers and hearts were quickly excised 30 h after DOX treatment and washed thoroughly with chilled phosphate-buffered saline (pH 7.4). The heart and liver of each animal was cut into three parts. A 10% tissue homogenate of one part of the heart and liver (Sonics Vibra-Cell, New Town, CT, USA) was prepared separately in 0.15 M Tris–HCl (pH 7.4), centrifuged at 12000 g for 15 minutes and the supernatant was col-

Table 1. Alteration in various serum enzymes by different doses of naringin in mice treated with 15 mg/kg body weight of doxorubicin (DOX). Data are mean ± standard error of the mean (SEM) of four animals. a = p < 0.05, b = p < 0.01 and c = p < 0.001, when compared with control.

Treatment	Serum enzyme levels (IU/ml) ±SEM			
	CK-MB	**LDH**	**GOT**	**GPT**
Control	7.24±0.538	131.92±2.3	34.03±1.39	9.73±0.79
2.5 mg/kg NIN	7.62±0.410	139.1±2.15	34.31±2.50	11.04±0.70
5 mg/kg NIN	6.98±0.21	128.0±3.12	35.6±1.24	9.45±0.22
7.5 mg/kg NIN	8.12±0.14	129.0±4.12	32.14±2.52	10.50±0.55
10 mg/kg NIN	6.54±0.54	135.0±5.68	30.44±1.98	8.97±0.44
DDW + DOX	32.99±0.93[c]	377.2±4.11[c]	85.83±1.94[c]	21.55±0.97[c]
2.5 mg/kg NIN + DOX	21.57±0.54	256.5±9.56	68.30±3.21	20.54±0.98
5 mg/kg NIN + DOX	16.24±0.24	200.2±8.47	59.87±4.21	16.54±1.44
7.5 mg/kg NIN + DOX	13.43±1.32[a]	191.8±4.02[b]	45.43±2.11[b]	15.03±0.17[c]
10 mg/kg NIN + DOX	13.58±1.44	180.0±5.23	45.57±2.54	16.12±1.11

lected for the estimation of GSH, CAT, SOD, lipid peroxidation (TBARS) and total proteins. The second part of heart and liver was homogenized separately for the estimation of 8-OHdG DNA adducts, where the DNA was isolated as described earlier (Devanaboyina & Gupta 1996). The third part of the heart and liver was used to prepare a single cell suspension (Klaunig *et al.* 1981, Goldspink *et al.* 1996) for the estimation of PARP activity.

Estimation of DNA adducts
The DNA adducts were estimated by using 8-OHdG Check ELISA Kit, Catalog # KOG-200SE, Gentuar, Belgium, according to the manufacturer's protocol. The 8-OHdG kit is a competitive in vitro enzyme-linked immunosorbent assay for quantitative measurement of oxidative DNA 8-OHdG adducts. The data are represented as means ± SEM (standard error of the mean) of quadruplicate samples for each drug dose from each concurrent group.

PARP assay
The sample was inoculated into the wells of a microplate and mixed with PARP reaction buffer (56 mM HEPES, 28 mM KCl, 28 mM NaCl, and 2 mM MgCl2) containing 0.01% digitonin and 10 μM biotinylated NAD. The microplates were incubated at 37°C for 30 minutes. Thereafter, the buffer was aspirated and cells were fixed for 10 minutes by the addition of prechilled 95% ethanol at -20°C. Endogenous peroxidase activity was blocked by addition of 0.5% hydrogen peroxide/methanol into each well followed by 15 minutes of incubation. Wells were washed once with PBS and then blocked with 1% BSA in PBS for 30 minutes at 37°C. BSA was replaced by the addition of peroxidase-labeled streptavidin (diluted 1:500 in 1% BSA-PBS) and incubated for 30 minutes at 37°C.

Thereafter, the microplates were washed three times with PBS and the reaction was developed. The optical density was measured in a microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Total proteins
The protein contents were determined using the modified method of Lowry.

Glutathione
GSH concentration was measured by the method of Moron *et al.* (1976). Briefly, proteins were precipitated by 25% TCA, centrifuged and the supernatant was collected. The supernatant was mixed with 0.2 M sodium phosphate buffer (pH 8.0) and 0.06 mM DTNB and incubated for 10 minutes at room temperature. The absorbance of the sample/s was read against the blank at 412 nm in a UV-VIS double beam spectrophotometer (Shimadzu Corporation, Tokyo, Japan) and the GSH concentration was calculated from the standard curve.

Catalase
The catalase activity was estimated by catalytic reduction of hydrogen peroxide as described earlier (Abei 1969). Briefly, cumene hydroperoxide was added to the sample and incubated at 37°C. The decomposition of hydrogen peroxide was monitored by recording the absorbance at 240 nm using a UV-VIS double beam spectrophotometer.

Superoxide dismutase
Total SOD activity was determined by the pyrogallol autooxidation method (Marklund & Marklund, 1974). Briefly, the sample was added to 62.5 mM triscacodylic acid buffer, containing 1 mM diethylenetriaminepentaacetic acid (DETAPA), followed by the ad-
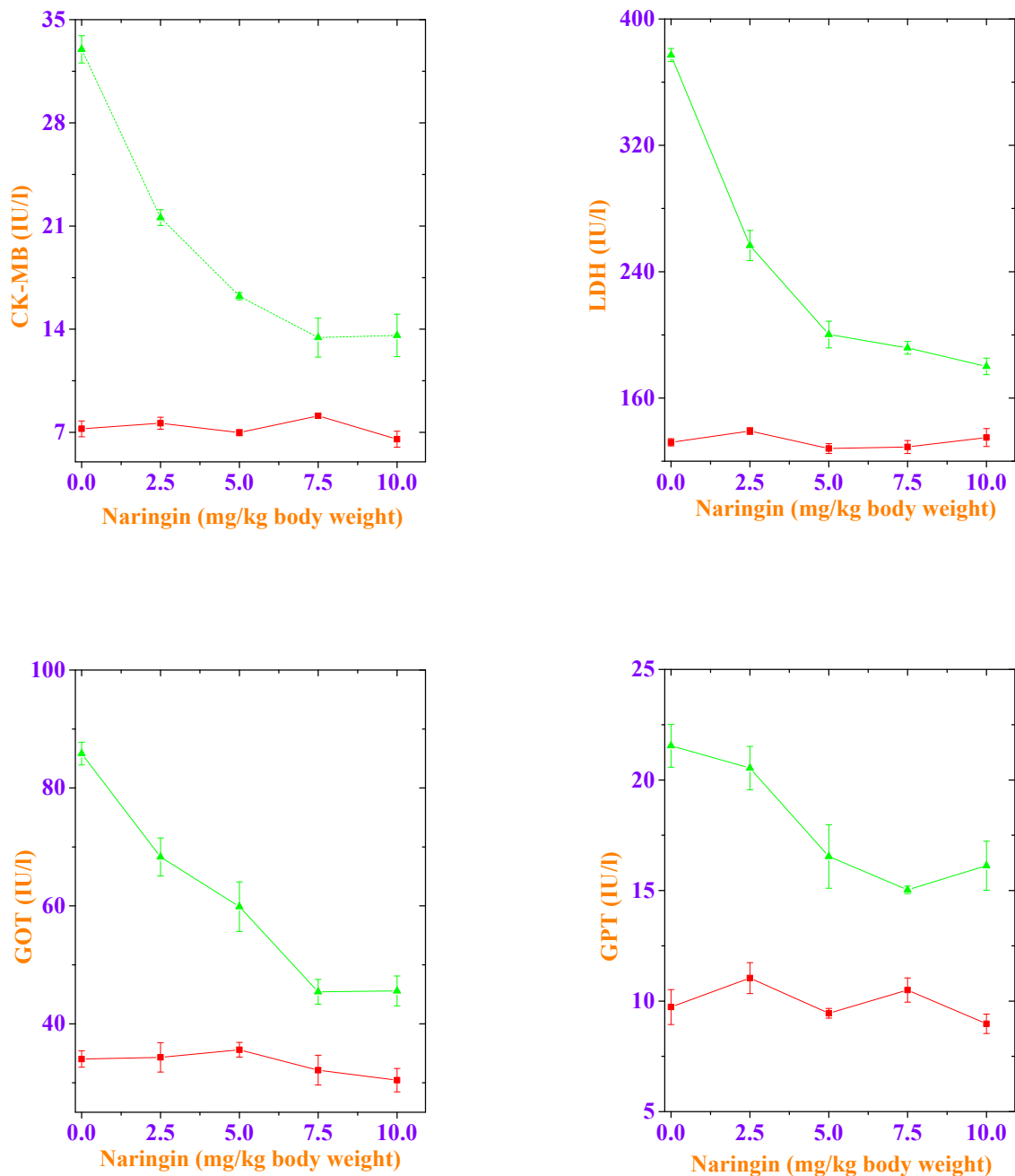
Figure 1. Alteration in the serum enzymes CK-MB, LDH, GOT and GPT in mice treated with or without naringin before doxorubicin treatment. Squares: control group; triangles: NIN + DOX group. P < 0.05 for all points, when compared to control or DOX alone compared with NIN + DOX group.

dition of 4 mM pyrogallol. The autooxidation of pyrogallol was monitored at 420 nm using a UV-VIS double beam spectrophotometer.

Lipid peroxidation (LOO)
LOO was performed according to the standard protocol (Gelvan & Saltman 1990). Briefly, the samples were incubated with a mixture of trichloroacetic acid

(15%), thiobarbituric acid (0.375%) and butylated hydroxytoluene (0.01%) in 0.25 N HCl at 95°C for 25 min. The reaction mixture was allowed to cool to room temperature and was centrifuged at 8,000 g. The supernatant was then collected and the absorbance was recorded using a UV-VIS double beam spectrophotometer. The lipid peroxidation has been expressed as TBARS that were determined against a standard curve
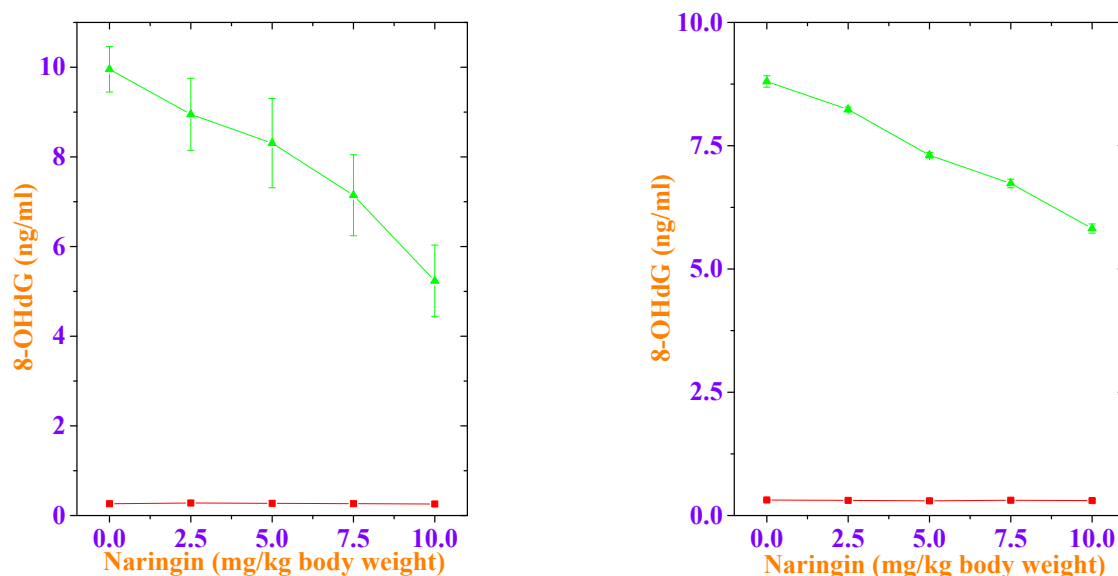
Figure 2. Effect of different doses of NIN on the DOX-induced 8-OHdG DNA adducts in the heart (left) and liver (right) of mice. Squares: NIN group; triangles: NIN + DOX group. $P < 0.01$ for all points, when compared to control or DOX alone compared with NIN + DOX group.

prepared with tetraethoxypropane. For all biochemical estimations duplicate samples were used from each animal for various estimations listed above. A minimum of four animals were used for each drug dose in each concurrent group and a total of forty animals were utilized for this experiment.

Effect of NIN in conjuntion with DOX in the tumor bearing mice

A separate experiment was carried out to evaluate the effect of NIN on the alteration in the anticancer activity of doxorubicin in the Ehrlich ascites carcinoma (EAC) bearing mice. They were treated with NIN or DOX or the combination of both NIN and DOX.

Ehrlich ascites carcinoma (EAC) procured from Cancer Research Institute (ACTREC), Mumbai, India was maintained and propagated by serial intraperitoneal transplantation in an aseptic environment. No spontaneous regression of EAC was observed during the entire study period. Usually, 10^6 viable EAC cells were injected into each animal intraperitoneally in sterile conditions and the day of tumour inoculation was considered as day 0.

Tumor bearing animals were divided into different groups: a) SPS group: Animals of this group received sterile physiological saline (SPS) intraperitoneally, consecutively for 9 days. DOX group: This group of animals was injected with 1.25 mg/kg b. wt. of DOX 24 hours after tumor inoculation, once daily consecutively for 9 days. B) NIN group: Animals of this group

were administered p. o. with 10 mg/kg b. wt. NIN 24 hours after tumor inoculation, once daily, consecutively for 9 days. C) NIN and DOX group: Animals of this group were administered orally with 10 mg/kg b. wt. NIN, 24 hours after tumor inoculation, and one hour before the administration of DOX, once daily consecutively for 9 days.

After the last administration of drug(s), the animals were monitored regularly for body weight changes, signs of toxicity and mortality. The animal weight was recorded every third day, up to 30 days after tumor inoculation in all the groups. The tumor response of DOX was assessed on the basis of median survival time and tumor free survival. A total of six animals were used in each group and a total of 24 animals were used for this experiment.

Statistical Analysis

The significance between the treatments was determined using one way analysis of variance (ANOVA) for 8-OHdG, PARP and antioxidant activities, while the log rank test was applied for the survival assay. The significance between treatments for estimation of GSH, and enzymes including CK-MB, LDH, GOT, GPT, catalase, SOD and LOO was determined by the Students' 'T' test. A p value of <0.05 was considered statistically significant. The Solo 4 statistical package (BMDP Statistical Software Inc, Los Angeles, CA, USA) was used for statistical analyses.
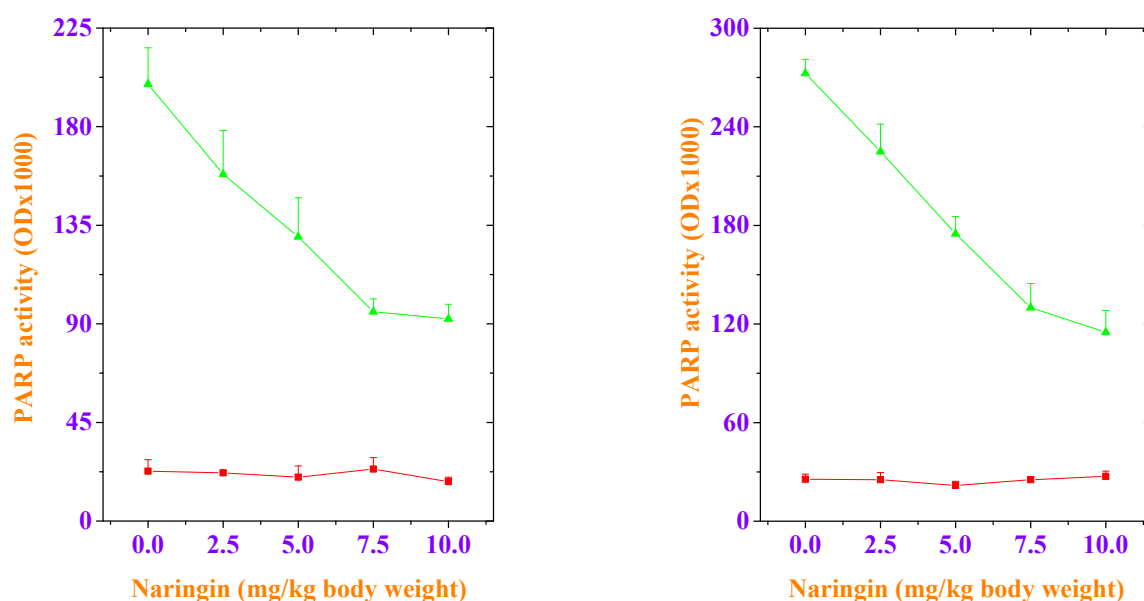
Figure 3. Effect of different doses of NIN on the DOX-induced PARP activity in the heart (left) and liver (right) of mice. Squares: NIN group; triangles: NIN+DOX group. P < 0.001 for all points, when compared to control or DOX alone compared with NIN+DOX group.

Results

Cardioprotection

Table 1 and Figure 1 show the effect of NIN on DOX-induced biochemical changes in the serum of mice. Treatment of mice with DOX significantly (p<0.001) elevated the activities of CK-MB, LDH GOT, and GPT indicating that DOX administration induced cardiotoxicity. The oral administration of various doses of NIN once daily for 5 consecutive days before DOX treatment significantly inhibited the increase in the activities of these enzymes in the serum of mice in the "NIN and DOX" group (Table 1 and Figure 1).

DNA adducts

Spontaneous levels of oxidative DNA adducts (8-OHdG) were low in heart and liver of non-drug treated control group. Treatment of mice with various doses of NIN did not significantly alter the spontaneous formation of 8-OHdG DNA adducts when compared with the control group in heart and liver (Figure 2). Administration of 15 mg/kg DOX significantly (p<0.001) increased the 8-OHdG DNA adduct levels in the heart and liver of mice, which was 38- and 65-fold higher in the heart and liver, respectively, when compared with the non-drug/s treated control group (Figure 2). Pre-treatment of mice with various doses of NIN once daily for five consecutive days before DOX administration caused a significant and NIN dose dependent decline in the levels of 8-OHdG DNA adducts. A

maximum reduction in DNA adduct formation was observed for 10 mg/kg NIN in the heart and liver when compared to the other doses of NIN (Figure 2). This reduction in DNA adducts was approximately 2-fold in the heart whereas 1.5-fold in the liver of animals receiving 10 mg/kg NIN before DOX treatment.

PARP

The ELISA method allows the precise quantification of PARP activity. Treatment of mice with various doses of NIN did not alter the spontaneous PARP activity. Exposure of mice to DOX significantly (p<0.001) raised the PARP activity, when compared with non-drug treated control group in the heart and liver. The PARP activation was almost 9- and 10-fold higher in the DOX-treated heart and liver of mice. Treatment of mice with various doses of NIN before DOX treatment caused a dose dependent decline in the PARP activity (Figure 3), when compared with the DOX treatment alone. A maximum of 2-fold reduction in PARP activity was observed in hearts and livers of mice treated with 10 mg/kg NIN before DOX treatment (Figure 3).

Glutathione

Mice treated with NIN alone did not alter the concentration of GSH when compared with the non-drug treated control group. Treatment of mice with DOX caused a significant decline (4-fold) in the GSH con-
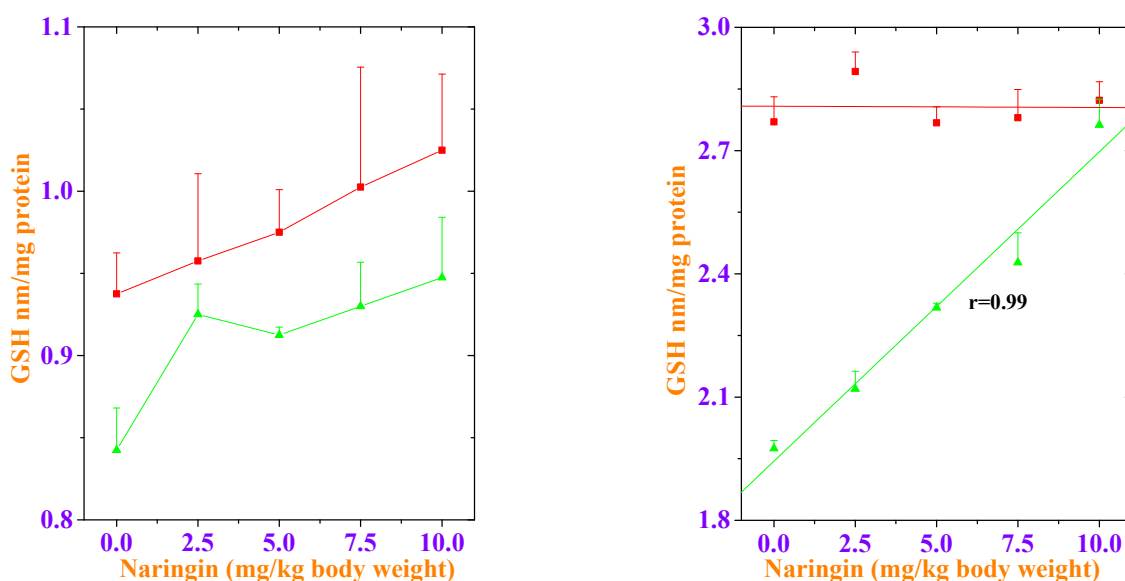
Figure 4. Alteration in the DOX-induced glutathione concentrations by different doses of NIN in the heart (left) and liver (right) of mice. Squares: NIN group; triangles: NIN+DOX group. P < 0.05 for all points, when compared to control or DOX alone compared with NIN+DOX group.

tents in the liver (p<0.001) and heart, whereas treatment of mice with NIN before DOX administration significantly elevated the GSH contents in the heart and liver in a dose dependent manner and a maximum rise in GSH concentration was observed for 10 mg/kg NIN before DOX treatment (Figure 4).

Catalase
Administration of different doses of NIN caused a significant elevation in the spontaneous level of catalase in the mouse heart and liver, when compared with the non-drug treated control group. DOX treatment caused a significant (p<0.01) decline in the catalase activity in the mouse heart and liver (Figure 5). Treatment of mice with different doses of NIN significantly arrested the DOX-induced decline in the catalase activity in a dose dependent manner in heart and liver. The highest increase in catalase activity was observed for 10 mg/kg NIN (Figure 5).

Superoxide dismutase
Treatment of mice with NIN alone elevated spontaneous SOD activity significantly (p<0.001), in a dose dependent manner in the heart and liver. The greatest elevation in SOD activity was observed for 10 mg/kg NIN. The DOX treatment caused a significant (p<0.001) decline in the spontaneous SOD activity in both the heart and liver of mice (Figure 6). Administration of mice with different doses of NIN before DOX treatment caused a significant and NIN dose de-

pendent elevation in the SOD activity in the heart and liver and the greatest increase was observed for 10 mg/kg NIN in the "NIN and DOX" group (Figure 6).

Lipid peroxidation (LOO)
The LOO was measured as thiobarbituric acid reactive substances (TBARS) in the heart and liver of mice. Mice treated with NIN alone did not alter the base levels of TBARS when compared with the non-drug treated control group. However, DOX treatment resulted in a significant (p<0.001) elevation in TBARS in the heart and liver of mice (Figure 7). The NIN administration before DOX treatment caused a significant reduction in TBARS in the mouse heart and liver in a NIN dose dependent manner and the greatest reduction (p<0.001) in the TBARS was observed for 10 mg/kg NIN in the "NIN and DOX" treatment (Figure 7).

Effect of NIN in conjunction with DOX in Ehrlich ascites carcinoma mice
The efffect of NIN on the antitumor efficacy of DOX was assessed in mice bearing Ehrlich ascites carcinoma. No spontaneous regression was observed in the mouse injected with EAC cells throughout the study and the animals exhibited a constant increase in the weight due to tumor cell multiplication. The first death was observed on day 15 and all the control animals died by day 24 post-tumor inoculation. The median survival time (MST) and the average survival time
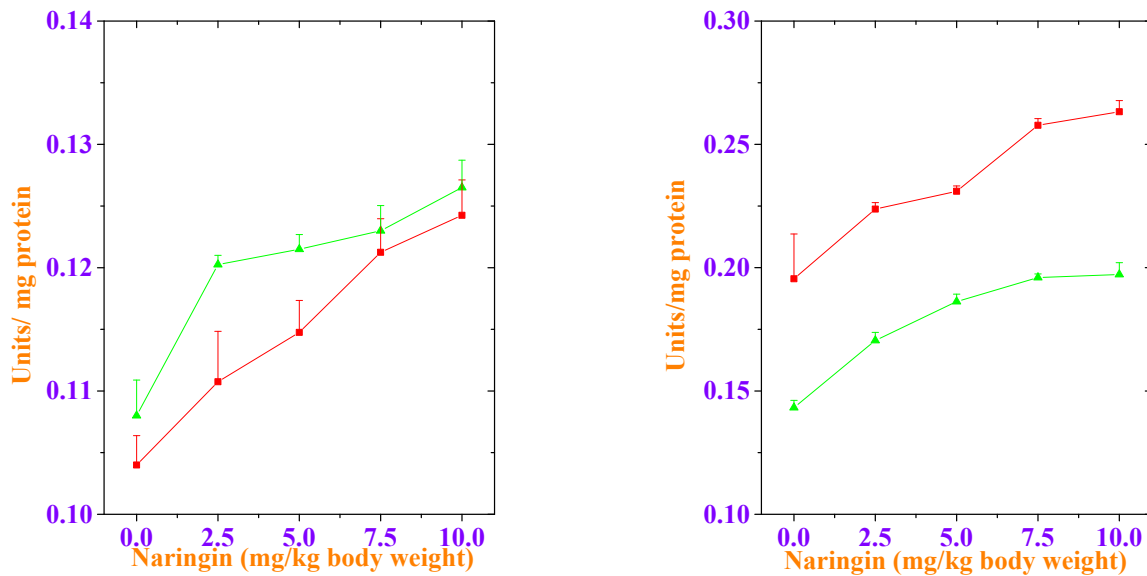
Figure 5. Alteration in the DOX-induced catalase activity by different doses of NIN in the heart (left) and liver (right) of mice. Squares: NIN group; triangles: NIN+DOX group. P < 0.05 for all points, when compared to control or DOX alone compared with NIN+DOX group.

(AST) were found to be 19.5 and 20.9 days, respectively (Table 2). Treatment of 24 h old tumors with 1.25 mg/kg DOX inhibited the weight gain in animals indicating arrest of tumor cell proliferation and growth (Table 2). The DOX treatment increased the MST and AST up to 28.5 and 28 days, respectively accompanied by an increase in the mean life span (IMLS) and the average life span (IALS) up to 46 % and 33 %, respectively. The treatment of 24 h old tumors with 10 mg/kg NIN marginally increased the MST (23 days) and AST (24 days) when compared with the control group (Table 2). However, when the animals were treated with 10 mg/kg of NIN and then with 1.25 mg/kg DOX a constant retardation in the weight gain was observed, indicating arrest of tumor cell multiplication and growth (Table 2). However, there was no significant alteration in the MST (27.5 days) and AST (27.6 days) when compared with the DOX treatment alone (Table 2). A similar effect was noticed for the increase in IMLS and IALS, that was 41% and 32%, respectively

Table 2. Effect of 10 mg/kg body weight naringin on median survival time (MST) and average survival time (AST) of EAC mice treated with DOX. Six animals were used in each group (n = 6).

Drug dose	MST	IMLS	AST	IALS
DDW	19.5		20.9	
NIN	23	17.9	24	14
DOX	28.5	46	28	33
NIN+DOX	27.6	41	27.6	32

(Table 2). A significant increase in survival was seen in the animals treated with DOX alone as well as with NIN and DOX, when compared with the DDW and NIN groups (Figure 8).

Discussion

The clinical use of anticancer anthracyclines is limited by the development of a distinctive and life-threatening form of cardiotoxicity. There are several hypotheses to explain DOX-induced cardiotoxicity. Among these, the hypothesis implicating the involvement of free radicals is the most widely accepted. DOX undergoes one-electron reduction through metabolic activation caused by NADPH-cytochrome-P-450 reductase, or other flavin-containing enzymes involving mitochondria (Bachur *et al.* 1978, Montaigne *et al.* 2012). This reduction generates a DOX semiquinone free radical, which reacts with the molecular oxygen, and reduces it to superoxide, with the regeneration of intact DOX. Superoxide is rapidly converted to hydrogen peroxide, either spontaneously or with the help of superoxide dismutase. The DOX semiquinone can react with hydrogen peroxide to yield a hydroxyl radical (Kalyanaraman *et al.* 1984). These highly toxic reactive oxygen species react with cellular molecules including nucleic acids, proteins and lipids and cause cell damage.
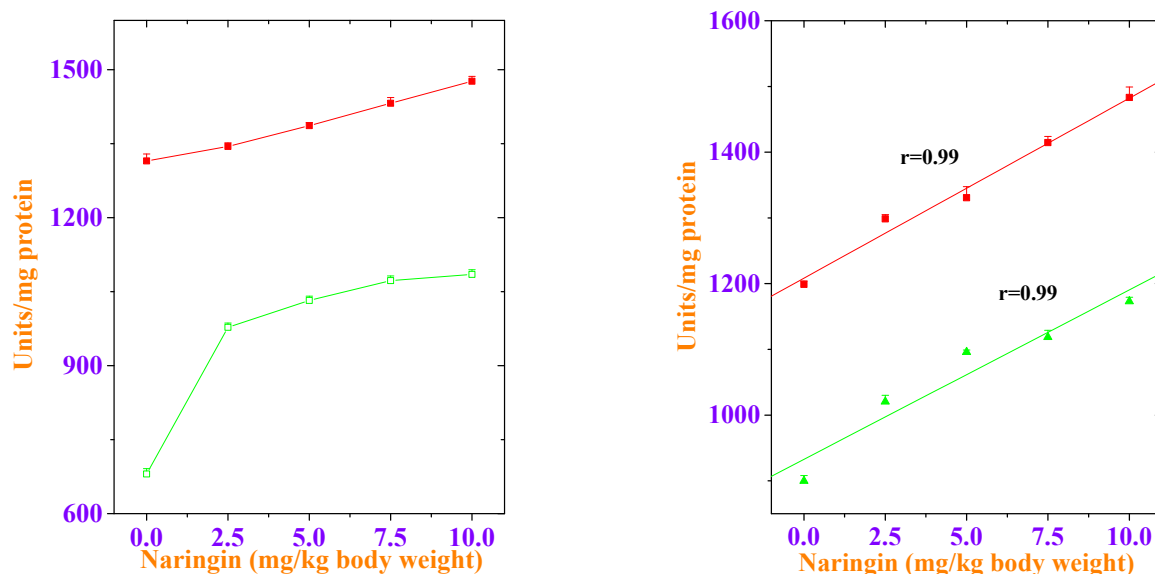
Anthracycline-induced cardiotoxicity has been

Figure 6. Alteration in the DOX-induced superoxide dismutase activity by different doses of NIN in the heart (left) and liver (right) of mice. Squares: NIN group; triangles: NIN+DOX group. P < 0.001 for all points, when compared to control or DOX alone compared with NIN+DOX group.

observed in 2 to 20% of patients at an early stage (Goebel & Kaplan 1992) and the reduction of DOX-induced cumulative cardiomyopathy represents a major goal in improving the clinical application of DOX. The iron chelator dexrazoxane (ICRF-187) has been shown to protect against doxorubicin induced cardiotoxicity; however, its clinical applications have been limited because of its hematological toxicity (Sparano 1998, Sepe *et al.* 2010). The administration of zinc or bismuth has also been reported to protect against DOX-induced myocardial toxicity by the induction of metallothionein (Satoh *et al.* 1998). The action of metallothionein seems to be based on their ability to scavenge hydroxyl radicals (Sato & Bremner, 1993). Angiotensin-converting enzyme inhibitors as well as 7-monohydroxyethylrutoside have been reported to protect against DOX-induced cardiotoxicity. The major drawback of 7-monohydroxyethylrutoside therapies has been the need of relatively high dose to obtain complete cardioprotection. Frederine has also been found to reduce the DOX-induced cardiotoxicity (Van Acker *et al.* 2001). It is necessary to screen for newer paradigms, which can reduce the toxicity of doxorubicin, without compromising its antineoplastic activity. Results of the present study demonstrate that NIN has been able to reduce the DOX-induced cardiac toxicity in mice, which was evident by a significant reduction in the serum levels of CK-MB, LDH, GPT and GOT. The elevation of serum CK has been reported to be a

reliable indicator of DOX-induced cardiotoxicity (Horacek *et al.* 2007). In line with this, narigin treatment has been previously reported to reduce DOX-induced cardiotoxicity (Arafa *et al.* 2005). Similarly curcumin and antarth, a poly herbal preparation, have been reported to reduce DOX-induced cardiotoxicity in vivo (Venkatesan 1998, Jagetia *et al.* 2005). Resveratrol (another polyphenol) has been reported to reduce the DOX-induced elevation in LDH and GOT in Wistar rats (Tatlidede *et al.* 2009) indicating alleviation of cardiotoxicity.

ROS can directly bind to DNA forming DNA adducts and alkali-labile sites. DNA strand breaks result following excision repair (Guirouilh-Barbat *et al.* 2008). It is therefore, conceivable that there is a close relationship between DNA adduct formation and DNA strand breaks. Under normal circumstances, DNA damage always takes place but is kept to a minimum by the cell's protective mechanisms that include a repertoire of antioxidants as well as efficient repair enzymes. However, under certain conditions, the fine balance between pro-oxidants and the protective mechanisms can be upset, resulting in oxidative stress. This can adversely affect the integrity of the cellular genome. Among the earliest cellular changes are the formation of DNA adducts, which are covalent binding products of oxidants with chemicals. These are formed when a DNA molecule is exposed to a pro-oxidant species. The DNA adducts could inhibit gene transcription on three different levels: a) by inhibiting
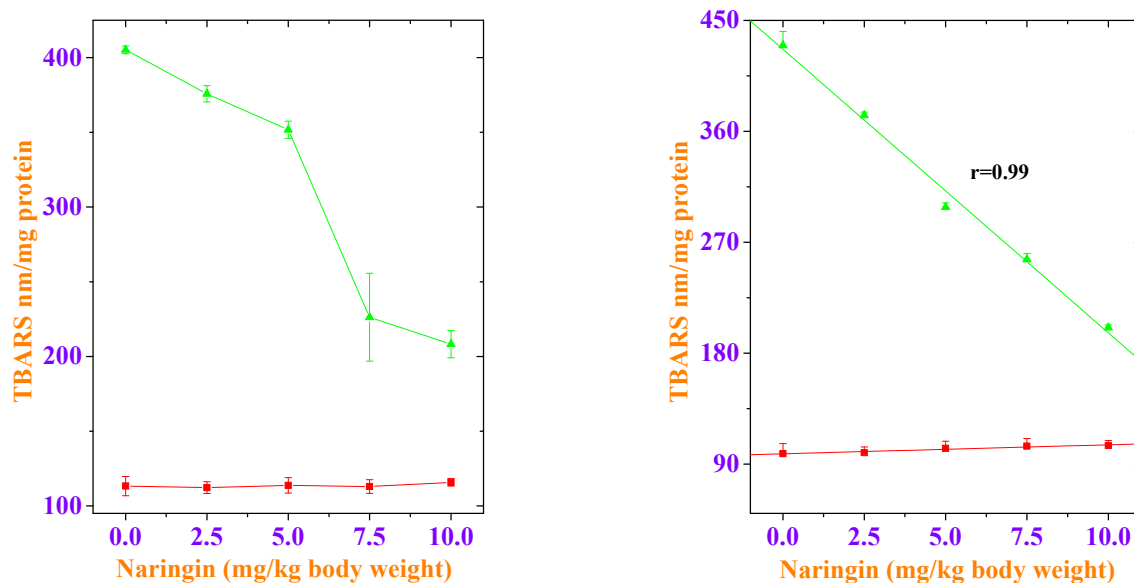
Figure 7. Alteration in the DOX-induced lipid peroxidation by different doses of NIN in the heart (left) and liver (right) of mice. Squares: Control group; triangles: NIN+DOX group. P < 0.001 for all points, when compared to control or DOX alone compared with NIN+DOX group.

DNA binding proteins through steric constraints, rendering DNA unrecognizable due to adduct-induced bending, or through subsequent mutation of the DNA bases required in the consensus sequence (Broggini & D'Incalci 1994, Pointon *et al.* 2010); b) by inhibiting the potential of the DNA to bend into the required conformation to bring distant regions together and initiate transcription (van der Vlet & Verrijzer 1993); and c) by posing a direct blockage to the path of RNA polymerase (Cullinane & Phillips, 1990, Cutts *et al.* 1994). Overall, the extent of inhibition of transcription facilitated by DNA adducts is potentially much greater. The possibility to determine specific DNA adducts quantitatively, and their comparison to other genotoxic parameters or biomarkers provides essential information on the mechanisms of potentially genotoxic agents. DNA adducts are of greater significance with respect to mutagenic or carcinogenic outcomes, and their role in mutagenesis is well documented (Vrtis *et al.* 2013).

Treatment of mice with various doses of DOX induced a significant and dose dependent increase in 8-OHdG in the heart and liver. A similar effect has been reported earlier, where doxorubicin treatment has been reported to induce 8-OHdG and mtDNA adducts in rats (Serrano 1999, Palmeira 1997, Zhou *et al.* 2001). Similarly, DOX treatment has been found to increase the DNA adducts in patients (Dorshow *et al.* 2001). A concentration dependent rise in DNA adduct formation has been observed in MCF-7 cells exposed to DOX

(Coldwel *et al.* 2008). Increased levels of 8-OHdG in the genomic DNA indicate DOX-induced oxidative damage. Pre-treatment of mice with various doses of NIN significantly reduced the levels of 8-OHdG in the heart and liver, when compared with the DOX group. Reports on the DOX-induced DNA adducts inhibition by NIN are unavailable. However, Resvreratrol has been reported to increase DNA adduct formation in DOX treated rats (Tatlidede *et al.* 2009). The alcohol free beer has been found to reduce DOX-induced DNA adducts (Valls-Belles *et al.* 2008).

PARP activation has been indicated in DOX-induced toxicity (Pacher *et al.* 2002). Over activation of PARP leads to tissue damage in various pathophysiological conditions associated with increased oxidative stress, including myocardial reperfusion injury as well as drug-induced cardiomyopathy (Pacher *et al.* 2002, Do & Chen 2013). DOX-treatment caused an overactivation of PARP in the mouse heart and liver, in the present study. A similar effect has been reported earlier in BALB/C mice treated with DOX (Patcher *et al.* 2002). Over activation of PARP is a hallmark of DOX-induced genomic damage. NIN significantly inhibited the DOX induced PARP synthesis in a dose dependent manner indicating that NIN protected the genome against DOX-induced insult and cardiotoxcity. PJ-34 has been reported to inhibit the activity of PARP induced by DOX. Pharmacological inhibition of PARP with PJ-34 has been reported to attenuate the DOX-
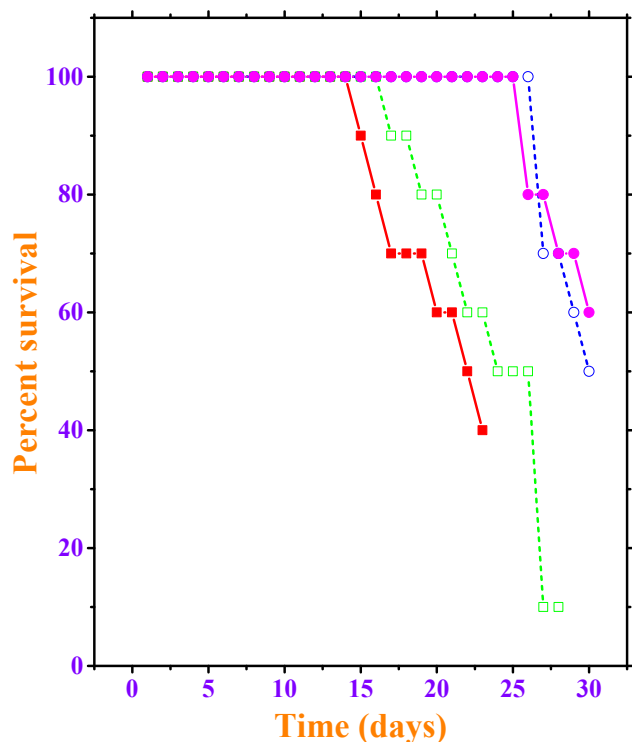
Figure 8. Kaplan Meirs's estimate of survival of Ehrlich ascites carcinoma bearing mice treated or not with naringin before doxorubicin treatment. Closed squares: SPS group; Open squares: NIN group; Closed circles: DOX group and Open circles: NIN+DOX group.

induced toxicity (Pacher *et al.* 2002, Szenczi *et al.* 2005, Magan *et al.* 2012). Similarly, AG014699 and tannic acid have been reported to inhibit PARP activity and reduce DOX-induced cardiotoxicity earlier (Ali *et al.* 2011, Tikoo *et al.* 2011).

It is clinically undesirable for the antitumor activity of DOX to be weakened by combining it with other pharmacological agents that protect against its cardiotoxicity. Therefore, we examined the influence of NIN on the antitumor activity of DOX in tumor bearing mice. The studies on EAC tumor bearing mice indicate that NIN treatment did not interfere with the antitumor activity of DOX as the MST and AST in the NIN and DOX-treated group remained unchanged when compared with the non-NIN treated controls. A similar effect was also discernible in the tumor bearing animals that received antarth before each DOX-treatment earlier (Jagetia *et al.* 2005). Our study demonstrates that use of NIN with DOX may be a good paradigm for tumor therapy, since NIN does not interfere with the antineoplastic activity of DOX and, at the same time, it also reduces cardiac and hepatic toxicities induced by DOX. Despite these encouraging results, it may be premature to look into the clinical implications of our findings since more detailed studies are required in different tumor models and the mecha-

nisms responsible for reduced DOX-induced cardio-toxicity by NIN need to be elucidated.

The exact mechanism of cardioprotection by NIN is not known; however, it may have used multiple mechanisms to exert its cardioprotective effects against DOX-induced caridotoxicity in the present investigation. DOX-induces free radical generation and elevates oxidative stress in the heart leading to cardiotoxicity (DeAtley *et al.* 1999, Gewirtz 1999). Therefore, inhibition of free radical generation by NIN in DOX-treated animals may be one important mechanism of its action. NIN has been reported to inhibit free radical generation *in vitro* (Jagetia *et al.* 2003). The increase in antioxidants by NIN in the DOX-treated group may be another mechanism by which NIN has reduced the DOX-induced cardiotoxicity. This contention is supported by the observation that NIN treatment before DOX-administration raised superoxide dismutase, catalase and GSH and reduced lipid peroxidation, which may be due to the upregulated gene expression of these enzymes. In line with this, an upregulation of mRNA expression of SOD, catalase and GSHPx has been reported by NIN-supplementation earlier (Jeon *et al.* 2001). The administration of NIN has reduced DOX-induced DNA adduct formation and inhibited the PARP activity, which may have also contributed to the alleviation of cardiotoxicity. NIN has been reported to chelate iron and this activity may have also been responsible for its effects in this study. Furthermore, DOX has been reported to increase the activation of NF-κB in cultured cardiomyocytes. Inhibition of NF-κB by NIN may have inhibited the inflammation and thereby reduced DOX-induced cardiotoxicity (Guo *et al.* 2013, Li *et al.* 2013).

Our study indicates that NIN alleviated cardiotoxicity in mice treated with DOX by inhibiting PARP activity and reducing the DNA adduct formation, scavenging of free radicals, increasing oxidant status and reduced lipid peroxidation. NIN may have also suppressed the NF-κB activation in the heart of mice receiving DOX.

Acknowledgements

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