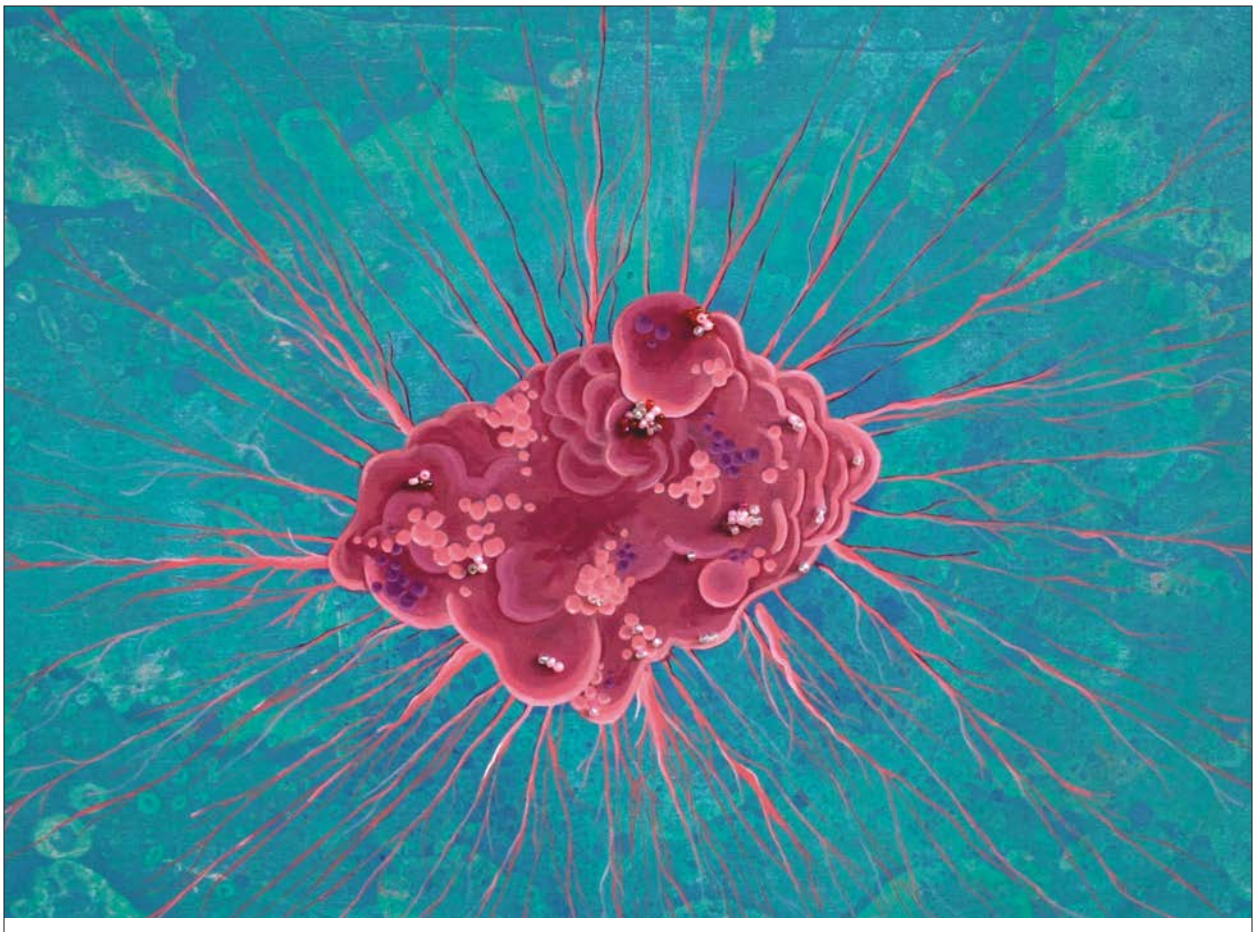

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Ethanolic Seed Extract of Grapefruit *(Citrus paradisi* Macfad) as an Effective Attenuator of Doxorubicin-Induced Oxidative Stress in the Rat Heart

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Abstract: In the present study, we examined the ameliorating effect of the 100% ethanol extract of *Citrus paradisi* (grapefruit) seed (GSE) on survival of doxorubicin treated rats and on DOX- induced cardiomyopathy. Whereas only 20% of the rats treated with DOX (20 mg kg^{-1} body weight intraperitoneally) survived at the end of 14 days, almost all the DOX-treatedrats survived when GSE (20 mg kg^{-1} body weight) was administered by gastric gavage. In the second experiment, GSE (20 mg kg^{-1} body weight) was administered daily by gavage for 14 consecutive days before a cumulative single dose of DOX (20 mg kg^{-1} body weight, intraperitoneally) was given. DOX induced marked biochemical alterations characteristic of cardiac toxicity. There was enhanced lipid peroxidation measured as malondialdehyde (MDA). The anthracycline antibiotic drug reduced the cardiac enzymatic activities of superoxide dismutase (SOD), glutathione S transferase (GST) and catalase (CAT). Besides, it reduced significantly the reduced glutathione (GSH) level; prior administration of grapefruit seed extract ahead of doxorubicin challenge ameliorated all these biochemical markers. Taken together, one could conclude that grapefruit seed extract has a protective role in the abatement of doxorubicin-induced cardiac toxicity that resides, at least in part, on its anti-radical effects.

Key words: Cardiotoxicity, doxorubicin, oxidative stress, grapefruit seed extract

INTRODUCTION

Doxorubicin (DOX) is an extremely effective antitumor anthracycline antibiotic (Armstrong and Lipsy, 1993). Unfortunately, DOX administration can induce a wide variety of acute cardiotoxic effects, including transient cardiac arrhythmia, nonspecific electrocardiographic abnormalities, pericarditis and acute heart failure (Billingham *et al*., 1978; Bristow *et al*., 1978). More frequently, DOX causes cardiomyopathy, which manifests as congestive heart failure months or even years after treatment. Several mechanisms have been postulated to account for the effects of DOX, both in terms of anticancer potential and cardiac toxicity. The proposed mechanisms are quite numerous, suggesting that DOX induced cardiotoxicity and drug action involve a cascade of multifactorial and complex processes. These include formation of free radicals, inhibition of enzymes and proteins, changes in cardiac muscle gene expression, alterations of mitochondrial membrane function, sensitization of Ca^{2+} release from sarcoplasmic reticulum channels, mitochondrial DNA damage and dysfunction (Olson and Mushlin, 1990) and induction of apoptosis (Arola *et al*., 2000).

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It is widely accepted that DOX induced cardiac myopathy resides for the most part on oxidative stress and the production of free radicals (Chularojmontri *et al*., 2005). DOX is known to generate free radicals either by the enzymatic pathway of redox cycling between a semiquinone form and a quinone form or by the non-enzymatic pathway of forming a DOX-Fe^{3+} complex (Davies and Doroshow, 1986). In both pathways, molecular oxygen is reduced to superoxide anion ($O^{\bullet}$), which is converted to other forms of reactive oxygen species such as hydrogen peroxide (H_2O_2) and the more toxic hydroxyl radical ($OH^{\bullet}$). These free radicals could then cause membrane and macromolecule damage, both of which lead to injury to the heart. The notion that the metabolic machinery in heart tissue is very active and the antioxidant resources such as superoxide dismutase (SOD) and catalase (CAT) are low in this organ compared with other organs in the body, made the heart quite vulnerable to free radical damage by doxorubicin (Doroshow *et al*., 1980; Quiles *et al*., 2002). Further, anthracyclines such as DOX appear to accumulate in the heart because of the high levels of cardiolipin.

Furthermore, studies have demonstrated that DOX-induced cardiotoxicity can be largely reduced by the over expression of the antioxidant enzymes MnSOD, metallothionein, or catalase (Kang *et al*., 1996, 1997). Indeed, a wealth of studies has emerged incorporating a plethora of antioxidants with doxorubicin in an attempt to prevent or attenuate its cardiac toxicity. These studies indicate that free radicals play a pivotal role in DOX-induced cardiotoxicity and that antioxidants may be used to minimize its side effects.

Numerous free radical scavengers, such as probucol, amifostine and dexrazoxane, have been shown to protect the heart against DOX-induced cardiotoxicity (Siveski-Iliskovic *et al*., 1994; Samelis *et al*., 1998; Nazeyrollas *et al*., 1999). Also, low-molecular-weight agents that scavenge reactive oxygen species such as melatonin, uric acid, lipoic acid, vitamin A, coenzyme Q10, selenium, vitamin C and vitamin E have also been addressed (Quiles *et al*., 2002; Conklin, 2005).

Unfortunately, most of these scavengers have pronounced clinical disadvantages. Probucol, a lipid-lowering antioxidant, confers significant protection against DOX-induced cardiotoxicity (Siveski-Iliskovic *et al*., 1995); however, concerns about its high-density lipoprotein-lowering property discourage its application in cancer patients. The cytoprotective drug amifostine is less potent than dexrazoxane (Zinecard), an iron chelator and it does not prevent the mortality and weight loss caused by DOX in spontaneously hypertensive rats (Herman *et al*., 1994). Finally, dexrazoxane, the only cardioprotective drug currently available clinically, only reduces 50% of DOX-related cardiac complications (Hasinoff, 1998). Moreover, it interferes with the antitumor activity of anthracycline antibiotics (Sehested *et al*., 1993) and also potentiates the hematotoxicity of DOX (Koning *et al*., 1991).

Grapefruit seed extract (GSE) from grapefruit (*Citrus paradisi*) contains high levels of vitamin C, vitamin E and bioflavonoids. These compounds are powerful antioxidants individually and collectively. Bioflavonoids form a class of benzo-gamma-pyrone derivatives that have high pharmacological potency. A great interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activity of these polyphenolic compounds (Diplok and Charleux, 1998). This is due primarily to their radical-scavenging and iron-chelating properties (Cook and Samman, 1996). They are found naturally in the leaves, bark, roots, flowers and seeds of plants (Cook and Samman, 1996).

One bioflavonoid with especially potent antioxidant capabilities is aringenin (Lee *et al*., 2002). It is the aglycone of the natural glycoside naringin present abundantly in grapefruit and which constitute its bitter principle. The antioxidant activities of bioflavonoids complement, extend and sometimes synergize the antioxidant activities of vitamin C, vitamin E and carotenoids, making them an important nutritional component in the body's defenses against free radical damage (Ho, 1994). Indeed GSE with its (pharmacological contents taken together) has since been touted as the most powerful natural antioxidant available (Sachs, 1997).

DOX-induced cardiotoxicity is principally mediated through oxidative pathway and that GSE possesses potential anti-radical effects. The aim of this study is therefore to evaluate the possible cardioprotective effects of this neutraceutical in male Sprague-Dawley rats challenged with a single cumulative dose of doxorubicin.

MATERIALS AND METHODS

Chemicals

Doxorubicin hydrochloride (®Adricin, Korea United Pharm. Inc., Chungnam, Korea) was obtained from Juli Pharmacy, Ikeja, Lagos State, Nigeria in the month of May, 2008.

Plant Material and its Extraction

In the third week of May, 2008, fresh parts of *Citrus paradisi* tree were collected from a cultivated farmland within the deciduous forest of Odorasanyi District, Ijebu-Igbo, Ogun State, Nigeria. The plant material was collected at this site because Adeneye (2008) had earlier collected plant material at same site which was identified and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher specimen with herbarium number FHI 107460 was deposited. Nigeria plant taxonomy was done by Mr. T.K. Odewo, Chief Superintendent Officer, Taxonomy Section, FRIN, Ibadan, Oyo State, Nigeria.

Fifteen grapefruits were cut into pieces and the seeds were separated out. These were thoroughly but gently rinsed in distilled water. The seeds were completely dried at room temperature for three weeks. The air dried seeds were reduced to a powdered substance by grinding. The sample was exhaustively extracted with 99.8% ethanol (NAAFCO scientific supplies Ltd., Lagos, Nigeria) by means of a Soxhlet apparatus and the extract was evaporated *in vacuo*. The residue was processed to give 10 g (7.5% yields) of dark yellowish solid crude mass, which was stored at 4°C o for the study. Fresh solution of the extract was prepared in peanut oil when required.

Animals and Interventions

Experimental procedures involving the animals and their care were conducted in conformity with international, national and institutional guidelines for the care of laboratory animals in Biomedical Research.

In this study, 50 healthy adult male Sprague-Dawley rats, weighing 200-250 g were used. They were obtained from Lagos State University College of Medicine (LASUCOM) Animal House, after approval was obtained from the ad-hoc Ethical Committee of the College. They were kept in the Animal House of Department of Anatomy, LASUCOM, Ikeja under standard laboratory conditions (12 h light and 12 h dark). They were allowed unrestricted access to water and food.

Four groups were used. The first group which served as the control was given peanut oil (vehicle) 0.5 mL daily by gastric gavage for 14 days. The second group had ethanol extract of GSE 20 mg kg^{-1} body weight (b. wt.)/day by gastric gavage for 14 days. The third group had intraperitoneal (i.p.) D0X 20 mg kg^{-1} b. wt. (single dose) following gastric gavage of GSE 20 mg kg^{-1} b. wt. daily for 14 days. The fourth and last group also had single dose i.p. DOX 20 mg kg^{-1} b. wt. but following administration of peanut oil 0.5 mL by gastric gavage daily for 14 days.

In the first experiment 20 animals were randomly but equally allocated and treated as stated above. They were observed for 14 days to evaluate the mean survival rates of the animals. The remaining 30 animals (6 for each group) were used for the second experiment which required their sacrifice.

Animal Sacrifice and Sample Collection

The rats were sacrificed on the second day after DOX administration. They were weighed and anaesthetized by placing them in a closed jar containing cotton wool sucked with chloroform

anaesthesia. The thoracic cavity was opened and the heart removed. The hearts were weighed with an electronic analytical and precision balance (BA 210S, d = 0.0001-Sartoriusen GA, Goettingen, Germany). The hearts volumes were measured by water displacement method.

Determination of Cardiac Anti-Oxidants

Heart homogenate (20% w/v) was prepared by sonication in ice-cold phosphate buffer (pH 8.0, 0.01 M). Aliquots were prepared and used for the assessment of different cardiac antioxidants; enzymatic and non-enzymatic.

Determination of Reduced Glutathione (GSH)

Reduced glutathione (GSH) was determined according to the method described earlier by Ellman (1959). The procedure is based on the reduction of Ellman's reagent by SH group to form 2-nitro-mercaptobenzoic acid, which has an intense yellow color which is measured spectrophotometrically at 412 nm using Shimadzu Spectrophotometer UV 1201 (Japan).

Assessment of Cardiac Anti-Oxidant Enzymes

Heart homogenate was centrifuged at 10000 rpm for 30 min at 4°C and the cytosolic fraction was used for the direct assay of the enzymatic activities of superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT). Cardiac activity of SOD was assessed according to the method of Marklund (1985). It simply resides on computing the difference between auto-oxidation of pyrogallol alone and in presence of the cytosolic fraction that contains the enzyme. Changes in the absorbance at 420 nm were recorded at 1 min interval for 5 min. Enzyme activity was expressed as U g^{-1} wet tissue. Heart GST activity was determined according to the method of Habig *et al*. (1974). In brief, the GST activity toward 1-chloro-2, 4-dinitrobenzene in presence of glutathione as a cosubstrate was measured spectrophotometrically at 25°C. The enzyme activity was determined by monitoring the changes in absorbance at 340 nm over 1 min intervals for 4 min. The enzymatic activity was expressed as nmol/min/g tissue. Catalase (CAT) activity was determined according to the method of Clairborne (1985). In short, the cytosolic fraction was added to a quartz cuvette containing 2.95 mL of 19 mmol L^{-1} H_2O_2 solution prepared in potassium phosphate buffer (0.1 M, pH 7.4). The change in absorbance was monitored every min at 240 nm over a 5 min period using a spectrophotometer (Shimadzu UV- 1201, Japan). Commercially available CAT was used as the standard. CAT activity was expressed as mmol/min/mg protein.

Determination of Lipid Peroxides

Malondialdehyde, a reactive aldehyde that is a measure of lipid peroxidation, was determined according to the method of Uchiyama and Mihara (1979). The adducts formed following the reaction of cardiac homogenate with thiobarbituric acid in boiling water bath, were extracted with n-butanol. The difference in optical density developed at two distinct wavelengths; 535 and 525 nm was a measure of the cardiac MDA content. Cardiac MDA content was expressed as nmol g^{-1} wet tissue.

RESULTS

General Observations and Survival Rate in Animals

The general appearances of rats from each group were observed after treatment. At the end of the experiment, the surviving rats in the DOX with vehicle and DOX with GSE groups had common symptoms, including weight loss, diarrhea and abdominal distension. However, these symptoms were more severe in the DOX alone group.

The survival rates of rats from each group are shown in Table 1. Whereas only 20% of the rats treated with DOX alone survived for 14 days, all animals thatreceived the vehicle alone and GSE alone survived for 14 days. Rats that had GSE prior to DOX challenge showed 80% survival rate.

Table 1: Animal survival rates

	Survival rate			
	7th day		14th day	
Groups	Number	Percent	Number	Percent
Control	5	100	5	100
GSE alone	5	100	5	100
GSE + DOX	4	80	4	80
Vehicle + DOX	2	40*	1	20*

(n = 5 rats), * Represents significant decrease at $p<0.05$ when compared to the control value

Body Weight Changes

Table 2 shows that rats that had the vehicle only and those that were given GSE alone showed less that significant gain in their weights. The animals that had DOX alone, however, exhibited a significant ($p<0.05$) loss in their weights. The rats that were pretreated with GSE had only a non significant ($p>0.05$) reduction in their body weights.

Weights and Volumes of Hearts

Table 2 also shows that the cardiac weights and volumes of the DOX alone rats were the least, being significantly lower ($p<0.05$) compared to the mean cardiac weights and volumes of the DOX rets that in addition cardiac had GSE which in turn lower but not significantly ($p>0.05$) lower than those of the control groups.

Cardiac Anti-Oxidants

The effects of DOX and/or GSE on the cardiac contents of GSH and MDA and enzyme activities of SOD, GST and CAT in male Sprague-Dawley rats are compiled in Table 3. Following treatment with GSE for 14 consecutive days, the cardiac GSH level was virtually the same as in the control group. A notable reduction ($p<0.05$) in GSH content was, however, observed after doxorubicin challenge. Administration of GSE before doxorubicin significantly elevated the cardiac content of GSH compared to animals that had the cytotoxic drug alone and returned it back to nearly its normal value.

Administration of GSE caused no significant ($p>0.05$) change in cardiac SOD activity, whereas, DOX provoked a statistically significant ($p<0.05$) decrease in SOD activity compared to control animals.

Pretreatment with GSE ahead of doxorubicin challenge significantly ($p<0.05$) increased the cardiac SOD activity compared to animals that received the anthracycline antibiotic alone. However, the enzyme activity was lower than normal value.

The GST activity following GSE administration was nearly the same as that of control animals. DOX, however, markedly decreased the enzyme activity compared to control value. Administration of GSE before DOX significantly increased the GST activity in cardiac tissue compared to animals treated with doxorubicin alone; a value that was significantly lower than control. The cardiac activity of CAT after GSE was almost the same as control value. DOX, however, resulted in reduction in cardiac CAT activity compared to control rats. Prior administration of GSE before DOX significantly increased the cardiac CAT activity by about 100% compared to doxorubicin- challenged animals. The CAT activity was approximately comparable to normal control value.

Cardiac Lipid Peroxidation

GSE had no effect on the cardiac content of lipid peroxides expressed as MDA when compared to control animals. Doxorubicin significantly elevated the cardiac MDA by about 5 folds compared to the control value (Table 3).

Prior administration of GSE exhibited a marked reduction in the cardiac MDA level compared to doxorubicin treated rats. However, the lipid peroxide level was still higher than control value (Table 3).

Table 2: The gross anatomical parameters result

Parameters	Control	GSE	GSE+DOX	Vehicle+DOX
Initial live body wt. (g)	250.30±15.25	255.20±20.20	255.25±30.25	260.20±20.35
Final live body wt. (g)	255.40±20.30	260.35±30.25	245.30±15.20	240.25±10.35
Body weight difference (g)	5.25(2%)	5.30(5%)	10.35(4%)	30.0(12%)*
Heart weight (g)	0.75±0.13	0.76±0.11	0.65±0.20	0.45±0.15*
Heart wt./body weight ratio	0.003	0.003	0.0036	0.002
Heart volume (mL)	0.83±0.15	0.85±0.13	0.60±0.14	0.50±0.11*

*Represents significant reduction at $p<0.05$ when compared to control value, %: Percentage change

Table 3: Cardiac contents of MDA, GSH and enzyme activities of cardiac SOD, GST and CAT

	Treatment regimen			
Parameters	Control	GSE	GSE+DOX	Vehicle+DOX
MDA (nmol g^{-1})	182.65±58.4	196.13±90.13	323.24±130.24	927.32±84.35*,+
GSH (μmol g^{-1})	0.64±0.04	0.62±0.23	0.64±0.15	0.38±0.32*
SOD (U g^{-1})	50.29±9 1.64	42.53±2.69	32.38±3.35	16.69±4.74*
GST (μmol/min/mg protein)	0.72±0.13	0.70±0.08	0.48±0.15	0.23±0.28*
CAT (mmol/min/mg protein)	13.78±2.43	15.20±3.52	14.34±7.60	6.70±2.84*

*,+ and * represents significant increase and reduction at $p<0.05$, respectively, when compared to control values

DISCUSSION

Cancer treatment is usually accompanied by diverse side effects to different body organs. The use of DOX, a potent anticancer agent with efficacy in a broad range of malignancies, is limited by severe cytotoxic side effects, the most important being cardiotoxicity (Arola *et al*., 2000). Oxidative stress is a major etiopathological factor in DOX induced cardiotoxicity. The molecular mechanism that has been suggested for the induction of DOX-induced cardiotoxic action is the production of free radicals and its related oxidant injury. DOX produces free radicals either through the enzymatic generation of semiquinone radical or by a non-enzymatic mechanism that includes a reaction with iron. In the enzymatic pathway DOX is converted into its toxic, short-lived metabolite semiquinone in cardiomyocyte by myocardial CYP450 and flavin monoxygenases, which interacts with molecular oxygen and initiates a cascade of reaction, producing reactive oxygen species. The latter reacts with hydrogen peroxide to produce hydroxyl ($OH^{\bullet}$) radical. Reactive oxygen species react with lipids, protein and other cellular constituents to cause damage to mitochondrial and cell membranes of cardiomyocytes (Wallace, 2003). Relatively low amounts of endogenous antioxidant make myocardium vulnerable to oxidative stress injury.

In the non-enzymatic mechanism, DOX will form a complex with iron that is able to reduce oxygen to different active oxygen species (Marklund, 1985). The role of free-radical formation and the consequent oxidative stress, in the induction of DOX induced cardiotoxicity is now widely accepted, as indicated by different studies that support this notion (Davies and Doroshow, 1986; Xu *et al*., 2001; Zhou *et al*., 2001; Tokarska-Schlattne *et al*., 2006).

The findings from the present study demonstrated that DOX inhibited growth of male Sprague Dawley rats. The gain in live body weight of the control rats could mean that the rats were still in the active growth phase. The deterioration in the anatomical parameters of live weights, heart weights and heart volumes of the experimental groups indicates that DOX has a negative effect on the body metabolic process (Lacy *et al*., 1993).

DOX-induced myocardial lesions have been well documented in patients as well as in experimental animals (Doroshow *et al*., 1980; Ytrehus and Hegstad, 1991).

The present study demonstrates several major findings regarding the effects of GSE on DOX-induced cardiotoxicity. First, we found that GSE can dramatically improve survival rates in rats treated with an acute high dose of DOX.

DOX administration induced oxidative stress in cardiac tissues as manifested by the alterations observed in cardiac antioxidant defense systems both enzymatic and non enzymatic. In this sense, the

DOX reduced significantly the cardiac GSH content, besides it notably lowered the cardiac enzymatic activities of SOD, GST and CAT. Further, DOX was associated with a marked increase in cardiac lipid peroxidation as manifested by increased TBARS (measured as MDA).

Though the exact mechanism(s) whereby DOX would induce cardiac toxicity is not fully explored, the principal mechanism could possibly be through free radical generation by the redox-cycling of the DOX molecule and/or by the formation of DOX- iron complexes (Hrdina *et al*., 2000). This concept of oxidative damage has been well documented in a plethora of earlier reports by Abd El-Aziz *et al*. (2001), Deepa and Varalakshmi (2003) and Buyukokuroglu *et al*. (2004). Pretreatment with GSE for 14 consecutive days significantly ameliorated all the biochemical parameters altered by DOX suggesting a profound anti-oxidant role for the GSE in this DOX -induced cardiomyopathy paradigm.

GSE contains vitamin C, vitamin E and bioflavonoids such as naringenin. These are potent antioxidants and free radical scavengers. In addition naringenin being a polyphenolic compound is also an effective iron chelator (Quiles *et al*., 2002). Acting individually and synergistically, these compounds in GSE provide the attenuation of DOX –induced cardiotoxicity demonstrated in this investigation.

In conclusion, prior administration of GSE ahead of doxorubicin challenge to male Sprague-Dawley rats reduced the DOX- induced mortality rate as well as abated all the biochemical parameters altered by the cytotoxic drug.

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