

Abatement by Naringenin of Doxorubicin-Induced Cardiac Toxicity in Rats

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ABSTRACT

Background: Doxorubicin is one of the most active cytotoxic agents in current use. It has proven efficacy in various malignancies either alone or combined with other cytotoxic agents. The clinical usefulness of the anthracycline drug has been precluded by cardiac toxicity. Many therapeutic interventions have been attempted to improve the therapeutic benefits of the drug. Few, however, have been efficacious in this setting.

Purpose: We have addressed in the current study the possible protective effects of naringenin, a flavonoid known to have anti-oxidant properties, on doxorubicin-induced cardiac toxicity in male Swiss albino rats.

Methods: Forty male Swiss albino rats were used in this study. Naringenin (25 mg/kg body weight) was administered daily by gavage for 7 consecutive days before a cumulative single dose of doxorubicin (15 mg/kg body weight, ip).

Results: Doxorubicin induced marked biochemical alterations characteristic of cardiac toxicity including, elevated activities of serum total lactate dehydrogenase (LDH) and creatine phosphokinase (CPK), enhanced lipid peroxidation measured as malondialdehyde (MDA). The anthracycline drug has also 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, but it increased the total NO content in heart tissue. Prior administration of naringenin ahead of doxorubicin challenge ameliorated all these biochemical markers.

Conclusions: Taken together, one could conclude that naringenin has a protective role in the abatement of doxorubicin-induced cardiac toxicity that resides, at least in part, on its anti-radical effects and regulatory role on NO production.

Key Words: *Cardiotoxicity - Doxorubicin - Oxidative stress - Naringenin.*

INTRODUCTION

Doxorubicin is an anthracycline antibiotic that has been used for a long time in therapy of an array of human malignancies either alone or combined with other cytotoxic agents [1]. The clinical usefulness of doxorubicin, however, has been hampered by its detrimental cardiac toxicity [2,3]. Several mechanisms have been postulated to account for the effects of doxorubicin, both in terms of anticancer potential and cardiac toxicity. These proposed mechanisms are quite numerous, suggesting that doxorubicin-induced cardiotoxicity and drug action involve a cascade of multifactorial and complex processes.

It is widely accepted that doxorubicin-induced cardiac myopathy resides for the most part on oxidative stress and the production of free radicals [3,4]. In this sense, doxorubicin has been reported to induce direct DNA damage [5], besides it stimulates lipid peroxidation [6]. Doxorubicin can generate free radicals through enzymatic (semiquinone free radicals) and non-enzymatic pathways [7]. The notion that the metabolic machinery in heart tissue is very active, and the antioxidant resources are low in this organ compared with other organs in the body, made the heart quite vulnerable to free radical damage by doxorubicin [1].

Indeed, a wealth of studies has been emerged incorporating a plethora of antioxidants with doxorubicin in an attempt to prevent or attenuate its cardiac toxicity. Amongst all the antioxidant strategies conducted, metal ion chelators like transferrins, metallothionein, desferrioxamine

and ceruloplasmin have been widely investigated. Also, low-molecular-mass agents that scavenge reactive oxygen species such as melatonin, uric acid, lipoic acid, vitamin A, coenzyme Q10, selenium, vitamin C and vitamin E have been also addressed [1,8].

Flavonoids 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 [9]. Due to their radical-scavenging and iron-chelating properties [1], flavonoids can be considered possible potential protectors against doxorubicin-induced cardiac toxicity.

Naringenin, the bitter principle of grapefruit (*Citrus paradisi*), has a multitude of pharmacological effects including, antithrombotic [10], anti-inflammatory [11], anti-estrogenic [12], as well as chemopreventive [13] actions. The concept that naringenin, like other flavonoids, possesses potential anti-radical effects [14,15], has prompted us to address in the current study the possible cardioprotective effects of this flavonoid in male Swiss albino rats challenged with a single cumulative dose of doxorubicin.

Serum total lactate dehydrogenase (LDH) and total creatine phosphokinase (CPK) activities, cardiac antioxidants; enzymatic activities of superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT), and non-enzymatic like reduced glutathione (GSH) content, as well as total cardiac levels of nitric oxide (NO) and lipid peroxides represented as malondialdehyde (MDA), were undertaken in the current study as markers of cardiac toxicity.

MATERIAL AND METHODS

Chemicals and Drugs:

Doxorubicin, supplied as ampoules (Adriablastina), was obtained from Farmitalia Carlo Erba (Italy). Naringenin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Reduced GSH, superoxide dismutase (SOD), glutathione-S-transferase (GST), 1-chloro-2,6-dinitrobenzene, 5,5-dithio-bis (2-nitrobenzoic acid), pyrogallol, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxy-propane were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the finest analytical grade.

Animals:

Male Swiss albino rats weighing 200-225 g were obtained from the Egyptian Organization for Biological Products and Vaccines (VACSERA, Giza, Egypt). The animals were housed in the animal Facility of the Faculty of Pharmacy, Al-Azhar University. The animals were fed a standard chow (El-Nasr Company, Abou-Zaabal, Cairo, Egypt) and tap water was supplied ad libitum. Animals were kept under controlled conditions of room temperature ($21 \pm 1^\circ\text{C}$), relative humidity ($55 \pm 5\%$) and a 12-h light/12-h dark cycle.

Design of Experiments:

A total of 40 rats were allocated to four groups, 10 animals in each. One group was administered naringenin dissolved in 30% propylene glycol w/w (25 mg/kg body weight, p.o.) for 7 consecutive days. A second group was given the same dose regimen of naringenin, followed by a single cumulative dose of doxorubicin (15 mg/kg body weight, i.p). A third group received the vehicle for 7 consecutive days and a single cumulative dose of doxorubicin (15 mg/kg body weight, i.p) thereafter. Finally, a fourth group was given 30% propylene glycol p.o. (vehicle) in a dosing volume of 5 ml/kg- and served as the control group. Thirty-six h after the last treatment, orbital blood samples were obtained under light ether anesthesia using heparinized microcapillaries. Serum was obtained by centrifugation at 3000 rpm for 10 min (Haereus Biofuge, Berlin, Germany) and was used later for the determination of serum total LDL and CPK activities. After terminal bleeding, animals were sacrificed by cervical dislocation. Hearts were rapidly dissected out, plotted dry, and kept frozen at -20°C till used.

Assessment of Serum Enzymes:

Serum total lactate dehydrogenase (LDH) and total creatine phosphokinase (CPK) activities were determined using commercial kits from Stanbio (TX, USA). Total LDH activity was assessed according to the method of Buhl and Jakson [16]. The method depends on the reaction of lactate with NAD, and NADH formed is measured at 340 nm using Shimadzu spectrophotometer UV 1201 (Japan). The increase in absorbance is measured at 1-min intervals for 3 min. Serum total LDH activity was calculated as units per liter (U/L).

Serum total creatine phosphokinase (CPK) activity was determined according to the method of Swanson and Wilkinson [17]. The method is based on the transphosphorylation of ADP to ATP through a series of coupled enzymatic reactions; NADH produced is directly proportional to the CPK activity. The increase in absorbance at 1-min intervals was recorded for 3 min at 340 nm.

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 anti-oxidants; enzymatic and non-enzymatic.

Determination of Reduced Glutathione (GSH):

Reduced glutathione (GSH) was determined according to the method described earlier by Ellman [18]. The procedure is based on the reduction of Ellman's reagent by SH group to form 2-nitro-5-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 [19]. It simply resides on computing the difference between autooxidation 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 wet tissue.

Heart GST activity was determined according to the method of Habig et al. [20]. In brief, the GST activity toward 1-chloro-2,4-dinitrobenzene in presence of glutathione as a co-substrate 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 [21]. In short, the cytosolic fraction (50 µl) was added to a quartz cuvette containing 2.95 ml of 19 mmol/l H₂O₂ 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. Total protein content in the cytosolic fraction was determined by the method earlier described by Lowry et al. [22].

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 [23]. 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 nm and 525 nm was a measure of the cardiac MDA content. Cardiac MDA content was expressed as nmol g⁻¹ wet tissue.

Assessment of Cardiac Total Nitric Oxide:

Cardiac total nitric oxide (NO) was determined using a commercial kit from R & D Systems (MN, USA) according to the method of Miles et al. [24]. This assay determines total NO based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess Reaction is based on the two-step diazotization reaction in which acidified NO₂ produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative, which absorbs visible light at 540 nm. Cardiac NO level was expressed as µmol/g wet tissue.

Statistical Methods:

The InStat version 2.0 (GraphPad, ISI Software, Philadelphia, PA, USA, 1993) computer program was used to conduct regression analysis and to compute statistical data. Data were expressed as means ± SD. Multiple comparisons were done using one-way ANOVA followed by

Bonferroni for selected pairs as a post-ANOVA test. The 0.05 level of probability was used as the criterion for significance.

RESULTS

Serum Enzymes:

Treatment with naringenin for 7 consecutive days had no significant effect on the serum activity of total LDH (Fig. 1). Doxorubicin, however, in a single cumulative dose significantly elevated the serum LDH activity by about 142% compared to normal serum value.

Prior administration of naringenin for 7 consecutive days ahead of doxorubicin dose markedly reduced the serum LDH activity by 46% compared to animals that received doxorubicin alone. Besides, the LDH value was not significantly different from that of control (Fig. 1).

Repeated dose administration of naringenin for a week did not affect the total CPK activity in the serum compared to control group (Fig. 2). Doxorubicin, however, markedly increased the CPK activity by about 226% compared to control serum value.

Pretreatment with the flavonoid for a week before doxorubicin administration notably reduced the activity of CPK by about 57% compared to doxorubicin-challenged group. The CPK activity was, however, not significantly different from control value (Fig. 2).

Cardiac Anti-Oxidants:

The effects of doxorubicin and/or naringenin on the cardiac contents of GSH and MDA and enzyme activities of SOD, GST and CAT in male Swiss albino rats are compiled in table (1).

Following treatment with naringenin for 7 consecutive days, the cardiac GSH level was virtually the same as in the control group. A notable reduction in GSH content amounted to about 44% was, however, observed after doxorubicin challenge.

Administration of naringenin before doxorubicin significantly elevated the cardiac content of GSH by about 42% compared to animals that had the cytotoxic drug alone and returned it back to nearly its normal value.

Administration of naringenin caused no significant change in cardiac SOD activity, whereas, doxorubicin provoked about 70% decrease in SOD activity compared to control animals.

Pretreatment with naringenin ahead of doxorubicin challenge increased the cardiac SOD activity by about 94% compared to animals that received the anthracycline alone. However, the enzyme activity was lower than normal value by approximately 42%.

The GST activity following naringenin administration was nearly the same as that of control animals. Doxorubicin, however, markedly decreased the enzyme activity by about 68% compared to control value.

Administration of naringenin before doxorubicin significantly increased the GST activity in cardiac tissue by about 96% compared to animals treated with doxorubicin alone; a value that was significantly lower than control one by about 37%.

The cardiac activity of CAT after naringenin was almost the same as control value. Doxorubicin, however, resulted in about 48% reduction in cardiac CAT activity compared to control rats.

Prior administration of naringenin before doxorubicin 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:

Naringenin had no effect on the cardiac content of lipid peroxides expressed as MDA compared to control animals. Doxorubicin significantly elevated the cardiac MDA by about 5-folds compared to the control value (Table 1).

Prior administration of naringenin exhibited a marked reduction in the cardiac MDA level amounted to 65% compared to doxorubicin-treated rats. However, the lipid peroxide level was still higher than control value by about 43% (Table 1).

Cardiac Total NO Level:

Administration of naringenin had no apparent change on the cardiac total NO content compared to normal level. Doxorubicin, however, significantly increased the heart total NO content by about 57% more than the control level (Fig. 3).

Prior treatment with naringenin for 7 consecutive days before doxorubicin challenge significantly reduced the heart total NO content by about 37% compared to rats that received the cytotoxic drug alone. The NO level was still higher than normal level by about 32% (Fig. 3).

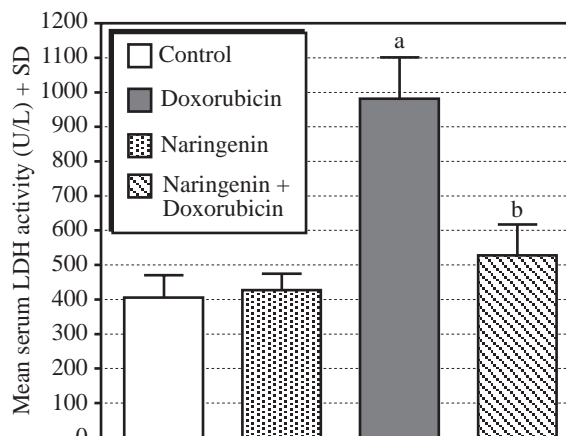


Fig. (1): Effects of doxorubicin and/or naringenin on the serum activity of total LDH in male Swiss albino rats.

a: Significantly different from control animals.
b: Significantly different from doxorubicin-treated animals.

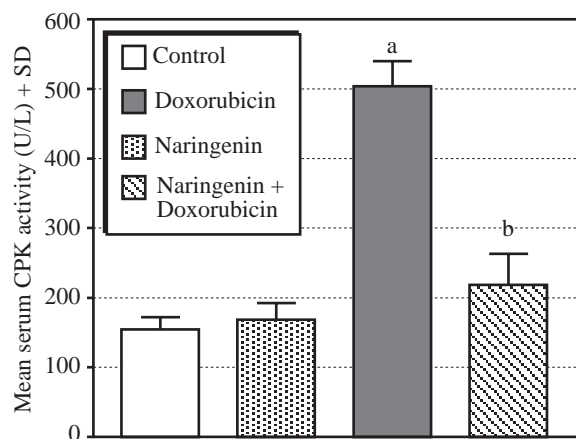


Fig. (2): Effects of doxorubicin and/or naringenin on the serum activity of total CPK in male Swiss albino rats.

a: Significantly different from control animals.
b: Significantly different from doxorubicin-treated animals.

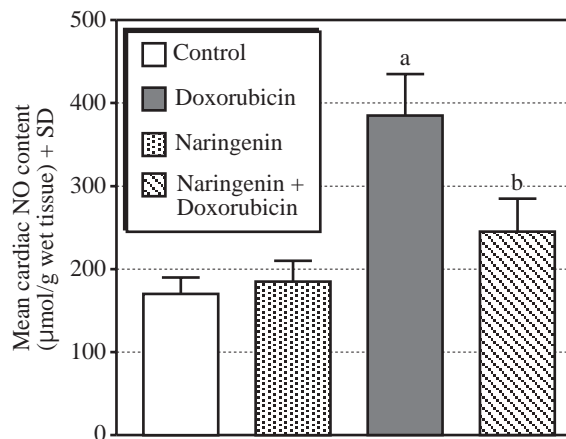


Fig. (3): Effects of Doxorubicin and/or naringenin on the cardiac NO content.

a: Significantly different from control animals.
b: Significantly different from doxorubicin-treated animals.

Table (1): Effects of doxorubicin and/or naringenin on the cardiac contents of GSH, MDA and enzyme activities of cardiac SOD, GST and CAT in male Swiss albino rats.

Parameter	Treatment regimen			
	Control ¹	Naringenin ²	Doxorubicin ³	Naringenin + Doxorubicin ⁴
GSH (µmol/g)	0.64±0.03	0.58±0.19	0.36 ^a ±0.28	0.62 ^b ±0.13
MDA (nmol/g)	184.76±73.6	296.64 ^a ±84.12	925.35 ^a ±81.64	323.33 ^{a,b} ±171.24
SOD (U/g)	55.29±91.64	32.53 ^a ±2.69	16.67 ^a ±4.74	32.28 ^{a,b} ±3.35
GST (µmol/min/mg protein)	0.71±0.13	0.68±0.06	0.23 ^a ±0.28	0.45 ^{a,b} ±0.13
CAT (mmol/min/mg protein)	12.87±2.53	14.20±3.48	6.70 ^a ±2.84	13.37 ^b ±6.38

Data are presented as Mean ± SD, n = 10

¹Animals received propylene glycol 30% w/v by gavage in a dosing volume of 5ml/kg and served as control.

²Animals received daily dose of naringenin (25mg/kg bd wt, po) by gavage for 7 consecutive days.

³Animals received a single cumulative dose of doxorubicin (15mg/kg bd wt, ip).

⁴Animals received a single daily dose of naringenin as before for 7 consecutive days followed by a single cumulative dose of doxorubicin as before.

Multiple comparisons were done using one-way ANOVA followed by Bonferroni test for selected pairs

^a: Significantly different from control.

^b: Significantly different from doxorubicin.

DISCUSSION

Doxorubicin is one of the most active cytotoxic agents in current use. It has proven efficacy in a variety of human malignancies [25]. The clinical efficacy of the anthracycline, however, has been precluded by acute and chronic cardiac toxicity [26]. Free radical generation and lipid peroxidation have been suggested to be responsible for doxorubicin-induced cardiac toxicity [3,27]. These oxygen-derived radicals cause severe damage of plasma membranes and interfere with cytoskeletal assembly [28]. Tissues with less developed antioxidant defense reserve such as the heart are highly susceptible to injury by anthracycline-induced oxygen radicals [29].

Amongst all the therapeutic modalities adopted to attenuate doxorubicin cardiac myopathy come the most promising results from combining the drug with a myriad of antioxidants in an attempt to abate oxidative damage in heart tissue and hence to abrogate the cardiac injury.

Flavonoids constitute a family of compounds that possess radical-scavenging and iron-chelating properties. Therefore, these compounds can be considered as possible potential protectors against doxorubicin cardiotoxicity.

The notion that naringenin, the aglycone of the natural glycoside naringin present abundantly in grapefruit, has shown previous anti-radical effects [14,15], has warranted the attention to address whether or not this flavonoid would ameliorate doxorubicin-induced cardiac toxicity following challenging rats with a cumulative dose of doxorubicin in the current study.

Doxorubicin challenge markedly increased the activities of serum LDH and CPK. Actually, these enzymes are considered important markers of early and late cardiac injury especially during clinical follow-up of doxorubicin therapy [25]. Many previous studies have demonstrated similar elevations in cardiac enzymes activities in rats following challenge with a single cumulative dose of doxorubicin (15-20 mg/kg) [2,30,31].

In this study prior administration of naringenin for 7 consecutive days ahead of doxorubicin notably reduced the activities of the cardiac enzymes in the serum of male Swiss albino rats and brought them back to the normal levels.

Doxorubicin 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 anthracycline drug reduced significantly the cardiac GSH content, besides it notably lowered the cardiac enzymatic activities of SOD, GST and CAT associated with a marked increase in cardiac lipid peroxidation as manifested by increased MDA level.

Though the exact mechanism (s) whereby doxorubicin would induce cardiac toxicity is not fully explored, the principal mechanism could possibly be through free radical generation by the "redox-cycling" of the anthracycline molecule and/or by the formation of anthracycline-iron complexes [32]. This concept of oxidative damage has been well documented in a plethora of previous reports [33-37]. Pretreatment with naringenin for 7 consecutive days significantly ameliorated all the biochemical parameters altered by doxorubicin suggesting a profound anti-oxidant role for the flavonoid in this doxorubicin-induced cardiomyopathy paradigm.

Being polyphenolic compounds, flavonoids have shown anti-radical and iron-chelating properties in experimental animals [1], and thus would be possible protectors against anthracyclines-induced cardiomyopathy. In this sense, Van Acker et al. [38,39] have demonstrated that 7-monohydroxyflavone (monoHER), a semi-synthetic flavonoid, provides a dose-dependent protection against doxorubicin-induced cardiotoxicity in mice.

Also, Kozluca et al. [40] described the catechin activity to prevent doxorubicin-induced free radical formation in rat cardiotoxicity models. The authors found that 20 mg/kg catechin reduced general and cardiac toxicity. The cardioprotective effects of the flavonoid have been attributed to its anti-oxidant and iron chelating properties.

Clinically, Sadzuka et al. [41] concluded in their study with g-rutin and luteolin flavonoids that the intake of food rich in flavonoids can ameliorate the side effects of doxorubicin, thus avoiding the necessity of taking other medications and improving the "quality of life".

Only four out of twenty synthetic flavonoids tested did protect against the negative inotropic effects caused by doxorubicin [42]. By the same token, total flavonoids from *Fructus Choerospondiatis* protected rat heart muscle both in vitro and in vivo against the toxic effects of doxorubicin as reflected from the improvement in the biochemical defense mechanisms as well as lipid peroxidation [43].

Recently, the flavonol quercetin and flavonolignan could protect heart microsomes and mitochondria against the iron-dependent doxorubicin-mediated lipid peroxidation [44]. Furthermore, Abou-El-Hassan et al. [45,46] reported that the flavonoid 7- monohydroxyflrutoside (monoHER) was more powerful than catalase and SOD gene therapy as cardioprotector in doxorubicin-induced cardiac damage in neonatal rat cardiac myocytes.

Actually the anti-oxidant effects of naringenin observed in the current study would account for its cardioprotective potential. Previous reports have demonstrated anti-radical and metal chelating effects of the flavonoid in different oxidant damage states [47-49].

Galvez et al. [50] have earlier demonstrated anti-peroxidative activities of naringenin against chemically-induced lipid peroxidation in rat liver. Also, naringenin was found to protect against hepatocytic autophagy and endocytosis induced by okadaic acid [51].

Mira et al. [52] investigated an array of flavonoids including naringenin, and found varying metal chelating properties towards iron and copper and suggested a role for these compounds in metal overload diseases and in oxidative stress conditions that involve transition metals.

Naringenin exhibited an inhibitory effect on MDA production from ethyl arachidonate [53], as well as a scavenging property of hydroxyl free radicals in kidney tissue [54] suggesting an anti-oxidant role of the flavonoid.

Later, Heo et al. [55] have reported that amyloid beta protein-induced free radical mediated neurotoxicity in ICR mice was inhibited by naringenin, and the neuroprotective effect was related to its antioxidant property.

Our findings showed that doxorubicin challenge significantly increased the cardiac level

of NO. Similar results have been previously reported demonstrating an apparent increase in cardiac NO levels following the administration of the anthracycline drug [56-59].

Nitric oxide (NO) is a key molecule involved in the pathophysiology of heart; dysregulation of activity of NO synthase (NOs) and of NO metabolism seems to be a common feature in various cardiac diseases. The contribution of NO to anthracycline cardiac damage is suggested by evidence demonstrating anthracycline-mediated induction of NOs expression and NO release in heart and the ability of NOs to promote anthracycline redox cycling to produce reactive oxygen species [60]. Though the oxidative damage and iron production are amongst the most popular candidate mechanisms for doxorubicin-induced cardiac toxicity, there is a possible role for NO in this cardiac myopathy [56].

Naringenin significantly reduced the cardiac total NO when administered for 7 consecutive days prior to doxorubicin challenge. Many flavonoids have been reported to diminish NO production following oxidant damage in different pathologic conditions such as infection and inflammation via regulating the induction of inducible nitric oxide synthase (NOs) [61].

Abd El-Gawad and Khalifa [62] have indicated that quercetin reduced NO generation in LPS-induced shock in rat brain. Similarly, pretreatment with the flavonoids kaempferole and apigenin dose-dependently inhibited nitric oxide synthase in murine macrophage cells [63]. Choi et al. [64] have demonstrated that NO production is responsible for the neurodegenerative damage of MPTP in mouse brain, and the prior administration of the green tea flavonoid, epigallocatechin-3-gallate had a neuroprotective effect that was mediated via inhibition of nitric oxide synthase.

In conclusion, prior administration of naringenin ahead of doxorubicin challenge to male Swiss albino rats ameliorated all the biochemical parameters altered by the cytotoxic agent. Apart from the regulatory role of naringenin on cardiac NO production observed in the current work, the cardioprotective effects of the flavonoid could possibly reside for the most part on its anti-radical effects. Thus, naringenin could possibly improve the therapeutic benefits of doxorubicin.

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