IJPT
1735-2657/09/82-61-66
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 8: 61-66, 2009

RIDR

RESEARCH ARTICLE

Protective Effect of *Ephedra nebrodensis* on Doxorubicin-Induced Cardiotoxicity in Rats

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Received July 9, 2008; Revised December 15, 2008; Accepted February 21, 2009

This paper is available online at *http://ijpt.iums.ac.ir*

ABSTRACT

Doxorubicin (DOX) is an anthracycline antibiotic with broad spectrum anti-tumour activity. Its effectiveness has been limited by the occurrence of dose-related myocardial and bone marrow toxicity. As oxidative stress is the main factor in DOX-induced cardiotoxicity, we presumed that agents which enhance endogenous antioxidants can prevent DOX induced cardiotoxicity. Animals received either DOX (3 mg/kg, i.p.) every other day or combination of *Ephedra nebrodensis* (100 mg/kg and 200 mg/kg, p.o.) and DOX or *Ephedra nebrodensis* (200 mg/kg, p.o.) extract alone for 2 weeks. Vitamin E (25 mg/kg, p.o.) was used as a positive standard. Cardiotoxicity was assessed by recording changes in ECG (increased QT interval), measuring the levels of cardiac marker enzymes such as lactic acid dehydrogenase (LDH), creatine phosphokinase (CPK), glutamic oxaloacetic transaminase (GOT), and the antioxidant defence enzyme such as reduced glutathione (GSH), superoxide dismutase (SOD), and lipid peroxidative value (LPO) at the end of treatment schedule. Changes in heart rate were also measured in all groups. Treatment with *Ephedra nebrodensis* (100 mg/kg and 200 mg/kg) significantly (p<0.05) decreased the levels of LPO and cardiac marker enzymes, increased the levels of other antioxidant defence enzymes, GSH and SOD, reversed the changes in ECG and prevented the decrease in heart weight in DOX-treated group. The results suggest that *Ephedra nebrodensis* has the potential in preventing the cardiotoxic effects induced by Doxorubicin.

Keywords: *Doxorubicin, Ephedra nebrodensis, Cardiac marker enzymes, Antioxidant enzymes, ECG*

Doxorubicin is an anthracyclin glycoside antibiotic that possesses a potent and broad spectrum antitumour activity against a variety of human solid tumours and haematological malignancies [1, 2]. Cardiotoxicity induced by DOX is usually mediated through lipid peroxidation and inhibition of long fatty acid oxidation in cardiac tissues [3-6]. DOX causes numerous changes in different metabolic reactions within cardiac cells with major adverse undesirable effects that involve cardiotoxicity and bone marrow suppression [7, 8]. Thus, oxidative stress, lipid peroxidation, and mitochondrial dysfunction have been associated with DOX induced cardiomyopathy [5].

Ephedra nebrodensis (Tineo.) (*Ephedraceae*) contain various medicinally-active alkaloids (notably ephedrine) and they are widely used in preparations for the treatment of asthma and catarrh [9]. This species is the richest source of ephedrine in India; the stems contain over 2.5% total alkaloids, of which about 75% is ephedrine [10]. The plant also has antiviral effects, particularly against influenza. They are used internally in the treatment of asthma, hay fever and allergic complaints [9]. The stems have diaphoretic, diuretic, febrifuge, hypertensive, nervine, tonic, vasoconstrictor and vasodilator properties [11].

The free radical scavenging effects of the *Ephedra nebrodensis* on DPPH (2, 2-diphenyl-1-picrylhydrazyl) was measured *in-vitro*. In view of this, since DOX-induced cardiotoxicity is linked to oxidative stress, we have investigated the possible protective effect of *Ephedra nebrodensis,* against DOX-induced cardiotoxicity in rats.

MATERIALS AND METHODS

Plant material

Aerial parts of *Ephedra nebrodensis* T. (*Ephedraceae*) were collected from Arzana Province, Sardenia, Italy and was identified and authenticated by Prof. Mauro Ballero of Department of Botonical Sciences, University of Cagliaria, Italy. The voucher specimen was deposited at the departmental herbarium.

Extract Preparation

The aerial parts of the plant were sun-dried and blended to coarse particles. About 0.5 kg of the blended aerial parts of the plant was extracted with 1:1 of acetone- ethanol for 72 h. The extract on removal of solvent (17.2 g) was allowed to cool. Appropriate concentration of the extracts was made in 0.2% acacia in distilled water. The phytoconstituents present in the crude extract are flavonoids and alkaloids.

Animals

The study was approved by the Institutional Animal Ethics Committee. Laboratory breed Wistar albino rats of either sex weighing between 150-200 g, maintained under standard laboratory conditions of 25 ± 1°C, and photo period (12 hr dark/12 hr light) were used for the experiment. Commercial pellet diet (Amrut laboratory rat and mice feed, Sangli, India) and water were provided *ad libitum*.

Chemicals

Doxorubicin (Oncodria, Sun Pharmaceutical Ind. Ltd. Gujarat, India) and Vitamin E capsule (Evinal, Alembic Ltd, Gujarat) was purchased from local market. All chemicals for sensitive biochemical assays were obtained from Sigma Chemicals Co. India, and Hi media Chemicals, Mumbai, India. Distilled water was used for biochemical assays. LDH and SGOT kits were obtained from Agappe Diagnostics Pvt. Ltd., India. and Span diagnostic Ltd., India respectively.

Experimental protocol

During the acclimatization period, the baseline ECG was recorded (Chart 5.0 AD Instrument, Austria). The animals were then randomly divided into the following experimental groups with 5 animals in each group.

Group 1: Vehicle-treated group,
Group 2: DOX (3mg/kg, i.p.) every other day for two weeks,
Group 3: Ephedra nebrodensis (100 mg/kg, p.o.) daily + DOX (3 mg/kg, i.p.) every other day for two weeks,
Group 4: Ephedra nebrodensis (200 mg/kg, p.o.) daily + DOX (3 mg/kg, i.p.) every other day for two weeks,
Group 5: Ephedra nebrodensis (200mg/kg, p.o.) daily for two weeks,
Group 6: Vitamin E (25 mg/kg, p.o.) daily + DOX (3 mg/kg, i.p.) every other day for two weeks.

At the end of two weeks, the animals were anaesthetized with diethyl ether, ECG recorded, and then sacrificed by a high dose of diethyl ether. Blood was withdrawn immediately for enzyme assays and the heart was dissected out and weighed. Heart tissue was washed with ice-cold 0.9% saline and homogenized quickly with ice cold 0.1 M Tris HCl buffer (pH-7.5) using Remi homogenizer to give a 10% homogenate.

Electrocardiography (ECG)

ECG was recorded before and after the treatment schedule. For ECG recording (Chart 5.0, ADI Instruments) rats underwent light ether anesthesia. Needle electrodes were inserted under the skin. For each ECG tracing, QRS complex, QT interval and ST interval were measured [12].

Preparation of Serum and tissue homogenate

Blood was collected and allowed to clot. Serum was separated by centrifugation of the clotted blood at 5000 rpm for 4 min and used for estimation of LDH, CPK and SGOT. Known amount of tissue was weighed and homogenized in ice cold 0.1 M Tris HCl buffer for estimation of lipid peroxidative indices and enzymatic and non-enzymatic antioxidants.

Antioxidant Parameters

Estimation of lipid peroxidative indices

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substance (TBARS) was measured by the method of Niehaus and Samuelsson [13] and Jiang *et al.* [14]. In brief, 0.1 ml of homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCL reagent (Thiobarbituric acid 0.37%, 0.25N HCl and 15% TCA) and placed in water bath for 15 min, cooled and centrifuged at room temperature for 10 min at 1000 rpm. The absorbance of clear supernatant was measured against reference blank at 535 nm.

Determination of non-enzymic antioxidant status

Estimation of Reduced glutathione (GSH)

Reduced glutathione was determined by the method of Ellman [15]. About 1.0 ml of homogenate was added to 1ml of 10% TCA and centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 1.0% sodium citrate) and 3 ml of phosphate buffer (pH 8.0). The color developed was measured at 412 nm.

Determination of enzymic antioxidant status

Superoxide dismutase activity (SOD)

The assay of SOD was based on the ability of SOD to inhibit spontaneous oxidation of adrenaline to adrenochrome [16, 17]. The supernatant (0.05 ml) was added to 2.0 ml of carbonate buffer and 0.5 ml of 0.01mM EDTA solution. The reaction was initiated by addition of 0.5ml of epinephrine and the auto-oxidation of adrenaline ($3x10^{-4}$ M) to adrenochrome at pH 10.2 was measured by following change in OD at 480 nm. The change in optical density every minute was measured at 480 nm against reagent blank. The results are expressed as units of SOD activity (mg/wet tissue). One unit of SOD activity induced approximately 50% inhibition of adrenaline.

Table 1. Effect of *E. nebrodensis* on ECG and heart weight at the end of two weeks

Treatment	QT interval (ms)	QRS complex (ms)	ST interval (ms)	Heart weight (mg)
Vehicle	28.5 ± 1.3	16 ± 0.6	11.5 ± 0.6	914 ± 34.58
OX	57.5 ±0.8$^{\#}$	23 ± 0.5$^{\#}$	35 ± 1.6$^{\#}$	566 ± 8.12$^{\#}$
E.N (100) + DOX (3)	43.5 ± 2.4$^{*\#}$	19 ± 1.0$^{*\#}$	24.5 ± 2.2$^{*\#}$	720 ± 13.78$^{*\#}$
E.N (200) + DOX (3)	41.5±1.5$^{*\#}$	16.5±0.6*	22.5±2.0$^{*\#}$	684 ± 23.15$^{*\#}$
E.N (200)	30.8±0.8*	15.8±0.8	14.2±0.8*	733.3 ± 33.33$^{*\#}$
Vit. E (25)	45 ± 1.8$^{*\#}$	16.3 ± 1.2*	28.8 ± 1.6$^{*\#}$	654 ± 16$^{*\#}$
F (5,24)	46.83	11.75	31.24	23.89

Values are mean±SEM; n=5 in each group; ANOVA followed by Dunnetts test; #P<0.05 when compared to vehicle,*P<0.05 when compared to DOX, DOX: Doxorubicin was given in a dose of 3 mg/kg, i.p. on alternate days for two weeks. E.N: *Ephedra nebrodensis* was given at a dose of 100 and 200 mg/kg, p.o. for two weeks. Vit. E: Vitamin E was administered 25 mg/kg, p.o. for two weeks.

Table 2. Effect of *E. nebrodensis* on LDH, SGOT and CPK at the end of two weeks

Treatment	LDH	CPK	SGOT
Vehicle	990 ± 30.84	36.04 ± 5.52	50.4± 10.87
DOX	3663 ± 12.1$^{\#}$	101.8 ± 13.48$^{\#}$	195.2 ± 12.37$^{\#}$
E.N (100) + DOX (3)	1814 ± 151.8$^{*\#}$	33.55 ± 1.91*	150.4 ± 0.46$^{*\#}$
E.N (200) + DOX (3)	1466 ± 63.78*	24.22 ± 0.99*	64.4±1.6*
E.N (200)	1237± 23*	47.19 ± 3.82*	49.33±3.71*
Vit. E (25)	3609 ± 87.78$^{\#}$	69.2 ± 1.83$^{*\#}$	53.6 ± 4.87*
F (5,24)	236.27	21.30	76.40

Values are in mean ±SEM; n=5 in each group; ANOVA followed by Dunnetts test; #P<0.05 when compared to vehicle, *P<0.05 when compared to DOX, DOX: Doxorubicin was given in a dose of 3 mg/kg, i.p. on alternate days for two weeks. E.N: *Ephedra nebrodensis* was given at a dose of 100 and 200 mg/kg, p.o. for two weeks. Vit. E: Vitamin E was administered 25 mg/kg, p.o. for two weeks.

Cardiac marker enzymes

Blood samples were collected from each animal and the serum obtained by centrifugation was used for determination of LDH (LDH kit-Agappe Diagnostics Pvt. Ltd., India), SGOT (SGOT kit-Span diagnostic Ltd., India) and CPK [18].

Statistical analysis

All data were expressed as the mean ± SEM. For statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) followed by Dunnett's test. The p values <0.05 was considered significant.

RESULTS

ECG and heart weight

Animals treated with DOX (3 mg/kg, alternate days for 2 weeks) showed a significant (p<0.05) prolongation of QT interval, ST interval, widening of QRS complex as compared to vehicle-treated animals. *E. nebrodensis* extract (100 and 200 mg/kg) significantly (p<0.05) decreased the prolongation of QT and ST intervals in animals treated with DOX as compared to DOX-treated group (Table 1). Treatment with *E. nebrodensis* extract (200 mg/kg) alone significantly (p<0.05) decreased the prolongation of QT and ST interval as compared to vehicle- and DOX-treated groups. There was a significant decrease in heart weight in DOX-treated animals which was prevented by *E. nebrodensis* extract (100 and 200 mg/kg) treatment for two weeks.

Cardiac marker enzymes

Chronic administration of DOX (3 mg/kg, alternate days for 2 weeks) to rats significantly increased the levels of cardiac injury markers i.e. serum LDH, CPK and SGOT levels. Treatment with *E. nebrodensis* extract (100 and 200 mg/kg) significantly (p<0.05) decreased the levels of LDH, CPK, and SGOT in DOX-treated animals as compared to DOX group (Table 2). In animals treated with *E. nebrodensis* extract (200 mg/kg) alone, the cardiac marker enzymes significantly decreased as compared with DOX-treated group.

Antioxidant enzymes

Chronic administration of DOX (3 mg/kg, alternate days for 2 weeks) to rats significantly (p<0.05) increased the levels of LPO and significantly (p<0.05) decreased the levels of GSH and SOD. Treatment with *E. nebrodensis* extract (100 and 200 mg/kg) significantly (p<0.05) decreased the levels of LPO and increased the levels of GSH and SOD in DOX-treated animals as compared to DOX group (Figures 1-3). Animals treated with *E. nebrodensis* extract (200 mg/kg) alone have significantly decreased the cardiac marker enzymes as compared to DOX treated group.

DISCUSSION

The mechanism of DOX-induced cardiomyopathy is not completely understood, but several hypothesis have been postulated which include inhibition of nucleic acid [19], protein synthesis [20], release of vasoactive amines [21], alterations in sarcolemmal Ca^{++} transport

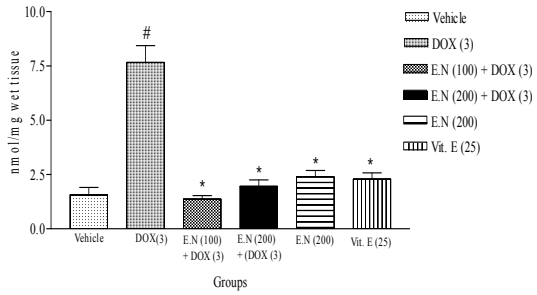

Fig 1. Effect of *E.nebrodensis* on LPO content in heart homogenate (n=5).Values are mean±SEM, ANOVA followed by Dunnetts test. $^{*}P<0.05$ compared with DOX, $^{\#}P<0.05$ compared with vehicle.E.N: *Ephedra nebrodensis* was administered at dose of 100 and 200 mg/kg, p.o. for two weeks; DOX: Doxorubicin was administered at dose of 3 mg/kg, i.p. on alternate days for two weeks; Vit. E: Vitamin E was administered at dose of 25 mg/kg, p.o.for two weeks.

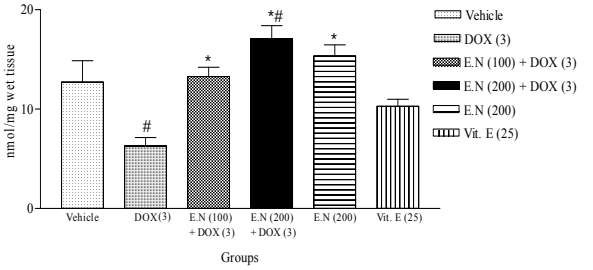

Fig 2. Effect of *E.nebrodensis* on GSH content in heart homogenate (n=5).Values are mean±SEM, ANOVA followed by Dunnetts test. $^{*}P<0.05$ compared with DOX,$^{\#}P<0.05$ compared with Vehicle E.N: *Ephedra nebrodensis* was administered at dose of 100 and 200 mg/kg, p.o. for two weeks; DOX: Doxorubicin was administered at dose of 3 mg/kg, i.p. on alternate days for two weeks; Vit. E: Vitamin E was administered at dose of 25 mg/kg, p.o. for two weeks.

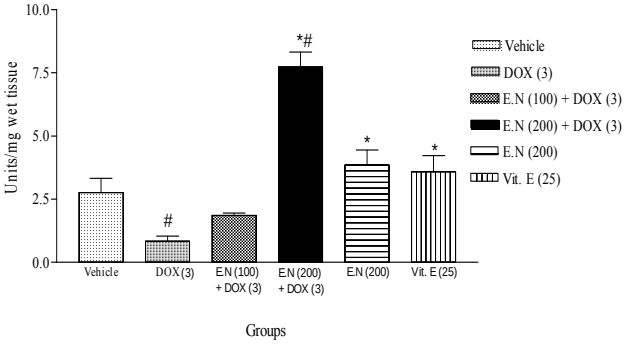

Fig 3. Effect of *E.nebrodensis* on SOD activity in heart homogenate (n=5).Values are mean±SEM, ANOVA followed by Dunnetts test. $^{*}P<0.05$ compared with DOX, $^{\#}P<0.05$ compared with Vehicle; E.N: *Ephedra nebrodensis* was administered at a dose of 100 and 200 mg/kg, p.o. for two weeks; DOX: Doxorubicin was administered at a dose of 3 mg/kg, i.p. on alternate days for two weeks; Vit. E: Vitamin E was administered at a dose of 25 mg/kg, p.o. for two weeks.

[22], alterations in membrane-bound enzymes [23], abnormalities in mitochondria and lysosomal alterations [24] and an imbalance of myocardial electrolytes [25]. The chronic cardiotoxicity is dose-dependent and causes irreversible myocardial damage resulting in cardiomyopathy with fatal congestive heart failure [26]. Data derived from several laboratories suggests that Ca transport abnormalities occur in cardiac [27, 28] tissues exposed to DOX and that the myocardium eventually becomes overloaded with calcium [29, 30]. This conclusion is based on total tissue calcium levels in hearts from treated animals and the observation of calcium phosphate accumulations in necrotic cells.

Repeated administration of DOX is likely to increase LPO levels in cardiac tissues. The generation of free radical by DOX in the form of DOX semiquinone has been suggested to play a major role in its cardiotoxic effects [31] by increasing oxygen free radical activity [32] and inducing the peroxidation of unsaturated lipids within the membranes [33]. Semiquinones are unstable under aerobic conditions thereby generating superoxide anion radicals. Antioxidants such as Vitamin E provide protection from cardiac cell damage with the decrease in lipid peroxidation [34].

The ECG changes induced by DOX consisted of prolongation of QT interval, ST interval and widening of QRS complex. These ECG changes have been partly explained by Jensen [35]. Whereas the QRS-intervals are directly related to cell depolarization, the QT interval is an expression of the late repolarization phase; the anthracyclines specifically prolong the later phase by disturbing the ion flux across the cellular membrane. It has been found that the above parameters are the most reliable ECG parameters for the assessment of DOX-induced cardiotoxicity [12]. One–way ANOVA followed by Dunnett's test showed that *E.nebrodensis* extract (100 and 200 mg/kg) significantly decreased the prolongation of QT [$F(5,24) = 46.83$] and ST intervals[$F(5,24) = 31.24$] in DOX treated groups. The heart weight [$F(5,24) = 23.89$] was significantly decreased in DOX treated group which was prevented by *E.nebrodensis* extract (100 and 200 mg/kg) treatment.

Chronic administration of DOX to rats significantly increased cardiotoxicity manifested by elevation in the levels of cardiac injury markers i.e. serum LDH, CPK and SGOT levels. Our results are in good agreement with those previously reported [36, 37]. One way ANOVA followed by Dunnett's test showed that treatment with *E.nebrodensis* extracts (100 and 200 mg/kg) has resulted in reversal of cardiac enzyme activities- LDH [$F(5,24) = 236.27$],CPK [$F(5,24) = 21.30$] and SGOT [$F(5,24) = 76.40$] (Table 2). The increase in cardiac marker levels in serum suggests an increased leakage of these enzymes from mitochondria as a result of toxicity induced by treatment with DOX.

The heart is particularly susceptible to free radical injury, because it contains less free radical detoxifying substances than do metabolic organs like liver or kidney [38, 39]. Moreover, DOX is known to have a higher affinity for cardiolipin, a major phospholipid component of the mitochondrial membrane in heart cells resulting in selective accumulation of doxorubicin inside cardiac cells [40].

Glutathione is considered to be the master anti-oxidant of the body and is found in almost all living cells. Reduced glutathione is an important endogenous antioxidant which controls the enzymatic and non-enzymatic detoxification of reactive oxygen species generated by many compounds [37]. LPO level is a direct indication of oxidative damage of cells as seen in aging, atherosclerosis and other pathological disorders. In our study, a decrease in concentration of cardiac GSH and SOD and an increase in LPO levels in DOX-treated group was observed. *E. nebrodensis* extract (100 and 200 mg/kg) treatment significantly reversed the changes in antioxidant levels induced by DOX treatment. We observed that SOD activity was significantly ($p<0.05$) increased by treatment with *E. nebrodensis* extract (200 mg/kg) as compared to control, but this rise was much more in DOX treated animals suggesting that the anti-oxidant enzyme activity is accelerated under oxidative stress. There is a general agreement that flavonoids act as scavengers of reactive oxygen species [41]. Flavonoids have been found to protect heart from DOX induced cardiotoxicity when co-administered with DOX in mice [42], which suggests that these compounds are potential cardioprotectors against DOX-induced chronic cardiotoxicity. Thus, in conclusion the above data suggests that *Ephedra nebrodensis* which is rich in flavonoids has the potential in preventing the cardiotoxic effects induced by Doxorubicin.

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