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Protective effect of *Nardostachys jatamansi* on oxidative injury and cellular abnormalities during doxorubicin-induced cardiac damage in rats

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Abstract

Nardostachys jatamansi is a medicinally important herb of Indian origin. It has been used for centuries in the Ayurvedic and Unani systems of medicine for the treatment of various ailments. We have evaluated the effect of *N. jatamansi* (rhizomes) on the biochemical changes, tissue peroxidative damage and abnormal antioxidant levels in doxorubicin (adriamycin)-induced cardiac damage. Preliminary studies on the effect of the graded dose of extract showed that 500 mg kg^{-1} orally for seven days was found to be optimum and hence all further study was carried out with this particular dose. Rats administered doxorubicin (15 mg kg^{-1}, i.p.) showed myocardial damage that was manifested by the elevation of serum marker enzymes (lactate dehydrogenase, creatine phosphokinase, aspartate aminotransaminase and alanine aminotransaminase). The animals showed significant changes in the antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase) and lipid peroxidation levels. Pretreatment with *N. jatamansi* extract significantly prevented these alterations and restored the enzyme activity and lipid peroxides to near normal levels. Restoration of cellular normality accredits the *N. jatamansi* with a cytoprotective role in doxorubicin-induced cardiac damage.

Introduction

Doxorubicin (adriamycin) is an anthracycline antibiotic that has major clinical significance because of its broad-spectrum antitumour activity. The isolation of doxorubicin was originally from a mutant *Streptomyces peucetius* obtained from the daunorubicin producing organism, *S. peucetius* (Gnanapragasam et al 2004).

Several studies have shown a cumulative dose-dependent and irreversible cardiotoxicity that limits its usefulness as a broad-spectrum anti-cancer drug (Kantrowitz & Bristow 1984; Buzadar et al 1985). The use of doxorubicin has been shown to cause development of cardiomyopathy, ultimately leading to congestive heart failure known as doxorubicin cardiomyopathy (Lefrak et al 1973).

The mechanism responsible for the cardiotoxic actions of doxorubicin has been extensively studied. The mechanism involves one-electron reduction of doxorubicin for generation of a semiquinone radical. Doxorubicin semiquinone radical reduces oxygen to produce superoxide and to regenerate doxorubicin. The net result of this process is that doxorubicin catalyses the reduction of oxygen by NADPH, to form a superoxide radical, which is subsequently reduced to hydrogen peroxide H_2O_2 by the antioxidant enzyme, superoxide dismutase. In the presence of Fe^{2+}, the H_2O_2 is further reduced to the extremely reactive hydroxyl radical ($^{\cdot}OH$), which can react with polyunsaturated fatty acids to yield lipid hydroperoxide. This initiates a lipid radical chain reaction, which can cause oxidative damage to cell membranes (Mimnaugh et al 1985; Nagi & Mansour 2000).

Many plants are in use for the treatment of heart related ailments (Mamtani & Mamtani 2005; Sun et al 2005). Even though modern drugs are effective in the control of cardiovascular disorders their use is often limited because of their side effects (Stollberger & Finsterer 2005).

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The search for an effective medicine to treat cardiovascular disorders without any side effects has led to the use of traditional plant based medicine. *Nardostachys jatamansi* belonging to the family valerianaceae is a perennial herb found in the Alpine Himalayas. *N. jatamansi* (commonly known as sambul-at-teeb or balchar) is a reputed Indian medicinal plant attributed with many CNS properties (Arora 1965; Gupta et al 1994). In ayurveda, rhizomes of *N. jatamansi* are used as a bitter tonic, stimulant and antispasmodic, and to treat epilepsy, hysteria and convulsions (Bagchi et al 1991). Reports have shown this plant to be a good antioxidant (Tripathi et al 1996) and neuroprotective (Salim et al 2003). In the Unani system of medicine this plant has been mentioned as a hepatotonic, cardiotonic, diuretic and analgesic (Ali et al 2000).

The active constituents present in the *N. jatamansi* are sesquiterpenes, lignans, neolignans and terpenoids (Chatterjee et al 2000) and these compounds present in various plants have been shown to possess antioxidant activity (Jodynis-Liebert et al 2000; Ghafoorunissa et al 2004; Vagi et al 2005). Plants such as *Phyllanthus urinaria*, and grape seed proanthocyanidin, which possess antioxidant properties, showed cardioprotective effects against doxorubicin-induced toxicity (Bagchi et al 2003; Chularojmontri et al 2005). Hence, we have explored and evaluated the cardioprotective activity of *N. jatamansi* extract by assessing the cardiac function through the assay of cardiac markers and have investigated the antioxidant status during doxorubicin-induced cardiomyopathy in experimental rats.

Materials and Methods

Collection of plant materials and chemicals

N. jatamansi was procured from a recognized and licensed ayurvedic shop in Chennai. The herb was identified and authenticated by Dr Sasikala Ethirajulu (Research Officer, Botany) in Central Institute for Siddha (CRIS), Arumbakkam, Chennai-600 101. Doxorubicin was procured from Dabur Pharmaceuticals (doxorubicin hydrochloride-Adrim), New Delhi, India. All other chemicals used were of analytical grade.

Preparation of the extract (Chatterjee et al 2000)

The rhizomes of the herb were washed, dried and crushed in an iron mortar. Crushed material was subjected to extraction in a Soxhlet apparatus at 60–70°C for 6 h continuously in 95% distilled ethyl alcohol. The extracted material was evaporated to dryness under reduced pressure (40–45°C). The dried ethanolic extract was suspended in distilled water which was then administered to rats orally at an optimum dosage of 500 mg kg^{-1}. This particular dosage was fixed after trying out different doses for different days in the same set of rats.

Animals

Healthy Wistar adult male albino rats (2–3-months old, 120–130 g) were used for the study. Housed immediately in polypropylene cages, maintained under standard conditions, the animals were fed with standard rat pellet diet (Hindustan Lever Ltd, Bangalore, India) and water was freely available. This study was ethically approved by the Ministry of Social Justices and Empowerment, Government of India and by the Animal Ethics Committee Guidelines of our institution.

Experimental protocols

The rats were divided into four groups (n = 6 in each group). Group 1, control; group 2, administered doxorubicin; group 3, administered *N. jatamansi* extract alone; group 4, administered doxorubicin + *N. jatamansi* extract. The ethanol extract of *N. jatamansi* was administered orally for seven days at the dose of 500 mg kg^{-1}. Doxorubicin was given at the dose of 15 mg kg^{-1} intraperitoneally (Nagi & Mansour 2000) on day 7 and the animals were killed 48 h later.

Biochemical parameters

At the end of the experimental period, the body weight was determined and rats were killed by cervical decapitation. Blood was collected and the separated serum was used for further estimation. The heart was excised immediately, rinsed in ice-cold saline, dried, weighed and homogenized in Tris-HCl buffer of pH 7.4 (0.1 M) using a Teflon homogenizer. The tissue homogenate was centrifuged in a cooling centrifuge at 500 *g* to remove the debris and the supernatant was used for the analysis of biochemical parameters. The tissue homogenate was kept at −20°C until further use. All the experiments were carried out in a dark cold room at 4°C.

Cardiac damage was assessed by measuring the activity of serum markers (lactate dehydrogenase (LDH) (King 1965), creatine phosphokinase (CPK) (Okinaka et al 1961), aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) (Bergmeyer & Bernt 1974)) and tissue antioxidants (superoxide dismutase (SOD) (Misra & Fridovich 1972), catalase (CAT) (Takahara et al 1960), glutathione peroxidase (GPx) (Rotruck et al 1973), glutathione-S-transferase (GST) (Habig et al 1974) and myocardial lipidperoxides (LPO) (Ohkawa et al 1979).

Assay of LDH

LDH was assayed according to the method of King (1965). To 0.1 mL buffered substrate, 0.1 mL enzyme preparation was added and the tubes were incubated at 37°C for 15 min. After adding 0.2 mL NAD^+ solution, the incubation was continued for another 15 min. The reaction was arrested by adding 0.1 mL DNPH (2,4-dinitrophenyl hydrazine). The tubes were incubated for a further 15 min at 37°C and then 7 mL 0.4 M NaOH (sodium hydroxide) solution was added. The colour developed was measured at 420 nm in a Shimadzu UV spectrophotometer. Suitable samples of the standards were analysed by the same procedure. The activity of the enzyme was expressed as μmol pyruvate liberated (mg protein)$^{-1}$ h^{-1}.

Assay of CPK

Serum CPK activity was determined by the method of Okinaka et al (1961). The reaction mixture comprised 0.05 mL serum, 0.1 mL substrate, 0.1 mL ATP solution and 0.1 mL cysteine-hydrochloride solution. The final volume was made

upto 2 mL with distilled water and incubated at 37°C for 30 min. The reaction was arrested by the addition of 1 mL 10% TCA (trichloroacetic acid) and the contents were subjected to centrifugation. To 0.1 mL supernatant, 4.3 mL distilled water and 1.0 mL ammonium molybdate were added and incubated at room temperature for 10 min. 1-Amino-2-naphthol-4-sulfonic acid (ANSA) 0.4 mL was added and the colour which developed was read at 640 nm after 20 min. The activity of the enzyme was expressed as μmol phosphorus liberated (mg protein)$^{-1}$ h^{-1}.

Assay of AST

The activity of AST was assayed by the method of Bergmeyer & Bernt (1974). To 1 mL substrate, 0.2 mL serum was added and incubated for 1 h at 37°C. To this was added 1 mL 0.02% DNPH and the tube kept at room temperature for 20 min. To the control tube, sample was added after arresting the reaction with DNPH, and then 5 mL 0.4 M NaOH was added. The colour which developed was read at 540 nm. The activity was expressed as μmol pyruvate liberated (mg protein)$^{-1}$ h^{-1}.

Assay of ALT

The activity of ALT was assayed by the method of Bergmeyer & Bernt (1974). To 1 mL substrate, 0.2 mL serum was added and incubated for 1 h at 37°C. To the control tubes, sample was added after the reaction was arrested by the addition of 1 mL DNPH. The tubes were kept at room temperature for 30 min, and then 5 mL 0.4 M NaOH was added. The colour which developed was read at 540 nm. The activity was expressed as μmol pyruvate liberated (mg protein)$^{-1}$ h^{-1}.

Assay of SOD

SOD was assayed by the method of Misra & Fridovich (1972). A 0.1-mL sample of tissue homogenate was added to the tubes containing 0.75 mL ethanol and 0.15 mL chloroform (chilled in ice) and centrifuged. To 0.5 mL supernatant was added 0.5 mL 0.6 mM EDTA solution and 1 mL 0.1 M carbonate-bicarbonate (pH 10.2) buffer. The reaction was initiated by the addition of 0.5 mL 1.8 mM adrenaline (freshly prepared) and the increase in absorbance at 480 nm was measure in a Shimadzu UV spectrophotometer. The enzyme activity was expressed as 50% inhibition of adrenaline autoxidation.

Assay of CAT

CAT activity was assayed by the method of Takahara et al (1960). To 1.2 mL 50 mM phosphate buffer pH 7, 0.2 mL tissue homogenate was added and the reaction was started by the addition of 1 mL 30 mM H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 30-s intervals for 3 min. The enzyme blank was run simultaneously with 1 mL distilled water instead of hydrogen peroxide. The enzyme activity was expressed as μmol H_2O_2 decomposed min^{-1} (mg protein)$^{-1}$.

Assay of GPx

GPx was assayed by the method of Rotruck et al (1973). The reaction mixture consisted of 0.2 mL 0.8 mM EDTA, 0.1 mL 10 mM sodium azide, 0.1 mL 2.5 mM H_2O_2, 0.2 mL reduced glutathione, 0.4 mL 0.4 M phosphate buffer pH 7, and 0.2 mL homogenate and was incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 mL 10% TCA and the tubes were centrifuged at 2000 rev min^{-1}. To the supernatant 3 mL 0.3 mM disodium hydrogen phosphate and 1 mL 0.04% 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were added. The colour which developed was read at 420 nm immediately. The activity of GPx was expressed as μmol glutathione oxidized min^{-1} (mg protein)$^{-1}$.

Assay of GST

GST was assayed by the method of Habig et al (1974). To 0.1 mL homogenate, 1 mL 0.3 M phosphate buffer pH 6.5, 1.7 mL water and 0.1 mL 30 mM CDNB (1-chloro-2,4-dinitrobenzene) were added. After incubation at 37°C for 15 min, 0.1 mL GSH was added and change in OD was read at 340 nm for 3 min at 30-s intervals. Reaction mixture without the enzyme was used as blank. The glutathione-S-transferase activity was expressed as U min^{-1} (mg protein)$^{-1}$.

Assessment of lipid peroxidation

Lipid peroxidation (LPO) was assayed by the method of Ohkawa et al (1979), in which the malondialdehyde (MDA) released served as the index of LPO. 1,1,3,3-Tetra ethoxypropane malondialdehye bis (diethyl acetal) was used as standard. To 0.2 mL homogenate, 0.2 mL 8.1% SDS, 1.5 mL 20% acetic acid (pH 3.5) and 1.5 mL 0.8% TBA were added. The mixture was made up to 4 mL with water and then heated in a water bath at 95°C for 60 min using a glass ball as a condenser. After cooling, 1 mL water and 5 mL n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rev min^{-1} for 10 min, the organic layer was taken and its absorbance was measured at 532 nm. The level of lipid peroxides was expressed as nmol MDA formed (mg protein)$^{-1}$.

Statistical analysis

All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance followed by least significant difference (LSD) test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ± s.d for six animals in each group.

Results

Effect of *N. jatamansi* extract on body weight and heart weight

Doxorubicin administration significantly decreased ($P < 0.05$) the body weight and heart weight when compared with control rats. *N. jatamansi* extract pretreatment significantly increased ($P < 0.05$) body and heart weight of rats when compared with the induced group (Table 1).

Table 1 Effect of *N. jatamansi* extract on body weight, heart weight and LPO levels in control and experimental rats

Parameters	Control	Doxorubicin	*N. jatamansi*	*N. jatamansi*+doxorubicin
Body weight (g)	140 ± 6.26	117 ± 3.77*	141 ± 6.26	138.33 ± 6.28*
Heart weight (mg)	498.66 ± 3.98	350.16 ± 6.21*	498 ± 3.34	488.16 ± 10.4*
LPO	3.46 ± 0.32	5.53 ± 0.21*	3.36 ± 0.32	3.9 ± 0.34*

Values are expressed as mean ± s.d for six animals in each group. *$P < 0.05$, comparisons were made between doxorubicin and control group; doxorubicin and *N. jatamansi* + doxorubicin group. LPO activity was expressed as nmol MDA formed (mg protein)$^{-1}$.

Effect of *N. jatamansi* extract on cardiac markers

Cardiac markers were assayed to assess the cardiac function. Doxorubicin induction significantly increased the cardiac markers LDH, CPK, AST and ALT at $P < 0.05$ levels when compared with the control group. Upon pretreatment with *N. jatamansi* extract this condition was reversed. Here, the LDH, CPK, AST and ALT were decreased ($P < 0.05$) when compared with the induced group (Table 2).

Effect of *N. jatamansi* extract on antioxidant status

Table 3 shows the activity of the antioxidants SOD, CAT, GPx and GST in the heart tissue of control and experimental rats. Doxorubicin induction showed a significant decrease ($P < 0.05$) of these antioxidants when compared with control rats. Pretreatment with *N. jatamansi* extract prevented these alterations by increasing the antioxidant activity significantly ($P < 0.05$) when compared with doxorubicin alone induced rats.

Effect of *N. jatamansi* extract on lipid peroxidation

MDA levels were significantly increased ($P < 0.05$) upon doxorubicin induction when compared with control rats (Table 1). Pretreatment with *N. jatamansi* extract significantly decreased ($P < 0.05$) the MDA levels when compared with doxorubicin-induced rats.

Discussion

Effective anti-cancer therapy with doxorubicin and other quinone anthracyclines is severely limited by severe cardiotoxicity, reportedly because semiquinone metabolites delocalize Fe (II) from ferritin and generate hydrogen peroxide, there by promoting hydroxyl radical formation and lipid peroxidation (Minotti et al 1996)). To reduce doxorubicin-induced cardiotoxicity, antioxidant substances have been investigated in experimental models (Lenzhofer et al 1983; Shimpo et al 1991; Lin et al 1992) and in humans (Vanella et al 1997).

Table 2 Effect of *N. jatamansi* extract on cardiac markers LDH, CPK, AST and ALT in control and experimental rats

Parameters	Control	Doxorubicin	*N. jatamansi*	*N. jatamansi*+doxorubicin
LDH	1074.83 ± 100.60	1631.33 ± 167.99*	1073.33 ± 100.42	1216.00 ± 119.41*
CPK	80.33 ± 8.73	178.49 ± 16.40*	79.46 ± 8.92	95.40 ± 8.92*
AST	78.89 ± 6.40	158.49 ± 15.17*	78.46 ± 6.43	96.72 ± 3.70*
ALT	22.41 ± 2.30	45.41 ± 4.51*	21.83 ± 2.31	29.82 ± 3.81*

Values are expressed as mean ± s.d for six animals in each group. *$P < 0.05$, comparisons were made between doxorubicin and control group; doxorubicin and *N. jatamansi* + doxorubicin group. Activity was expressed as: LDH, AST and ALT, μmol pyruvate liberated (mg protein)$^{-1}$ h^{-1}; CPK, μmol phosphorus liberated (mg protein)$^{-1}$.

Table 3 Effect of *N. jatamansi* extract on tissue antioxidants SOD, CAT, GPx and GST in control and experimental rats

Parameters	Control	Doxorubicin	*N. jatamansi*	*N. jatamansi*+doxorubicin
SOD	8.91 ± 0.86	4.70 ± 0.44*	9.30 ± 0.98	8.07 ± 0.83*
CAT	84.51 ± 8.75	64.54 ± 6.00*	85.48 ± 8.23	80.66 ± 8.78*
GPx	1.85 ± 0.09	1.24 ± 0.11*	1.80 ± 0.09	1.71 ± 0.09*
GST	0.33 ± 0.01	0.17 ± 0.11*	0.34 ± 0.13	0.28 ± 0.02 *

Values are expressed as mean ± s.d. for six animals in each group. *$P < 0.05$, comparisons were made between doxorubicin and control group; doxorubicin and *N. jatamansi* + doxorubicin group. Activity was expressed as: SOD, 50% inhibition of adrenaline autoxidation; CAT, μmol H_2O_2 decomposed min^{-1} (mg protein)$^{-1}$; GPx, μmol GSH oxidized min^{-1} (mg protein)$^{-1}$; GST, U min^{-1} (mg protein)$^{-1}$.

Doxorubicin toxicity reduces food intake and inhibits protein synthesis which results in the reduction of body weight (Tong et al 1991), which was seen in this investigation. *N. jatamansi* extract pretreatment prevented the change in body weight showing no inhibition to protein synthesis. In this study, a single dose of doxorubicin (15 mg kg^{-1}, i.p.) induced cardiotoxicity manifested biochemically by a significant increase in serum cardiac markers after 48 h. During administration of doxorubicin, a collapse in the circulatory system may have been produced which ultimately deprived the supply of fresh blood with oxygen and nutrients, which ultimately caused leakage of the plasma membrane thereby elevating the levels of serum markers in the blood stream (Ithyarasi et al 1996). These results were consistent with other investigators (Satliel & McGuire 1983; Singal et al 1995), who found that doxorubicin induced cardiotoxicity in normal rats. The net results of this study clearly demonstrated that *N. jatamansi* extract, when given orally, protected rats from doxorubicin-induced cardiotoxicity as evident from the significant reduction in the levels of the serum enzymes, LDH, CPK, AST and ALT. The biochemical mechanism involved in the development of doxorubicin cardiotoxicity has been well studied and documented. It is now believed that the formation of superoxide radical from doxorubicin recycling is a crucial factor in the pathogenesis of doxorubicin cardiotoxicity (Sarvazyan et al 1995; Singal et al 1995). Three important antioxidant enzymes, SOD, CAT and GPx, are important in mitigating free radical-induced cell injury. Cellular antioxidants such as SOD, CAT, GPx and GST are critical in combating the reactive oxygen species induced cell death and tissue injury. The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles (Andrew & Mathew 1989). A number of studies have reported diverse results for the changes of the activity of these antioxidant enzymes in different animal species treated with different doses and schedule of doxorubicin. Administration of doxorubicin resulted in a dose- and time-dependent decrease in antioxidant status in different experimental models (Ji & Mitchell 1994; Siveski-Illiskovic et al 1995), which concurred with this study. Pretreatment with the extract of *N. jatamansi* showed an increase in the activity of the antioxidant defense mechanism enzymes, thus preventing the toxic effect caused by doxorubicin in rats. In our study, lipid peroxidation was significantly increased, suggesting an increase in oxidative stress. In the presence of transition metal ions, the chain reaction continues and free iron appears to play a particularly important role in doxorubicin-induced lipid peroxidation. Without free iron, LPO formation is minimal and even a low concentration of free iron can lead to a substantial LPO production (Griffin-Green et al 1988). Doxorubicin may act by transferring an electron directly to Fe^{3+} and the Fe^{2+} produced can reduce oxygen to hydrogen peroxide. This redox cycling of doxorubicin and its iron chelate generate free radical metabolites and active oxygen species (Gianni et al 1985; Sinha & Polliti 1990). This study showed an increase in LPO upon doxorubicin intoxication. Upon pretreatment with *N. jatamansi* extract the LPO levels were reduced. This suggested the possible involvement of the extract by inhibiting the transfer of electron to Fe^{3+}. These findings showed that *N. jatamansi* extract could act against oxidative stress by maintaining the equilibrium in antioxidant defense mechanism enzymes.

A number of plants have been proved to possess antioxidants (Gorinstein et al 2005; Heimler et al 2005; Kalra et al 2005; Wang et al 2005) and antiperoxidative properties, which have been proved to be protective against a number of ailments especially heart related problems (Sun et al 2005). *N. jatamansi* is one such plant which has been indicated in ayurvedic text to be a potent cardiotonic agent (Ali et al 2000). A number of active components are present in this plant (Chatterjee et al 2000), which include lignans, sesquiterpenes and terpenoids. It has been reported that plants with these compounds possess cardioprotective effect through an antioxidant defense mechanism (Pattanaik & Prasad 1998; Sumitra et al 2001; Yamauchi et al 2005).

It can be concluded from the results of this study that *N. jatamansi* prevented cardiac damage by reduction of lipid peroxidation levels, which might have been through inactivation of free radical and by increasing the antioxidant levels which constitutes the foremost defense system that limit the toxicity associated with free radicals, formed during doxorubicin-induced myocardial injury.

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