

Stress modulating antioxidant effect of *Nardostachys jatamansi*

Nazmun Lyle¹, Dipankar Bhattacharyya^{1*}, Tapas K Sur¹, Santanu Munshi², Suhrita Paul³, Suparna Chatterjee¹
and Antony Gomes⁴

¹Department of Pharmacology, Institute of Post-Graduate Medical Education & Research,
244-B, A. J. C. Bose Road, Kolkata 700 020

²Department of Pharmacology, Calcutta National Medical College, 32, Gorachand Road, Kolkata 700 014

³Department of Pharmacology, Calcutta Medical College, 88, College Street, Kolkata 700 012

⁴Department of Physiology, University College of Science and Technology,
University of Calcutta, 92, A. P. C. Road, Kolkata 700 009

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The rhizomes of *Nardostachys jatamansi*, the plant commonly known as Jatamansi have been described in Ayurveda for their soothing and sedative action on the central nervous system. In the present study, the anti-stress effect of hydro-ethanolic extract (70%) of *N. jatamansi* (NJE) was evaluated in reference to its antioxidant property. Wistar rats were divided into four groups: naïve, stressed, and T-200 and T-500 stressed with oral pre-treatment of NJE 200 and 500 mg/kg, respectively. Restraint of rats in metallic chambers for 4 h at 4°C was followed by sacrifice and assessment of stress-induced alterations in biochemical parameters, incidence and severity of ulcers. Lipid peroxidation (LPO) and NO levels in stomach and LPO, NO levels and catalase activity in brain, plasma corticosterone level and adrenal ascorbic acid were measured. *In vitro* antioxidant activity of NJE was studied by measuring the free radical scavenging activity. NJE showed potent antioxidant activity and significantly reversed the stress-induced elevation of LPO and NO levels and decrease in catalase activity in the brain. It inhibited the incidence of gastric ulcerations and reversed the alterations in biochemical parameters/markers of stress-induced gastric ulceration. NJE also significantly altered stress-induced increase in adrenal and spleen weights and decrease in level of ascorbic acid in adrenal gland. Elevation of plasma corticosterone level was negated dose- dependently. The findings suggest that the NJE possesses significant anti-stress activity, which may be due to its antioxidant activity.

Keywords: Adaptogen, Corticosterone, Lipid peroxidation, *Nardostachys jatamansi*, Restraint stress, ROS, Ulcer.

Stress may alter variety of central nervous system (CNS)-mediated processes including locomotor activity, sleep, mood, feeding behaviour, sexual activity, neuroendocrine and cognitive function etc. The major phenomena related to stress response is the activation of the hypothalamo-hypophyseal-adrenal axis¹. Modern psychotropic drugs used for the treatment of mental illnesses have side effects, ranging from sedation and hypotension to extra-pyramidal symptoms². Many medicinal plants quoted in Ayurveda have been shown to possess anti-stress

effects³. *Nardostachys jatamansi* (family *Valerianaceae*), an indigenous medicinal plant induces in organisms a state of resistance against stress. It helps to promote physical and mental health, augment resistance of the body against disease and has shown potent antioxidant activity. It also shows marked tranquillizing activity, as well as hypotensive, hypolipidemic, anti-ischemic, antiarrhythmic, hepatoprotective, anticonvulsant, neuroprotective activities⁴⁻⁷.

Restraint stress (RS) has been one of the most popular stressors in experimental medicine⁸. It elicits the purest form of psychological frustration accompanied by vigorous struggling which means muscular exercise. It is very effective in producing typical non-specific stress manifestations^{9,10}. In the present study, the potential of *N. jatamansi* has been explored on RS-induced changes in different parameters and possible modulatory role exhibited by its antioxidant property.

*Author for correspondence:

E-mail: lyle.nazmun@gmail.com; lyle_n@rediffmail.com

Tel: +919432287171 (M)/ 033-22234135 (O)

Abbreviations: CMC, carboxymethylcellulose; CNS, central nervous system; DNPH, 2,4, N-dinitrophenyl hydrazine; DPPH, 1-diphenyl-2-picryl-hydrazyl; LPO, lipid peroxidation; NJE, *Nardostachys jatamansi* extract; NO, nitric oxide; PO, per oral; ROS, reactive oxygen species; RS, restraint stress; TCA, trichloro acetic acid.

Materials and Methods

Drug and vehicle

The plant material i.e rhizomes of *Nardostachys jatamansi* was collected from Syntrex Company Ltd., Kolkata and identified and authenticated taxonomically by a taxonomist of Botanical Survey of India. Voucher specimen of the collected samples (DB/ICMR/05-06/04) was preserved in the departmental museum. Rhizomes were coarsely powdered, extracted with 70% ethanol at room temperature, concentrated in reduced temperature and pressure on rotary evaporator, lyophilized and stored (yield, 9%). The chemical constituents of decoction were identified by qualitative analysis and confirmed by thin layer chromatography¹¹.

The *Nardostachys jatamansi* extract (NJE) was administered orally once a day for 5 days prior to the experimentation in two doses i.e. 200 and 500 mg/kg, selected from pilot dose response trial. The test and standard drugs were suspended in 0.3% carboxymethyl cellulose (CMC).

Animals and treatment

Male Wister rats weighing between 120-150 g were procured from Indian Institute of Chemical Biology, Kolkata and housed in normal temperature and humidity with alternate 12 h light and dark cycle according to guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, and provided free access to water and food pellets (Tetragon Chemie Pvt. Ltd.). They were acclimatized to the laboratory conditions for 5 days before behavioral studies¹². All the experiments were performed during the same time of the day. The experimental protocol was approved by Institutional Animal Ethics Committee.

The animals were divided into four groups: Group-I or (normal control) received vehicle only, Group-II (stressed control) received only stress and vehicle, Group-III (T-200) and Group-IV (T-500) were given 200 mg/kg and 500 mg/kg NJE orally in vehicle, in addition to stress.

In vitro antioxidant activity

The antioxidant activity of the NJE was determined by using a method based on the reduction of ethanolic solution of colored-free radical 1, 1-diphenyl-2-picryl-hydrazyl (DPPH). The ethanolic solution of DPPH (0.1 mM, 1 ml) was incubated with 3 ml of

different concentrations of the extract at room temperature for 30 min and at the end of incubation period, the optical density was determined at 520 nm. The radical scavenging activity of tested sample was expressed as an inhibition percentage¹³. Ascorbic acid was used as reference standard.

Experimental procedures

Acute toxicity

The studies on the selection of lethal dose of each plant extract had been conducted. NJE was administered orally in different doses (50-2000 mg/kg) to normal rats. During the first 8 h after drug administration, the animals were observed carefully for signs of toxicity, morphological and behavioral abnormalities and mortality and then kept under observation for next 14 days. The plant extract up to 2 g/kg body weight by oral route did not cause any mortality¹⁴. Further increments of doses could not be possible due to less solubility of the extract.

Cold restraint stress

On the 5th day of drug treatment after 1 h of the last dose, animals were subjected to stress by immobilization. The fore and hind limbs were tied separately and then together and finally securing them with adhesive tape. The rats were then individually put in restraint chambers; the tail was taped to the side to completely immobilize the animal for 4 h at 4°C^{15,16}. Thereafter, they were sacrificed by cervical dislocation. The intensity of stress-induced effects was assessed using the following parameters:

Plasma corticosterone estimation

Plasma corticosterone was measured using HPLC-UV detection. The mobile phase was acetonitrile-water-acetic acid-TEA (22:78:0.1:0.03, v/v), and pumped at a flow rate of 1.0 ml/min at ambient temperature¹⁷.

Dissection and homogenization of brain

On 5th day, animals were sacrificed by cervical dislocation, followed by decapitation. The whole brains were removed and 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer, pH 7.4, centrifuged for 25 min at 15000 rpm at 4°C and the supernatant was used for estimation of the following biochemical assays.

Lipid peroxidation (LPO) assay

The quantitative measurement of LPO was performed as described previously¹⁸. The amount of

malondialdehyde (MDA) was measured by reaction with thiobarbituric acid reactive substances at 532 nm spectrophotometrically. The values were calculated by multiplying with extinction coefficient of chromophore ($1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) and expressed as nM/mg protein.

Estimation of nitrite and catalase

The accumulation of nitrite in the supernatant is an indicator of production of nitric oxide (NO), which is produced due to oxidative stress occurring in the brain. Production of NO was determined by spectrophotometric assay with Griess reagent (0.1% N-1-naphththyl ethylenediamine dihydrochloride, 1% sulphanilamide and 2.5% phosphoric acid)¹⁹. Equal volumes of brain homogenate and Griess reagent were mixed, the mixture was incubated for 10 min at room temperature and the absorbance was measured at 540 nm. The concentration of nitrite in the supernatant was determined from the standard curve and expressed in μM .

Catalase activity was measured spectrophotometrically as previously described¹⁹. The method is based on the fact that catalase causes breakdown of H_2O_2 ($1.25 \times 10^{-2} \text{ M}$). The H_2O_2 was mixed in 3 ml phosphate buffer (pH 7.4) and then 0.02 ml of brain homogenate (10%) was added and the changes in absorbance at 240 nm were recorded up to 3 min at the interval of 15 s. Enzyme activity was calculated using the extinction coefficient of H_2O_2 (0.045) and expressed as micromoles of H_2O_2 decomposed min^{-1} mg of protein⁻¹.

The amount of protein was estimated according to the method of Lowry *et al*²⁰.

Gastric ulceration

The stomach was opened along with the greater curvature. The numbers of discrete ulcers were noted with the help of a magnifying glass. Severities of ulcers were scored after histological confirmation²¹.

Estimation of LPO and nitrite in stomach

Mucosal layer of stomach was scrapped and 10% homogenate was prepared as described earlier. LPO was measured as mentioned in brain homogenate¹⁸. Production of NO in stomach was estimated with Griess reagent, as estimated in rat brain¹⁹.

Adrenal gland and spleen weights and adrenal ascorbic acid

The weights of adrenal gland and spleen were taken by high precision Metler weighing balance. Adrenal ascorbic acid concentration was measured as

described previously²². In the adrenal gland homogenate, 6% TCA was added and centrifuged properly. The supernatant was then coupled with 2,4, N-dinitrophenyl hydrazine (DNPH) in presence of thiourea as a mild reducing agent. Conc. H_2SO_4 was added drop by drop slowly, which converts DNPH into a red compound assayed spectrophotometrically at 540 nm.

Statistical analysis

Results were expressed as mean \pm SEM. Comparisons between the control and treated groups were performed by analysis of variance (ANOVA), followed by Student's t- test. In all the tests, criterion for statistical significance was $P < 0.05$.

Results

Phytochemical analysis of the NJE indicated the presence of alkaloids, flavonoids, steroids, triterpenoids, saponin, gums, etc.

In vitro antioxidant activity

NJE tested for *in vitro* using DPPH showed potent free radical scavenging activity, as evidenced by low IC_{50} values. Fig. 1 depicted that the IC_{50} value of the NJE was 3.85 mg/ml, whereas ascorbic acid used as standard showed an IC_{50} of $19.90 \pm 2.3 \mu\text{g/ml}$.

Acute toxicity

Rats that received different doses of NJE did not manifest clinical signs of toxicity. None of the doses tested could produce mortality in rats during the treatment period up to 14 days. The doses of NJE up to 2 g/kg b. wt. were found to be non-toxic.

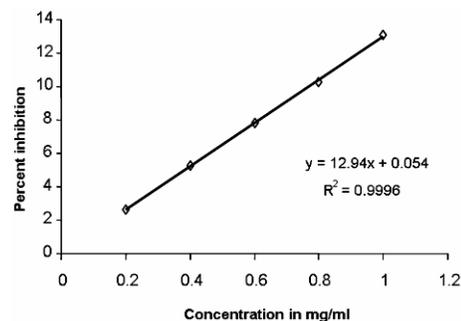


Fig. 1—Free radical (DPPH) scavenging activity of *Nardostachys jatamansi* (NJE) in *in vitro* systems. Graphical representation of the concentration of *N. jatamansi* required inhibiting 50 percent of free radicals [Each point represents the mean percentage inhibition of six experiments. Regression coefficient = 0.9996, IC_{50} = 3.85 mg/ml. Ascorbic acid used as standard showed an IC_{50} of $19.90 \pm 2.3 \mu\text{g/ml}$]

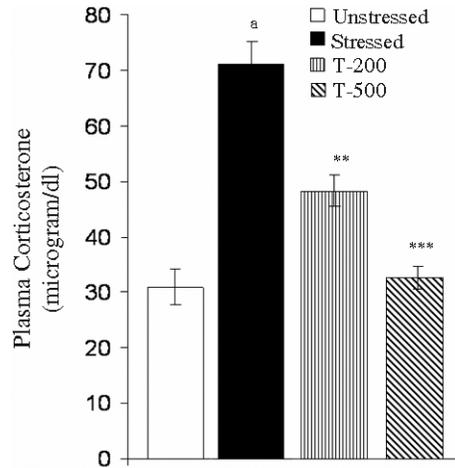


Fig. 2—Effect of *N. jatamansi* (NJE) on plasma corticosterone level in the cold restraint stress induced model [Values expressed as mean \pm S.E.M. ^a $P < 0.001$ as compared with unstressed or naïve ($n = 7$), ** $P < 0.01$ as compared to stressed group with treated group i.e. T-200 and *** $P < 0.001$ with T-500 ($n = 8$)]

Table 1—Effect of NJE on oxidative measurements of brain in restraint stress

[Values expressed as mean \pm S.E.M.]

Treatments	n	LPO (nM/mg protein)	NO (μ M)	Catalase (U/ml/mg/protein)
Naïve (without stress)	7	5.98 \pm 0.2	4.00 \pm 0.58	21.72 \pm 1.83
Control (RS)	8	12.32 \pm 2.29 ^a	18.95 \pm 6.14 ^a	15.5 \pm 0.58 ^a
T-200 + RS	8	7.135 \pm 1.21 *	6.75 \pm 0.56 **	17.45 \pm 0.52 **
T-500 + RS	8	4.95 \pm 0.48 ***	6.38 \pm 0.26 **	23.45 \pm 2.58 ***

RS = Restraint stress, T-200 (NJE 200 mg/kg), T-500 (NJE 500 mg/kg). P values: ^a $P < 0.05$ as compared to control stressed (RS) with unstressed or naïve. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to control (RS) group

Table 2—Effect of NJE on oxidative measurements of stomach in restraint stress

[Values expressed as mean \pm S.E.M.]

Treatments	n	LPO (nM/mg protein)	NO (μ M)	Ulcer incidence (%)	Severity of ulcers
Naïve (Without stress)	7	4.51 \pm 0.2	6.11 \pm 0.41	—	—
Control (RS)	8	8.94 \pm 1.81 ^a	14.91 \pm 4.19 ^a	100%	20.16 \pm 3.8
T-200 + RS	8	5.39 \pm 0.23 **	10.73 \pm 1.42 **	40%	7.54 \pm 0.26 **
T-500 + RS	8	4.75 \pm 0.13 **	7.02 \pm 0.13 **	25%	5.08 \pm 1.09 ***

RS = Restraint stress, T-200 (NJE 200 mg/kg), T-500 (NJE 500 mg/kg). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared to control (RS) group. ^a $P < 0.05$ as compared to control (RS) with naïve (without stress)

Plasma corticosterone

Plasma corticosterone level increased significantly during the stressed condition (Fig. 2). Pre-treatment with NJE 200 mg/kg, as well as 500 mg/kg negated the increment of plasma corticosterone level after stress.

LPO, nitrite level and catalase activity in brain

Restraint stress significantly increased LPO and nitrite levels and decreased the catalase activity in brain, as compared to normal control. Pre-treatment with NJE-200 mg/kg and 500 mg/kg significantly reversed the increase in LPO and nitrite levels and significantly restored the catalase activity (Table 1).

Gastric ulcer

Restraint stress resulted in increased ulcer incidence, as also the number and severity of gastric ulcers. These effects were significantly attenuated in dose-dependent manner by pre-treatment with NJE (200 and 500 mg/kg bw). The gastric mucosal lesions occurred in stressed animals and the treatment with NJE (200 and 500 mg/kg bw) prevented the decay of mucosal layer (Table 2).

LPO in stomach and gastric nitrite level

Stress-induced gastric ulceration increased the LPO level in the stomach. Restraint stress also significantly increased the nitrite level in mucosal layer of stomach as compared to normal control. Treatment with NJE (200 and 500 mg/kg) significantly reduced the LPO and nitrite levels compared to stressed animals (Table 2).

Adrenal gland and spleen

Stress caused hypertrophy of the glands. Weights of the adrenal glands and spleen were increased significantly in the stressed group, compared to unstressed naïve group. NJE (200 and 500 mg/kg) treatment mitigated the hypertrophy of the glands significantly (Table 3).

Table 3—Effect of NJE on glands in restraint stress induced rats
[Values expressed as mean \pm S.E.M.]

Treatments	n	Adrenal gland wt (mg/120 g)	Spleen wt (mg/120 g)	Ascorbic acid (adrenal) (μ g/mg)
Naïve (Without stress)	7	15.61 \pm 1.89	0.299 \pm 0.5	206.6 \pm 30.2
Control (RS)	8	19.56 \pm 2.78 ^a	0.402 \pm 0.16 ^a	114.3 \pm 34.45 ^a
T-200 + RS	8	16.6 \pm 1.32*	0.339 \pm 0.17	169.83 \pm 18.51***
T-500 + RS	8	15.85 \pm 0.95**	0.293 \pm 0.06	227.83 \pm 11.33*** ^a

RS = Restraint stress, T-200 (NJE 200 mg/kg), T-500 (NJE 500 mg/kg). * P < 0.05; ** P < 0.01; *** P < 0.001 as compared to control group, ^a P < 0.01 as compared to naïve rats.

Adrenal ascorbic acid

Adrenal ascorbic acid was depleted during the stress. Prior treatment with NJE (200 and 500 mg/kg) significantly attenuated the stress-induced effect on this biochemical marker of adrenocortical activity (Table 3).

Discussion

Cold restraint stress (RS) or forced immobilization stress is one of the effective and explored models of stress in rats. Painful stimuli are not involved in RS and it is more akin to physiological stress, as it combines emotional stress (escape reaction) with physical stress (muscle work), resulting in both restricted motility and aggression²³. Experimental stress, including immobilization affects central neurotransmitters in several animal species, including rats²⁴. A number of centrally acting drugs exert their pharmacological actions through one or more of these putative neurotransmitters²⁵. Previously, we observed that RS for 4 h enhances the level of corticosterone in plasma²⁶. The ascending serotonergic neurons from raphe nuclei are reported to innervate hypothalamic and limbic sites and have an overall role in the secretion of corticotrophic hormone (ACTH) during stress²⁷. Counteracting stress with psychotropic drugs has shown that they are not always effective or do not fully correct the pathology associated with stress, as also they have a number of side effects. There is no drug in modern medicine that has specific anti-stress effect, though anti-anxiety agents are available.

The present study demonstrates that NJE shows both *in vitro* and *in vivo* antioxidant activity. It attenuates stress-induced elevation of biochemical changes such as membrane LPO, elevated NO production in brain as well as stomach, antioxidant enzyme like catalase, which are consistent with its

anti-stress properties. *In vitro* study shows that NJE has potent free radical scavenging action. Antioxidant property of NJE may be attributed to the presence of flavonoids and polyphenols and which in turn may be responsible for its anti-stress effect²⁸.

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