Amelioration of immobilization stress-induced biochemical and behavioral alterations and mitochondrial dysfunction by naringin in mice: possible mechanism of nitric oxide modulation

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Objective: The present study was undertaken to evaluate the effects of naringin on immobilization stress-induced biochemical-behavioral changes and mitochondrial dysfunction in mice.

Methods: Mice were randomized and grouped based on body weights. Respective drug treatments were given for 14 d., and on the 15th day all the animals were subjected to a 6-hour immobilization stress; then all the animals were subjected to various behavioral paradigms and were sacrificed. Various biochemical parameters and mitochondrial functions were analyzed using brain homogenate.

Results: The 6-hour acute immobilization stress significantly altered the behavioral (anxiety and memory) and biochemical parameters coupled with mitochondrial dysfunction in mice. Fourteen days pretreatment with naringin (50 and 100 mg/kg, per oral) significantly inhibited the behavioral and biochemical alterations and mitochondrial dysfunction caused by acute immobilization stress (P<0.05). Further, pretreatment with L-arginine (50 mg/kg, intraperitoneally), a nitric

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oxide precursor, reversed the protective effect of naringin (P < 0.05). In addition, pretreatment with N\textsuperscript{G}-nitro-L-arginine methyl ester (5 mg/kg, intraperitoneally) caused potentiation in the protective effect of naringin.

**Conclusion:** These results suggest the possible involvement of nitricergic pathway in the protective effect of naringin against immobilization stress-induced behavioral, biochemical and mitochondrial dysfunctions in mice.

**Keywords:** naringin; restraint, physical; stress; nitric oxide; lipid peroxidation

Stress is a very crucial factor in maintenance of health and occurrence of disease\cite{11}. Stress induces changes in emotional behavior and anxiety-like state, which are associated with oxidative damage, namely, free radical damage\cite{5, 13}. Acute immobilization stress (IS) triggers numerous cellular cascades that lead to a increase in reactive oxygen species (ROS) production\cite{14}. Because of brain’s high oxygen consumption, abundant lipid content and relative paucity of antioxidant enzymes, the central nervous system is highly vulnerable to free radical damage\cite{5}. IS has also been reported to induce two to three fold higher rise of plasma cortisol level and such an increased cortisol level has been linked with anxiety-like behavior\cite{6, 7}. It has been reported that stress triggers the motor alteration in different animal models, and central nucleus of amygdala is important in modulating affective response to stress\cite{8, 10-12}. Furthermore, evidence suggests that mitochondria are both producers as well as targets of ROS which increases oxidative damage\cite{13}. As a consequence, damaged mitochondria progressively become less efficient, lose their functional integrity and release more reactive oxygen molecules\cite{13}. Increasing oxidative burden deteriorates functional mitochondria during aging. Mitochondria are the major source of energy or adenosine triphosphate (ATP) for the normal functioning of eukaryotic cells. Dysfunction of mitochondria is well known to generate ROS, reduce mitochondrial ATP production, increase mitochondrial DNA mutations and abnormal mitochondrial cristae structures and impair intracellular calcium level\cite{13}. Increased ROS generation with compromised mitochondrial function ultimately affects neurons and accelerates neurodegenerative process\cite{13}.

Natural products such as bioflavonoids possess very good antioxidant property\cite{14, 15} and could inhibit lipid peroxidation in biological membranes\cite{16}. Naringin (4’, 5, 7-trihydroxyflavanone-7-rhamnoglycoside) is a well-known flavanone glycoside of fruits such as *Citrus paradise*, *Citrus sinensis*, *Citrus unshiu* and *Artemisia selengensis*\cite{17}, roots of *Cudrania cochinchinensis* and fruits of *Poncirus*. Naringin has been reported to possess potent antioxidant, superoxide-scavenging, antiapoptotic, antiatherogenic and metal-chelating activities\cite{18}. Orally administered naringin is metabolized to naringenin (4’, 5, 7-trihydroxyflavanone)\cite{19}, which could cross the blood-brain barrier\cite{19}. Despite many studies on the beneficial effects of naringin, its therapeutic potential as a neuroprotectant against mitochondrial dysfunction and free radical-mediated toxicity has not been well understood and it has been proved to be very effective in various neurobehavioral diseases.

With this background, the present study was designed to investigate the possible neuroprotective effect of naringin against acute IS-induced anxiety-like behavior and associated oxidative damage in mice. The functional interaction of naringin with nitricergic signaling was investigated using nitric oxide (NO) precursor, L-arginine, and non-selective NO synthase inhibitor, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME).

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1 Materials and methods

1.1 Drugs and chemicals Naringin, L-arginine and L-NAME were purchased from Sigma Aldrich Bangalore, India. All other drugs and chemicals used for the study were of analytical grade and purchased from local firms.

1.2 Experimental animals Male Swiss Albino mice weighing between 20 to 25 g were purchased from Bioneeds Preclinical Services, Bangalore, India. They were housed under standard laboratory conditions and maintained in a 12 h light/dark cycle and had free access to food and water. Animals were acclimatized to standard laboratory conditions before the experiment. All the experiments were carried out between 9:00 and 17:00. The experimental protocols were approved by the Institutional Animal Ethics Committee of People’s Education Society College of Pharmacy, Bangalore, India (No. PESC/IAEC/06/2006-07) and conducted according to the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) Guidelines for the use and care of experimental animals.

1.3 Treatment schedule Male Swiss Albino mice were randomly divided into 10 groups (G1-G10) based on body weight (n = 10). G1 is normal control group without any treatment and without IS; G2 is IS control treated with 0.5% carboxy methyl cellulose (CMC); G3 and G4 were treated with naringin 50 and 100 mg/kg, respectively; G5 and G6 were treated with L-arginine (50 mg/kg, intraperitoneally (i.p.)) and L-NAME (5 mg/kg, i.p.), respectively; G7 and G8 were treated with combination of L-arginine with naringin 50 and 100 mg/kg, respectively; G9 and G10 were treated with combination of L-NAME with naringin 50 and 100 mg/kg, respectively. The suspension of naringin in 0.5% CMC in distilled water and L-NAME and L-arginine in normal saline were freshly prepared and administered 10 min before naringin treatment (50 and 100 mg/kg, per oral (p.o.)) for two weeks and animals were subjected to IS on the 15th day.

1.4 IS induction On the 15th day, all animals (except mice in G1) were immobilized for 6 h by taping all four limbs on a board, after putting them on their backs using zinc oxide hospital tape. Release was affected by unraveling the tape after moistening with acetone in order to minimize pain or discomfort. In G1, the mice were kept in animal cage with soft bedding in the experimental room.

1.5 Behavioral assessment

1.5.1 Measurement of locomotor activity Animals were kept in actophotometer for 3 min for acclimatization, followed by 5 min of actual recording. The apparatus was placed in a dark, light-sound-attenuated and ventilated testing room. Each animal was observed over a period of 5 min in a square (30 cm²) closed arena equipped with infrared light-sensitive photocells using digital photoactometer and values were expressed as counts per 5 min².

1.5.2 Measurement of anxiety

1.5.2.1 Mirror chamber test The mirror chamber consisted of a wooden chamber having a mirror cube enclosed within it. Animal was placed at the distal corner of the mirror chamber at the beginning of the test. During the 5-minute test session, following parameters were noted, namely, the latency to enter into the mirror chamber, and the average time spent per entry in the mirror chamber. An anxiogenic response was defined as decreased number of entries and time spent in the mirror chamber.

1.5.2.2 Elevated plus-maze test The elevated plus-maze test was described elsewhere. The apparatus comprised of two open arms (35 cm × 5 cm) and two closed arms (50 cm × 5 cm × 15 cm) that extended from a common central platform (5 cm × 5 cm). The floor and walls of the closed arms were wooden and painted black. The entire maze was elevated to a height of 50 cm above the floor level. Mice were housed in pair for 10 d prior to testing in the apparatus. During this time animals were handled by the investigator on alternate days to induce stress. On the 15th day after IS, each mouse was placed in the center of the maze facing one of the open arms. During a 5-minute test period the following parameters were recorded: the number of entries into the arm, time spent in the open and the closed arms and time spent in the central zone and the rear area. The procedure was conducted preferably in a sound-attenuated room.

1.6 Biochemical parameters All the animals were sacrificed by decapitation on the same day immediately after behavioral assessment. The brains were removed, rinsed in isotonic saline and weighed. A 10% (weight volume ratio) tissue homogenate was prepared with 0.1 mol/L phosphate buffer (pH 7.4). The post nuclear fractions were obtained by centrifugation of the homogenate at 12 000 × g for 20 min at 4 °C.

1.6.1 Lipid peroxidation assay The quantitative measurement of lipid peroxidation in the whole brain was assessed as per method of Wills. The amount of malondialdehyde (MDA) formed was measured by the reaction with thiobarbituric acid at 532 nm using Perkin-Elmer Lambda 20 spectrophotometer. The results were expressed as nanomole of MDA per milligram protein using the molar extinction coefficient of chromophore (1.56×10^5 M⁻¹ cm⁻¹).

1.6.2 Estimation of reduced glutathione Reduced glutathione (GSH) in the brain was estimated according to the method of Ellman. One mL of homogenate was precipitated with 1.0 mL of 4% sulfosalicylic acid by keeping the mixture at 4 °C for 1 h and the samples were immediately centri-
fuged at 1 200 × g for 15 min at 4 °C. The assay mixture contains 0.1 mL of supernatant, 2.7 mL of phosphate buffer with pH 8.0 and 0.2 mL of 0.01 mol/L dithio-bis-nitrobenzoic acid (DTNB). The absorbance of the reaction product was immediately measured at 412 nm using a Perkin-Elmer Lambda 20 spectrophotometer. The results were expressed as micromole GSH per milligram protein.

1.6.3 Nitrite estimation Nitrite is the stable end product of NO in living system. Accumulation of nitrite was measured in cell-free supernatants from brain homogenates by spectrophotometer assay based on Greiss reagent (1% sulphanilamide /0.1% naphthylylendiamine dihydrochloride /2.5% phosphoric acid) and incubated at room temperature for 10 min to yield a chromophore. Absorbance was measured at 543 nm spectrophotometrically. The nitrite concentration was calculated from a standard curve using sodium nitrite as standard and expressed as micromole nitrite per milligram protein.[21]

1.6.4 Protein estimation The protein content was measured according to the method of Lowry using bovine serum albumin as a standard[68].

1.6.5 Catalase estimation Catalase activity was assayed by the method of Luck[29], wherein the breakdown of hydrogen peroxide (H2O2) was measured at 240 nm. Briefly, the assay mixture consisted of 3 mL of H2O2 in phosphate buffer and 0.05 mL of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm. The results were expressed as micromole H2O2 decomposed per minute of protein per minute.

1.7 Mitochondrial complex estimation

1.7.1 Isolation of brain mitochondria Brain mitochondria were isolated by the method of Berman and Hastings[30]. The striatum regions were homogenized in isolation buffer with ethylene glycol tetraacetic acid (EGTA) (215 mmol/L mannitol, 75 mmol/L sucrose, 0.1% bovine serum albumin, 20 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mmol/L EGTA, pH 7.2). Homogenate was centrifuged at 13 000 × g for 5 min at 4 °C. Pellet was resuspended in isolation buffer with EGTA and spun again at 13 000 × g for 5 min. The resulting supernatants were transferred to new tubes and topped off with isolation buffer with EGTA and again spun at 13 000 × g for 10 min. Pellets containing pure mitochondria were resuspended in isolation buffer without EGTA.

1.7.2 Nicotinamide adenine dinucleotide dehydrogenase activity Nicotinamide adenine dinucleotide (NADH) dehydrogenase activity was measured spectrophotometrically (UV-Pharmaspec 1700 Shimadzu, Japan) by the method of King and Howard[31]. The method involves catalytic oxidation of NADH to NAD+ with subsequent reduction of cytochrome c.

1.7.3 Succinate dehydrogenase activity Succinate dehydrogenase (SDH) activity was measured spectrophotometrically (UV-Pharmaspec 1700 Shimadzu, Japan) according to King[22]. The method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide.

1.7.4 MTT assay The method employed in the present study is based on the in vitro studies to evaluate mitochondrial redox activity through the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium salt to formazan crystals by mitochondrial respiratory chain reactions in isolated mitochondria by the method of Liu et al.[32]. The absorbance of the resulting medium was measured with an enzyme-linked immunosorbent assay reader at 580 nm wavelength.

1.7.5 Cytochrome oxidase activity Cytochrome oxidase activity was assayed according to the method of Sottocassa et al.[33].

1.8 Statistical analysis All the values were expressed as mean ± standard error of mean. The data were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey’s test. P < 0.05 was considered statistically significant.

2 Results

2.1 Behavioral assessments The normal control animals showed consistent and stable locomotor activity and no anxiety-like behavior. The 6-hour acute IS significantly reduced the locomotor activity (as indicated by decreased ambulatory movements) and induced anxiety-like behaviors (delayed latency to enter into the mirror chamber, decreased number of entries and time spent in the mirror chamber, decreased number of entries and time spent in the open arm in the elevated plus-maze test) as compared to unstressed normal control group (P < 0.05). Fourteen days pretreatment with naringin (50 and 100 mg/kg) significantly inhibited the anxiety-like behavior (shortened the latency to enter into the mirror chamber, increased average time spent in the mirror chamber, increased number of entries and time spent in the open arm in the elevated plus-maze test) and improved locomotor activity (increased ambulatory movements) as compared to the IS control animals. However, naringin treatment could not restore the performance of stressed mice to the levels of the normal control group (Figures 1 to 5).

Pretreatment with L-arginine (50 mg/kg, i.p.), an NO precursor, produced significant anxiety-like behavior on actophotometer (Figure 1), elevated plus-maze (Figures 2 and 3) and mirror chamber test (Figures 4 and 5) as compared to the normal control group (P < 0.05). In addition, L-arginine (50 mg/kg, i.p.) treatment reversed the protective effect of naringin (50 and 100 mg/kg) in all the behavioural tasks as compared to the
naringin per se ($P < 0.05$). L-NAME (5 mg/kg, i. p.) as an NO synthase inhibitor, showed significant effect on actophotometer, elevated the plus-maze and mirror chamber test indexes as compared to the IS control ($P < 0.05$). Furthermore, L-NAME (5 mg/kg, i. p.) given in combination with naringin (50 and 100 mg/kg) potentiated the protective effect of naringin (increased ambulatory movements, decreased the latency to enter into the mirror chamber, increased the number of entries and time spent in the mirror chamber, increased the number of entries and time spent in the open arm in the elevated plus-maze test) as compared to their effect per se ($P < 0.05$) (Figures 1 to 5).

2.2 Biochemical measurements The 6-hour acute IS significantly increased MDA and nitrite concentration, decreased reduced GSH and catalase activity as compared to the normal control animals. Fourteen days of pretreatment with naringin (50 and 100 mg/kg) significantly attenuated the rise in MDA and nitrite concentration and caused restoration of reduced GSH and catalase activity as compared to the IS control group ($P < 0.05$) (Table 1).

Treatment with naringin (50 and 100 mg/kg) significantly attenuated elevated lipid peroxidation and nitrite activity and restored reduced GSH and catalase activity as compared to the IS control ($P < 0.05$). Pretreatment with L-arginine (50 mg/kg, i. p.) significantly increased lipid peroxidation and nitrite levels as compared to the IS control ($P < 0.05$) (Table 1).

![Figure 1](image1)  
**Figure 1** Effects of naringin on IS-induced altered locomotor activity  
All the values are expressed as mean±SEM, $n=10$. * $P < 0.05$, vs normal control; △ $P < 0.05$, vs IS control; $\text{△△} P < 0.01$, vs L-arginine (50 mg/kg) plus naringin (50 mg/kg); $\text{△△△} P < 0.05$, vs L-arginine (50 mg/kg) plus naringin (100 mg/kg); $\text{△△△△} P < 0.05$, vs L-arginine (50 mg/kg). IS: immobilization stress; Nar: naringin; SEM: standard error of mean; L-NAME: $N^\circ$-nitro-L-arginine methyl ester.

![Figure 2](image2)  
**Figure 2** Effects of naringin on IS-induced anxiety-like behavior observed by mirror chamber test (latency to enter into the mirror chamber)  
All the values are expressed as mean±SEM, $n=10$. * $P < 0.05$, vs normal control; △ $P < 0.05$, vs IS control; $\text{△△} P < 0.01$, vs L-arginine (50 mg/kg) plus naringin (50 mg/kg); $\text{△△△} P < 0.05$, vs L-arginine (50 mg/kg) plus naringin (100 mg/kg); $\text{△△△△} P < 0.05$, vs L-arginine (50 mg/kg). IS: immobilization stress; Nar: naringin; SEM: standard error of mean; L-NAME: $N^\circ$-nitro-L-arginine methyl ester.
Figure 3  Effects of naringin on IS-induced anxiety-like behavior observed by mirror chamber test
(average time spent per entry in the mirror chamber)
All the values are expressed as mean±SEM, n=10. * P<0.05, vs normal control; △ P<0.05, vs IS control; ☆☆ P<0.01, vs L-arginine (50 mg/kg) plus naringin (50 mg/kg); ○○ P<0.05, vs L-arginine (50 mg/kg) plus naringin (100 mg/kg); ■ P<0.05, vs L-arginine (50 mg/kg). IS: immobilization stress; Nar: naringin; SEM: standard error of mean; L-NAME: N^6-nitro-L-arginine methyl ester.

Figure 4  Effects of naringin on IS-induced anxiety-like behavior observed by elevated plus-maze test (number of entries into the open arm)
All the values are expressed as mean±SEM, n=10. * P<0.05, vs normal control; △ P<0.05, vs IS control; ☆☆ P<0.01, vs L-arginine (50 mg/kg) plus naringin (50 mg/kg); ○○ P<0.05, vs L-arginine (50 mg/kg) plus naringin (100 mg/kg); ■ P<0.05, vs L-arginine (50 mg/kg). IS: immobilization stress; Nar: naringin; SEM: standard error of mean; L-NAME: N^6-nitro-L-arginine methyl ester.

Figure 5  Effects of naringin on IS-induced anxiety-like behavior observed by elevated plus-maze test (time spent in the open arm)
All the values are expressed as mean±SEM, n=10. * P<0.05, vs normal control; △ P<0.05, vs IS control; ☆☆ P<0.01, vs L-arginine (50 mg/kg) plus naringin (50 mg/kg); ○○ P<0.05, vs L-arginine (50 mg/kg) plus naringin (100 mg/kg); ■ P<0.05, vs L-arginine (50 mg/kg). IS: immobilization stress; Nar: naringin; SEM: standard error of mean; L-NAME: N^6-nitro-L-arginine methyl ester.
The combination of L-arginine (50 mg/kg, i.p.) with naringin (50 and 100 mg/kg) reversed the neuroprotective effect of naringin (50 and 100 mg/kg) on lipid peroxidation, nitrite activity, reduced GSH and catalase activity as compared to their effect per se (P < 0.05) (Table 1). L-NAME (5 mg/kg, i.p.) per se produced significant effect on oxidative stress parameters as compared to the IS control (P < 0.05). Furthermore, the combination of L-NAME (5 mg/kg, i.p.) pretreatment with naringin (50 and 100 mg/kg) caused further attenuation of lipid peroxidation and nitrite activity and restored the reduced GSH levels and catalase activity which was significant as compared to their effect per se (P < 0.05) (Table 1).

2.3 Effects of naringin on mitochondrial enzymes complexes (I, II and IV) in IS-induced intoxicated mice

There were no significant alterations in mitochondrial enzyme complexes I, II and IV and mitochondrial redox activity of the normal control group. However, 6 h of IS significantly impaired mitochondrial complex I (NADH dehydrogenase), II (succinate dehydrogenase) and IV (cytochrome c oxidase) enzyme activity as well as mitochondrial redox activity in the IS control group as compared to the normal control group (P < 0.05) (Table 2). Pretreatment with naringin (100 mg/kg) for 14 d significantly restored the alterations in mitochondrial enzyme complex activities and mitochondrial redox activity as compared to the IS control group (P < 0.05). While, pretreatment with naringin (50 mg/kg) alone moderately restored the alterations in mitochondrial enzyme complex activities and mitochondrial redox activity when compared to the IS control (P < 0.05).

Pretreatment with L-arginine (50 mg/kg, i.p.) significantly increased lipid peroxidation and nitrite levels as compared to the IS control (P < 0.05). The combination of L-arginine (50 mg/kg, i.p.) with naringin (50 and 100 mg/kg) reversed the protective effect of naringin on mitochondrial enzyme complex activities and mitochondrial redox activity as compared to their effect per se (P < 0.05). L-NAME (5 mg/kg, i.p.) per se restored the normal mitochondrial functions as compared to the IS control (P < 0.05). Furthermore, the combination of L-NAME (5 mg/kg, i.p.) with naringin (50 and 100 mg/kg) caused further restoration of alterations in mitochondrial enzyme complex activities and mitochondrial redox activity which was significant compared to their effect per se (P < 0.05).

3 Discussion

Stress is a physical, environmental and psychological stimulus, that is capable of altering the physiological homeostasis of the body, and hence coping up with such stress condition makes it a crucial determinant in health and disease[61]. Stress activates hypothalamus-pituitary-adrenal axis and also causes changes in catecholamine levels and thereby influences several neurological functions at both central and peripheral levels. Any kind of stress could influence brain functions by causing long-term changes in the multiple neural systems which leads to various neurodegenerative disorders[62]. Exposure to chronic IS in animals and psychological stress in human has implicated pathophysiology of anxiety and mood disorders[63]. Acute IS has been reported to influence motor activity, anxiety-like behaviors and depression-like behaviors in the animals[64]. In the present study, the 6-hour IS caused significant anxiety-like behavior and impaired motor activity, indicating stress-induced neurobehavioral alterations due to acute and chronic stress[65]. In the present

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Reduced GSH (μmol/mg protein)</th>
<th>MDA (nmol/mg protein)</th>
<th>Catalase (μmol H₂O₂/(min-mg protein))</th>
<th>Nitrite (μmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>10</td>
<td>0.094±0.003</td>
<td>0.115±0.001</td>
<td>0.795±0.004</td>
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<td>10</td>
<td>0.012±0.005</td>
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<td>670.30±12.24</td>
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<td>Naringin (50 mg/kg)</td>
<td>10</td>
<td>0.035±0.002</td>
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<td>0.421±0.003</td>
<td>425.80±8.53</td>
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<td>Naringin (100 mg/kg)</td>
<td>10</td>
<td>0.051±0.003</td>
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<td>362.50±7.92</td>
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<td>L-arginine (50 mg/kg)</td>
<td>10</td>
<td>0.011±0.004</td>
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<td>0.125±0.001</td>
<td>683.40±15.41</td>
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<tr>
<td>L-NAME (5 mg/kg)</td>
<td>10</td>
<td>0.062±0.001</td>
<td>0.276±0.002</td>
<td>0.569±0.003</td>
<td>342.51±12.41</td>
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<td>L-arginine (50 mg/kg) plus naringin (50 mg/kg)</td>
<td>10</td>
<td>0.014±0.002</td>
<td>0.597±0.004</td>
<td>0.135±0.002</td>
<td>668.20±14.56</td>
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<tr>
<td>L-arginine (50 mg/kg) plus naringin (100 mg/kg)</td>
<td>10</td>
<td>0.018±0.003</td>
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<td>0.142±0.002</td>
<td>657.60±11.25</td>
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<tr>
<td>L-NAME (5 mg/kg) plus naringin (50 mg/kg)</td>
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<td>0.064±0.002</td>
<td>0.391±0.005</td>
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<td>467.60±13.65</td>
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<tr>
<td>L-NAME (5 mg/kg) plus naringin (100 mg/kg)</td>
<td>10</td>
<td>0.075±0.003</td>
<td>0.189±0.003</td>
<td>0.658±0.004</td>
<td>433.90±15.81</td>
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* P<0.05 vs normal control; $P<0.05$ vs IS control; $P<0.01$ vs L-arginine (50 mg/kg) plus naringin (100 mg/kg); 0.05 P<0.05 vs L-arginine (50 mg/kg) plus naringin (100 mg/kg); $P<0.05$ vs L-arginine (50 mg/kg); IS: immobilization stress; SEM: standard error of mean; L-NAME: N^2^-nitro-L-arginine methyl ester; GSH: glutathione; MDA: malondialdehyde.
Table 2 Effects of naringin on IS-induced mitochondrial dysfunction in mice (mean±SEM, %)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Complex I</th>
<th>Complex II</th>
<th>MTT assay</th>
<th>Complex IV</th>
</tr>
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<tbody>
<tr>
<td>Normal control</td>
<td>10</td>
<td>100.00±4.63</td>
<td>100.00±5.13</td>
<td>100.00±5.35</td>
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<tr>
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<td>10</td>
<td>64.65±2.56*</td>
<td>52.25±4.62*</td>
<td>64.52±6.31*</td>
<td>49.25±7.21*</td>
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<tr>
<td>Naringin (50 mg/kg)</td>
<td>10</td>
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<td>75.44±6.32△</td>
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<tr>
<td>L-arginine (50 mg/kg)</td>
<td>10</td>
<td>59.98±4.74*</td>
<td>48.51±3.31*</td>
<td>62.29±4.84*</td>
<td>51.86±3.78*</td>
</tr>
<tr>
<td>L-NAMES (5 mg/kg) *</td>
<td>10</td>
<td>105.26±5.81▲▲</td>
<td>104.30±7.23▲▲</td>
<td>103.58±9.16▲▲</td>
<td>101.45±6.16▲▲</td>
</tr>
<tr>
<td>L-arginine (50 mg/kg)</td>
<td>10</td>
<td>61.85±5.74*</td>
<td>50.24±4.32*</td>
<td>63.74±3.96*</td>
<td>52.75±4.75*</td>
</tr>
<tr>
<td>plus naringin (50 mg/kg)</td>
<td>10</td>
<td>63.74±3.61*</td>
<td>53.45±5.21*</td>
<td>66.58±4.87*</td>
<td>54.54±3.56*</td>
</tr>
<tr>
<td>L-NAMES (5 mg/kg)</td>
<td>10</td>
<td>105.56±4.81▲▲</td>
<td>106.30±7.23▲▲</td>
<td>105.64±9.16▲▲</td>
<td>106.86±7.21▲▲</td>
</tr>
<tr>
<td>plus naringin (50 mg/kg)</td>
<td>10</td>
<td>110.31±6.22▲▲</td>
<td>112.40±6.19▲▲</td>
<td>114.74±8.45▲▲</td>
<td>115.28±9.27▲▲</td>
</tr>
</tbody>
</table>

Data are expressed as the percentage of the normal control group. *P<0.05, vs normal control; △P<0.05, vs IS control; ▲▲P<0.01, vs L-arginine (50 mg/kg) plus naringin (50 mg/kg); ▲P<0.05, vs L-NAMES (5 mg/kg) plus naringin (100 mg/kg); ■P<0.05, vs L-arginine (50 mg/kg). IS: immobilization stress; SEM: standard error of mean; L-NAME: N^6-nitro-L-arginine methyl ester; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

study, observed behavioral changes might be due to alteration in the brain regions which control the motor activity and anxiety-like behavior, while impaired motor activity could be due to stress-induced depression.

In the present study, naringin (50 and 100 mg/kg, p.o.) offered significant protection against IS-induced neurobehavioral alterations and oxidative damage to the brain, suggesting its neuroprotective effect against stressful conditions.

IS causes drastic increase in the production of ROS and consequent oxidative damage with a proportionate decrease in in vivo antioxidant defense. Oxidative stress causes cellular damage and accelerates neuro-degeneration by inducing the ROS that oxidize vital cellular components such as lipids, proteins and DNA. In the present study, 6-hour IS caused significant oxidative damage as indicated by raise in lipid peroxidation and nitrite concentration and depleted reduced GSH and catalase activity. Pretreatment with naringin (50 and 100 mg/kg), significantly attenuated the lipid peroxidation and nitrite concentration and restored GSH and catalase activity, suggesting its antioxidant effect. Clinical trial also indicated increased level of MDA in patients with affective disorders. Besides, it is demonstrated that IS may induce expression of the inducible isofrm of NO synthase (NOS-2) in rat brain, and its inhibition may protect the stress-induced cell damage in this model, which leads to up-regulation of NOS expression in the brain of the stressed animals. Formation of large amount of nitrogen reactive species may account for the oxidation of cellular components.

In accordance with the above findings, in the present study, naringin elevated the antioxidant enzyme defense system against IS-induced oxidative damage. The possible mechanism behind the protective effect of naringin may be the inhibition of NO synthesis in the brain.

Mitochondria are considered as the major source of ROS, which include superoxide anion, H_2O_2, and the hydroxyl free radical. The evidence suggests that, oxidative stress-induced damage to mitochondria is a major cause of brain cell death. Damage in various mitochondrial complexes was measured in terms of respiratory enzyme oxidative properties and our findings in the present study are in accordance with the literature reports.

Naringin (50 and 100 mg/kg) pretreatment significantly restored the mitochondrial enzyme activity, suggesting its role in mitochondrial enzyme functions. Further, L-arginine pretreatment with naringin (50 and 100 mg/kg) caused reversal of naringin’s protective effect (impairment of mitochondrial enzyme complexes), while L-NAME pretreatment with naringin caused potentiation of its protective effect which further confirms the involvement of NO mechanism.

4 Conclusion

The findings of the present study suggest that, naringin (50 and 100 mg/kg) as a bioflavonoid is found to ameliorate the IS-induced oxidative-nitricergic stress, biochemical alterations, mitochondrial dysfunction and associated neurobehavioral alterations in mice and also suggest the involvement of nitricergic pathway in the protective effect of naringin against IS-induced neurobehavioral alterations.

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6 Declaration of interest

The authors report no declarations of interest.
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柚皮苷改善束缚性应激引起的小鼠生化指标及行为变化的一氧化氮调节机制

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目的：研究柚皮苷对束缚性应激引起的小鼠生化指标改变、行为变化及线粒体功能紊乱的影响。

方法：将小鼠按照体重随机分组，分别给予不同药物治疗14 d。在第15天让所有小鼠接受束缚性应激刺激6 h，然后在接受各种不同的行为测试后处死。取小鼠的大脑匀浆进行各种生化指标测量和线粒体功能分析。

结果：6 h的急性束缚性应激刺激能显著改变小鼠的行为（焦虑和记忆），引起生化指标变化和线粒体功能紊乱。用柚皮苷预处理（50或100 mg/kg 腹腔注射）14 d则显著减轻急性束缚性应激刺激引起的鼠行为和生化指标变化以及线粒体功能紊乱（P<0.05）；而使用一氧化氮的前体左旋精氨酸酶预处理（50 mg/kg腹腔注射）可以拮抗柚皮苷的保护效果（P<0.05）。此外，采用左旋精氨酸甲酰酶预处理（5 mg/kg 腹腔注射）则导致柚皮苷的保护效果增强。

结论：柚皮苷对束缚性应激刺激引起的小鼠行为和生化指标变化以及线粒体功能紊乱的保护作用与氮能神经通路有关。

关键词：柚皮苷；约束，身体的；应激；一氧化氮；脂质过氧化作用