Evaluation of antioxidant effect of Nerium indicum in anxious rats

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Abstract:
Aim: The aim of this study was to analyze the ethyl acetate extract of Nerium indicum (NIE) flower for its antioxidant effect in anxious Sprague–Dawley rats.

Materials and Methods: Animals were divided into six groups (n = 6) and treated with 200 mg/kg and 400 mg/kg p.o. of NIE for 21 days to assess its preventive and curative effects. Anxiety was induced by isolating animals socially for 21 days. Elevated plus maze (EPM) and light and dark model were used for measuring anxiety in animals. Oxidative stress parameters such as lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) in blood and brain tissue homogenate were monitored after 21 days of social isolation in animals.

Results: Rats were treated with NIE 200 mg/kg and 400 mg/kg p.o. Both the treatments showed a significant (P < 0.001) increase in the number of open arm entries and time spent in open arm in EPM when compared with the negative control. Results also demonstrated that there was a significant (P < 0.001) increase in the number of lightbox entries and time spent in light box in light and dark model when compared with negative control. There was a significant (P < 0.001) improvement in endogenous anti-oxidants such as SOD, CAT, reduced GSH, and decreased levels of LPO in blood and brain tissue when compared with the negative control.

Conclusion: The present study suggests the role of NIE in the treatment of anxiety, possibly by modulating the oxidative stress.

Key words: Antioxidant, anxiolytics, kaner, Nerium indicum, oxidative stress

Anxiety is the most prevalent psychiatric disorder.[1] It is generally concerned with depression, obsessive-compulsive disorder, generalized anxiety disorder (GAD), panic attacks, phobias, and post-traumatic stress disorder.[2] It is the most common neuropsychiatric disorder in many countries including the USA.[3,4] The prevalence of anxiety is very high in India, i.e. 8.5%, which is about 1% from 7% to 9% of worldwide occurrence.[5] Nearly, 16.6% of worldwide population is affected by anxiety disorder.[6] GAD is the most common anxiety disorder, but not severe than panic disorder and coexists with mental disorder. The anxiety ratio of female to male is about 2:1. Gamma-aminobutyric acid, serotonergic, noradrenergic, dopaminergic, and histaminergic systems are concerned with anxiety. Diazepam (DZM) is most frequently used in the treatment of anxiety, but it has many problems such as occurrence of tolerance, dependence, impaired alertness, and cognition. Withdrawal symptoms also occur after the cessation of DZM. Nerium indicum plant belongs to the family Apocynaceae has been reported with a broad spectrum of activities. Seed, bark, and root of this plant contain cardiac glycosides. It is reported for antibacterial, antileprotic, anticancer, and cardiotonic activities, skin disease, epilepsy, and central nervous system depression,[2,7] but no data is available for its anti-anxiety activity.

Materials and Methods

Plant Extract
Flowers of N. indicum were collected from the local area of Vidharbha region and authenticated from the Department of Botany, Amravati University, Amravati. Flowers were dried in shade and coarsely powdered weighing 1 kg was extracted with ethyl acetate by maceration process for 7 days. The extract of N. indicum flower (NIE) was found to be 10.7 g with percentage practical yield of 1.070% w/w.
Animal
Healthy male Sprague–Dawley rats (200–250 g) were used for the pharmacological screening. The animals were housed in polypropylene cages with wire mesh top and husk bedding and maintained under standard environmental conditions (25°C ± 2°C, relative humidity 60% ± 5%, light-dark cycle of 12 h each) and fed with standard pellet diet (Trimurti feeds, Nagpur) and water ad libitum for the entire animal study. The experiments were performed during day (08:00–16:00 h). Animals were housed and treated according to the rules and regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and Institutional Animal Ethics Committee (IAEC). The protocol for all the animal studies was approved by the IAEC with research project number 650/02/c/CPCSEA/04/2009.

Experimental
Animals were grouped into six groups of six animals each, as follows:
1. Control group: Animals were not subjected for social isolation and not treated with any drug
2. Negative control group: Animals were subjected for 21 days isolation in dark room without any treatment
3. Low dose group (NIE 200 mg/kg p.o.): Animals were subjected to social isolation and treated with 200 mg/kg of *N. indicum* extract once daily p.o.
4. High dose group (NIE 400 mg/kg p.o.): Animals were subjected for social isolation and treated with 400 mg/kg of *N. indicum* extract once daily p.o.
5. DZM group: Animals were subjected for social isolation and treated with DZM (2 mg/kg) alone i.p. before 1 h prior to the experiment
6. VE Group: Animals were subjected for social isolation and treated with Vitamin E (100 mg/kg) alone p.o. once daily for 21 days.

Behavioral and oxidative stress parameters (in blood and tissue) were studied in socially isolated rats and normal rats (social isolation for 21 days). After 21 days of social isolation, behavioral study was carried out in animals using various behavioral models such as elevated plus maze (EPM) and light and dark model.

Behavioral Study
**Elevated plus maze**
Three main parameters were studied, namely, percent number of open arm entries, time spent in open arms, and percent number of closed arm entries. Increase in percent number of open arm entries and time spent in open arms indicated a reduction of anxious behavior.[9]

**Light and dark model**
This model consists of two areas, both connected through a small opening. One area is small, black, nonilluminated and other is larger, white, and brightly light. Number of entries and time spent in the light compartment were used for the estimation of anxiety.[10]

**Determination of Oxidative Stress Parameters in Blood and Tissue**
**Blood sample preparation**
Suspension of red blood cells (RBCs, 5%) was prepared by adding phosphate buffer saline (PBS) (8 ml) to packed cells. About 0.5 ml of 5% RBC was mixed with 5 ml of distilled water, shaken for 5 min, and then kept at 4°C for 5 min. Subsequently, 0.4 ml of 3.5% chloroform–ethanol mixtures was added, shaken vigorously to precipitate hemoglobin, and then 0.15 ml of distilled water was added. The mixture was centrifuged to get a clear erythrocyte lysate.

**Tissue preparation**
After receiving the treatments for 21 days, animals were sacrificed using deep ether anesthesia. Brain was removed and thoroughly washed with ice-cooled 0.1 M PBS containing 0.1 mmol/L phenyl methanesulfonyl fluoride. This tissue was blotted dry and homogenized in 0.1 M PBS in an ice bath to prepare a 10% suspension. This suspension was then centrifuged at 16,000 × rpm for 1 h in a cooling centrifuge at 0°C. The supernatant was employed to assess the parameters of oxidative stress after estimating the protein content.[11]

Lipid peroxidation (LPO) was determined by the molar extinction coefficient of malondialdehyde (MDA) (1.56/105) and expressed in terms of nanomoles of MDA/gHb.[12] The activity of catalase (CAT) enzyme was determined in the erythrocyte lysate, as the decrease in absorbance was measured spectrophotometrically at 240 nm for 1 min.[13] The activity of superoxide dismutase (SOD) was determined spectrophotometrically in the erythrocyte lysate; increase in the absorbance was measured at 420 nm for 3 min. One unit of enzyme activity represents 50% inhibition of the rate of auto-oxidation of pyrogallol, as determined by change in absorbance/minute at 420 nm.[14] Blood-reduced glutathione (GSH) was measured by addition of 0.2 ml of whole blood to 1.8 ml of distilled water followed by 3.0 ml of precipitating mixture. It was centrifuged at 2000 rpm for 5 min, and 1 ml of supernatant was added to 1.5 ml acid; 5 ml of phosphate solution, followed by the addition of 0.5 ml of Dithionitrobenzoic, 5'-Dithiobis (2-nitrobenzoic acid) reagent. The absorbance was measured at 412 nm.[15]

**Results**
Table 1 shows that there was a significant (*P* < 0.001) increase in the closed arm entries and decrease in the open arm entries of negative control as compared to the control group. NIE 200 mg/kg p.o., NIE 400 mg/kg p.o., and DZM group show a significant (*P* < 0.001) decrease in closed arm entries and a significant (*P* < 0.001) increase in the open arm entries as compared to negative control group. There was a significant (*P* < 0.001) increase in the time spent in the closed arm and decrease in time spent in the open arm of negative control group as compared to control group. NIE 200 mg/kg p.o., NIE 400 mg/kg p.o., and DZM group show a significant (*P* < 0.001) decrease in time spent in the closed arm and a significant (*P* < 0.001) increase in the time spent in the open arm as compared to the negative control group.

Table 2 shows that there was a significant (*P* < 0.001) increase in the dark box entries of negative control as compared to control group. NIE 200 mg/kg p.o., NIE 400 mg/kg p.o., and DZM group show a significant (*P* < 0.001) decrease in dark box entries and a significant (*P* < 0.001) increase in light box entries as compared to the negative control group. There was a significant (*P* < 0.001) increase in the time spent in dark box and decrease in the time spent in the light box of negative
control as compared to control group. NIE 200 mg/kg p.o., NIE 400 mg/kg p.o., and DZM group show a significant ($P < 0.001$) decrease in time spent in dark box and a significant ($P < 0.001$) increase in the time spent in light box as compared to the negative control group.

Table 3 shows that there was a significant ($P < 0.001$) increase in the LPO and decrease in SOD, GSH, and CAT levels of negative control as compared to control group, and in NIE 200 mg/kg p.o., NIE 400 mg/kg p.o., and VE group, there was a significant ($P < 0.001$) decrease in LPO and increase in SOD, GSH, and CAT levels in blood.

Table 4 shows that there was a significant ($P < 0.001$) increase in the LPO and decrease in SOD, GSH, and CAT levels of negative control as compared to control group in brain tissue. In NIE 200 mg/kg p.o. treated group, there was a significant ($P < 0.05$) decrease in the LPO and increase in CAT level in brain tissue. In NIE 400 mg/kg p.o. and VE group, there was a significant ($P < 0.001$) decrease in the LPO and increase in SOD, GSH, and CAT levels in brain tissue.

### Table 1: Effect of Nerium indicum flower extract on anxiety by elevated plus maze test after 21 days of social isolation

| Groups          | 0 day                  | 21 days                 |
|-----------------|------------------------|-------------------------|
|                 | Number of entries in   | Number of entries in    | Time spent in closed arm (%) | 30.16±0.78 | 52.41±0.52 | 54.71±0.25 |
|                 | closed arm (%)         | open arm (%)            | Time spent in closed arm (s) | 42.50±0.64 | 50.43±1.85 |
|                 |                        |                        | Time spent in open arm (s)   | 39.50±3.00 | 49.87±1.14 |
| Control group   | 69.84±0.66             | 36.73±1.99             | 34.34±2.38 | 68.92±0.58 | 72.92±9.63a |
| Negative control group | 63.27±1.22             | 44.25±1.10             | 48.25±1.36 | 59.75±5.91** | 53.80±7.96** |
| Low dose group  | 65.66±2.59             | 34.34±1.44             | 51.31±1.84 | 61.61±15.44** | 48.39±4.44** |
| High dose group | 67.11±2.71             | 28.00±3.12             | 48.50±2.49 | 61.51±15.44** | 48.39±4.44** |
| DZM group       | 67.05±0.49             | 32.95±1.33             | 42.52±3.18 | 58.67±4.00** | 48.39±4.44** |

### Table 2: Effect of Nerium indicum flower extract on anxiety by light and dark model after 21 days of social isolation

| Groups          | 0 day                  | 21 days                 |
|-----------------|------------------------|-------------------------|
|                 | Number of entries in    | Number of entries in    | Time spent in closed arm (%) | 30.16±0.78 | 52.41±0.52 | 54.71±0.25 |
|                 | dark box (%)           | light box (%)           | Time spent in closed arm (s) | 42.50±0.64 | 50.43±1.85 |
|                 |                        |                        | Time spent in open arm (s)   | 39.50±3.00 | 49.87±1.14 |
| Control group   | 60.50±0.76             | 39.50±2.18             | 51.25±2.81 | 61.25±1.25 | 59.50±8.25 | 246.0±7.25 |
| Negative control group | 63.83±0.98             | 36.17±4.81             | 56.25±1.15 | 68.67±4.83** | 31.33±5.72* |
| Low dose group  | 62.89±0.94             | 37.11±1.35             | 50.75±3.63 | 63.92±0.75** | 30.08±1.03** |
| High dose group | 63.88±0.70             | 36.12±2.05             | 78.00±3.24 | 61.67±2.00** | 38.17±1.11** |
| DZM group       | 62.66±0.88             | 37.34±4.54             | 64.50±3.54 | 58.67±4.00** | 41.33±4.94** |

### Table 3: Effect of Nerium indicum flower extract on oxidative stress parameters in blood after 21 days of social isolation

| Groups          | 0 day                  | 21 days                 |
|-----------------|------------------------|-------------------------|
|                 | LPO (nMMDA/gHb)        | SOD U/mg protein        | CAT U/mg protein | GSH (µM/mg protein) | LPO (nMMDA/gHb) | SOD U/mg protein | CAT U/mg protein | GSH (µM/mg protein) |
| Control group   | 5.27±0.37              | 443.1±14.11             | 35.49±2.50       | 40.23±0.55          | 5.03±0.22       | 436.6±8.61       | 39.19±0.30       | 41.18±0.94          |
| Negative control group | 4.37±0.53              | 354.3±13.50             | 32.89±1.66       | 35.85±0.75          | 7.39±3.01**     | 272.3±81.15      | 25.90±6.99       | 26.23±7.62          |
| Low dose group  | 4.98±0.77              | 322.8±20.05             | 30.88±1.41       | 33.75±0.65          | 4.61±0.36**     | 346.7±19.54      | 32.64±1.75       | 31.05±2.70          |
| High dose group | 5.19±0.52              | 356.7±15.91             | 39.43±3.79       | 36.14±0.48          | 4.48±0.70**     | 376.2±19.54      | 37.95±1.48       | 33.37±2.77          |
| VE group        | 6.17±0.77              | 356.19±10.23            | 36.65±1.94       | 39.58±0.62          | 6.25±0.85**     | 417.6±11.71      | 37.42±0.76       | 38.42±1.16**        |

Discussion

The present study evaluates the anxiolytic activity of NIE by the virtue of its antioxidant potential. In this study, anxiety was induced by isolating the animals socially for 21 days. There are different models used for the induction of anxiety such as novelty-induced hyponeophagia, social interaction, and open field exploration.[16] Social isolation enhances the oxidative stress and thereby alters and causes anxiety-like behavior. Here, in the present investigation, it was observed that there was a significant increase in the oxidative stress and anxiety level in socially isolated animals. The EPM and dark and light model are most commonly used models for the study of anxiogenic or anxiolytic effect of drug in rodents.[17-19] The results of the present study showed that NIE exhibited a significant ($P < 0.001$) anxiolytic activity by an increase in the percent of entries and time spent in open arms and decrease in the percent of entries and time spent in closed arms in socially isolated animals, as compared to negative control. Whereas in dark and light test, treatment with NIE showed a significant ($P < 0.001$) increase in the number of entries and time spent...
### Table 4: Effect of *Nerium indicum* flower extract on oxidative stress in brain after 21 days of social isolation

| Groups         | LPO activity (nMMDA/g protein) | SOD activity (U/mg protein) | CAT activity (U/mg protein) | GSH activity (µM/mg protein) |
|----------------|-------------------------------|-----------------------------|----------------------------|-------------------------------|
| Normal control | 24.27±4.41                    | 134.1±9.18                  | 138.6±11.7                 | 44.92±0.81                    |
| Negative control | 38.31±3.60*                    | 83.50±1.11*                 | 53.34±2.25*                | 27.67±0.76*                   |
| Low dose group | 23.26±2.78*                    | 104.0±4.57*                 | 75.35±3.48*                | 35.67±1.05*                   |
| High dose group | 17.17±2.42**                   | 122.5±2.56**                | 94.47±1.05**               | 45.67±0.98**                  |
| VE group       | 24.09±3.58*                    | 133.3±6.53**                | 156.3±8.09**               | 42.33±1.61**                  |

*P<0.001 when compared with normal control group, *P<0.05 when compared with negative control group, **P<0.001 when compared with negative control group.* All values are shown as means±SD and n=6. SD=Standard deviation, LPO=Lipid peroxidation, CAT=Catalase, GSH=Glutathione, VE=Vitamin E

at light box when given at 200 mg/kg p.o. and 400 mg/kg p.o. doses. Literature also suggest that social isolation alters glucocorticoid production and neurotransmitter system, which induce stress.[19] Increased stress condition alters catecholamine metabolism and thereby increases free radical production.[20] It was also observed that production of reactive oxygen species is increased in anxiety, and the drug possessing antioxidant property effectively manages the anxiety-like behavior. This has enforced the research in finding antioxidants in foods and medicinal plants; as a result, approximately, 4000 antioxidants have been identified.[21] This study shows that social isolation significantly (P < 0.001) increases the LPO level, and there is a decrease in the levels of SOD, CAT, and GSH found in the blood and brain of anxious rats compared to normal rats. Whereas the treatment with NIE to anxious rats significantly (P < 0.001) decreases the LPO level and increases the levels of SOD, CAT, and GSH.

### Conclusion

The study confirms the involvement of oxidative stress in anxiety. The results suggest that NIE upregulates the activities of some antioxidant enzymes’ intracellular activities in anxiety. It also suggests that these plant products could offer protective roles against oxidative damage. The beneficial antioxidant effects of *N. indicum* could be a result of inhibition of specific pathways that are activated as a consequence of increased oxidative stress in the progression of anxiety. Therefore, antioxidant therapy may be helpful in relieving anxiety and related complications.

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### Conflicts of Interest

There are no conflicts of interest.

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