Chapter 5

Evaluation of anxiolytic effects of *Nardostachys jatamansi* in mice
Chapter 5 - Anxiolytic effects of Nardostachys jatamansi

Introduction

Anxiety and fear can be defined as the response of a subject to real or potential threats that may impair his/her homeostasis that can significantly mar the function and quality of life. This response could include physiological (increase in heart rate, blood pressure etc.), as well as behavioural (inhibition of ongoing behaviours, scanning, avoidance of the source of danger, etc.) changes. When this response gets excessive or maladaptive it is referred to as ‘pathological’ anxiety (Belzung and Griebel, 2001). Anxiety becomes a predicament only when it interferes with our daily activities. The introduction of benzodiazepines has set the stage for greater understanding of the biochemistry of anxiety. Benzodiazepines interact with the GABA\textsubscript{A} subtype of GABA receptor, primarily postsynaptically, and mediate changes in neuronal membrane potential by opening chloride ion (Cl\textsuperscript{-}) channels (Barchas and Altemus, 1999). In the present scenario, there is an upsurge in research on herbs as supplements for treating psychiatric disorders with minimal adverse effects and it focuses on elucidation of their mechanism of action.

In the present study we tried to investigate what duration of dosage of NJE (250 mg/kg) effectively alleviated anxiety in mice. Mice were orally fed with NJE for 3, 7, 14 days followed by assessment of their potential anxiolytic effects using mouse-models of anxiety viz., EPM, OFT, LDB and VCT. The brain tissue was immediately excised out for studying NJE’s probable role as a neuromodulator by analysing the brain neurotransmitters and tissue antioxidant parameters.

Materials and methods

Animals

Swiss albino mice (25-30 g) were used for the experimental study. The experimental protocols were accepted at the 14\textsuperscript{th} Institutional Animal Ethics Committee (IAEC) meeting held on 17\textsuperscript{th} November, 2009. The animals were housed in groups of eight mice per cage with conventional housing conditions as mentioned in chapter 2.

Drugs

Diazepam (1 mg/kg) was procured from M/s Ranbaxy laboratories limited, India. NJE (250 mg/kg) was prepared freshly and administered orally.
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**Experimental design**

**Anxiolysis experiments**

Mice were administered 70% ethanolic extract of *Nardostachys jatamansi* (NJE) or diazepam for 3, 7 and 14 days followed by anxiolytic experiments viz. OFT, EPM, LDB and VCT on the 3rd, 7th and 14th day. After EPM, mice were sacrificed by cervical dislocation and the brain tissue was immediately dissected out, washed with ice cold isotonic sodium chloride and drained thoroughly and stored at -80ºC until further use (Fig. 5.1).

**Fig. 5.1**

**Fig. 5.1: Schematic representation of the experimental design for anxiolysis experiments**

**Behavioural analysis**

The effect on anxiety was assessed using Open field test, Elevated plus maze, Light dark box test and Vogel’s conflict test. The changes in behaviour were recorded using the ANY - maze software, Stoelting Co., USA.

**Elevated plus maze test (EPM)**

EPM test is the commonly used behavioural paradigm to assess anxiolytic behaviour in rodents. The apparatus and the experimental set up was as described in chapter 2.
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Spontaneous locomotor activity in open field test (OFT)

Open field test is a measure of spontaneous locomotor activity by measuring the total number of line crossings/total ambulatory distance. The test was performed as described in chapter 2.

Light - dark box test

The light - dark box is partitioned into a small dark compartment (15 × 20 cm) and a large illuminated white compartment (30 × 20 cm). A small opening within the partition, 5 × 7 cm, allows mice to move freely between chambers. The latency to the dark compartment, transitions between the light and dark compartment, time spent in light compartment was recorded for 5 min (Bourin and Hascoet, 2003).

Vogel’s conflict test (VCT)

The test was performed by the method described by Vogel et al., (1971) with modifications. The test box (50 × 12 × 40 cm) was made of plexiglass and a grid floor of stainless steel and contained drinking bottle with water (Columbus, USA). An electric shock (0.4 mA, 1 s duration) was applied between the grid floor and the drinking spout. The animals received the first shock after 30 seconds of drinking. During the subsequent 3 min test period shocks were delivered every 20th lick. The number of shocks accepted was recorded. The procedure was controlled by a microcomputer. Mice were deprived of water for 48 h before the test. After the first 22 h of water deprivation each mouse was placed individually in the test box and was allowed to drink water for 10 min without an electric shock. Then, the mice were given water ad libitum in their home cage for 2 h. After another 20 h of water deprivation each mouse was placed once again into the test cage and animals which did not start to drink during the first 2 min or which did not drink continuously for 2 min were excluded from further experiment. Again animals had free access to water for 2 h in their home cage. On the third day, after another 20 h of water deprivation the test was performed.

Neurotransmitters estimation by HPLC

GABA

Reverse phase HPLC analysis was performed on a Waters 2465 RP-HPLC (Milford MA, USA) with 4.6 x 250 mm analytical column. Components were eluted
isocratically using a Waters 515 HPLC pump. A Waters 464 pulsed electrochemical detector in conjunction with a glassy carbon electrode combined with Ag/AgCl reference electrode was operated at 0.8 V. The mobile phase consisted of 0.1 M monosodium phosphate, 0.1 mM EDTA, 40 % methanol and the pH was adjusted to 4.6 with 1M phosphoric acid. The mobile phase was filtered twice through 0.22 µm hydrophilic polypropylene membrane filters and degassed for 20 min before using (Rowley et al., 1995). The derivatizing agent was made according to Smith and Sharp (1994). Briefly, 22 mg of o-phthalaldehyde was dissolved in 500 µL of ethanol absolute followed by addition of 500 µL of 1M sodium sulfite and 900 µL of 0.1 M tetraborate buffer and pH adjusted to 10.4 with 5 M sodium hydroxide. The reagent was prepared freshly everyday and stored at 4 °C. Standard GABA was prepared at 100 nmol/L and stored at 4 °C. The derivatization process was allowed to proceed between the derivatizing solution and 1mL of standard solution (GABA) or sample at room temperature for 10 min before injection into HPLC.

**Monoamine neurotransmitters and their metabolites**

Monoamine neurotransmitters viz. 5-hydroxytryptamine (5-HT) or serotonin, norepinephrine (NE) and dopamine (DA) levels and their metabolites viz 5-hydroxyindoleacetic acid (5-HIAA) and 3, 4-dihydroxyphenylacetic acid (DOPAC) in mouse brain were estimated by RP-HPLC coupled to an electrochemical detector (Alburges et al., 1993). The brain tissue (100 mg) was homogenised in an ice-cold solution of 0.4 M perchloric acid containing 5 mM sodium bisulfite and 0.04 mM EDTA for avoiding oxidation and then centrifuged at 30,000 x g for 15 min at 4 °C. The mobile phase consisted of 17.6 % methanol (v/v) and 82.4 % distilled water containing 0.0876 mM EDTA disodium, 1.512 mM triethylamine, 9 mM DL-10-camphorsulfonic acid, 20 mM Na₂HPO₄·12H₂O and 15 mM citrate at a flow rate of 0.5 mL/min. The estimations were done at electrode potentials of a glassy carbon electrode +650 mV vs Ag/AgCl reference electrode with Waters 1645 electrochemical detector. 5-HT, NA, DA, 5-HIAA and DOPAC and were identified and quantified by comparing their retention time and peak areas to those of standards. The concentrations of 5 HT, NA, DA, 5-HIAA and DOPAC were expressed in ng/g wet brain tissue. Standard monoamines were prepared as 1mg/mL stock solutions in 0.4 M perchloric acid.
Brain antioxidant status

Reduced glutathione

Reduced glutathione (GSH) was estimated according the method of Ellman, 1959. Briefly, to an equal volume of homogenate, 10% TCA was added, mixed and centrifuged at 8000 x g for 20 min to separate the proteins. To 500 µL of this supernatant, 2 mL of disodium hydrogen phosphate (0.3 M) was added followed by 250 µL of DTNB (di-thio bis nitro benzoic acid) were added and mixed properly. The yellow colour developed was read at 412 nm. The result was calculated from the standard curve and expressed as µM GSH/mg protein.

Glutathione Peroxidase

Glutathione peroxidase was assayed according to Glutathione peroxidase kit (Ransel Cat # RS505), Randox Laboratories Ltd, UK. The methodology was based on Paglia and Valentine, 1967. GPx catalyses the oxidation of GSH (4 mmol/L) by cumene hydroperoxide (4.3 mmol/L). In the presence of GR (≥ 0.5 U/L) and NADPH (0.34 mmol/L), the oxidised GSH (GSSG) is immediately converted to reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance is measured at 340 nm. The results are expressed as U/mg protein and one unit converts one µmol of NADPH to NADP⁺ in one minute.

Glutathione Reductase

Glutathione reductase (GR) activity was measured according to Glutathione reductase assay kit (Item # 703202), Cayman chemical company, MI, USA. GR activity is assayed by measuring the rate of NADPH oxidation which is accompanied by a decrease in absorbance at 340 nm, which is directly proportional to the GR activity of the sample. One unit is defined as the amount of enzyme that oxidises 1.0 nmol of NADPH to NADP⁺ per minute at 25 ºC. The reaction rate is determined using the NADPH extinction coefficient of 0.00373 µM⁻¹ and the GR activity is represented as nmol/min/mg protein.

Glutathione S-transferase (GST)

GST activity was measured according to the method of Habig et al., 1974. Briefly, to 1 mL of phosphate buffer (0.3 M, pH 6.5), 0.1 mL of CDNB (1-chloro-2, 4-dinitrobenzene; 30 mM) and 0.1 mL of homogenate (enzyme) was added and the
volume was adjusted to 2.9 mL with water. After incubation for 5 min at 37 °C, the reaction was started by addition of 0.1 mL of 30 mM reduced glutathione and the increase in absorbance at 340 nm was measured for 5 min in a spectrophotometer. The results were expressed as µmol of CDNB-GSH conjugate formed/min/mg protein.

**Superoxide Dismutase**

Superoxide dismutase activity was analysed using Superoxide dismutase assay kit (Item # 706002), Cayman chemical company, MI, USA. The kit uses a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50 % dismutation of superoxide radical. The absorbance is measured at 440 nm and the results are expressed as U/mg protein.

**Catalase**

Briefly, to 3 mL of H\textsubscript{2}O\textsubscript{2} in phosphate buffer (1.25 × 10\textsuperscript{-2} M H\textsubscript{2}O\textsubscript{2}), 50 µL of homogenate was added and the change in absorbance was measured at 240 nm. Catalase enzyme activity was calculated using the extinction coefficient of H\textsubscript{2}O\textsubscript{2} (43.6 M\textsuperscript{-1} cm\textsuperscript{-1}). The results were expressed as µM H\textsubscript{2}O\textsubscript{2} degraded/min/mg protein (Luck, 1965).

**Acetyl choline esterase activity**

Acetyl choline esterase activity was assayed following the method of Ellman *et al.*, 1961 with minor modifications. Briefly, to 100 µL of homogenate, phosphate buffer was added (0.1 mM, pH 8.0) followed by addition of DTNB (0.4 mg/mL with 1 % sodium citrate) and 20 µL of substrate (acetylthiocholine iodide) and changes in absorbance were recorded and the change in absorbance per minute was calculated.

**Protein estimation**

The amount of protein in the samples was estimated according to the method of Lowry *et al.*, 1951. Briefly, different concentrations of sample or standards were added to 5 mL of Lowry’s reagent comprising of 2 % Na\textsubscript{2}CO\textsubscript{3} in 0.1 M NaOH, incubated for 10 min, followed by addition if 1:1 diluted Folin-Ciocalteau reagent. This mixture was incubated for 30 min before its absorbance was recorded at 660 nm. The protein content of the sample was calculated by comparing with standards.
Statistical analysis

All the results obtained were expressed as mean ± SD (n=8). Data was evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s Honestly Significant Difference Post hoc test using SPSS 16.0 software. $p$ value less than 0.05 was considered significant.

Results

Anxiolysis experiments

OFT

The behavioural parameter measured in OFT was the number of line crossings. NJE-7 and diazepam (1 mg/kg) showed maximum effect with increased locomotor activity in the OFT apparatus measured as the number of line crossings made, with significantly higher time spent in the central zone of the apparatus. NJE-3 and NJE-14 also showed significant results ($p < 0.05$) but, no significant difference ($p > 0.05$) was observed between NJE-7 and NJE-14 Post hoc test showed a significant difference between control vs all the groups ($p < 0.05$). (Fig. 5.2).

Fig. 5.2

![Fig. 5.2: Effects of administration of NJE (250 mg/kg) for 3, 7 and 14 days and diazepam (1 mg/kg) on the behaviour of mice in open field test. * $p < 0.05$ versus control.](image)

EPM

A widely used behavioural assay for assessing anti-anxiety effects of pharmacological agents (Walf and Frye, 2011) is the EPM test. One-way ANOVA revealed significant differences between the groups with respect to latency to enter the
closed arm \( [F (4, 35) = 328.4, p < 0.05] \), total number of transitions between the closed and open arm \( [F (4, 35) = 263.04, p < 0.05] \), time in centre zone \( [F (4, 35) = 161.9, p < 0.05] \), and number of dips into open \( [F (4, 35) = 170.8, p < 0.05] \) and closed arm \( [F (4, 35) = 56.8, p < 0.05] \) and the time spent in open arms \( [F (4, 35) = 202.7, p < 0.05] \).

Mice pre-treated with NJE for 7 days and diazepam (1mg/kg) took significantly more time to enter the closed arm, were more active with increased locomotor activity measured as the number of transitions between open and closed arm, spent significantly less time on the central zone \( (p < 0.05) \) of the apparatus in comparison to control and made more dips into the open arms, but, made significantly lesser number of dips into the closed arm. Mice pre-treated with NJE for 3 and 14 days also showed significant results \( (p < 0.05) \) but NJE-7 was the most effective with results comparable to diazepam (Fig. 5.3 A, B, C, D and E).

**Fig. 5.3**

![Graph A](image1.png)  
**A** Latency to enter closed arm (seconds)  
- Control  
- NJE-3  
- NJE-7  
- NJE-14  
- Dzp  

![Graph B](image2.png)  
**B** Total number of transitions between compartments  
- Control  
- NJE-3  
- NJE-7  
- NJE-14  
- Dzp
Fig. 5.3: Effects of administration of NJE (250 mg/kg) and diazepam (1 mg/kg) for 3, 7 and 14 days on the behaviour of mice in elevated plus maze test. (A) Latency to enter the closed arm (B) Total number of transitions between open and closed compartments (C) Time spent by mice on the central zone (D) Number of dips into open and closed arm (E) Time spent in open arm zone. *p < 0.05 versus control.
LDB

LDB test kindles the natural conflict between the tendency to explore and innate tendency to avoid the unfamiliar (neophobia) (Bourin and Hascoet, 2003). Mice pre-treated with NJE-7 showed significantly ($p < 0.05$) increased latency to enter the dark compartment, were more active and showed more locomotion observed as increased number of transitions between the light and dark compartments, and spent significantly greater time in the lit compartment, with results similar to diazepam (1 mg/kg). NJE-3 and NJE-14 also showed significantly ($p < 0.05$) better results in comparison to control. NJE-14 treated mice, however, showed less number of transitions between the compartments may be because this dose was sedating the mice (Fig. 5.4 A, B and C).

Fig. 5.4
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Fig. 5.4: Effects of administration of NJE (250 mg/kg) and diazepam (1 mg/kg) for 3, 7 and 14 days on the behaviour of mice in light dark box test. (A) Latency to enter the dark compartment (B) Total number of transitions between light and dark compartments (C) Time spent by mice in the lit compartment. * $p < 0.05$ versus control.

VCT

One-way ANOVA revealed significant difference between the groups with respect to the number of licks made [$F (4, 35) = 108.2, p < 0.05$] and the subsequent shocks accepted [$F (4, 35) = 147.1, p < 0.05$] by mice. Mice treated with NJE-7 significantly increased the number of licks and subsequently accepted shocks. Similarly, diazepam (1 mg/kg) also increased the number of punished licks and accepted more shocks. Post-hoc test showed a significant difference between NJE-7 vs NJE-3 and 14, but no difference was observed between NJE-3 and NJE-14 (Fig. 5.5 A and B).

Fig. 5.5
Effect of NJE on whole brain monoamines, monoamine metabolites and GABA levels

The effects of oral administration of NJE on whole brain monoamine neurotransmitters (serotonin, dopamine, norepinephrine), monoamine metabolites (DOAPC and 5HIAA) and GABA levels are shown in Table 5.1 and Fig. 5.6 respectively. Significant increase in brain GABA levels was observed after 7 day treatment ($p < 0.05$) comparable to diazepam. Serotonin, dopamine, norepinephrine levels were also elevated ($p < 0.05$) in the NJE treated groups with the 7 day treatment showing a significant augmentation in monoamine neurotransmitter levels. The 5-HT metabolite, 5-HIAA levels were higher in the control group, with a lower 5-HIAA/5-HT ratio in NJE and diazepam groups. Dopamine metabolite, DOPAC showed lower levels in NJE treated groups in comparison to control.
Table 5.1: Effects of administration of NJE (250 mg/kg) and diazepam (1 mg/kg) on the brain monoamines and monoamine metabolite levels. *p < 0.05 versus control

<table>
<thead>
<tr>
<th></th>
<th>Serotonin</th>
<th>Norepinephrine</th>
<th>Dopamine</th>
<th>5HIAA</th>
<th>DOPAC</th>
<th>5HIAA/5HT ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>751±42</td>
<td>542±29</td>
<td>799±41</td>
<td>325±18</td>
<td>591±33</td>
<td>0.413±0.029</td>
</tr>
<tr>
<td><strong>NJE-3</strong></td>
<td>765±47</td>
<td>606±32*</td>
<td>824±45*</td>
<td>314±17</td>
<td>429±27*</td>
<td>0.352±0.019*</td>
</tr>
<tr>
<td><strong>NJE-7</strong></td>
<td>801±51*</td>
<td>646±34*</td>
<td>896±47*</td>
<td>292±13*</td>
<td>397±21*</td>
<td>0.311±0.017*</td>
</tr>
<tr>
<td><strong>NJE-14</strong></td>
<td>778±49*</td>
<td>622±33*</td>
<td>887±51*</td>
<td>310±20</td>
<td>408±19*</td>
<td>0.340±0.021*</td>
</tr>
<tr>
<td><strong>Dzp</strong></td>
<td>816±53*</td>
<td>636±30*</td>
<td>907±58*</td>
<td>305±21</td>
<td>403±17*</td>
<td>0.309±0.016*</td>
</tr>
</tbody>
</table>

Brain antioxidant status

Glutathione, a principal tripeptide thiol, is involved in antioxidant cellular defense (Balendiran et al., 2004). GSH levels depleted in control mice were elevated in NJE treated groups. NJE-7 and diazepam (1 mg/kg) showed significantly (p < 0.05) higher GSH levels. The levels of GSH enzymes viz., glutathione peroxidase, glutathione reductase and glutathione s-transferase were also significantly elevated (p
< 0.05) in NJE fed mice in comparison to control. Catalase and superoxide dismutase, endogenous antioxidant enzymes which can neutralize free radicals also showed elevated levels in NJE fed mice. Diazepam also showed significantly ($p < 0.05$) elevated levels with respect to these two enzymes (Table 5.2). Acetyl choline esterase, a hydrolase, catalyses the hydrolysis of acetylcholine a key neurotransmitter of the CNS. The levels of acetyl choline esterase were modulated in NJE treated mice in comparison to diazepam (Fig. 5.7).

**Table 5.2: Antioxidant status of the brain after oral administration of NJE (250 mg/kg) and diazepam (1 mg/kg).** * $p < 0.05$ versus control

<table>
<thead>
<tr>
<th></th>
<th>Glutathione (µmol/mg protein)</th>
<th>Glutathione Reductase (nmol/min/mg protein)</th>
<th>Glutathione peroxidase (U/mg protein)</th>
<th>Glutathione-s-transferase (µmol CDNB -GSH/min/mg protein)</th>
<th>Catalase (mM H$_2$O$_2$ degraded/min/mg protein)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.57±0.18</td>
<td>2.90±0.13</td>
<td>0.127±0.0035</td>
<td>32.84±1.09</td>
<td>13.62±0.63</td>
<td>8.11±0.53</td>
</tr>
<tr>
<td>NJE-3</td>
<td>6.07±0.21*</td>
<td>3.34±0.19*</td>
<td>0.133±0.0050</td>
<td>34.82±1.10</td>
<td>14.83±0.72*</td>
<td>9.11±0.42*</td>
</tr>
<tr>
<td>NJE-7</td>
<td>7.13±0.19*</td>
<td>3.51±0.21*</td>
<td>0.167±0.0041</td>
<td>37.19±1.10*</td>
<td>19.10±0.66*</td>
<td>13.99±0.35*</td>
</tr>
<tr>
<td>NJE-14</td>
<td>6.66±0.21*</td>
<td>3.41±0.22*</td>
<td>0.142±0.0035</td>
<td>35.43±1.20*</td>
<td>16.34±0.73*</td>
<td>11.81±0.35*</td>
</tr>
<tr>
<td>Dzp</td>
<td>6.99±0.18*</td>
<td>3.44±0.25*</td>
<td>0.162±0.0035</td>
<td>36.20±1.16*</td>
<td>18.06±0.65*</td>
<td>12.85±0.39*</td>
</tr>
</tbody>
</table>

**Fig. 5.7**

**Fig. 5.7: Acetyl choline esterase levels of brain after oral administration of NJE (250 mg/kg) and diazepam (1 mg/kg).** * $p < 0.05$ versus control.
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Discussion

The results of the present study confirm the anxiolytic-like effects of NJE with seven day oral treatment and this dosage also caused an increase in brain monoamine and GABA levels and modulated the levels of antioxidant enzymes.

Mice were administered 250 mg/kg of NJE orally for 3, 7 and 14 days. This dose was selected based on previous reports (Salim et al., 2003) and preliminary experiments by us (Razack and Khanum, 2012). Acute administration of the drug for 3 days and chronic administration for 14 days showed significant results but the maximum anxiolytic-like effect was observed after 7 day treatment by increasing the time spent in the open arms in EPM, the locomotor activity in OFT, increased time spent in the lit box in LDB and more number of licks and shocks accepted in VCT. Joshi and Parle, (2006) administered the 95 % ethanolic extract of Nardostachys jatamansi (200 mg/kg) for 8 days and this duration was efficacious in reversing amnesia (chemical and age-induced) in mice.

EPM has been ascribed as a simple method for assessing anxiety responses of rodents (File, 2001). It relies on the rodent’s proclivity towards dark, enclosed spaces (approach) and an unconditional fear of heights or open spaces (avoidance) (Barnett, 2007). Further, EPM can be used as a behavioural assay to study the brain sites (e.g., limbic regions, hippocampus, amygdala, dorsal raphe nucleus, etc) (Gonzalez and File, 1997; Silveira et al., 1993) and mechanisms (e.g., GABA, glutamate, serotonin, hypothalamic–pituitary–adrenal axis neuromodulators, etc.) (Pellow et al., 1985; Handley and Mithani, 1984; Lister, 1987; Cortese and Phan, 2005; Overstreet et al., 2003; Korte and De Boer, 2003; Rodgers et al., 1992; Silva and Brandao, 2000) underlying anxiety behaviour. Diazepam as reported showed an increase in the number of entries into the open arms as well as the time spent on open arms and also caused a significant increase in total ambulatory activity as observed by the open field test (Pellow et al., 1985; Fernandez-Guasti et al., 2001; Wilson et al., 2004; Silva and Brandao, 2000). NJE also showed similar results by increasing the percent time spent in open arms and the number of entries into open arms. Other ethological behaviours recorded further supported the role of NJE as an anxiolyte viz. latency to enter closed arm, total number of transitions between open and closed arm, time in central zone.
and number of dips into any arm. These results clearly suggest that NJE at 250 mg/kg administered for 7 days was efficacious in alleviating anxiety in mice in EPM.

The open field test (OFT) is a common measure of exploratory behaviour and general activity in both mice and rats (Gould et al., 2009), a primary unconditioned indicator of emotionality in laboratory investigations. As observed NJE increased the locomotor activity in the open arena of open field test. Diazepam also caused a significant increase in the locomotor activity. The dosage administered for 7 - days showed maximum locomotor activity in OFT.

LDB is based on the innate aversion of rodents to brightly illuminated areas and on spontaneous exploratory behaviour of rodents in response to mild stressors, i.e., novel environment and light (Crawley and Goodwin, 1980). A natural conflict situation occurs when an animal is exposed to an unfamiliar environment or novel objects. The conflict is between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia). NJE helped overcome the innate tendency to avoid novel environments by increasing the time spent in the lit compartment and also increasing number of transitions between the two compartments.

VCT utilises a conditional response to assess the neurobiological underpinnings of anxiety. The results are expressed as the number of punished licks made by subjects, with an increase in the number of licks reflecting antianxiety-like behaviour (Walf and Frye, 2011). The water deprived mice for 24 h were subjected to VCT apparatus for 10 min without any shock. After another 24 h they were again exposed to the apparatus but with an electric shock. Mice treated with NJE made maximum licks and subsequently accepted more shocks, with results comparable to diazepam (1 mg/kg), standard anxiolyte.

An increase in brain GABA levels was observed in NJE treated group for both 7 and 14 days. NJE belongs to family Valerianaceae and Valeriana officinalis (the most popular medicinal herb of the family valerianaceae) extract was shown to contain high levels of GABA and Glutamine (Santos et al., 1994; Cavadas et al., 1995). Substantial amounts of free amino acids particularly GABA, tyrosine, arginine and glutamine are also present in aqueous extracts of roots of valerian (Houghton, 1999). The ethanolic extract prepared by us also showed a significantly high level of glutamate content (precursor for GABA), substantial amounts of GABA, glutamine,
tyrosine and arginine contents (elaborate explanation in chapter 4) probably the main cause for the herb to work as an effective anxiolyte. Valerenic acid, a sesquiterpenoid reported in *Valeriana* spp. is known to inhibit the enzyme system causing the breakdown of GABA in the brain (Houghton, 1999) and thereby a subsequent increase in brain GABA levels aids in regulating the neuronal excitability and serves as a ‘brake’ on the neuronal circuitry during stress (Weeks, 2009).

The brain monoamine levels in NJE treated group were significantly higher. The dopamine levels were also high. This neurotransmitter has a role in motivated behaviours clearly suggesting that its low levels hampered motivation and correspondingly made the mice more anxious. The levels of norepinephrine were also high. A study by Prabhu *et al.*, (1994) has shown similar results with 15 - day treatment with alcoholic extract of *Nardostachys jatamansi* resulted in a significant increase in the levels of norepinephrine, dopamine, 5-HT and GABA.

Brain is susceptible to free radical damage because it is a highly oxygenated organ and consumes one-fifth of the oxygen used by the body. Additionally, brain contains large amounts of iron and polyunsaturated fatty acids and relatively low levels of antioxidants like catalase (Halliwell, 1989). Kuloglu *et al.*, (2002) have reported significant differences between lipid peroxidation product (MDA) and antioxidant enzyme (SOD, GSH, GPx) activity levels in patients with schizophrenia and bipolar disorder compared to control (Atmaca *et al.*, 2008). The levels of antioxidant enzymes were found to be elevated following treatment with St. John’s wort (50 and 100 mg/kg) (Kumar *et al.*, 2010). NJE pre-treatment also elevated levels of antioxidant enzymes, clearly suggesting that any oxidative onslaught could be dealt with. Therefore, antioxidants from a normal diet or phytonutrients could help prevent from anxiety development (Bouayed, 2011)

Awad *et al.*, (2007), suggest anxiolytic plants to interact with either glutamic acid decarboxylase (GAD) or GABA transaminase (GABA-T) and ultimately influence brain GABA levels and neurotransmission. GAD facilitates synthesis of GABA from glutamate, a rate limiting step in GABA synthesis (elaborate explanation in chapter 1), GABA is catabolised into succinic semialdehyde by GABA-T. The mode of action of valerian and its active constituents are strongly linked to GABA system (Riedel *et al.*, 1982; Santos *et al.*, 1994; Yuan *et al.*, 2004). Studies have
shown that neural action of valerenic acid, a sesquiterpene and active constituent of valerian, is involved in the GABA system of the brain and to a lesser extent the serotonergic system (Khom et al., 2007; Dietz et al., 2005). It appears that valerenic acid interacts with GABA_A neurons similar in action of benzodiazepenes, by binding to specific units of GABA_A receptor complex. Stimulation of GABA_A receptors directly opens chloride channels, thereby producing neural inhibition. NJE also showed enriched GABA (80.24 mg/g), and Glx content (18.95 ± 2.05 g/100g), analysed by HPTLC and HPLC.

Al-Awadi et al., (2006) have shown that GABA crossed the blood brain barrier. They administered exogenous GABA into the femoral artery and compared GABA levels in the brains of hypertensive and non-hypertensive rats. GABA levels, measured in CSF as well as several brain regions by HPLC showed an increase in a dose-dependent manner. GABA uptake was greater in hypertensive rats, while non-hypertensive rats also showed uptake.

We contemplate that NJE also belonging to the same plant family as valerian i.e. valerianaceae, exerts a similar mechanism in anxiolysis. Analysis of the brain for GABA, showed elevated levels in NJE fed groups of mice, hinting at the involvement of GABAergic systems. Now, that we are sure about its anxiolytic actions, we moved on to check whether the GABA system or serotonergic system was responsible for the anxiolytic actions of NJE. We studied the anxiolytic actions of NJE using antagonists of GABA receptors, picrotoxin and flumazenil, whose elaborate studies have been reported in the succeeding chapter.

LC-MS aided in identification of major metabolites of NJE. Most of the identified metabolites included sesquiterpenes and monoterpenes. Sesquiterpenes have been reported to be potent anxiolytes (Galdino et al., 2012; Hu et al., 2011; Becker et al., 2014). Further studies into the identification of sesquiterpenes crossing the blood brain barrier, to exert anxiolytic effects in mice is elaborated in the succeeding chapter.

Thus, the results clearly suggest the anxiolytic-like effects of NJE, evidenced by behavioural anxiolytic assays viz., EPM, OFT LDB and VCT. NJE was able to increase the time spent in open arm of EPM, NJE increased the latency to enter the closed arm as soon as the mice was placed in the central zone, it increased the number
of transitions made between the open and closed arm, it decreased the time spent by mice on the central zone and also increased the number of dips made into the open arm, NJE also increased the time spent in the lit box of LDB and also elevated the number of licks made and shocks accepted in VCT. Further, analysis of brain for neurotransmitters showed an elevated brain GABA and monoamine neurotransmitter levels. NJE effectively modulated the levels of key antioxidant enzymes in the brain. These results clearly point out that NJE could be useful as an anxiolyte in providing solace to the anxious.