Chapter 7

*Nardostachys jatamansi* abrogates oxidative stress induced anxiety in mice
Introduction

Oxidative stress (OS) has been implicated in a majority of diseases inflicting humans. Reports state OS to be a primary mediator of various neuropsychiatric disorders including anxiety and depression. Anxiety disorders are known to affect humans at some point during their lifetime causing uneasiness, discomfort and fearfulness in their lives. In the current scenario, there is an upsurge in research on herbs in treating psychiatric disorders owing to their wide therapeutic pertinence and minimal adverse effects.

Anxiety is a central nervous system (CNS) disorder, and a critical component of the CNS involved, is the brain. Brain controls several activities and functions like emotion, correct functioning of the nervous system mediated by interplay of several neurotransmitters and an adequate interaction with the other regulatory systems including the endocrine system and the immune system (Bouayed, 2011). The brain is highly susceptible to oxidative stress owing to its high oxygen consumption (utilizes 20% of the oxygen consumed by the body) its modest antioxidant defences and its lipid-rich content (Ng et al., 2008; Halliwell, 2006; Clarke and Sokoloff, 1999). There are several reports demonstrating a link between oxidative stress and neurodegenerative disorders (Albarracin et al., 2012; Rao and Balachandran, 2002). Reduction in content of brain vitamin E, a fat soluble antioxidant has been shown to increase anxiety in mice (Desrumaux et al., 2005). Another, indirect evidence is induction of anxiety-like behaviour in rats wherein increased protein oxidation was found in frontal cortex (Souza et al., 2007). Hovatta et al., (2005) identified the expression of two genes, glutathione reductase 1 and glyoxalase 1 involved in the antioxidative metabolism to be highly correlated with anxiety-related phenotypes. The activity of these enzymes was highest in most anxious mice and lowest in least anxious mice. Glyoxalase 1 is an enzyme of the glyoxalase system, which protects against carbonyl stress and glutathione is a determinant cofactor for the enzymatic reaction catalysed by glyoxalase 1 (Thornalley, 2006).

L-Buthionine-(S,R)-sulfoximine (BSO), induces oxidative stress by inhibiting γ-glutamylcysteine synthetase, a key enzyme in glutathione synthesis thereby depleting cells of endogenous glutathione (GSH), a tripeptide (gamma-glutamyl cysteinyl glycine) which has a direct antioxidant function in the elimination of ROS.
Neurons are highly susceptible to GSH deficiency. Indeed, GSH depletion in neurons in culture is accompanied early in the process by a strong, transient increase in ROS concentration and causes apoptotic (Merad-Boudia et al., 1998; Ratan et al., 1994) or necrotic (Kane et al., 1995) death of the cells (Fahn and Cohen, 1992). GSH has been shown to give protection against the induction of cell death in neurons (Ben-Yoseph et al., 1996).

In the present study the potential anxiolytic effects of 70% ethanolic extract of NJE (250 mg/kg) after induction of oxidative stress using BSO (300 mg/kg), an inducer of oxidative stress for 7 days (Salim et al., 2010) was evaluated in mice using mouse-models of anxiety (EPM, OFT, LDB and VCT), followed by assessment of OS parameters in the brain and serum/plasma of mice, neurotransmitters in the brain, and western blotting of OS induced genes of anxiety, glyoxalase 1 and glutathione reductase 1 in the brain.

Materials and methods

Plant Material and preparation of the Extract

The 70% ethanolic fraction of *Nardostachys jatamansi* was prepared as described in chapter 2.

Animals

Swiss albino mice (25-30g) were used for the experimental study. The experimental protocols were accepted at the 14th Institutional Animal Ethics Committee (IAEC) meeting held on 17th November, 2009. The animals were housed under conventional conditions as described in chapter 2.

Chemicals and reagents

L-Buthionine-(S,R)-sulfoximine, GABA, serotonin, norepinephrine, dopamine were procured from Sigma-Aldrich, St. Louis, MO, USA. Diazepam (Calmpose) utilised as a standard anxiolytic was procured from M/s Ranbaxy Laboratories, India. All other reagents were of standard laboratory grade.
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Experimental design

OS induced anxiety

Mice received extract (250 mg/kg) p.o for seven days. From the eighth day upto the 14\textsuperscript{th} day mice simultaneously received i.p doses of BSO (300 mg/kg) (Fig. 7.1). This dose and pre-treatment duration was according to Salim \textit{et al.}, (2010).

Fig. 7.1

![Diagram showing experimental design]

**Fig. 7.1: Schematic representation of the experimental design for OS induced anxiety**

Animals

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

Elevated plus maze test (EPM)

EPM test is the commonly used behavioural paradigm to assess anxiolytic behaviour in rodents. The elaborate description of the apparatus is given in detail in chapter 2.

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 elaborate description of the apparatus is given in detail in chapter 2.
Light dark box test

The light dark box test was performed as described in chapter 5.

Vogel’s conflict test (VCT)

The Vogel’s conflict test was performed as described in chapter 5.

Preparation of homogenate

After Vogel’s conflict test, mice were sacrificed by cervical dislocation and liver and brain tissues were immediately dissected out, washed with ice cold isotonic sodium chloride and drained thoroughly. For liver/brain tissue parameters the homogenate was prepared (10 % w/v) using phosphate buffer (pH – 7.4) followed by centrifugation at 15,000 x g for 20 min. The supernatant was utilised for all the assays.

Neurotransmitters estimation by HPLC

GABA

GABA estimation was done on a Waters 2465 RP-HPLC (Milford MA, USA). The protocol described in chapter 5 was used for estimation GABA in the brain homogenate.

Monoamine neurotransmitters

Monoamine neurotransmitters viz 5-hydroxytryptamine (5-HT) or serotonin, norepinephrine (NE) and dopamine (DA) levels in mouse brain were estimated by RP-HPLC coupled to an electrochemical detector as described in chapter 5.

Reduced glutathione

Reduced glutathione, a major endogenous thiol was estimated as described in chapter 5.

Cortisol

The cortisol estimation in plasma was done according to Cortisol EIA kit (Item # 500360), Cayman Chemical Company, MI, USA. The assay is based on detection of Ellman’s reagent at 412 nm and the amount of cortisol in the sample was expressed as ng/mL.
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Lipid peroxidation

Malondialdehyde (MDA), a product of lipid peroxidation can be measured by TBARS (thiobarbituric acid reactive substances) method (Nelson et al., 1994). Briefly, to brain/liver homogenates 0.5 mL trichloroacetic acid (10 %) was added followed by 2 mL of thiobarbituric acid mixture (TBA 0.35 %, SDS 0.2 %, FeCl₃ 0.05 mM and BHT in glycine-HCl buffer 100 mM, pH 3.6). This was boiled for 30 min and then cooled. The mixture was then centrifuged at 8,000 x g for 10 min and the absorbance of the supernatant was measured at 532 nm. The MDA equivalents were calculated using extinction co-efficient of $1.56 \times 10^{-5} \text{ M}^{-1} \text{cm}^{-1}$.

Protein carbonyls (PCOs)

PCOs formed were measured by the method described by Reznick and Packer (1994). 1 mL of 10 mM DNPH in 2 M HCl was added to the reaction mixture (2 mg protein). Samples were incubated for 1 h at room temperature and were vortexed every 15 min. To this reaction mixture 1 mL of cold trichloroacetic acid (10 % w/v) was added and centrifuged at 3000 x g for 10 min. The protein pellet was washed three times with 2 mL of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 mL of guanidine hydrochloride (6 M, pH 2.3) and incubated for 10 min at 37 ºC while mixing. The absorbance was measured at 370 nm and the carbonyl content was calculated based on molar extinction coefficient of DNPH ($\varepsilon = 2.2 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$).

Glutathione Peroxidase

Glutathione peroxidase was assayed according to glutathione peroxidase kit (Ransel Cat # RS505), Randox Laboratories Ltd, UK following the protocol described in chapter 5.

Glutathione-S-Transferase (GST)

GST activity was measured according to the method of Habig et al., (1974), as described in chapter 5.

Superoxide Dismutase

Superoxide dismutase activity was analysed using Superoxide dismutase assay kit (Item # 706002), Cayman Chemical Company, MI, USA, as described in chapter 5.
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Catalase

Catalase was estimated following the method of Luck (1965) as described in chapter 5.

ABTS

Total antioxidant activity was measured by ABTS cation radical decolourization assay (Re et al., 1999). Stock ABTS solution was prepared by mixing ABTS (14 mM) and potassium persulfate (4.9 mM) together and leaving in dark for 12-16 h. The working ABTS solution was diluted from stock ABTS with deionized water, until absorbance reached 0.7 ± 0.02 at 734 nm. To 990 µL of this working ABTS solution, 10 µL of plasma was added and the absorbance was monitored at 734 nm for 3 min. The total antioxidant status was determined by molar extinction coefficient of ABTS (1.5 x 10^4 M^-1 cm^-1).

Acetylcholine esterase

Acetyl choline esterase activity was assayed following the method of Ellman et al., 1961 with minor modifications as described in chapter 5.

Acetylcholine

Acetylcholine was quantified using choline/acetylcholine quantification kit (cat # K615-100), BioVision, USA. Briefly, to 50 µL of reaction mix (44 µL choline assay buffer, 2 µL choline probe, 2 µL acetylcholine esterase, 2 µL enzyme mix), sample or standards were added to each well and mixed. Following incubation for 30 min in dark the absorbance was measured at 570 nm. The acetylcholine activity of the sample was represented as nmol/g tissue.

Glutamate

Glutamate quantification was done using Glutamate assay kit, (cat # K629-100), BioVision, USA. Briefly, to each well 100 µL of reaction mix (90 µL assay buffer, 8 µL glutamate developer 2 µL glutamate enzyme mix), standard or samples were added and mixed followed by incubation at 37 ºC in dark. The absorbance was measured at 450 nm. The results were expressed as U/g tissue.
**Monoamine oxidase A and B (MAO-A and MAO-B)**

Mouse brain mitochondrial fraction was prepared following the procedure described by Schurr and Livne (1976). Briefly, 100 mg of mouse brain tissue was homogenised in 1 mL of 0.25 M sucrose - 0.1 M Tris - 0.02 M EDTA buffer (pH 7.4) and centrifuged twice at 800 x g for 10 min at 4 ºC. The supernatant was collected and centrifuged at 12000 x g for 20 min at 4 ºC. The precipitates were washed twice with sucrose – tris - EDTA buffer and suspended in 9 volumes of sodium phosphate buffer (pH 7.4; 10 mM with 320 mM sucrose) and mingled well at 4 ºC for 20 min followed by centrifugation at 15000 x g for 30 min at 0 ºC and the pellet was then suspended in cold sodium phosphate buffer. The protein concentration was determined by the Lowry’s method (Lowry et al., 1951) using bovine serum albumin as standard and adjusted to 1 mg/mL. MAO activity was analysed spectrophotometrically as described previously (Charles and Mc Ewan, 1977). Briefly, for analysing the MAO-A activity, 2.75 mL sodium phosphate buffer (100 mM, pH 7.4) and 100 µL of 4 mM 5-hydroxytryptamine were mixed in the cuvette and placed in a double beam spectrophotometer (Shimadzu, Japan). This was followed by addition of 150 µL solution of mitochondrial fraction to initiate the enzymatic reaction and change in absorbance for 5 min was recorded against a blank containing sodium phosphate buffer and 5-HT at 280 nm. For analysing MAO-B activity 2.75 mL sodium phosphate buffer (100 mM, pH 7.4) and 100 µL of 0.1 M benzylamine were mixed in the cuvette and placed in a double beam spectrophotometer (Shimadzu, Japan). This was followed by addition of 150 µL solution of mitochondrial fraction to initiate the enzymatic reaction and the kinetics of change in absorbance for 5 min was recorded at 249.5 nm against a blank containing sodium phosphate buffer and benzylamine (Dhingra and Goyal, 2008).

**Protein estimation**

The amount of protein in the samples was estimated according to the method of Lowry et al., (1951) as described in chapter 5.

**Western blotting**

Brain samples were lysed in ice-cold lysis buffer with protease and phosphatase inhibitor cocktail. The supernatants were collected by centrifugation at 12,000 x g for 10 min at 4 ºC and the protein content was determined by Lowry et al., (1951). The protein (25 mg) was separated on 8 – 12 % SDS-PAGE and transferred to
nitrocellulose membranes. The membranes were blocked with 5% (v/v) non-fat dry milk in tris-buffered saline + tween-20 (TBS-T) (10 mM tris–HCl, 150 mM NaCl, and 0.1% tween-20, pH 7.5). The membranes were incubated with primary antibodies namely GAPDH (sc-5286), glyoxalase 1 (sc-651) glutathione reductase (sc-66048) and superoxide dismutase (Santa Cruz Biotechnology, CA, USA) at 1:1000 dilution for 3 h with shaking. After washing thrice with TBS-T, the membranes were incubated for 2 h at room temperature in dark with horseradish peroxidase (HRP) conjugated rabbit anti-goat, goat anti-mouse and goat anti-rabbit secondary antibodies (DAKO, Denmark) at 1:10,000 dilutions. Further after three final washes with TBS-T the immunoreactivity of the membranes was detected using enhanced chemiluminescence peroxidase substrate kit (CPS-160, Sigma, St. Louis, MO, USA) and the band intensity was calculated using ‘Image-J’ software.

**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**

**Effect of chronic treatment with BSO on the behaviour of mice in elevated plus maze test**

Treatment with BSO for 7 days produced significant changes in the behaviour of mice in EPM (Fig. 7.2 A, B C and D). One way ANOVA revealed a significant difference between the groups with respect to latency to enter closed arm [F (3, 28) = 55.08; p < 0.05], number of open arm entries [F (3, 28) = 64.58; p < 0.05], time spent in central zone [F (3, 28) = 259.16; p < 0.05] and percent time spent in open arm zone [F (3, 28) = 130.74; p < 0.05]. Treatment of mice with BSO caused a significant decrement in latency to enter the closed arm, number of open arm entries, percent time spent on open arms and increased the time spent on central zone of the apparatus. Pretreatment of mice with NJE/Dzp helped in overcoming BSO induced effects by increasing the latency to enter closed arm, number of open arm entries and the percent time spent on open arms. Tukey’s HSD showed significant difference between BSO vs all groups for all the behavioural parameters, whereas no significant difference was observed between control vs Dzp+BSO with respect latency to enter closed arm and the percent time spent in open arms (p ≠ 0.05).
Fig. 7.2: Effects of coadministration of BSO (300 mg/kg) with NJE (250 mg/kg) and Dzp (1 mg/kg) on the behaviour of mice in elevated plus maze test. (A) Latency to enter the closed arm (B) Number of open arm entries (C) Time spent by mice on the central zone (D) Percent time spent in open arm zone. * p < 0.05 versus control; # p < 0.05 versus BSO.
Open field test

Another crucial behavioural analysis measuring locomotor activity as the number of line crossings is OFT. One way ANOVA revealed a significant difference between the groups \( [F (3, 28) = 338.41; p < 0.05] \), with respect to the number of line crossings. BSO treated mice showed less number of line crossings in OFT. Pretreatment with NJE or Dzp significantly enhanced the locomotor activity with mice spending more time in the centre of the apparatus (Fig. 7.3).

Fig. 7.3

![Bar chart showing number of line crossings in different groups](image)

**Fig. 7.3**: Effects of coadministration of BSO (300 mg/kg) with NJE (250 mg/kg) and Dzp (1 mg/kg) on the behaviour of mice in open field test. \* \( p < 0.05 \) versus control; \# \( p < 0.05 \) versus BSO.

Light-dark box test

ANOVA revealed a significant difference between the groups with respect to latency to enter dark compartment \( [F (3, 28) = 266.14; p < 0.05] \), total number of transitions between the compartments \( [F (3, 28) = 149.37; p < 0.05] \) and time spent in light compartment \( [F (3, 28) = 249.92; p < 0.05] \). Mice pretreated with NJE or Dzp were able to reverse the BSO induced effects by increasing the latency to enter dark compartment, the number of transitions between the two compartments and the time spent in the lit compartment. BSO on contrary caused a significant decrement \( (p < 0.05) \) in all these behavioural parameters measured on LDB. Tukey’s HSD showed a significant difference between control or BSO vs all groups with respect to all the behavioural parameters. No significant difference \( (p > 0.05) \) was observed between NJE+BSO vs Dzp+BSO in the total number of transitions between compartments (Fig. 7.4 A, B and C).
Fig. 7.4: Effects of coadministration of BSO (300 mg/kg) with NJE (250 mg/kg) and Dzp (1 mg/kg) 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; # $p < 0.05$ versus BSO.
**Vogel’s conflict test**

In the thirsty rat conflict paradigm or VCT, one way ANOVA revealed significant difference between the groups with respect to number of licks made \( F(3, 28) = 107.24; p < 0.05 \) and number of shocks \( F(3, 28) = 35.25; p < 0.05 \) accepted (Fig. 7.5 A and B). An increase in the number of licks made and shocks accepted reflects anti-anxiety behaviour. An increase in the number of licks made and shocks accepted was observed in NJE+BSO and Dzp+BSO pre-treated groups. BSO pretreatment caused a significant reduction in the licks and shocks accepted (\( p < 0.05 \)). Tukey’s HSD showed no significant difference between control vs Dzp+BSO with respect to the number of shocks accepted.

**Fig. 7.5**

![Graph A](image)

**A**

*Number of licks made*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>BSO</th>
<th>NJE+BSO</th>
<th>Dzp+BSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of licks</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
</tbody>
</table>

* p < 0.05 versus control; # p < 0.05 versus BSO

![Graph B](image)

**B**

*Number of shocks accepted*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>BSO</th>
<th>NJE+BSO</th>
<th>Dzp+BSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of shocks</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* p < 0.05 versus control; # p < 0.05 versus BSO

**Fig. 7.5:** Effects of coadministration of BSO (300 mg/kg) with NJE (250 mg/kg) and Dzp (1 mg/kg) on the behaviour of mice in Vogel’s conflict test. (A) Number of licks made (B) Number of shocks accepted. * p < 0.05 versus control; # p < 0.05 versus BSO.
Effect of BSO induced oxidative stress on monoamine and GABA neurotransmitters, antioxidant enzymes and indices of oxidative stress.

BSO induced oxidative stress also caused a significant decrement \( (p < 0.05) \) in brain monoamine neurotransmitter levels. The levels of serotonin, norepinephrine and dopamine depleted in BSO treated groups were elevated by co-treatment with NJE and Dzp (Fig. 7.6). Another, neurotransmitter with critical roles in anxiety is GABA. Significant increase in brain GABA levels was seen in NJE and Dzp co-treated groups. BSO caused a significant \( (p < 0.05) \) decrement in brain GABA levels (Fig. 7.7).

**Fig. 7.6**

![Chart showing monoamines levels in control, BSO, NJE+BSO, and Dzp+BSO groups with significance markers]

**Fig. 7.7**

![Chart showing GABA levels in control, BSO, NJE+BSO, and Dzp+BSO groups with significance markers]

**Fig. 7.7**: Effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on the brain GABA levels. \( p < 0.05 \) versus control; \# \( p < 0.05 \) versus BSO.
BSO caused a significant decrease in the levels of reduced glutathione in the brain ($p < 0.05$), a well known endogenous antioxidant. However, NJE was effective in restoring the levels to normal. Several antioxidant enzyme levels were also lowered in the BSO treated groups. The levels of catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, and glutathione S-transferase were reduced significantly ($p < 0.05$). However, both diazepam and NJE were able to restore the levels of these antioxidant enzymes to normal (Table 7.1 and 7.2). These enzymes play critical roles in neutralising the free radicals by scavenging them effectively. The total antioxidant capacity measured using ABTS radical cation was also enhanced following NJE treatment (Table 7.2).

Lipid peroxides formed measured as malondialdehyde and cortisol are biomarkers of stress. BSO caused a significant increase in their production affirming that it is an oxidative stress inducer. NJE however reversed this effect by lowering the lipid peroxides formed and also decreasing the cortisol levels as measured in the plasma. Diazepam was also effective in combating oxidative stress induced anxiety (Table 7.2).

Emerging evidence states that glutamate transmission to have anxiolytic effects. NJE was effective in increasing the brain glutamate levels depleted by BSO thereby exerting its anxiolytic action (Table 7.3). Acetylcholine, a neurotransmitter, levels are enhanced in anti-anxiety states. As observed, the levels were significantly enhanced in NJE treated groups in comparison to BSO. Acetylcholine esterase is crucial for memory retention. BSO caused an increase in acetyl choline esterase levels whereas NJE was effective in decreasing the brain acetyl choline esterase thereby improving memory. Diazepam was ineffective in decreasing the brain acetyl choline esterase levels thereby aiding in memory impairment (Fig. 7.8).
Table 7.1: Effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on the brain antioxidant markers and enzymes viz glutathione, GR, GPx and GST. * \( p < 0.05 \) versus control; # \( p < 0.05 \) versus BSO

<table>
<thead>
<tr>
<th></th>
<th>Glutathione (( \mu M/mg ) protein)</th>
<th>Glutathione reductase (U/mg protein)</th>
<th>Glutathione peroxidase (U/mg protein)</th>
<th>Glutathione S-transferase (( \mu mol ) CDNB-GSH/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.84±0.12</td>
<td>145.14±2.90</td>
<td>3.45±0.12</td>
<td>46.93±2.74</td>
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<tr>
<td>BSO</td>
<td>2.78±0.13*</td>
<td>117.41±2.61*</td>
<td>1.77±0.09*</td>
<td>34.83±2.24*</td>
</tr>
<tr>
<td>NJE+BSO</td>
<td>4.25±0.16*#</td>
<td>143.12±4.14*</td>
<td>3.65±0.11*</td>
<td>49.31±2.61*</td>
</tr>
<tr>
<td>Dzp+BSO</td>
<td>3.92±0.19*#</td>
<td>137.96±3.60*</td>
<td>3.30±0.13*</td>
<td>51.47±1.97*</td>
</tr>
</tbody>
</table>

Table 7.2: Effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on the brain antioxidant markers and enzymes viz catalase, SOD, ABTS, lipid peroxides and protein carbonyls. * \( p < 0.05 \) versus control; # \( p < 0.05 \) versus BSO

<table>
<thead>
<tr>
<th></th>
<th>Catalase (mM H(_2)O(_2) degraded/ min/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>ABTS (( \mu M/cm/) protein)</th>
<th>Lipid peroxides (( \mu mol/) g tissue)</th>
<th>Inhibition of Protein carbonyls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.07±1.24</td>
<td>8.91±0.29</td>
<td>4.17±0.20</td>
<td>127.09±5.2</td>
<td>100±2.5</td>
</tr>
<tr>
<td>BSO</td>
<td>22.88±1.62*</td>
<td>7.12±0.27</td>
<td>2.94±0.17*</td>
<td>161.75±6.3*</td>
<td>131.60±2.6*</td>
</tr>
<tr>
<td>NJE+BSO</td>
<td>33.92±1.70#</td>
<td>11.81±0.31**</td>
<td>4.07±0.21*</td>
<td>129.72±7.4#</td>
<td>108.97±2.1**</td>
</tr>
<tr>
<td>Dzp+BSO</td>
<td>34.66±1.60#</td>
<td>10.99±0.28**</td>
<td>3.29±0.15**</td>
<td>133.34±6.8#</td>
<td>115.17±2.9**</td>
</tr>
</tbody>
</table>

Table 7.3: Effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on the stress markers and neurotransmitters viz cortisol, acetylcholine and glutamate. * \( p < 0.05 \) versus control; # \( p < 0.05 \) versus BSO

<table>
<thead>
<tr>
<th></th>
<th>Cortisol (ng/mL)</th>
<th>Acetylcholine (nmol/g)</th>
<th>Glutamate (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>261.84±15</td>
<td>42.89±1.71</td>
<td>175±9.2</td>
</tr>
<tr>
<td>BSO</td>
<td>307.98±21*</td>
<td>38.25±2.13*</td>
<td>213±9.8*</td>
</tr>
<tr>
<td>NJE+BSO</td>
<td>263.08±19*</td>
<td>43.12±1.57*</td>
<td>179±7.8*</td>
</tr>
<tr>
<td>Dzp+BSO</td>
<td>269.84±17*</td>
<td>42.45±1.92*</td>
<td>182±8.8*</td>
</tr>
</tbody>
</table>
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Fig. 7.8

![Graph showing effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on acetylcholine esterase levels.]

Fig. 7.8: Effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on acetylcholine esterase levels. *p < 0.05 versus control; # p < 0.05 versus BSO.

MAO-A and B

MAO-A and B catalyse the breakdown of monoamines. The levels of MAO A and B in NJE + BSO and Dzp + BSO were restored to normal (Fig. 7.9).

Fig. 7.9

![Graph showing effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on MAO-A and MAO-B levels.]

Fig. 7.9: Effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on MAO-A and MAO-B levels. *p < 0.05 versus control; # p < 0.05 versus BSO.
Western blotting

Glyoxalase 1 and glutathione reductase 1 are protein biomarkers playing roles in mediating OS induced anxiety. The protein expression of glyoxalase 1 and glutathione reductase 1 was over expressed in BSO treated groups. However, NJE and Dzp were able to downregulate the expression of glyoxalase 1 and glutathione reductase 1. SOD 1, an important endogenous antioxidant enzyme whose expression depleted in BSO treated mice was upregulated in NJE and Dzp co-treated mice (Fig. 7.10 A, B, C and D).

Fig. 7.10

A

![Western blot images of Glyoxalase 1, Glutathione Reductase, and Superoxide Dismutase](image)

B

![Bar graph showing relative expression of Glyoxalase 1](image)
Discussion

Oxidative stress is a state where oxidation exceeds the antioxidant systems because the balance between them has been lost (Yoshikawa and Naito, 2002). Under physiological conditions, multiple lines of defence exist to protect against these free radicals, including the restriction of their production through the maintenance of a high oxygen gradient between the ambient and cellular environments, their removal by non-enzymatic and enzymatic antioxidants, and reparation of oxidative damages by structural repair and replacement mechanisms (Davies, 2000). Several studies have surfaced evidencing the role of oxidative stress in anxiety. This drove us to postulate the present study and analyse the role of NJE in alleviating oxidative stress induced anxiety.

Fig. 7.10: Effects of co-administration of BSO (300 mg/kg) with NJE (250 mg/kg) and diazepam (1 mg/kg) on the oxidative stress biomarkers of anxiety viz glyoxalase 1, glutathione reductase 1 and superoxide dismutase 1 as analysed by western blotting. B, C and D correspond to band intensities expressed as relative protein expression calculated by Image-J software. *p < 0.05 versus control; #p < 0.05 versus BSO.
anxiety and study its plausible mechanism of action. So in the present study oxidative stress was induced using BSO (300 mg/kg) for 7 days after pretreatment with *Nardostachys jatamansi* extract (250 mg/kg) followed by assessing anxiety levels in mice using behavioural indices and biochemical parameters as well as western blot of two important biomarkers linking oxidative stress with anxiety viz glyoxalase 1 and glutathione reductase 1.

Animal models allow the study of mechanisms of specific behaviours and their pathophysiology and aid in developing and predicting therapeutic responses to pharmacological agents. The behavioural tests of anxiety are useful in understanding the potential activity of pharmacological agents in humans and the mechanism of action of drugs (Bourin *et al*., 2007).

The anxiolytic tests employed showed that BSO induced oxidative stress for 7 days caused a significant decrement in the time spent in open arm of EPM, in the exploratory behaviour in OFT and light compartment of LDB and also in number of licks and shocks accepted in VCT. However, treatment with BSO for 2 days did not bring about significant results (results not shown). These results are in accordance with previous reports (Salim *et al*., 2010). Several studies have reported similar effects of BSO in these anxiolytic tests (Masood *et al*., 2008, Salim *et al*., 2010). NJE was able to combat oxidative stress mediated anxiety by reversing the effects of BSO as evidenced by behavioural tests. Allam *et al*., (2013), have also showed the ability of grape powder supplementation to overcome oxidative stress mediated anxiety by BSO. Diazepam was ineffective in overcoming anxiety in EPM (similar results were shown by Masood *et al*., 2008) but results from other behavioural tests showed that it too could effectively overcome oxidative stress mediated anxiety. Salim *et al*., (2010) have reported that oxidative stress in adult rat hippocampus was reported to be anxiogenic with decreased locomotion and exploration of rats in open field test.

BSO induces oxidative stress by depleting cells of glutathione. Glutathione, a tripeptide is the most abundant thiol antioxidant in mammalian cells. It directly neutralises free radicals or acts as a cofactor for antioxidant enzymes like GPxs. Additionally, GSH keeps sulfhydryl groups of cytosolic proteins in reduced form by maintaining thiol redox potential in cells (Dringen *et al*., 2000) and regulates cellular signalling pathway in apoptosis (Chandra *et al*., 2000; Hall, 1999). The requirement
of GSH and total antioxidant capacity is particularly high in the brain (Chen et al., 2005). The levels of GSH were depleted in BSO treated group whereas NJE could restore their levels to normal. The levels of other antioxidant enzymes viz. catalase, SOD, GPx, GST, GR, and the total antioxidant status was also restored. The lipid peroxides, protein carbonyls and cortisol levels elevated in oxidative stress states were controlled by NJE supplementation. Several studies have reported similar results where Atmaca et al., (2004) showed that, in patients with social phobia, sub-chronic treatment with citalopram decreased antioxidant enzymes and malondialdehyde levels and that they could be regarded as state markers of social phobia as their levels return to normal with treatment. Yasunari et al., (2006), showed that trait anxiety may increase plasma norepinephrine and increase ROS formation by mononuclear cells. Souza et al., (2007) suggested that consumption of highly palatable diet enriched with sucrose leads to an obese phenotype, increases protein oxidation in frontal cortex and appears to induce anxiety-like behaviour in rats. Bouayed et al., (2007) showed that chlorogenic acid (a common constituent of plums, apples, cherries) induced a decrease in anxiety-related behavior suggesting the anxiolytic-like effect of the polyphenols.

Acetylcholine esterase plays critical roles in maintaining normal functions of the central cholinergic system. Elevated levels terminate the cholinergic transmission by degradation of acetylcholine to acetate and choline in the synaptic cleft that impairs the normal functioning of the nervous system (Ballard et al., 2005; Pandareesh and Anand, 2014). An elevated level of acetylcholine esterase was observed in BSO and Dzp treated groups which were significantly attenuated in NJE treated mice with a subsequent elevation of acetylcholine levels.

Exposure to severe stress has been associated with glutamate excitotoxicity, which, in turn, can cause neuronal damage and/or death (Cortese and Phan, 2005). As observed elevated levels of glutamate in BSO treated mice were alleviated by NJE and Dzp supplementation suggesting their neuroprotective roles.

MAO-A and MAO-B oxidize neurotransmitters and dietary amines and their regulation is important in maintaining normal mental states (Chen et al., 2004). Inhibition of this enzyme causes a reduction in catabolism and a subsequent increase in the concentration of biogenic amines (Dhingra and Goyal, 2008). NJE and Dzp
were able to restore MAO A and MAO B levels to normal. This could be the reason for elevated levels of monoamines in groups pretreated with NJE. Chronic restraint stress has been shown to decrease levels of serotonin, with increased 5-HIAA/5-HT ratio in the hippocampus and decreased levels of dopamine in the hippocampus (Torres et al., 2002). As observed, BSO depleted levels of monoamines which were elevated and restored to normal with NJE and Dzp supplementation. This is in concordance with previous reports (Lyle et al., 2012). Chronic stress exposure has been reported to result in attenuation of GABAergic signalling (Liu et al., 2014).

Exposure to stress has been shown to exert wide actions on GABA_A receptors, ranging from changing their orthosteric and allosteric binding sites, to modulating the mRNA and protein expression of GABA_A receptors subunits. In the amygdala, chronic stress has been reported to regulate the expression of several GABA_A receptor subunits (Cuadra and Molina, 1993; Hsu et al., 2003; Verkuyl et al., 2004; Jacobson-Pick and Richter-Levin, 2012; Orchinik et al., 1995). The levels of GABA attenuated in BSO treated mice were elevated in NJE and Dzp pretreated mice. This is in accordance with previous reports (Pandareesh and Anand, 2014).

Hovatta et al., (2005) linked the expression levels of genes – glyoxalase 1 (a cellular detoxification enzyme) and glutathione reductase 1 (antioxidant enzyme) both of which take part in the oxidative stress pathway to anxiety. The authors concluded that changes in the expression levels of glyoxalase 1 and glutathione reductase 1 in the brain led to a significant effect on anxiety-related behaviour, and established a causal role for these genes, which are both part of a pathway that regulates oxidative stress, in the genesis of anxiety-like behaviour. Western blotting of these two genes showed an overexpression of glyoxalase 1 and glutathione reductase 1 in oxidative stress induced group.

SOD 1, a scavenger of oxygen free radicals is a copper and zinc metalloenzyme. Defects in gene encoding SOD 1 have been implicated in progression of neurological diseases including amyotrophic lateral sclerosis, Down’s syndrome and Alzheimer’s disease (Levanon et al., 1985; Bewley, 1988). Western blotting of SOD 1 showed an upregulated expression in NJE supplemented groups in comparison to BSO.
Further, analysis of melatonin in the roots of NJE showed enriched content (chapter 4), melatonin and its metabolites are effective radical scavengers and they have pleiotropic actions on various pathways involved in inflammation and oxidative stress, thereby an effective neuroprotectant (Espino et al., 2012; Tan et al., 1993; Galano et al., 2013). Melatonin in the roots of NJE could be exerting its role as an efficient radical scavenger and neuroprotectant thereby serving NJE as an effective agent in combating oxidative stress mediated anxiety.

These results in totality suggest that NJE could prove to be an effective agent in combating oxidative stress mediated anxiety via its antioxidant machinery and could be an effective remedy in treating neuropsychiatric disorders like anxiety mediated by oxidative stress.