

## Ameliorating Effect of Bacopa-Monnieri against Nicotine Induced Cerebellar Toxicity in Male Sprague-Dawley Rats

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Nicotine present in the tobacco leaves are activated through nicotinic acetylcholine receptors and are involved in neurobehavioral activity. Despite creating awareness, there is an increasing trend in the consumption of tobacco. Few plant products have been documented to protect the pathological consequences of nicotine. The present study is aimed to find the beneficial effects of Bacopa-Monnieri (BM) against nicotine induced physical, psychological, neurobehavioral and biochemical changes in cerebellum. Thirty-two male Sprague-Dawley rats (220-235g) were equally randomized into four groups: Group I: Control; received normal Saline. Group II: received Nicotine (5mg/kg Body-Weight) for 90 days. Group III: received nicotine (5mg/kg Body-Weight) for initial 90 days followed by Bacopa-Monnieri (100mg/kg Body-Weight) for next 90 days. Group IV: received Bacopa-Monnieri (100 mg/kg Body-Weight) for 90 days. All compounds were administered through oral gavage. Rats were subjected to Open Field Test, Elevated Plus Maze and Beam Walking Test. Following behavioral tests, rats were anesthetized with ketamine (80 ml/kg Body-Weight) and xylocaine (100 ml/kg Body-Weight), brain was dissected and cerebellum was separated. Concentration of Malondialdehyde, Nitric Oxide and activity of Glutathione Peroxidase were measured spectrophotometrically in the supernatant of cerebellum tissue homogenates. Nicotine increases the Malondialdehyde (MDA) and Nitric-Oxide (NO) level in cerebellar tissue compared to control. Nicotine induced increase in Malondialdehyde and Nitric-Oxide level were prevented by Bacopa-Monnieri. The Glutathione Peroxidase (GPx) activity was lower in nicotine treated rats whereas oral supplementation of Bacopa-Monnieri significantly increases the activity of Glutathione-Peroxidase. Bacopa-Monnieri supplementations significantly reverse the Nicotine induced reduction in locomotion activity, exploratory behavior, anxiety, motor impairment and balance. Bacopa-Monnieri confers the protective effects against nicotine induced neurobehavioral alteration and oxidative stress in rats.

**Keywords:** Bacopa-Monnieri; Behavioral activities; Cerebellum; Nicotine; Oxidative Stress Markers.

Cigarette smoking; a major source of tobacco consumption; is an addictive agent which is increasing across the younger generation and middle-aged population<sup>1</sup>. Once, a person gets the taste of tobacco, the active ingredients help addiction and it is hard to withdraw from

it. Although, tobacco smoking is recognized as a risk factor for adverse health outcomes, the daily smoking of tobacco has been increasing rapidly across the world<sup>2</sup>. It has been observed that chronic consumption of tobacco products has been linked with wide spectrum of diseases

especially the disease involving nervous system such as cognition-related or neurodegeneration related pathological changes and also alters the brain morphology and neurochemistry<sup>3,4</sup>.

Nicotine; an important constituent of tobacco; is a natural alkaloid with chemical name  $C_{10}H_{14}N_2$ , obtained from the leaves and stems of *Nicotiana tabacum* and *Nicotiana rustica*<sup>5</sup>. The major form of nicotine consumption is by cigarette smoking. During cigarette smoke or oral consumption as tobacco chewing, nicotine enters the body which is quickly absorbed into the circulatory system and then reaches the brain within 10-20 seconds and gets concentrated in the brain showing alterations in multiple brain regions, such as thalamus, prefrontal cortex and cerebellum<sup>6</sup>. Nicotine might also cause a cerebellar deficit which has many effects on its function, cognition and behavior<sup>7,8</sup>. In rodents, nicotine exposure shows several neurobehavioral deficits such as attention, learning and memory sensory processing defects and also causes analgesia, depression or anxiety-like behavior<sup>9</sup>. Presently, the information available regarding the scientific correlation linking cigarette smoke with the cerebellar dysfunction is limited. Though, nicotine causes postural imbalance, showing that nicotine might be the circuitry involving the cerebellum in smokers<sup>10</sup>. Nicotine causes significant loss of white matter and reduction in number of Purkinje cells of cerebellum, with possible predisposition to progressive impairment in the structural integrity and function of the cerebellum<sup>11,12</sup>. Therefore, chronic exposure to nicotine negatively impacted on the cerebellum, leading to impaired behavior and cognitive function. The excessive production of free radicals and reactive oxygen species by nicotine induced brain can produce a condition of oxidative stress which may cause structural alterations in various regions of the brain and also neurobehavioral impairments<sup>13,14</sup>.

In Indian traditional medicine, *Bacopa-Monnieri* (BM) is considered as a magical medicinal plant, known to have a brain tonic adaptogenic effect<sup>15</sup>. BM has a vast number of active compounds including several alkaloids and saponins. Saponins, Bacosides A and B; the principle components of BM, are mainly attributed for cognitive effect and learning ability<sup>16</sup>. Bacosides and bacopasaponins constituents

of BM also showed anti-oxidant properties<sup>17</sup>. Crude plant extract and bacosides have also shown neuropharmacological activities such as anticonvulsant, neuroprotection, antidepressant, anxiolytic effect and antioxidant activity<sup>18</sup>.

Although, numerous studies have acknowledged the various pharmacological activities of BM *intoto*, very little is known about its interaction with the Nicotine induced cerebellar neurotoxicity. Keeping in view, the ameliorative effect of *Bacopa-Monnieri* which is studied less and is a dilapidated molecule, the present study addressed to investigate the neuroprotective effect of the crude form of *Bacopa-Monnieri* against nicotine induced oxidative stress in cerebellum of rats and also on locomotor and exploratory behavior, motor coordination and balance in rats.

## MATERIALS AND METHODS

### Chemical

Study was conducted by using nicotine commercially available molecule as Nicotine Bi-L-tartrate Dihydrate supplied by TCI chemical India.

### Experimental Animals and housing

The present study was approved Institutional Animal Ethics Committee (IAEC) with a reference No. IAEC/PHARMA/SDUMC/2018-19/12a. Experimental Animals were humanely cared with guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). Male Sprague-Dawley rats aged 10-12 weeks weighing 220 to 235 grams were obtained from Biogen Bangalore, Karnataka, India. The rats were kept in well-maintained temperature ranging from 20°C-26°C and preserved in polypropylene cages confirming 2-rats per cage. The cage was bedded with dust free paddy husk. Rats were provided with free access to standard feed pellets and drinking water *Ad-libitum*.

### Selections of Animals

#### Inclusion criteria

Healthy male Sprague-Dawley rats aged 10-12 weeks with 220-235 gram in weight were included.

#### Exclusion criteria

Females rats and rats with any sign/symptoms of illness were excluded from the study.

### Study Design

The study was an experimental study

conducted in Central Animal House of Sri Devaraj Urs Academy of Higher Education and Research (SDUAHER). After 2-weeks of acclimatization, the study rats were equally distributed into 4-groups.

Group-I (N=8): Control rats; received only normal saline

Group-II (N=8): rats received nicotine (5mg/kg BW) orally through oral gavage once in a day for 90 days

Group-III (N=8): rats received nicotine (5mg/kg BW) for 90-days followed by *Bacopa-Monnieri* (100mg/kg BW) for next 90 days

Group-IV (N=8): rats received only *Bacopa-Monnieri* for 90 days

### Preparation and administration of drugs

#### Nicotine

Nicotine Bi-L-Tartrate dihydrate 5mg/kg Body-Weight was freshly prepared on day of experiment. The salt form of the nicotine was dissolved in RO/UV-treated water and administered to rats. The pH of nicotine solution was adjusted to 7.0 and administered orally through oral gavage once in a day for 90 days.

#### *Bacopa-Monnieri* (BM)

The aerial part of *BM* was collected. The

identification and authentication of *BM* was done by qualified Botanist of University of Agriculture Sciences, Bengaluru. The specimen was deposited in the herbarium with a voucher specimen number *UASB4608*.

The plant was washed with normal tap water followed by double distilled water and finally with ethanol to eliminate the bacterial contamination from the plant. The washed plants were air-dried at room temperature. The plants were powdered; crude form of extract was used for the study. Freshly prepared *BM*, 100 mg/kg Body-Weight was given daily throughout the study period.

#### Neurobehavioral study

After the last dose of treatment, all rats were subjected to behavioral test in the room where the temperature was maintained between 20°C-26°C with all requisite precautions against noise and dust. The experiment was carried out between 09:00 am to 03:00 PM. Rats were acclimatized in the test room for 30-minutes before the conduct of behavioral test.

#### Open field test (OFT)

Rats were tested to evaluate locomotion, anxiety and behavior activities in the OFT as per the

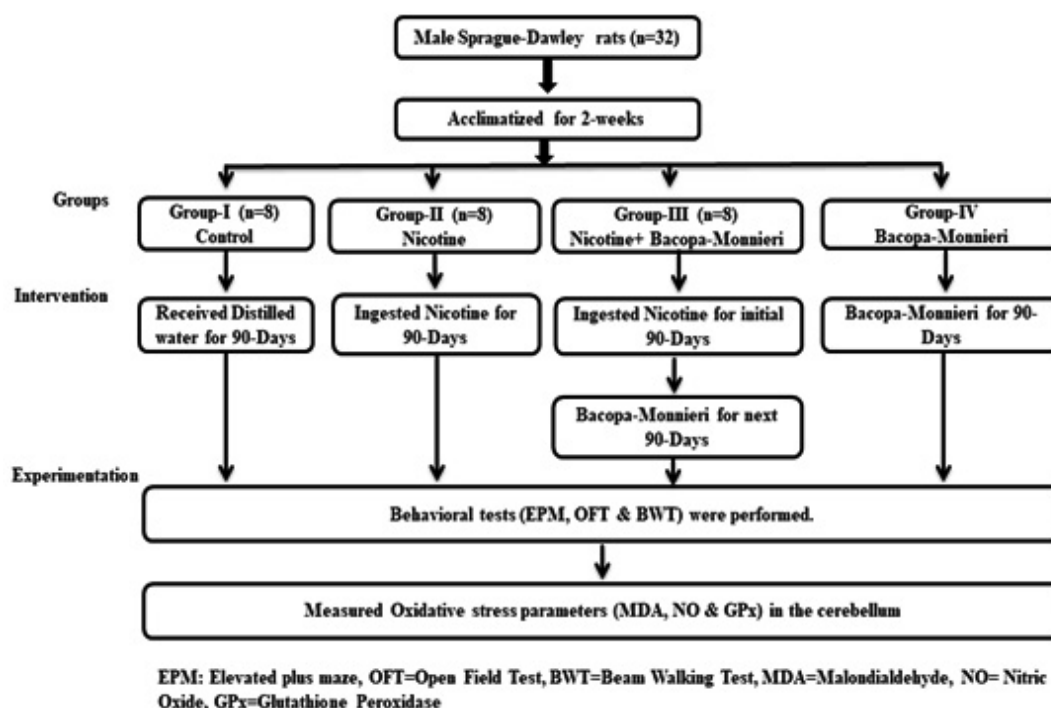


Fig. 1. Overview of Experimental methodology followed in the present study

study conducted by Arika WM *et.al.*<sup>19</sup>. **[Figure-2]**

Rats were picked by tail from the home cage and dropped at the center of arena, allowed to explore the open field for 300 seconds; video recording was done to confirm the activities of rats.

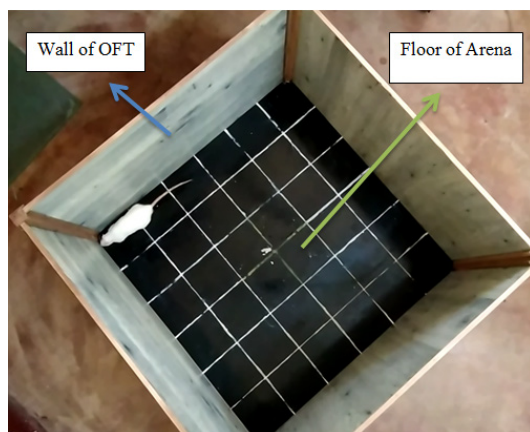
The following parameters were recorded:

I. Motor activities were confirmed by locomotion across the lines drawn in the OFT box. Results were considered only if the rats had moved across with all four paws

II. Behavioral activities was confirmed by grooming, rearing, wall-rearing, frequency of entries on the central part of arena and freezing

#### **Elevated Plus Maze (EPM)**

This is an animal model for measuring anxiety as proposed by Donatti AF *et. al.*<sup>20</sup>. The test was done during light phase of light-dark cycle. For testing, rats were individually placed in the central square of the maze with head facing open arm and were allowed free exploration for 300 sec. The behavioral activities were recorded by a video camera. **[Figure-3]**



**Fig. 2.** Open Field Test (OFT)

The following parameters were recorded:

I. Open and Closed arm entry

II. Time spent in each arm

III. Freezing time

IV. Number of entry in center and time spent in it.

V. Frequency and duration of rearing and grooming were also assessed

#### **Beam walking test (BWT)**

Ataxia and dystonic movements were evaluated by BWT and confirmed by motor coordination and balance similar to the study conducted by Silveira EMS *et. al.* [21]. **[Figure-4]**

The parameters evaluated were confirmed by video recordings and monitored for

I. Time taken to complete the beam

II. Number of foot slips

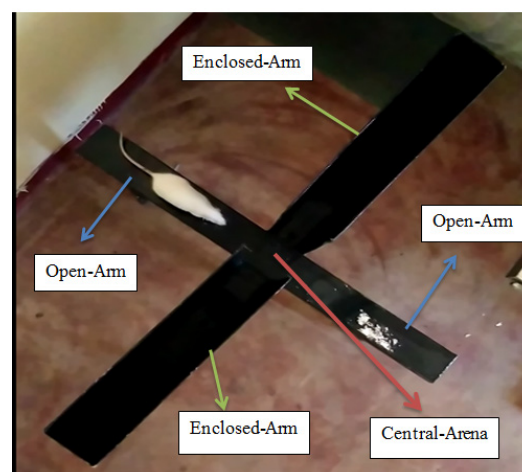
III. Number of near fall

IV. Actual fall.

#### **Biochemical Analysis**

##### **Collection of sample**

• Following behavioral tests, all rats were fasted



**Fig. 3.** Elevated Plus Maze (EPM)



**Fig. 4.** Beam Walking Test (BWT)

**Table 1.** Physical and physiological observations in comparison with Group I.

| Group     | Parameters | 0-4 Days | 5-10 Days  | 11-15 Days | 16-42 Days | 43-90 Days | 91-110 Days          | 111-180 Days |
|-----------|------------|----------|------------|------------|------------|------------|----------------------|--------------|
| Group II  | Food       | Normal   | Reduction  | Reduction  | Normal     | Reduction  | Rats were sacrificed |              |
|           | Water      | Normal   | Reduction  | Reduction  | Normal     | Reduction  |                      |              |
|           | Defecation | Normal   | Semi-solid | Semi-solid | Normal     | Normal     |                      |              |
|           | Hair-gloss | Normal   | Normal     | Normal     | Normal     | Shedding   |                      |              |
| Group III | Food       | Normal   | Reduction  | Reduction  | Normal     | Reduction  | Rats were sacrificed | Normal       |
|           | Water      | Normal   | Reduction  | Reduction  | Normal     | Reduction  |                      | Normal       |
|           | Defecation | Normal   | Semi-solid | Semi-solid | Normal     | Normal     |                      | Normal       |
|           | Hair-gloss | Normal   | Normal     | Normal     | Normal     | Shedding   |                      | Shedding     |
| Group IV  | Food       | Normal   | Normal     | Normal     | Normal     | Normal     | Rats were sacrificed |              |
|           | Water      | Normal   | Normal     | Normal     | Normal     | Normal     |                      |              |
|           | Defecation | Normal   | Normal     | Normal     | Normal     | Normal     |                      |              |
|           | Hair-gloss | Normal   | Normal     | Normal     | Normal     | Normal     |                      |              |

[Group-I=Control, Group-II=Nicotine, Group-III=Nicotine+Bacopa-Monnieri, Group-IV=Bacopa-Monnieri]

overnight except water.

- All rats were anesthetized by injecting ketamine and xylocaine intraperitoneally.
- Rats were sacrificed by cervical decapitation.
- Brains were immediately taken out and washed with ice cold saline and Phosphate buffer solutions (PBS) to remove blood.
- The cerebellum was rapidly dissected from the intact brain carefully.
- The cerebellum was frozen quickly into liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

#### Estimation of Oxidative stress markers in cerebellum tissue

Tissue homogenates were obtained from a 150-200mg piece of cerebellar tissue, homogenized by glass Teflon homogenizer in PBS with pH-7.4, in the ratio of 1:10 (W/V) on the ice for 10-15 minutes.

The homogenates were centrifuged to separate nuclear debris at 10,000g X 20 minutes at  $4^{\circ}\text{C}$  and supernatant was collected and stored at  $-80^{\circ}\text{C}$  until use. Supernatant was used to estimate levels of Malondialdehyde, Nitric-Oxide and the activity of Glutathione-Peroxidase.

#### Estimation of Malondialdehyde (MDA)

The MDA level was estimated by thiobarbituric acid reactive substances assay (TBARS) methods<sup>22</sup>. Briefly, 0.25ml of supernatant was mixed with 0.75% thiobarbituric acid (TBA) and 25% trichloroacetic acid (TCA) solution and kept in boiling water bath for 15 minutes. After cooling, the supernatant was taken and measured the absorbance of developed pink color at 530nm. The concentration of MDA was expressed as nmol/mg of protein in brain tissue according to a standard graphic, which was prepared with serial dilutions of standards 1,1,3,3-tetramethoxypropane.

#### Estimation of Nitric-Oxide (NO)

Modified Griess's method was used to assess the levels of Nitric-Oxide<sup>23</sup>. Briefly, 0.25 ml of supernatant was added to 25  $\mu\text{l}$  of sulphosalicylic acid (70%) and mixed it every 5 minutes for 30 minutes then centrifuged it at 3000 rpm for 20 minutes. The supernatant was collected. 50  $\mu\text{l}$  of supernatant was added to 7.5  $\mu\text{l}$  of NaOH (10%) and 75  $\mu\text{l}$  of tris-HCl buffer (pH-9) and then added 132.5 $\mu\text{l}$  of Griess reagent. All the reagents were kept in dark place for 15 minutes and the absorbance was read at 540nm against blank.

Griess reagent was prepared freshly by mixing 5 ml each of Sulphanilamide (3%) and N-(Naphthelene)-Ethylene Diamine Dihydrochloride (0.3%). The results were expressed as nmoles/mg protein.

#### Estimation of Glutathione Peroxidase (GPx)

Glutathione peroxidase (GPx) activity was measured following the procedure of Rotruck *et.al.*<sup>24</sup>. 1 ml of the reaction mixture was prepared which contained 0.2 ml each of phosphate buffer (0.4 M, PH 7), 0.1 ml of sodium azide (10mM), 0.2 ml of EDTA (0.4 mM), 0.1 ml of H<sub>2</sub>O<sub>2</sub> (0.2 mM), 0.2 ml of GSH (2 Mm) and 0.2 ml of samples were mixed and incubated at 37°C for 10 minutes. The reaction was terminated by addition of 0.5 ml of 10% TCA. The tubes were centrifuged and the supernatant was collected. 1.8 ml of Ellman's reagent (DTNB) was added to 0.2 ml of reaction supernatant. After mixing, the absorbance was read (within 2-3 minutes after the addition of DTNB) at 412 nm. The specific activity of GPx was expressed as mmoles of GSH oxidized/min/mg of protein for tissue.

#### Statistical Analysis

Licensed version of SPSS-20 developed by IBM was used for data analysis. Parametric variables were represented as Mean $\pm$ SD and non-parametric variables were indicated as median (25<sup>th</sup>-75<sup>th</sup> Percentile). p-Value for parametric variables were calculated by ANOVA. Mann-Whitney U test was applied for non-parametric variables. p-Value<0.05 considered significant.

## RESULTS

#### Physical and physiological Observations

Physical and physiological observations are presented in the Table 1. Group II and III rats reduced their food and water intake for 5-15 days of exposure and between 16-42 days, they were normal when compared to Group I and IV rats. Rats showed reduction in food and water intake between 43-90 days of exposure in Group II and III rats. Further, we also noted that there was a reduction in food and water intake from 91-110 days and it was found normal after 110 days.

We found that rats showed alteration in the fecal texture in group II and III for 5-15 days of exposure and was found to be normal after 15 days.

Hair shedding was observed in Group II and III after 42 days of exposure and continued to shed till their decapitation. Group I and IV showed no changes in any of the observations.

#### Effect of *Bacopa-Monnieri* on body-weight and brain weight

Body weight and Brain weight are presented in the Table 2A & 2B. We have observed a good body weight gain of 91.87 grams in group I compared to other groups. The net weight gain in group II and Group III in the first half (0- 90days) was almost the same as in the second half of the Group III (91-180 days). This clearly indicates that rats treated with combination of nicotine and BM has improved the body weight to an extent of 28 grams. To our surprise, rats that were only given BM showed a net weight gain of 70.63 grams. However, the brain weight in all the four groups was consistent between 2.13 to 2.23 grams; which was observed after decapitation. This gives an idea that the gross brain weight is not altered in any of the four groups, irrespective of permutation and combination of nicotine and *Bacopa-Monnieri*. This does not stop thinking of the morphological or intracellular changes that is happening within the brain and also the molecular mechanisms need to be addressed to construe the decision. With the results that we have observed with respect to body weight gain, hypothetically, we comment that *BM* has got an antagonistic effect on the nicotine.

#### Effect of *Bacopa-Monnieri* (BM) on Nicotine induced Oxidative Stress Parameters

Lipid peroxidation (MDA), Nitric Oxide (NO) and Glutathione Peroxidase (GPx) were assessed to evaluate the protective role of *BM* against nicotine-induced oxidative stress in the cerebellum of rats. (Table 3)

MDA level in Group III and Group IV rats showed no any marked changes and are comparable to that of Group I. There was a significant increase in MDA level in Group II [6.65 (6.37-8.18)] rats compared to Group I [3.02(2.35-3.61), Group III [3.58 (2.74-4.20)] and Group IV [3.3(2.6-4.41)]. The observed lower concentration of MDA in Group III rats showed the protective effects of BM against Nicotine induced degenerative changes.

The cerebellar Nitric Oxide (NO) level showed a significant increase in the rats of Group II [23.5(20.74-25.29)] compared to Group I [13.93(11.01-16.16)], Group III [11.61 (10.4-

15.17)] and Group IV [16 (15.14-18.86)]. We also found that rats that Group III had a significantly lower value than Group IV with a p-value of <0.018. There was no significance difference between the Group I [13.93(11.01-16.16)], Group III [11.61 (10.4-15.17)] and Group IV [16 (15.14-18.86)] rats. This suggests that BM may have the

protective effect of preventing nicotine-induced excess nitric oxide.

The activity of Glutathione Peroxidase (GPx) showed a significantly lower value in rats of Group II [3.95 (2.71-4.92)] than Group I [7.63(7.08-9.19)], Group III [5.7 (5.1-6.62)] and Group IV [11.86 (10.34-13.37)]. On the other hand,

**Table 2A.** Comparison of weight of Rats following exposure to different duration.  
[Data were expressed as Mean±SD]

| Group     | Initial body weight (0-days) (Gram) | Body Weight After 90-days (Gram) | Body Weight After (180-days) (Gram) | Net Body Weight gain (Gram)                      |
|-----------|-------------------------------------|----------------------------------|-------------------------------------|--|
| Group I   | 225.38±6.78                         | 317.25±6.985                     | -                                   | 91.87±0.204                                      |
| Group II  | 234.88±5.12                         | 291.63±6.664                     | -                                   | 56.75±1.539                                      |
| Group III | 230.50±5.26                         | 284.00±6.477                     | 365.5±7.03                          | 53.5±1.213(0-90 days)<br>81.5±0.554(90-180 days) |
| Group IV  | 224.00±9.05                         | 294.63±10.3                      | -                                   | 70.63±1.246                                      |

**Table 2B.** Brain-weight; Data were Mean±SD

| Parameters   | Group-I   | Group-II  | Group-III | Group-IV  | p-Value |
|--------------|-----------|-----------|-----------|-----------|---------|
| Brain-Weight | 2.17±0.19 | 2.21±0.13 | 2.23±0.09 | 2.13±0.15 | 1       |

p>0.05 considered no significant difference. The present results were not significant between any of the groups. [Group I=Control, Group II= Nicotine, Group III= Nicotine+Bacopa-Monnieri, Group IV= Bacopa-Monnieri]

**Table 3.** Oxidative Stress Parameters, Data were Median [25<sup>th</sup>-75<sup>th</sup> Percentile]

| Parameter                                      | Group I (N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group II (N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group III (N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group IV (N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | p-Value |
|--|---|--|---|--|---------|
| MDA [nmol/mg of protein]                       | 3.02<br>[2.35-3.61]   | 6.65<br>[6.37-8.18] <sup>a</sup>   | 3.58<br>[2.74-4.20] <sup>b</sup>  | 3.3<br>[2.6-4.41] <sup>b</sup>   | 0.001   |
| NO [nmol/mg of protein]                        | 13.93<br>[11.01-16.16]  | 23.5<br>[20.74-25.29] <sup>a</sup>   | 11.61<br>[10.4-15.17] <sup>b</sup>  | 16<br>[15.14-18.86] <sup>bc</sup>  | 0.005   |
| GPx [mmoles of GSH oxidized/min/mg of protein] | 7.63<br>[7.08-9.19]   | 3.95<br>[2.71-4.92] <sup>a</sup>   | 5.7<br>[5.1-6.62] <sup>ab</sup>   | 11.86<br>[10.34-13.37] <sup>abc</sup>  | 0.005   |

Groups with values that have different superscript small-letter (a,b,c) differ significantly from other groups [a=compared to Group-I, b=compared to Group-II and c=compared with Group-III]. Mann-Whitney U test was used to compare the between groups. p<0.05 was considered significant.

we also found a significant increase in Group IV [11.86 (10.34-13.37)] rats when compared with Group I [7.63(7.08-9.19)] and Group III [5.7 (5.1-6.62)].

#### **Effect of *Bacopa-Monnieri* on Nicotine induced neurobehavioral changes**

##### **Effect of *Bacopa-Monnieri* on OFT parameters**

Nicotine induced rats showed significant decrease in the frequency of square line crossed, rearing, wall-rearing and grooming compared to Group I with a p-value of <0.001. The oral ingestion of BM significantly improved in all the elements of the OFT in nicotine treated rats. High frequency of square line crossed and rearing and reduction in freezing time indicates high level of locomotion and low level of anxiety. It was observed that nicotine treated rats remained inactive for more duration, which leads into reduction of activities in OFT. BM treated rats showed no marked changes and resembled the control group in rearing, wall rearing, time spent in center and grooming. [Table-4].

##### **Effect of *Bacopa-Monnieri* on Elevated Plus Maze parameters**

Group II rats showed a significant

reduction in number and time spent in open arm, center, rearing, grooming compared to Group I with a p-value <0.001. On other hand, freezing time, time spent in center, significantly increased in Group II rats compared to Group I with a p-value of <0.001. The oral administration of BM prevented this degenerative changes that was treated with both nicotine and BM, as it was observed that the frequency and duration of open arm, center square, rearing and grooming were significantly increased than those of the Group II with a p-value of <0.001. (Table-5).

##### **Effect of *Bacopa-Monnieri* on BWT parameters**

The nicotine treated rats took significantly longer time to traverse the beam when compared with Group I with a p-value of <0.001. Oral administration of BM significantly reduces the time to traverse the beam in Group II with a p-value of <0.008. We also noted that Group II rats significantly increased the number of foot slips and near fall compared with Group I (<0.001), Group III (<0.001), Group IV (<0.002). Number of foot slips and near fall in Group I and Group III rats were nil. There were no rats which had a fall from the beam in any of the groups. (Table-6).

**Table 4.** Open field test values, Data were presented as Median [25<sup>th</sup>-75<sup>th</sup> Percentile]

| Parameters       | Group I<br>(N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group II<br>(N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group III<br>(N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group IV<br>(N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | p-Value |
|------------------|--|---|--|---|---------|
| Number of Square | 128  | 35  | 71.3   | 102   | 0.001   |
| Line Crossed     | [121.7-141.7]  | [34.4-36.25] <sup>a</sup>   | [68.4-74.4] <sup>ab</sup>  | [89.8-105.3] <sup>abc</sup>   |         |
| Number of        | 13.7   | 3.3   | 12.2   | 13.2  | 0.001   |
| Rearing          | [11.8-16.2]  | [2.7-5.5] <sup>a</sup>  | [11.3-12.9] <sup>b</sup>   | [12-17] <sup>b</sup>  |         |
| Number of        | 25.2   | 9.3   | 19   | 18.5  | 0.05    |
| Wall-Rearing     | [22.3-27.3]  | [7.8-10.6] <sup>a</sup>   | [16.9-23.2] <sup>ab</sup>  | [17.3-22.3] <sup>b</sup>  |         |
| Time Spent in    | 5.3  | 0.67  | 1.5  | 3.5   | 0.05    |
| Center (Seconds) | [3.5-13.75]  | [0-1.92] <sup>a</sup>   | [0-2.25] <sup>a</sup>  | [1.5-7.5] <sup>b</sup>  |         |
| Freezing Time    | 47.84  | 216.5   | 76.3   | 75.17   | 0.006   |
| (Seconds)        | [39.5-62]  | [194-231.4] <sup>a</sup>  | [73.1-82.25] <sup>ab</sup>   | [67-81.08] <sup>ab</sup>  |         |
| Number of        | 10.5   | 4.5   | 8.17   | 8.3   | 0.006   |
| Grooming         | [9.7-11.9]   | [4-5.25] <sup>a</sup>   | [7-8.3] <sup>ab</sup>  | [7.5-8.3] <sup>ab</sup>   |         |
| Time Spent       | 26.3   | 26.7  | 35.2   | 32.2  | 0.006   |
| in Grooming      | [21.4-28.8]  | [24.1-28.8]   | [32.5-38.9] <sup>ab</sup>  | [20.7-34.3]   |         |
| (Seconds)        |  |   |  |   |         |

Groups with values that have different superscript small-letter (a,b,c) differ significantly from other groups [a=compared to Group-I, b=compared to Group-II and c=compared with Group-III]. Mann-Whitney U test was used to compare the between groups. p<0.05 was considered statistically significant.



**Table 5.** Elevated Plus Maze values, Data were expressed as Median [25<sup>th</sup>-75<sup>th</sup> Percentile]

| Parameters                     | Group I (N=8)<br>Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group II<br>(N=8)Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group III<br>(N=8)Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group IV<br>(N=8)Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | p-Value |
|--------------------------------|---|---|--|---|---------|
| Number Of Entry in Closed Arm  | 11.8 [10.7-14.2]  | 5.2 [4.7-6.3] <sup>a</sup>  | 5.3 [5.1-6.8] <sup>a</sup>   | 10.5 [8.5-11.8] <sup>bc</sup>   | 0.001   |
| Time Spent in Closed Arm (Sec) | 35.5 [29-39.2]  | 228.8[227.4-232.3] <sup>a</sup>   | 82.3[75.8-98.8] <sup>ab</sup>  | 33.3 [21.9-35.4] <sup>bc</sup>  | 0.001   |
| Number of Entry in Open Arm    | 26.7[25.2-28.2]   | 7.7[6.7-8.2] <sup>a</sup>   | 22.2 [20-27.1] <sup>b</sup>  | 29.3 [28.2-31.5] <sup>abc</sup>   | 0.001   |
| Time Spent in Open Arm (Sec)   | 212 [204.5-216]   | 50.3 [44.7-52.7] <sup>a</sup>   | 170.5 [156.4-174.4] <sup>ab</sup>  | 215.3 [213.7-230.7] <sup>bc</sup>   | 0.001   |
| Freezing Time (Sec)            | 38.7[34.9-43.4]   | 208.3 [198-211.7] <sup>a</sup>  | 80.8 [78.5-86.4] <sup>ab</sup>   | 36 [29.8-41.4] <sup>bc</sup>  | 0.001   |
| Number of Entry in Center      | 39.2 [37.7-41.4]  | 12.8 [12.1-13.3] <sup>a</sup>   | 27.7 [25.8-32.3] <sup>ab</sup>   | 40 [39.7-41.1] <sup>bc</sup>  | 0.001   |
| Time Spent in Center (Sec)     | 57.8 [55.7-64.2]  | 20 [19.4-21.3] <sup>a</sup>   | 46.5 [41.8-50.2] <sup>ab</sup>   | 50.34[47.8-52.4] <sup>ab</sup>  | 0.001   |
| Number of Rearing              | 18.7 [18.1-21.8]  | 6.5 [6.1-7] <sup>a</sup>  | 16.5 [15.2-17.2] <sup>ab</sup>   | 27 [25.3-29.9] <sup>abc</sup>   | 0.001   |
| Time Spent in Rearing (Sec)    | 33.5 [31.7-36.8]  | 13 [12.7-13.9] <sup>a</sup>   | 27.8 [24.4-28.6] <sup>ab</sup>   | 53.2 [52.4-55] <sup>abc</sup>   | 0.001   |
| Number of Grooming             | 14.5 [13.1-16.6]  | 4.8 [4.2-5.3] <sup>a</sup>  | 9.5 [9-10.6] <sup>ab</sup>   | 14.7[12.7-16.2] <sup>bc</sup>   | 0.001   |
| Time Spent in Grooming (Sec)   | 27.3[25.4-32.2]   | 7.3 [6.8-7.9] <sup>a</sup>  | 20.7 [18.4-23.1] <sup>ab</sup>   | 24.7[23.9-25.2] <sup>abc</sup>  | 0.001   |

Groups with values that have different superscript small letter (a,b,c) differ significantly from the other groups. [a=compared with Group-I, b=Compared with Group-II, c=compared with Group-III]. p<0.05 was considered significance difference.

**Table 6.** Beam Walking Test values, Data were Median [25<sup>th</sup>-75<sup>th</sup> Percentile]

| Parameters                         | Group I<br>(N=8)Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group II<br>(N=8)Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group III<br>(N=8)Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | Group IV<br>(N=8)Median<br>[25 <sup>th</sup> -75 <sup>th</sup><br>percentile] | p-Value |
|------------------------------------|--|---|--|---|---------|
| Time taken to cross the beam (Sec) | 9.0[7.25-10.0]   | 33.33[28.42-47.34] <sup>a</sup>   | 25.0[22.08-27.67] <sup>ab</sup>  | 14.83[11.42-22.09] <sup>abc</sup>   | 0.05    |
| Number of foot slips (No)          | 0  | 6.33[5.00-7.67] <sup>a</sup>  | 0 <sup>b</sup>   | 2.67[2.33-3.59] <sup>abc</sup>  | 0.001   |
| Number of near fall (No)           | 0  | 2.33[1.42-2.33] <sup>a</sup>  | 0 <sup>b</sup>   | 0 <sup>b</sup>  | 0.001   |

Groups with values that have different superscript small letter (a,b,c) differ significantly from the other groups. [a=compared with Group-I, b=Compared with Group-II, c=compared with Group-III]. p<0.05 was considered significance difference.

## DISCUSSION

The present study highlights that the daily administration of nicotine for 90 days induced excessive oxidative stress, which can be observed by the high significant increase in the MDA and NO levels. Also, a significant inhibition of GPx activity was observed. Nicotine exposure also showed impairment in motor coordination and balance (by increasing number of foot-slips and near-fall as observed in BWT), reduction in locomotory and exploratory behavior (as assessed in OFT by reducing number of square-line crossed, rearing, wall-rearing and grooming) and more anxious (by increased freezing time and time spent in closed arm as evaluated in EPM). On the contrary, oral supplementation of *Bacopa-Monnieri* (100 mg/kg BW) ameliorates the oxidative stress markers and neurobehavioral activities against nicotine treated rats.

We found that the nicotine treated rats reduced their body weight compared to the control group. This may be due to reduction in food intake. Our observation findings have shown that the weight gain in control group is almost double to those rats that were administered only nicotine. Further, we observed that the reduction was consistent and similar to those rats which were treated with a combination of Nicotine and *Bacopa Monnieri*, during the initial 90-days. However, the weight gain is similar to that of the control group of those rats which was treated with combined exposure of Nicotine and *Bacopa Monnieri* after 180 days. The values that we have observed in the second half of those rats treated with Nicotine and *Bacopa-Monnieri* may be because of the antagonistic effect of *Bacopa-Monnieri* verses Nicotine. This indicates that the compounds of *Bacopa-Monnieri* shall mask the adverse effects of Nicotine. These results are in agreement with the rats which have been treated with *Bacopa-Monnieri* depriving them with Nicotine and the difference we could observe is around 32%. This experimental data were supported by the findings where rats exposed to nicotine showed decrease in body weight<sup>25,26</sup>. This could be due to increased lipolysis, energy expenditure and metabolic rate or loss of appetite or reduction in insulin sensitivity<sup>26</sup>. However, the oral administration of

BM prevented the decrease in body weight against nicotine induced rats which can occur as a result of increased food consumption.

As the nicotine pass through BBB, it binds to nicotinic acetylcholine receptor and disturbs the cholinergic systems and disrupts brain development, neuronal migration and synaptogenesis and neurotransmitter release<sup>27</sup>. These adverse effects may produce oxidative stress and neurobehavioral impairments. Oxidative stress was evaluated on the basis of changes in the activity of GPx and concentration of MDA and NO in cerebellar tissues of rats.

Nicotine promotes the over production of free radicals such as reactive oxygen species (ROS) both in-vivo and in-vitro conditions and alter the brain tissue. Many experimental data from the animal study have shown that nicotine potentiated the oxidative stress indices including MDA in different organs such as brain, testes and liver<sup>2,28</sup>.

Our results disclosed that nicotine elevates the lipid peroxidation in the cerebellum tissue; observed as an increase in MDA levels and elevated MDA level may lead to cell disruption, oxidative damage to cell membrane and hence increase the susceptibility to lipid-peroxidation<sup>29</sup>. Our results are similar with the finding of Omotoso GO et al. and Das S et al. who documented that the nicotine treated group has a higher MDA expression in cerebellar tissue compared with other groups<sup>7,30</sup>. Notably, the oral supplementation of BM significantly neutralizes the free radical generation and thus protects the biomembranes damage caused by lipid-peroxidation due to nicotine.

Nitric-oxide is a signaling molecule that plays a pivotal role in the pathogenesis of inflammation. The data from our study showed that nicotine treated rats produced a marked increase in NO level in the cerebellum which may be due to upregulation of induced NOS (iNOS) expression induced by nicotine<sup>31</sup>. The increase in NO level may specify the initiation of neuro-inflammation due to nicotine. Interestingly, treatment with BM supplementation has significantly brought down the NO level to the normal suggesting that the beneficial effect of BM may either involve suppression of iNOS or attenuation of pro-inflammatory cytokine expression<sup>32</sup>.

The present study also clarifies that nicotine treated rats significantly decreased the GPx activity in cerebellar tissue. The lowered GPx activity may impair protection against ROS, causing damage to fatty acids, proteins or DNA and can suggest an inhibitory action of nicotine on anti-oxidant enzyme in brain and brain neurons which are sensitive to oxidative stress<sup>33</sup>. This oxidative stress leads to an imbalance between oxidation/reduction reactions (redox state) within the cells, which cause a progressive loss in cellular functions. In the present study, alterations in the GPx activity in cerebellum tissue due to Nicotine were restored to a great extent on oral administration of *BM*. It is traditionally well-known anti-oxidant and controls the expression of enzymes involved in generation and scavenging of reactive oxygen species<sup>34</sup>.

To substantiate the protective effect of *BM* in motor co-ordination, balance, locomotion and anxiety related behavior in nicotine treated rats, Elevated Plus maze (EPM), Open Field test (OFT) and Beam Walking Test (BWT) was performed.

The EPM is relatively simple behavioral assessment method which was developed for the investigation of quantifying anxiety related behaviors in rodents<sup>20</sup>. Many of the available data indicate that nicotine increases the neuropsychiatric symptoms such as anxiety<sup>29,35</sup>.

The results of EPM experiment showed that the frequency and duration in open arm and center in nicotine treated rats were lower than those of the other groups, whereas freezing time and time spent in enclosed arm were higher in nicotine treated rats than those of the other groups. These alterations in nicotine treated rats (i.e. Decreased frequency and duration in open arm and center and increased freezing time and time spent in enclosed arm) indicate anxiogenic like effect for the drug in EPM. The pathogenesis of anxiety may be associated either with alteration in free radicals or activation of nicotinic-acetylcholine receptors by nicotine which enhances the release of many neurotransmitters such as dopamine, noradrenalin, glutamate and 5-HT involved in anxiety<sup>36</sup>. The obtained results are in line with the previous report showing that nicotine exerted an anxiogenic response<sup>29,35,37</sup>. Our study is contradictory to the study done by Budzynska B. et al. and Benwell ME et.al.<sup>33,38</sup>. It is interesting to note that *BM* abolished these alterations caused by nicotine treatment;

pointing anxiolytic impact of *BM* against nicotine treated rats. The study demonstrates for the first time the anxiolytic effect of *BM* in nicotine induced rats.

To assess the locomotion, exploratory and also anxiety related behavior, we performed OFT. Animal behaviors such as frequency of line crosses, frequency of rearing, central square entries and latency in the central square are used as measures of locomotion and exploration and vice-versa<sup>39</sup>.

In the present study, frequency of square line crossed, rearing and wall rearing and also time spent in center were significantly reduced in nicotine-treated rats and also inactive for longer duration of time, which illustrates nicotine exposed rats decreases the locomotion. The decrease in locomotion could be due to alteration in neuronal motor function or anxiety or reduction in body-weight which may decrease the muscle strength or inactiveness<sup>40</sup>. However, our study agrees with the results obtained by Omotoso GO et.al.<sup>7</sup>. On contrary to the studies conducted by Clarke and Kumar where they stated that chronic exposure of Nicotine increases the locomotor activity<sup>41</sup>. This non-consistent observation may be because of the doses and/or duration of nicotine exposure.

In addition, we also observed nicotine induced rats significantly reduces the rearing frequency and duration in EPM and OFT, which could be due to impairing motor coordination and balance<sup>42</sup>. The present study is in agreement with Iñiguez SD et. al. He described intraperitoneal administration of nicotine decreased the frequency of rearing in adult rats<sup>43</sup>. Furthermore, we evaluated the grooming activity and found that number and duration of grooming was significantly decreased in nicotine treated rats. This alteration in grooming might be related to dysfunction in serotonergic, glutamatergic and dopaminergic neurotransmission within corticostriatal circuitry<sup>42</sup>. Interestingly, co-exposure of *BM* along with nicotine significantly improves the behavioral activities altered due to nicotine administration as evidenced in EPM and OFT, establishing its neuropharmacological impact on the nicotine.

The BWT requires non-paretic paws and unimpaired inter-limb organization for maintaining the balance and motor coordination to traverse a narrow beam<sup>21</sup>. The present result showed that nicotine exposed rat took longer time to traverse

the beam and also increased number of foot-slips and near-falls than all other groups. The abnormal performances (longer duration to cross the beam and increased frequency of near-fall and actual fall) in BWT indicate impaired motor coordination and balance in nicotine treated rats signifying cerebellar dysfunction due to their disability to integrate sensory input with appropriate motor action to balance posture and at the same time adjust the limb movements on the beam<sup>44</sup>. Further, inability to judge distance and timing, loss of motor movement is also an indicative of cerebellar impairment<sup>44</sup>.

Conversely, the treatment with BM considerably reduced the time of crossing and number of foot-slips and near-fall against nicotine treated rats. This strongly suggests that BM improved motor coordination and balance possibly due to improving oxidative stress and oxidative stress and neurotransmitters. This is the first study exhibited on the effect of BM against nicotine in motor coordination and balance in BWT.

### CONCLUSION

The current study investigated that BM-supplementation prevents body weight loss caused by nicotine exposure. The findings of our study showed that BM treatment possesses a potential to control levels of oxidative stress markers and thus prevent the oxidative damage and neurotoxicity caused by nicotine administration. The data also indicate that impairment in motor coordination and balance, reduction in locomotor activities and high level of anxiety was observed in nicotine treated rats which were improved by oral administration of crude form of BM. Therefore, based on the present evidence, we conclude that BM has protective effect against nicotine on the cerebellum of rat brain. Further to draw a line in conclusion, Molecular studies may confirm whether BM administration prevents the structural degeneration of cerebellum in nicotine treated rats.

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### Conflict of interest

Authors have no conflicts of interest to declare.

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