Protective effect of *Withania somnifera* Dunal on the behavioral and biochemical alterations in sleep-disturbed mice (Grid over water suspended method)

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Sleep disruption involves extensive changes in physiological function, including EEG, motor, metabolic, autonomic processes physiological homeostasis and psychological balance that are necessary for physical health. Benzodiazepines are the most widely used drugs for the sleep related problems in spite of their limitations and side effects. Objective of the study was to investigate the protective effect of *W. somnifera* on the behavioral and biochemical alterations in sleep-disturbed mice. Pretreatment with *W. somnifera* root extract (100, 200 mg/kg) and diazepam (0.5 mg/kg) significantly protected reduction in body weight, improved the reduced locomotor activity and anxiety levels in animals. Biochemical studies also revealed that *W. somnifera* (100 and 200 mg/kg) and diazepam (0.5 mg/kg) pretreatment for five days decreased significantly lipid peroxidation, nitrites levels and improved catalase, and reduced glutathione levels. Co-administration of *W. somnifera* (100 mg/kg) with diazepam (0.5 mg/kg) improved significantly all the biochemical parameters as compared to their effect per se. Preliminary results suggest that *Withania* root extract can be used in the management sleep loss and associated oxidative stress.

**Keywords:** Anxiety, Locomotor activity, Oxidative stress, Sleep disturption, *Withania somnifera*

Sleep is normally a period of relaxation and repair, important for the maintenance of physiological homeostasis and psychological balance. Sleep loss is considered a health risk that contributes to several disease processes and leads to behavioral, hormonal and neurochemical alterations. Insomnia (i.e. difficulty in falling asleep) and other sleep-related disorders are common complaints among the middle-aged populations in different countries. Sleep deprivation is a kind of stress which leads to decrease in body weight in spite of increase in food intake, initial hyperthermia followed by hypothermia, increase in anxiety levels and decrease in locomotor activity, behavioral alternation (irritability and poor performance). Sleep deprivation is known to impair cognitive functions, oxidative stress and decreased anti-oxidative defense. Sleep deprivation causes oxidative stress by generating free radicals and non-radical derivatives of oxygen and nitrogen. These products are generally involved in normal cell regulation and signal transduction, an imbalance between their generation and the antioxidant defense system results in oxidative stress. The hypermetabolism and immunopathology in sleep-deprived animals are reported to produce excessive metabolic and oxidative burdens. Benzodiazepines and their analogues are the most widely used hypnotic drugs since the 1960s. However, it is well known that benzodiazepines have many untoward reactions, such as drug dependence, tolerance, rebound insomnia, amnesia and muscle relaxation. Therefore, it seems likely that these hypnotics need to be used cautiously. On the other hands, herbs are extensively used as traditional medicine for treatment of various nervous system disorders. *Withania* root extract and its constituents have mild to moderate hypnotic action and antioxidative property in animals. But no study yet available that established its effects in sleep-disturbed mice. Therefore, the aim of present study is to explore the protective effect of *Withania* root extract in sleep-disturbed mice.

**Materials and Methods**

*Animals*—Male Laca mice bred in Central Animal House of Panjab University, Chandigarh and weighing between 25-30 g were used. The animals were kept under standard laboratory conditions, maintained on 12 hr light/dark cycle and had free access to food and water. Animals were acclimatized...
to laboratory conditions before the test. Each animal was used once in the experiments. All the experiments were performed between 0900 and 1700 hrs. The experimental protocols were approved by Institutional Animal Ethics Committee and were conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

**Sleep deprivation**—Animals were sleep deprived for 48 hr by placing on the grid suspended over water method as described by Sinomiya et al. Animals were placed on a grid floor (29×15×7 cm) inside the plastic cage filled with water to 1 cm below the grid surface for 48 hour. The stainless steel rods of the grid (3 mm wide) were set 2 cm apart from each other. Food and water were provided *ad libitum*.

**Drug treatment**—Following drugs were used in the study- *Withania* root extract (Himalyan Drugs; Bangalore; 100 mg/kg and 200 mg/kg, po), diazepam (Ranbaxy Research Lab, Gurgaon; 0.5 mg/kg, ip). Drugs were dissolved in distilled water before administration. Animals were divided in six groups (six animals in each group). First and second groups were treated as naïve and stressed animals (suspended on grid). Third and fourth groups were treated with *Withania* root extract (100 and 200 mg/kg, po) for five days, starting three days before 48 hr sleep deprivation. Similarly fifth and sixth group were diazepam (0.5 mg/kg, ip) treated and its combination with withania (100 mg/kg, po) groups.

**Parameters measured**

**Body weight**—The body weights of animals were recorded before the start of experiment and thereafter before each behavioral quantification.

**Measurement of anxiety levels**—Elevated plus maze, developed by Pellow and File and modified by Kulkarni is a novel test for the selective anxiogenic and anxiolytic drug effect in rodents. The plus maze apparatus consist of two open (16 × 5 cm for mice) and two closed arm (16 × 5 ×12 cm), and is placed at a height of 25 cm for mice. The animals are placed individually at the center of the elevated plus maze with their head facing toward an open arm. During the 5 min test, the number of entries into the open and closed arm and the time spent in each arm of the maze is recorded.

**Locomotor activity**—The locomotor activity was monitored by using actophotometer (IMCORP, India). Before subjecting the animal for locomotor task they were individually placed in activity meter and the total activity count was registered for 5 min. The locomotor activity was expressed in terms of total photobeams counts/5 min per animal.

**Biochemical tests**

**Dissection and homogenization**—On day 5, the animals were sacrificed by decapitation immediately after behavioral assessments. The whole brains were removed and 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenate were centrifuged for 20 min at 15000 rpm and supernatant was used for estimation of lipid peroxidation and reduced glutathione levels. The post nuclear fractions for catalase assay were obtained by centrifugation of the homogenate at 1000 g for 20 min, at 4°C and for other enzyme assays centrifuged at 12,000 g for 60 min at 4°C.

**Lipid peroxidation assay**—The quantitative measurement of lipid peroxidation was performed according to the method of Wills. The amount of malondialdehyde (MDA), a measure of lipid peroxidation was measured by reaction with thiobarbituric acid (532 nm) using Shimadzu spectrophotometer. The values are were calculated using molar extinction coefficient of chromophore (1.56×10^5 M^-1 cm^-1) and expressed as percentage of control.

**Estimation of reduced glutathione**—Reduced glutathione in brain was estimated according to the method described by Ellman. Supernatant (1 ml) was precipitated with 1ml of 4% sulfosalicylic acid and cold digested at 4°C for 1hr. The sample was centrifuged at 1200 g for 15 min at 4°C. To supernatant (1 ml), 2.7 ml of phosphate buffer (0.1M, pH 8) and 0.2 ml of 5,5-dithiobis (2-nitrobenzoic acid)(DTNB) were added. The yellow color developed was read immediately at 412 nm using Shimadzu spectrophotometer. Results were calculated using molar extinction coefficient of chromophore (1.36 × 10^5 M^-1 cm^-1) and expressed as percentage of control.

**Estimation of nitrite**—The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid] as per Green et al. Equal volumes of supernatant and Greiss reagent were mixed, the mixture was incubated for 10 min at room temperature and the absorbance was measured at
540 nm using Shimadzu spectrophotometer. The concentration of nitrite in the supernatant was determined from a standard curve and expressed as percentage of control.

**Protein estimation**—The protein content was measured according to the method of Lowry et al.\(^{25}\) using bovine serum albumin as standard.

**Estimation of catalase**—Catalase activity was assayed by method of Luck\(^{26}\), wherein the breakdown of \(\text{H}_2\text{O}_2\) was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of \(\text{H}_2\text{O}_2\) phosphate buffer (1.25 \(\times\) \(10^{-2}\) M \(\text{H}_2\text{O}_2\)) and 0.05 ml of supernatant of the brain homogenate (10%), and the change in absorbance was recorded at 240 nm using the Shimadzu Spectrophotometer. Enzyme activity was calculated using the millimolar extinction coefficient of \(\text{H}_2\text{O}_2\) (0.07). The result was expressed as micromoles of \(\text{H}_2\text{O}_2\) decomposed/min/mg protein.

**Statistical analysis**—All the values are expressed as mean ± SE. The data were analyzed using analysis of variance (ANOVA) followed by Tukey test. In all the test criterion for statistical significance was \(P<0.05\).

**Results**

**Effects of Withania root extract on body weight of sleep-disturbed mice**—Body weights of 48hr sleep-deprived mice were significantly reduced as compared to naïve mice (without sleep deprived). Pretreatment with withania root extract (100 and 200 mg/kg) and diazepam (0.5 mg/kg) for 5 days significantly reversed the reduction in body weight. Co-administration of diazepam (0.5 mg/kg) with withania (100 mg/kg) could not significantly further reverse the body weight reduction as compared to effect per se \((P<0.05; \text{Table 1})\).

**Effects of Withania root extract on locomotor activity of sleep-disturbed mice**—Sleep loss (48 hr) caused significant decrease in locomotion in mice, which was significant as compared to naïve mice (without sleep deprived). Pretreatment with withania root extract (100 and 200 mg/kg) and diazepam (0.5 mg/kg) improve significantly the locomotor activity as compared to sleep deprived mice group. However, co-administration of diazepam (0.5 mg/kg) with withania (100 mg/kg) could not significantly further improve the locomotor activity as compared to effect per se \((P<0.05; \text{Table 1})\).

**Effects of Withania root extract on anxiety of sleep-disturbed mice**—Sleep deprivation for 48 hr increased anxiety level in mice (increased number of entries as well as duration in closed arm and decrease number of entries as well as duration in open arm of plus maze test), which was significant as compared to naïve mice. Pretreatment with withania root extract (100 and 200 mg/kg) and diazepam (0.5 mg/kg) increased the number of entries and duration in open arm significantly as compared to control (sleep deprived) as well as decrease the duration in closed arm. However, co-administration of Withania (100 mg/kg) with diazepam (0.5 mg/kg) drug treatment could not produce any further significantly effect as compared to their effect per se \((P<0.05; \text{Table 2})\).

**Effects of Withania root extract on antioxidant parameters of sleep-disturbed mice**—Glutathione and catalase activity: Sleep deprivation (48 hr) decreased glutathione level and catalase activity significantly as compared to naïve (without sleep deprived) mice. Pretreatment with withania root extract (100 and 200 mg/kg) and diazepam (0.5 mg/kg) reversed the depleted reduced glutathione and catalase activity, which was further potentiated, on combination of withania (100 mg/kg) and diazepam (0.5 mg/kg; \text{Table 1}).

**Lipid peroxidation and nitrite level:** Sleep loss for 48 hr caused significantly increased in lipid peroxidation and nitrite level, which was significant

<table>
<thead>
<tr>
<th>S N</th>
<th>Treatment (mg/kg)</th>
<th>No of entries</th>
<th>Duration</th>
<th>No of entries</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naive</td>
<td>4.16±0.54</td>
<td>244±2.14</td>
<td>3.66±0.33</td>
<td>36.00±2.14</td>
</tr>
<tr>
<td>2</td>
<td>Control (sleep deprived)</td>
<td>6.00±1.0(^a)</td>
<td>296.16±1.27(^a)</td>
<td>0.66±0.21(^a)</td>
<td>4.16±1.42(^a)</td>
</tr>
<tr>
<td>3</td>
<td>Diazepam (0.5)</td>
<td>6.33±1.52</td>
<td>226.0±6.63(^b)</td>
<td>3.83±0.65(^b)</td>
<td>74.0±11.63(^b)</td>
</tr>
<tr>
<td>4</td>
<td>ASH (100)</td>
<td>5.33±0.66</td>
<td>258±1.57(^b)</td>
<td>2.83±0.3(^b)</td>
<td>20.0±1.57(^b)</td>
</tr>
<tr>
<td>5</td>
<td>ASH (200)</td>
<td>5.33±0.66</td>
<td>242.33±5.32(^b)</td>
<td>3.83±0.6(^b)</td>
<td>37.66±7.32(^b)</td>
</tr>
<tr>
<td>6</td>
<td>ASH (100) + Dia (0.5)</td>
<td>5.33±0.71</td>
<td>286±2.43</td>
<td>2.33±0.42(^b)</td>
<td>14.0±2.43(^b)</td>
</tr>
</tbody>
</table>

\(P<0.05; ^a\)as compared to naïve, \(^b\)as compared to control (sleep deprived)
as compared to naïve or base line (without sleep deprived group \( P<0.05 \)). Pretreatment with withania root extract (100 and 200 mg/kg) and diazepam (0.5 mg/kg) reduced lipid per oxidation and nitrite activity as compared to the control (sleep deprived). However co-administration of withania (100 mg/kg) and diazepam (0.5 mg/kg) could further potentiated nitrite level only. There was not significant effect on the lipid peroxidation in the combination group (Table 2).

### Discussion

Sleep deprivation is considered a risk for disease. Several lines of evidences suggest that sleep has an important role in learning and memory, motor control and anxiogenesis level. Sleep deprivation caused various behavioral disturbances such as motor activity, anxiety level, memory dysfunction, and metabolic function such as reduced anabolic hormones, body weight etc.\(^6\,27\). The behavioral and biochemical changes produced by sleep deprivation that results in health consequences however are largely unknown.

In the present study also, 48 hr sleep deprivation significantly impaired behavioral (locomotors activity and anxiety level) as well as oxidative stress parameters (lipid peroxidation, glutathione, catalase and nitrite level). Marked behavioral changes may be due to alteration in the brain regions involve in sleep and wakefulness. However, marked reduction in body weight was also noticed in animals. Sleep deprivation has been reported to cause hypermetabolism.\(^3\,14\).

*Withania somnifera* root extract is widely used traditionally for number of problems because of its antioxidant, adaptive, immune enhancing, and anabolic properties and also has weak or mild hypnotic effect.\(^28\,30\). Benzodiazepines are among the most commonly consumed drug worldwide for sleep and sleep related problems. In the present study, administration of *Withania somnifera* extract for five days significantly protected the reduction in body weight, reversed the reduction of motor activity and anxiety in sleep-deprived mice. This suggests beneficial effect of withania in sleep disturbances. However, co-administration of withania with diazepam did not improve further locomotor activity as well as anxiety in animals. However, both *Withania* and diazepam have been known to act by GABA/BZD mechanism. The recorded anxiety and reduced locomotor activity might be either due to their additive effect in term of hypnosis or increased inhibitory effect that led to hypnosis resulting in decreased activity in plus maze and actophotometer tests.

The 48 hr sleep deprivation also produced oxidative stress.\(^31\). Oxidative stress parameters namely lipid peroxidation, glutathione, catalase and nitrite level may be useful to understand the biochemical events in sleep deprivation. However, little is known whether stress is an important consequence of sleep deprivation. Reimund theorized\(^32\) that sleep increases the efficiency of antioxidant mechanisms in the brain. Present study suggests that withania root extract significantly reduced the lipid peroxidation, nitrite level and restored the catalase and glutathione level in animals. Ramanathan *et al.*\(^12\) also reported a significant decrease in superoxide dismutase activity in the hippocampus and brain stem in sleep-deprived rats. This indicates that beneficial effect of withania in sleep deprivation associated oxidative stress and beneficial effect of withania might be due to its antioxidative property. However co-administration of withania with diazepam did not potentiated the

### Table 2—Effects of Withania root extract on locomotors, body weight and oxidative parameters of sleep-disturbed mice

[Values are mean±SE from 6 animals in each group]

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Locomotor Activity in 5 min</th>
<th>Body weight (% reduction)</th>
<th>LPO (% of the naïve)</th>
<th>GSG (% of the naïve)</th>
<th>Catalase (% of the naïve)</th>
<th>Nitrite (% of the naïve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>271.83±4.41</td>
<td>0±0</td>
<td>100±5.3</td>
<td>100±1.63</td>
<td>100±17.12</td>
<td>100±15</td>
</tr>
<tr>
<td>Control</td>
<td>76.62±2.56 (^a)</td>
<td>25.3±1.5 (^a)</td>
<td>188±5.7 (^a)</td>
<td>36.06±3.3 (^a)</td>
<td>18.58±2.75 (^a)</td>
<td>290±2.5 (^a)</td>
</tr>
<tr>
<td>Diazepam (0.5)</td>
<td>212±22.7 (^b)</td>
<td>14.7±1.8 (^b)</td>
<td>115.5±5.3 (^b)</td>
<td>93.44±0.9 (^b)</td>
<td>52.77±7.48 (^b)</td>
<td>96.66±11.6 (^b)</td>
</tr>
<tr>
<td>ASH (100)</td>
<td>106.0±4.5 (^b)</td>
<td>14.32±0.81 (^b)</td>
<td>126.22±5.3 (^b)</td>
<td>70.49±1.6 (^b)</td>
<td>37.77±5.07 (^b)</td>
<td>188.33±19.9 (^b)</td>
</tr>
<tr>
<td>ASH (200)</td>
<td>122.5±1.2 (^b)</td>
<td>12.15±0.97 (^b)</td>
<td>92.88±5.3 (^b)</td>
<td>106.55±1.6 (^b)</td>
<td>75.55±4.9 (^b)</td>
<td>155±18.3 (^b)</td>
</tr>
<tr>
<td>ASH (100) + Dia (0.5)</td>
<td>29.33±7.09</td>
<td>10.51±0.69</td>
<td>92±4.44</td>
<td>113.11±1.6 (^c)</td>
<td>111.01±17.5 (^c)</td>
<td>83.33±9.9 (^c)</td>
</tr>
</tbody>
</table>

\(P<0.05\); \(^a\) as compared to naïve, \(^b\) as compared to control (sleep deprived), \(^c\) as compared to diazepam (0.5mg/kg) and ASH (100 mg/kg)
antioxidative effect. Effect was comparable to that of diazepam. This suggests that both drugs might have different or other unknown antioxidative action.

To conclude, the present study suggests that withania root extract can be used in the management of sleep loss and their related behavioral and biochemical alterations.

References