Effect of *Withania somnifera* Dunal in ethanol-induced anxiolysis and withdrawal anxiety in rats

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*Withania somnifera* (WS) or its psychotropic preparation is known to play a critical role in morphine, alcohol and benzodiazepines addiction. This study investigates the role of WS in acute ethanol and withdrawal from chronic ethanol consumption using elevated plus maze paradigm in rats. Acute administration of ethanol (1.5-2 g/kg, ip) triggered anxiolytic effect and withdrawal from prolonged ethanol (9% v/v ethanol, 15 days) consumption elicited enhanced behavioral despair (anxiety). Acute administration of WS (50 mg/kg, oral) potentiated the anxiolytic action of subeffective dose of ethanol (0.5 or 1 g/kg, ip). Moreover, the ethanol withdrawal anxiety was markedly antagonized in dose dependent manner by WS at 200 and 500 mg/kg or higher dose of ethanol (2.5 g/kg). However, co-administration of subeffective doses of WS (50 mg/kg, oral) and ethanol also attenuated withdrawal-induced anxiety due to chronic ethanol (9% v/v ethanol, 15 days) consumption. The results suggest the protective effect of WS in the management of ethanol withdrawal reactions.

**Keywords:** Elevated plus maze, Ethanol, *Withania somnifera*, Withdrawal anxiety

Alcoholism is a major public health problem that not only causes enormous damage to health and quality of life, but also undermines the well-being of family and society. The anxiolytic property of ethanol following its acute administration has been well demonstrated in humans and in rodent models of anxiety. Anxiety is one of the most pervasive and troubling symptoms of ethanol withdrawal in humans and rodents. Although benzodiazepines have been used to treat ethanol withdrawal disorders, their addictive potentials and sedative sideeffects limit the use. Since, the chronic treatment with benzodiazepines often proves more harmful in the longer run, there has been an increasing thrust worldwide to opt for safer and effective plant-derived anxiolytics and hypnosedatives mentioned in the traditional medical systems.

*Withania somnifera* Dunal (WS) (family, Solanaceae), known as ashwagandha in Ayurveda, the ancient Hindu system of medicine, has been in use for more than 2500 years. Historically, WS, or its major active principles, has been used as an antioxidant, adaptogen, anxiolytic, antidepressant, memory enhancer, antiinflammatory, antiulcerogenic and anticarcinogenic agents. The active principles of WS, consisting of sitoindosides VII–X and withaferin-A, have been shown to exhibit significant anti-stress and antioxidant effect in rat brain frontal cortex and striatum. The experimental studies in animals have extensively demonstrated a GABA-mediated action of WS.

Ethanol withdrawal anxiety has already been reported to be antagonized by various agents such as tri-substituted benzoflavone moiety of *Passiflora incarnata* Linnaeus, serotonin receptor antagonist, GABA mimetic agents, corticotropin-releasing factor (CRF) receptor antagonist, neurosteroids and melanocortin receptor antagonist. Benzodiazepines and opioids induced tolerance and withdrawal has been blocked by a polyherbal preparation, BR-16A (Mentat), which has WS as a one of its ingredient. Importantly, WS also blocked morphine dependence and tolerance to its analgesic effect. Interestingly, ethanol withdrawal anxiety and convulsion have also been blocked by BR-16A (Mentat) in rodents. Therefore, the present study has been undertaken to investigate the effects of WS on ethanol-induced anxiolysis and withdrawal anxiety in rats.

**Materials and Methods**

*Animals*—Male, Wister albino rats weighing 150–180 g (90 to 110 days old) were housed in group of 4 per cage under controlled light (12:12 light: dark...
cycle, light on at 0700 hr) and temperature (25° ± 2°C) environment and behavioral assessment was conducted during the light cycle. Food (Rat chow, Lipton, India) and water were provided ad libitum. All procedures were carried out under strict compliance with ethical principles and guidelines of the Institutional Animal Ethical Committee constituted as per the direction of the Committee for the Purpose of Control and Supervision of Experimental Animals, Chennai (Reg. No. 870/ac/05/CPCSEA).

Drugs—Commercial ethanolic WS root extract (Dabur, New Delhi, India) was suspended in 0.5% w/v carboxy methyl cellulose (CMC) in distilled water and administered via oral route. The stock solution contained 100 mg/ml of WS. Absolute ethanol was procured from MSSIDC, Mumbai, India.

Elevated plus maze test (EPM) for rats—The model has been validated pharmacologically and currently considered the ‘gold standard’ test of anxiety-related behavior. The model modified by Kokare et al., was used in the present study. After drugs treatment individual rat was placed at the center of the maze, head facing an open arm. During the 5 min test period, the number of entries and time spent on the open arm as well as closed arm were measured. An entry was defined as placing all four paws of the animal on an arm.

Experimental design—Each experimental group (n=7) had a separate set of rats being allotted randomly. All subjects were experimentally naive at the beginning of each study and used only once to avoid “one-trial tolerance” to anxiolytic efficiency of drugs in EPM test.

Effect of ethanol and WS in EPM test (acute study)—Animals from different groups were administered with vehicle (0.5 ml of 0.5% w/v CMC in distilled water, oral, n=7) or WS (50-500 mg/kg, oral, n=7) and ethanol (0.5-2.5 g/kg, ip, single injection of 8% w/v ethanol, n=7 per group). One hour after oral and 30 min after ip injection, individual rat was placed in EPM test.

Influence of WS on ethanol in EPM (acute study)—To assess the influence of WS on anxiolytic action of ethanol, separate groups of rats administered with subeffective dose of WS (50 mg/kg, oral, n=7), 30 min prior to subeffective doses of ethanol (0.5, or 1 g/kg, ip, single injection of 8% w/v ethanol, n=7) treatment. After 30 min, individual rat was subjected to EPM test.

Ethanol withdrawal anxiety (chronic study)—Animals were given free access to ethanol continuously for 15 days through liquid diet as described earlier. Briefly, different groups of rats housed individually, were given nutritionally balanced control liquid diet (Novartis India Ltd., Mumbai) for 2 days to allow adaptation to the novel food. Water was made freely available. After adaptation in some groups of animals, ethanol was gradually introduced into the liquid diet containing 4.5% (v/v) ethanol on the first day, 7.5% (v/v) ethanol on the second day and were maintained on 9% (v/v) ethanol from third day onwards for 15 days (ethanol-fed group). Rest of the animals continued on the nutritionally balanced control liquid diet (pair-fed control group). Fresh aliquot of ethanol diet and/or control liquid diet (100 ml/rat) was introduced in the cage each morning. At the end of 15 days of treatment, pair-fed groups were continued on the same diet, whereas to ethanol fed groups, ethanol free liquid diet was introduced from 16th day (0900 hr) onwards until the termination of the experiment (ethanol withdrawal). Three days post withdrawal condition, during which the abstinence anxiety was observed; the animals were challenged with different doses of WS (100, 200 or 500 mg/kg, oral, n=7 per group). After one hour, individual rat from all groups was subjected to the EPM test. Some of these ethanol-withdrawn groups (n=7) were challenged with a combination of ethanol (8% w/v, 1.5-2 g/kg, ip) plus WS (50 mg/kg, oral) before the EPM test. Control animals that received only ethanol (1.5-2.5 g/kg, ip) were also subjected to the EPM test. Effect of chronic ethanol administration on weight gain was also assessed during the study. In all experiments vehicle, WS and ethanol treated groups were run in parallel.

TLC study of WS root extract—Thin-layer chromatography (TLC) was used to identify the steroidal lactones (withanolides) present in WS. The solvent system used was chloroform:methanol:water (64:50:10, v/v) and spots were finally identified with vanillin–phosphoric acid.

Statistical analysis—The results are presented as mean ± SE. The effects of different doses of acute ethanol or WS were statistically analyzed by one-way analysis of variance (ANOVA) with repeated measures on drug treatments followed by post hoc Dunnett’s test. The data obtained from ethanol-withdrawn and pair fed rats were compared by unpaired t-test. The acute combination protocols and
treatment of drugs in ethanol-withdrawn rats was analyzed by one-way repeated-measures ANOVA, and individual means were compared by Student-Newman-Keuls post hoc test. Differences were considered to be significant at $P<0.05$.

**Results**

**Effect of ethanol and WS in EPM**—Acute administration of ethanol 1.5-2 g/kg, i.p., significantly increased the time spent [$F(4,34)=37.504, P<0.01$] and number of entries [$F(4,34)=19.257, P<0.01$] into the open arms as compared to vehicle-treated group (Table 1). However, at higher dose (2.5 g/kg, ip), ethanol significantly decreased the time spent ($P<0.05$) and number of entries in both open and closed arms ($P<0.05$) due to mild sedation or motor incoordination. Rats injected with ethanol, at the dose of 0.5 or 1 g/kg, showed insignificant change in the time spent or in the number of entries into the open arms.

WS (100, 200 or 500 mg/kg, oral) also showed the significant increase in the time spent and the number of entries into the open arms as compared to vehicle treated rats. However, the lower dose of WS (50 mg/kg, oral) showed insignificant change in time spent and number of entries into the open arms as compared to vehicle (Table 1). Insignificant change in the number of closed arms entries were observed irrespective of the treatment, ethanol [$F(4,34)=0.4392, P>0.05$, n.s.] or WS [$F(4,34)=0.5410, P>0.05$, n.s.] as compared to the vehicle treated group, except animals injected with 2.5 g/kg ethanol that caused mild sedation or motor incoordination (Table 1).

**Effect of acute ethanol following treatment with WS in EPM**—Prior treatment with subeffective dose of WS (50 mg/kg, oral) significantly increased anxiolytic effect of subeffective doses of ethanol (0.5 or 1 g/kg, ip), by increasing time spent [$F(3,27)=7.293, P<0.05$ or $F(3,27)=14.342, P<0.001$] and number of entries [$F(3,27)=17.143, P<0.001$ or $F(3,27)=15.000, P<0.001$] into the open arms as compared to corresponding ethanol given alone in EPM test (Table 1). The number of closed arms entries [$F(3,27)=0.9086, P>0.05$, ns] and [$F(3,27)=1.857, P>0.05$, ns] did not change significantly irrespective of the ethanol (0.5 or 1 g/kg, ip) given alone (Table 1).

**Effect of WS on the ethanol withdrawal anxiety**—In 72 h of chronic ethanol withdrawn rats, the time spent 9.286 ± 1.796 [unpaired $t$-test; $t=3.109, df=12, P<0.01$] and number of entries into the open arms 0.142 ± 0.1429 [unpaired $t$-test; $t=3.536, df=12, P<0.01$] and number of closed arms entries 1.857 ± 0.2608 [unpaired $t$-test; $t=4.801, df=12, P<0.001$] were significantly reduced as compared to time spent (22.571 ± 3.878), number of entries into the open arms (0.857 ± 0.1429) and number of closed arms entries (3.714 ± 0.2857) in pair-fed rats respectively. The ethanol-Table 1—Effects of acute ethanol or WS on behavior of rats in elevated plus maze test

[Values are mean ± SE over 5 min duration of 7 rats in each group]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Open arm time (seconds)</th>
<th>Open arm entries (number)</th>
<th>Closed arm entries (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>22.571 ± 3.878</td>
<td>0.857 ± 0.1429</td>
<td>3.714 ± 0.2857</td>
</tr>
<tr>
<td>Ethanol 0.5 g/kg, ip</td>
<td>28.571 ± 1.556</td>
<td>1.286 ± 0.1844</td>
<td>4.143 ± 0.6701</td>
</tr>
<tr>
<td>Ethanol 1.0 g/kg, ip</td>
<td>31.429 ± 1.601</td>
<td>1.429 ± 0.2020</td>
<td>4.571 ± 0.6117</td>
</tr>
<tr>
<td>Ethanol 1.5 g/kg, ip</td>
<td>47.143 ± 2.219**</td>
<td>2.429 ± 0.2974**</td>
<td>4.229 ± 0.3689</td>
</tr>
<tr>
<td>Ethanol 2.0 g/kg, ip</td>
<td>60.000 ± 2.047**</td>
<td>3.286 ± 0.2857**</td>
<td>4.286 ± 0.5216</td>
</tr>
<tr>
<td>Ethanol 2.5 g/kg, ip</td>
<td>10.857 ± 2.121**</td>
<td>0.285 ± 0.1844**</td>
<td>1.571 ± 0.3689**</td>
</tr>
<tr>
<td>WS 50 mg/kg, oral</td>
<td>28.857 ± 5.470</td>
<td>1.143 ± 0.1429</td>
<td>3.429 ± 0.2020</td>
</tr>
<tr>
<td>WS 100 mg/kg, oral</td>
<td>40.429 ± 3.491**</td>
<td>1.857 ± 0.2608**</td>
<td>3.571 ± 0.3689</td>
</tr>
<tr>
<td>WS 200 mg/kg, oral</td>
<td>46.714 ± 2.533**</td>
<td>2.286 ± 0.2857**</td>
<td>3.429 ± 0.2020</td>
</tr>
<tr>
<td>WS 500 mg/kg, oral</td>
<td>51.714 ± 3.076**</td>
<td>3.143 ± 0.2608**</td>
<td>3.143 ± 0.2608</td>
</tr>
<tr>
<td>Ethanol 0.5 g/kg, ip + WS 50 mg/kg, oral</td>
<td>45.000 ± 1.786a</td>
<td>2.429 ± 0.2020b</td>
<td>4.429 ± 0.4809</td>
</tr>
<tr>
<td>Ethanol 1 g/kg, ip + WS 50 mg/kg, oral</td>
<td>55.429 ± 1.901b</td>
<td>2.714 ± 0.2857b</td>
<td>4.714 ± 0.4206</td>
</tr>
</tbody>
</table>

WS - *Withania somnifera*

$P$ values: *<0.05, **<0.01, ***<0.001 as compared with vehicle, Dunnett’s test.

$P$ values: a<0.05, b<0.001 as compared with respective ethanol control, Student-Newman-Keuls test.
withdrawn rats, when challenged with WS (100-500 mg/kg, oral) displayed significant anxiolytic effect at 200 and 500 mg/kg doses (Table 2). This was evident from the greater time spent \[F(2,20)=25.806, P<0.001\] or \[F(2,20)=43.267, P<0.001\] and entries into open \[F(2,20)=7.548, P<0.01\] or \[F(2,20)=19.500, P<0.001\] arms. The number of closed arms entries remained unchanged \[F(3,27)=0.3974, P>0.05, \text{ns}\]. The withdrawal anxiety was not reversed by acute challenge with moderate dose (1.5 or 2 g/kg, ip) of ethanol (ns), although higher dose (2.5 g/kg, i.p.) significantly reversed by increasing the time spent \[F(3,27)=43.619, P<0.001\] and entries \[F(3,27)=13.306, P<0.001\] into the open arms (Table 2). However, the closed arms entries was not significantly influenced \[F(3,27)=1.333, P>0.05, \text{ns}\].

Effect of combination of WS and ethanol on abstinence anxiety—In ethanol withdrawn animals, subeffective doses of ethanol (1.5 or 2 g/kg, ip), given in combination with subeffective dose of WS (50 mg/kg, oral), exhibited marked augmentation of anxiolytic effect as evident by increased time spent \[F(3,27)=64.224, P<0.001\] or \[F(3,27)=127.17, P<0.001\] and entries \[F(3,27)=18.000, P<0.001\] or \[F(3,27)=28.364, P<0.001\] into the open arms. However, the closed arms entries was not significantly influenced \[F(5,41)=0.9184, P>0.5, \text{n.s.}\] as compared with respective ethanol control (Table 2).

In the weight gain study, each rat consumed about 12 ± 2.5 g/kg ethanol through liquid diet daily and the treatment does not seem to alter the body weight. On the last day of liquid diet, the mean ± SE body weight (g) for the control and ethanol fed animals was 167 ± 7.0 g and 178 ± 5.4 g, respectively.

**TLC study of WS root extract**—TLC showed the presence of four blueish violet spots (Rf value: 0.8–0.9)22.

**Discussion**

In the present study, WS increased ethanol induced anxiolysis and inhibited the chronic ethanol withdrawal anxiety as revealed in the EPM paradigm of anxiety. Acute administration of ethanol at dose 1.5 or 2 g/kg showed significant, while the lower dose of ethanol (0.5 and 1 g/kg, ip) showed insignificant anxiolytic effects. The higher dose of ethanol (2.5 g/kg, ip) resulted in mild sedation or motor in-coordination as previously reported2,3. Acute administration of WS at 100, 200 or 500 mg/kg dose significantly increased time spent and number of entries in the open arms as compared to vehicle and showed anxiolytic effect, similar to previously reported work22,23. The anxiolytic effect of WS and ethanol has been extensively studied in rodents employing a range of paradigms of anxiety and action is mediated via the GABA A receptors2,7. In this study, subeffective dose of WS positively modulated the subanxiolytic doses of ethanol (0.5 and 1 g/kg, ip) through potentiation of its response in EPM test. This suggests that anxiolytic effect of ethanol may be potentated by GABA mimetic effect of WS7.

Several previous reports based on the EPM test suggest that withdrawal of ethanol following chronic treatment generates anxiety3,9,20, which is measure of psychological dependence. The present studies confirm

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**Table 2—Effect of WS and ethanol on ethanol abstinence anxiety in elevated plus maze test**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Open arm time (seconds)</th>
<th>Open arm entries (number)</th>
<th>Closed arm entries (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>9.286 ± 1.796</td>
<td>0.142 ± 0.1429</td>
<td>1.857 ± 0.2608</td>
</tr>
<tr>
<td>WS 100 mg/kg, oral</td>
<td>15.000 ± 2.820</td>
<td>0.571 ± 0.2020</td>
<td>2.000 ± 0.2182</td>
</tr>
<tr>
<td>WS 200 mg/kg, oral</td>
<td>30.571 ± 1.288**</td>
<td>1.143 ± 0.2608</td>
<td>2.143 ± 0.4041</td>
</tr>
<tr>
<td>WS 500 mg/kg, oral</td>
<td>37.857 ± 1.100**</td>
<td>1.714 ± 0.1844</td>
<td>2.286 ± 0.1844</td>
</tr>
<tr>
<td>Ethanol 1.5 g/kg, ip.</td>
<td>13.000 ± 1.345</td>
<td>0.428 ± 0.2020</td>
<td>2.000 ± 0.2182</td>
</tr>
<tr>
<td>Ethanol 2.0 g/kg, ip</td>
<td>15.000 ± 1.431</td>
<td>0.714 ± 0.1844</td>
<td>2.143 ± 0.4041</td>
</tr>
<tr>
<td>Ethanol 2.5 g/kg, ip</td>
<td>32.857 ± 1.625**</td>
<td>1.714 ± 0.1844</td>
<td>2.571 ± 0.2020</td>
</tr>
<tr>
<td>Ethanol 1.5 g/kg, ip + WS 50 mg/kg, oral</td>
<td>30.429 ± 1.784*</td>
<td>1.571 ± 0.2020(^a)</td>
<td>2.286 ± 0.1844</td>
</tr>
<tr>
<td>Ethanol 2 g/kg, ip + WS 50 mg/kg, oral</td>
<td>43.714 ± 1.911*</td>
<td>2.143 ± 0.2608(^a)</td>
<td>2.429 ± 0.3689</td>
</tr>
</tbody>
</table>

WS - *Withania somnifera*

\(P\) values: \(<0.01, "<0.001 as compared with vehicle, Student-Newman-Keuls test.\)

\(^{a}\)\(P<0.001\) as compared with respective ethanol control, Student-Newman-Keuls test.
these observations. Termination of ethanol ingestion precipitated withdrawal anxiety as revealed by the significant reduction in open arm exploration. The closed arms entries were also markedly decreased in ethanol-withdrawn animals. Anxiety response induced by ethanol withdrawal is almost always associated with a decrease in general activity as measured by closed or total arms entries. It is interesting to note that very high dose of ethanol (2.5 g/kg, ip) was required to reverse ethanol withdrawal anxiety. However, this dose in acute studies, in ethanol naive rats produced sedation or motor incoordination instead of anxiolytic effect. The present experimental design was similar to the previous findings in terms of the duration of treatment, dose and species of animal. In the present study, WS reversed the ethanol withdrawal anxiety and also potentiated the anxiolytic responses following lower doses of ethanol. The downregulation of GABA receptors and/or decrease in the GABAergic transmission have been implicated in the withdrawal symptoms of ethanol. This suggests that GABA mimetic and adaptogenic effect of WS may decrease further downregulation of GABA receptors.

The number of total arm entries has been criticized as a measure of locomotor or general activity, since it changes following anxiolytic and anxiogenic agents. On the other hand, the absolute number of closed arms entries alone was suggested as a critical measure for locomotion in EPM paradigm. Since acute or ethanol-withdrawn rats receiving WS failed to show any influence on the number of closed arms entries, the involvement of the locomotor component in their action on modulation of anxiety may be disregarded.

In conclusion, WS potentiate the anxiolytic effect of ethanol. Similarly, the treatment with WS and/or its combination with lower dose of ethanol may prevent the ethanol withdrawal anxiety.

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References

