

# Withaferin A attenuates Alcohol Abstinence Signs in Rats

Nandkishor Ramdas Kotagale, Ankit Kedia, Rupali Gite, Shubham Nilkanth Rahmatkar, Dinesh Yugraj Gawande, Milind Janraoji Umekar, Brijesh Gulabrao Taksande\*

## ABSTRACT

**Background:** *Withania somnifera* (WS) have been reported to inhibit acquisition and expression conditioned place preference, self-administration and withdrawal anxiety of psychostimulants. In the present work, we have assessed the effect of Withaferin A on somatic and affective symptoms of ethanol withdrawal syndrome in rats. **Methods:** Animals had given free access to ethanol uninterrupted for 21 days through liquid diet. Withaferin A (5, 10 and 20 mg/kg) was injected (ip) either during the development of ethanol dependence phase (days 15 – 21 or 30 min before ethanol withdrawal assessment. Withdrawal signs characterized by changes in somatic signs were measured in the open field followed by evaluation of anxiety parameters, locomotion, and depressive behavior. **Results:** Withaferin A treatment 30 min before 24 h post-ethanol withdrawal assessment did not alter the scores of somatic behavioral signs in ethanol abstinence animals. However, withaferin A (10 and 20 mg/kg, ip) from day 15-21 prevented the ethanol withdrawal-induced elevated scores of somatic behaviors, hyperlocomotion, depressive behavior, and anxiety. Withaferin A treatment did not influence the blood ethanol levels in dependent and withdrawn animals. However, withaferin A administration attenuated the elevated plasma corticosterone and ACTH levels in ethanol-withdrawn rats, suggesting withaferin A induced anti-stress effect and stabilization of HPA axis activity could have facilitated the inhibitory effect of withaferin A on ethanol withdrawal syndrome. **Conclusion:** The finding supports further investigation of Withaferin A and other bioactive components of WS in alcohol addiction.

**Key words:** Anxiety, Corticosterone, Ethanol withdrawal, HPA axis, Withaferin A.

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## History

- Submission Date: 11-12-2017;
- Review completed: 03-05-2018;
- Accepted Date: 06-08-2018

DOI : 10.5530/pj.2018.6.204

## Article Available online

<http://www.phcogj.com/v10/i6>

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## INTRODUCTION

Alcohol withdrawal syndrome is potentially life-threatening in addicted people and associated maladies constitute a serious health and social issues.<sup>1</sup> Abstinence from chronic ethanol consumption leads to the manifestation of a variety of somatic and affective symptoms attributed to central nervous system hyperexcitability, like irritability, anxiety, restlessness and dysphoria.<sup>2</sup> Despite the tremendous advances made in the treatment of alcoholism and/or its abstinence, remarkably, the majority of these agents, including naltrexone and benzodiazepines etc. have unpleasant side effect.<sup>3,4</sup>

Withaferin A is a steroidal lactone, an active compound isolated from *Withania somnifera* (WS) (Family- Solanaceae). WS, known as ashwagandha in Ayurveda or its active principles, including withaferin A has been used as an antioxidant, adaptogen, antistress,<sup>5,6</sup> anti-inflammatory, neuroprotective, anxiolytic, anti-depressant, immunomodulatory, memory enhancer, anti-ulcer and anti-carcinogenic agents.<sup>7-12</sup> In addition, WS extract has been inhibited the morphine-induced acquisition and expression in conditioned place preference,<sup>13</sup> ethanol conditioned place preference<sup>14</sup> and self-administration,<sup>15</sup> ethanol withdrawal-induced anxiety in rats.<sup>16</sup> In the present work, we have assessed

the effect of withaferin A on somatic and affective symptoms of ethanol withdrawal syndrome in rats.

## MATERIALS AND METHODS

### Subjects

Adult healthy Sprague Dawley rats weighing 200-220 g (3-4 months old) were group housed (four per cage) under controlled temperature (25±2°C) and light (12 h light/dark cycle, light on at 07.00 am) environment with free access to food and water. Experimental protocols were approved by the Institutional Animal Ethical Committee and executed in strict accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India. The behavioral assessments were conducted during the light cycle.

### Drugs

Withaferin A was purchased from Natural Remedies Private Limited, Bangalore, India and administered intraperitoneally (ip) as a solution (1 ml/kg) in dimethylsulphoxide (DMSO) prepared just before the experiments. Ethanol (99% w/v) (Merck, India)

**Cite this article:** Kotagale NR, Kedia A, Gite R, Rahmatkar SN, Gawande DY, Umekar MJ, Taksande BG. Withaferin A attenuates Alcohol Abstinence Signs in Rats. *Pharmacogn J.* 2018;10(6):1190-5.

was given through liquid diet. Doses and timing of the drug injections with respect to the behavioral testing employed in the protocols were selected on the basis of previous experiments carried in our laboratory and available literature.<sup>17,18</sup>

### Induction of ethanol dependence and withdrawal

All different groups of rats ( $n = 16 - 20$ ) housed individually were given free access to nutritionally balanced liquid diet (Novartis India Ltd., Mumbai) without ethanol for 7 days to allow adaptation to the novel food. Water was made freely available. From 8<sup>th</sup> day onwards, half of the animals ( $n = 6$ ) had given free access to ethanol uninterrupted for 21 days through liquid diet.<sup>19</sup> Initially, 2.4% v/v ethanol was introduced into the liquid diet for 3 days, which was increased to 4.8% v/v for the following 4 days and finally to 7.2% v/v for 14 days (ethanol-fed group). The remaining rats kept on the control liquid diet (nutritionally balanced) (pair-fed group). Every day fresh ethanol and control liquid diet (100 ml/rat) was introduced into the cage each morning at 0900 h. The weight of each rat was recorded everyday and daily ethanol intake was monitored and expressed as g/kg/day. From day 22 (0900 h), pair-fed groups were continued on the same diet, whereas to ethanol-fed groups, ethanol diet was stopped and animals were fed with isocaloric liquid diet (without ethanol) until the end experiment. Control rats ( $n = 6$ ) were pair-fed with an isocaloric liquid diet containing sucrose as a caloric substitute to ethanol.

This protocol had two segments: In the first design, withaferin A (5, 10 and 20 mg/kg) was injected (ip) during the development of ethanol dependence phase (days 15 - 21). In the second design, withaferin A (5, 10 and 20 mg/kg) was administered to rats 30 min before ethanol withdrawal assessment. Separate groups ( $n = 6$ ) of rats were assigned to different doses. Appropriate vehicle-treated controls were also maintained simultaneously.

### Behavioral changes during ethanol withdrawal

At 24 h post ethanol withdrawal, the pair-fed groups, as well as ethanol-fed groups, were subjected to evaluation of ethanol withdrawal syndrome. This time point was selected since peak withdrawal somatic and affective signs were apparent at this time point.<sup>17,18, 20,21</sup> Withdrawal signs characterized by changes in somatic signs were measured in open field for 15 min.<sup>19</sup> This was followed by evaluation of anxiety parameters in elevated plus maze (EPM),<sup>18</sup> locomotion (actophotometer) and depressive behavior (Porsolt's forced swim test, FST) each for 5 min duration.<sup>17</sup> All the animal experiments were performed during the light cycle between 0900 and 1200 h.

### Somatic signs

At 24 h of ethanol withdrawal, animals were observed individually for 15 min and the frequency of various stereotyped behavioral signs that included grooming, sniffing, head weaving, gnawing, chewing, scratching, genital licking and body shake were recorded. Ethanol abstinence score (EAS) consisting of the sum of all the stereotyped behaviors was compiled and expressed as mean  $\pm$  SEM.<sup>22</sup> Wet dog shakes, tremors, tail stiffness, abnormal gait and posture, abdominal constriction and teeth chattering were also assessed for the incident.

### Anxiety related behavior

The anxiety in animals was evaluated in elevated plus maze (EPM), the earlier reported experimental protocol was followed.<sup>18</sup> Briefly, after 24 h of withdrawal rats were placed on the central platform of EPM with open arm facing. In EPM following variables were recorded during 5 min, frequencies and time spent in closed and open arms respectively. After each test, the platform of the maze was wiped and cleaned with damp

cotton. All subjects were experimentally naive at the beginning of each study and used only once to avoid 'one trial tolerance' to EPM test.

### Locomotor activity

Locomotor activities of the rats were recorded by an automatic actophotometer (38  $\times$  38  $\times$  14 cm) (VJ instruments, Karanja, India). Any movement of the rat that interrupted photo beams was recorded as a total of horizontal, vertical and ambulatory activities and expressed as mean motor counts  $\pm$  SEM. Locomotor counts were not recorded for first 5 min after placement and the spontaneous locomotor count of each animal was recorded for following 5 min. After each animal's recording, the grid floor of actophotometer was carefully cleaned. The data are expressed as a mean number of counts for 5 min.

### Depressive behavior

Porsolt's forced swim test (FST) was used to determine the depressive behavior in ethanol-withdrawal rats.<sup>23-24</sup> Animals were individually allowed for forced to swim in a cylindrical glass tank (46 cm  $\times$  20 cm) containing 30 cm of water and the time of immobility was recorded. Two swimming sessions were carried out with an initial 15 min 'pre-test' followed by a 6 min test after 24 h. Reduction in the duration of immobility was considered as attenuation of the depressive behavior of ethanol withdrawal. A trained observer blind to the treatments monitored the immobility time, which is the measure of depression.

### Determination of blood ethanol, corticosterone and ACTH levels

Blood samples were collected from a separate group of animals at 1 h before (0800 h) and at 24 h after (0900 h) ethanol withdrawal from a rat tail vein in heparinized tubes and centrifuged at 13000  $\times$  g for 15 min at 4°C to separate plasma and stored at -20°C. The separated plasma samples were then used to determine ethanol, corticosterone and adreno-corticotrophic hormone (ACTH). Plasma ethanol concentrations were determined by nicotinamide adenine dinucleotide-alcohol dehydrogenase enzymatic assay (Sigma-Aldrich, St. Louis, MO, USA).<sup>25</sup> Plasma corticosterone concentrations were measured using a quaternary gradient HPLC system equipped with a Crestpak C18T-5 column and PDA detector (MD2010 plus) (Jasco, Japan) as per the earlier described procedure.<sup>26,27</sup> ACTH concentrations in plasma were measured by two-site ELISA assay using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA).<sup>28</sup>

### Data Analysis

The results are presented as mean  $\pm$  SEM. The data obtained from ethanol-withdrawal and pair-fed rats were compared by unpaired t-test. The effects of different acute drug treatments were statistically analyzed by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test or Bonferroni multiple comparison tests. A value of  $P < 0.05$  was considered significant.

## RESULTS

### Ethanol consumption, body weight, and ethanol levels

Daily ethanol consumption of the rats in ethanol-fed control and withaferin A treated groups ranged from 13.6  $\pm$  1.3 to 15.8  $\pm$  2.1 g/kg during exposure to ethanol (7.2 % w/v) of last 2 weeks. However, this difference was not statistically significant. Average liquid food consumption of the above two groups was 42.5  $\pm$  4.2 and 44.3  $\pm$  6.1 ml/rat from day 1 - 21 and was not different from the pair-fed control animals.

On the 21<sup>st</sup> day of the experiment, the mean body weight of pair-fed and ethanol-fed animals were 207.2  $\pm$  4.9 g (against the average body weight

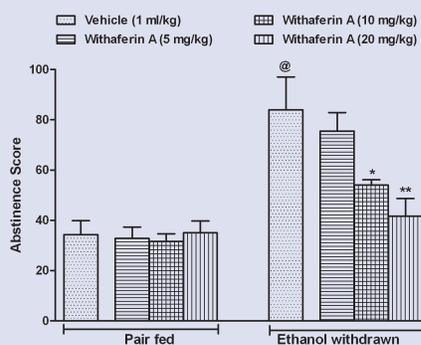
of  $204.3 \pm 3.5$  g on day 1) and  $208.5 \pm 3.8$  g (against the average body weight of  $211.6 \pm 6.8$  g on day 1) respectively and these body weight changes in pair-fed and ethanol-fed animals were statistically insignificant. Blood ethanol concentration was found to be  $173.79 \pm 7.68$  mg/dl and  $6.31 \pm 0.99$  mg/ml, 1 h before (0800 h) and at 24 h after (0900 h) ethanol withdrawal respectively (Table 1). The decreased blood ethanol level 24 h after withdrawal was found significantly correlated with the abstinence signs.

### Behavioral changes during ethanol withdrawal

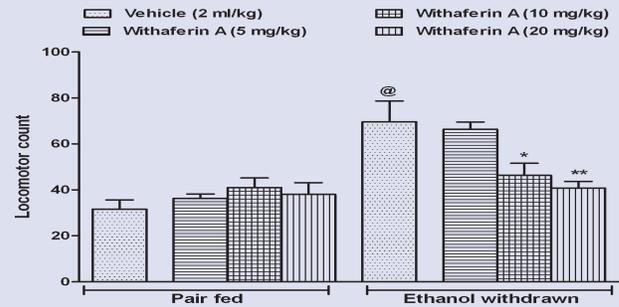
The significant stereotyped behaviors (somatic behaviors) such as grooming ( $t = 3.055$ ,  $df = 2$ ,  $P = 0.0185$ ), scratching ( $t = 4.085$ ,  $df = 2$ ,  $P = 0.0047$ ), head shake ( $t = 3.705$ ,  $df = 2$ ,  $P = 0.0076$ ), genital licking ( $t = 3.831$ ,  $df = 2$ ,  $P = 0.0064$ ), chewing ( $t = 3.055$ ,  $df = 2$ ,  $P = 0.0185$ ), body shakes ( $t = 3.020$ ,  $df = 2$ ,  $P = 0.0194$ ) etc. were evident in ethanol dependent rats when compared with control pair fed animals at 24 h post withdrawal. The sum of all the stereotyped behaviors expressed as EAS was significantly higher in ethanol withdrawn rats as compared to pair fed animals ( $t = 3.49$ ,  $df = 10$ ,  $P < 0.01$ ; unpaired  $t$  test) (Figure 1). However, tail stiffness, abnormal gait, abnormal posture, abdominal constriction, wet dog shakes, tremor or teeth chattering were not observed in the withdrawn, pair fed as well as treated animals.

**Table 1: Blood ethanol concentration in withaferin A treated ethanol withdrawn rats. Separate groups of rats received ethanol in the liquid modified diet for 21 days and treated with withaferin A (5 – 20 mg/kg, ip) from day 15 - 21 of ethanol presentation. Blood ethanol concentration was measured 24 h before and after withdrawal. Data expressed as mean ethanol concentration (mg/dl)  $\pm$  SEM (n=6).**

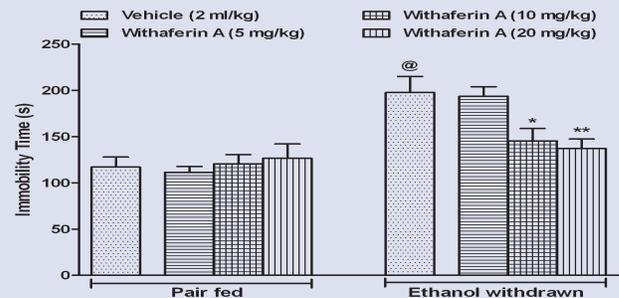
Treatment groups	Blood ethanol concentration (mg/dl)	
	Before withdrawal	24 h after withdrawal
Vehicle (1 ml/kg, ip)	$173.79 \pm 7.68$	$6.31 \pm 0.99$
Withaferin A (5 mg/kg, ip)	$169.32 \pm 6.73$	$5.44 \pm 0.71$
Withaferin A (10 mg/kg, ip)	$174.44 \pm 8.15$	$7.86 \pm 0.84$
Withaferin A (20 mg/kg, ip)	$170.79 \pm 7.08$	$6.76 \pm 0.81$



**Figure 1:** Effect of withaferin A on ethanol withdrawal-induced somatic behaviors. Rats were given free access to ethanol through liquid modified diet for of 21 days and from the 22<sup>nd</sup> day onwards they were deprived of ethanol. Animals were treated with withaferin A (5 – 20 mg/kg, ip) once daily from day 15 -21 of ethanol dependence. Somatic behaviors (grooming, jumping, genital licking, leg licking, head shakes etc.) were determined for 15 min at 24 h following ethanol withdrawal. Each bar indicates mean EAS  $\pm$  SEM (n = 6). @P < 0.001 vs vehicle-treated pair-fed rats; \*P < 0.05, \*\*P < 0.01 vs vehicle-treated ethanol-withdrawn animals (One way ANOVA post hoc Bonferroni test).



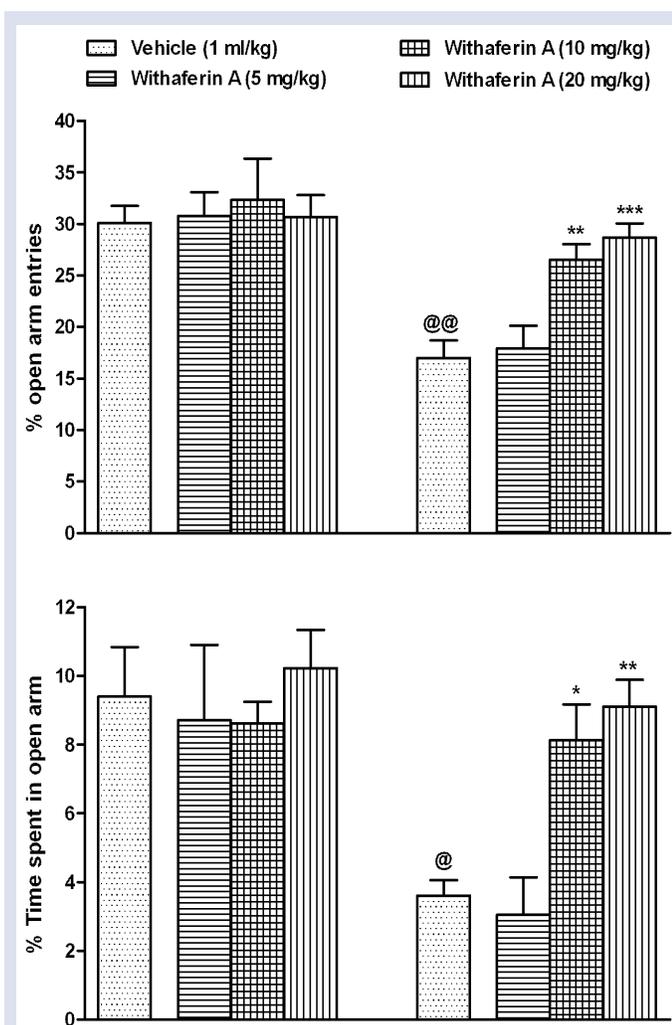
**Figure 2:** Effect of withaferin A on ethanol withdrawal-induced locomotor hyperactivity. Rats were given free access to ethanol through liquid modified diet for 21 days and from the 22<sup>nd</sup> day onwards they were deprived of ethanol. Animals were treated with withaferin A (5 - 20 mg/kg, ip) once daily from day 15 -21 of ethanol dependence. Locomotor activity was determined for 5 min in actophotometer at 24 h following ethanol withdrawal. Each bar indicates mean locomotor counts  $\pm$  SEM (n = 6). @P < 0.01 vs vehicle-treated pair-fed rats; \*P < 0.05, \*\*P < 0.01 vs vehicle-treated ethanol-withdrawn animals (One way ANOVA post hoc Bonferroni test).



**Figure 3:** Effect of withaferin A on ethanol withdrawal-induced depressive behaviors. Rats were given free access to ethanol through liquid modified diet for of 21 days and from the 22<sup>nd</sup> day onwards they were deprived of ethanol. Animals were treated with withaferin A (5 - 20 mg/kg, ip) once daily from day 15 -21 of ethanol dependence. Immobility duration was determined for 5 min in FST at 24 h following ethanol withdrawal. Each bar indicates mean immobility time (s)  $\pm$  SEM (n = 6). @P < 0.01 vs vehicle-treated pair-fed rats; \*P < 0.05, \*\*P < 0.01 vs vehicle-treated ethanol-withdrawn animals (One way ANOVA post hoc Bonferroni test).

A significant locomotor hyperactivity was observed in ethanol-withdrawn animals at the 24 h of post-withdrawal testing as compared to control animals ( $t = 3.86$ ,  $df = 10$ ,  $P < 0.01$ ; unpaired  $t$ -test) (Figure 2). Similarly, when tested in FST, immobility time was increased as compared to pair-fed animals ( $t = 3.93$ ,  $df = 10$ ,  $P < 0.01$ ; unpaired  $t$ -test) (Figure 3).

As depicted in Figure 4, animals withdrawn from the chronic ethanol exhibited the anxiogenic effect indicated by animal behavior in the maze. Significant decrease in % time spent in open arm ( $t = 5.49$ ,  $df = 10$ ,  $P < 0.001$ ; unpaired  $t$ -test) and % open arm entries ( $t = 3.84$ ,  $df = 10$ ,



**Figure 4:** Effect of withaferin A on ethanol withdrawal-induced anxiety-related behaviors. Rats were given free access to ethanol through liquid modified diet for of 21 days and from the 22<sup>nd</sup> day onwards they were deprived of ethanol. Animals were treated with withaferin A (5 - 20 mg/kg, ip) once daily from day 15 -21 of ethanol dependence. The number of entries (A) and time spent (B) were determined for 5 min in EPM at 24 h following ethanol withdrawal. Each bar indicates mean % entries/time spent in open arm ± SEM (n = 6). @@P < 0.001 vs vehicle-treated pair-fed rats; \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs vehicle-treated ethanol-withdrawn animals (One way ANOVA post hoc Bonferroni test).

P < 0.01; unpaired t-test) was observed in ethanol-withdrawn rats when compared with pair-fed group.

### Withaferin A attenuates ethanol withdrawal-induced behaviors

Withaferin A (5 - 20 mg/kg, ip) treatment 30 min before 24 h post-ethanol withdrawal assessment did not alter the scores of somatic behavioral signs in ethanol abstinence animals. Moreover, a single administration of withaferin A (5 - 20 mg/kg, ip) before withdrawal assessment did not influence the locomotion, immobility time as well as EPM indices as compared to ethanol-withdrawn rats (Data not shown).

Chronic treatment with withaferin A (10 and 20 mg/kg, ip) from day 15-21 significantly prevented the ethanol withdrawal induced elevated scores of somatic behaviors [F (4, 29) = 7.34; P < 0.001] (Figure 1) and hyperlocomotion [F (4, 29) = 9.46; P < 0.001] (Figure 2) as well as

depressive behavior [F (4, 29) = 7.83; P < 0.001] (Figure 3). The posthoc Dunnett mean comparisons demonstrated that withaferin A (10 and 20 mg/kg) treatment for 7 days in ethanol dependent rats significantly attenuated the elevated somatic scores (P < 0.05 and P < 0.01), locomotor activity (P < 0.05 and P < 0.01 respectively) and immobility time (P < 0.05 and P < 0.01 respectively) and when compared against vehicle treated ethanol withdrawn rats.

Similarly, as shown in Figure 4, ethanol-withdrawn rats treated with withaferin A (10 - 20 mg/kg, ip) from day 15-21 along with ethanol exposure displayed a significant reduction in the withdrawal-induced anxiety as evident from decreased percent entries into the open arms [F (4, 29) = 12.84; P < 0.001] (Figure 4A) and percent time spent [F (4, 29) = 9.17; P < 0.001] (Figure 4B) as compared to ethanol-withdrawn rats administered with vehicle for 7 days. Post-hoc Dunnett analysis demonstrated a significant reversal of ethanol withdrawal-induced anxiety behaviors [% entries in open arm (P < 0.01 and P < 0.01) and % time spent in open arm (P < 0.05 and P < 0.01) in animals treated with withaferin A (10 and 20 mg/kg, respectively) in ethanol-dependent rats compared to vehicle-treated controls. Administration of withaferin A in pair-fed animals did not alter basal somatic behaviors, immobility time, locomotor activity and plus-maze behaviors.

### Effect of withaferin A on ethanol withdrawal-induced elevated plasma ACTH and corticosterone levels

As shown in Table 1, blood ethanol levels in withaferin A (5-20 mg/kg, ip) administered ethanol-withdrawn rats were not significantly different from vehicle-treated abstinence animals. However, as depicted in Table 2, ethanol withdrawal results in a significant elevation in plasma

**Table 2:** Plasma corticosterone concentration in withaferin A treated ethanol withdrawn rats. Separate groups of rats received ethanol in the liquid modified diet for 21 days and treated with withaferin A (5 - 20 mg/kg, ip) from day 15 - 21 of ethanol presentation. Plasma corticosterone concentration was measured 30 min before and 24 h after withdrawal. Data expressed as mean corticosterone level (ng/ml) ± SEM (n=6). #P < 0.001 vs vehicle treated ethanol fed rats 30 min before ethanol withdrawal, \*P < 0.001 vs vehicle treated ethanol fed rats 24 h after ethanol withdrawal.

Treatment groups	Plasma corticosterone concentration (ng/ml)	
	Before withdrawal	24 h after withdrawal
Vehicle (1 ml/kg, ip)	61.82 ± 3.63	166.8 ± 15.91 <sup>#</sup>
Withaferin A (5 mg/kg, ip)	76.59 ± 4.50	140.4 ± 8.35
Withaferin A (10 mg/kg, ip)	74.93 ± 6.31	102.9 ± 8.07*
Withaferin A (20 mg/kg, ip)	75.02 ± 7.89	89.81 ± 11.49*

**Table 3:** Plasma ACTH concentration in Withaferin A treated ethanol withdrawn rats. Separate groups of rats received ethanol in the liquid modified diet for 21 days and treated with withaferin A (5 - 20 mg/kg, ip) from day 15 - 21 of ethanol presentation. Plasma ACTH concentration was measured 24 h before and after withdrawal. Data expressed as mean ACTH level (pg/ml) ± SEM (n=6). #P < 0.001 vs vehicle treated ethanol fed rats 30 min before ethanol withdrawal, \*P < 0.05 vs vehicle treated ethanol fed rats 24 h after ethanol withdrawal.

Treatment groups	Plasma ACTH concentration (pg/ml)	
	Before withdrawal	24 h after withdrawal
Vehicle (1 ml/kg, ip)	122.2 ± 13.82	252.4 ± 23.91 <sup>#</sup>
Withaferin A (5 mg/kg, ip)	148.4 ± 16.50	205.2 ± 35.78
Withaferin A (10 mg/kg, ip)	140.7 ± 15.31	167.9 ± 8.66*
Withaferin A (20 mg/kg, ip)	145.2 ± 10.89	164.3 ± 15.07*

corticosterone levels were as compared to their concentration before withdrawal ( $t = 6.44$ ,  $df = 10$ ,  $P < 0.001$ ; unpaired  $t$ -test). In order with the behavioral observations, Withaferin-A [10 ( $P < 0.001$ ) and 20 mg/kg ( $P < 0.001$ )] administration significantly attenuated the elevated plasma corticosterone levels in ethanol-withdrawn rats [ $F(4, 29) = 16.20$ ;  $P < 0.001$ ].

In addition, ELISA analysis revealed a significant rise in the levels of ACTH follows ethanol withdrawal ( $t = 4.72$ ,  $df = 10$ ,  $P < 0.001$ ; unpaired  $t$  test). Treatment withaferin A [10 ( $P < 0.05$ ) and 20 mg/kg ( $P < 0.05$ )] dose dependently reversed the elevated plasma ACTH levels as compare to control rats [ $F(4, 29) = 5.11$ ;  $P < 0.01$ ] (Table 3). Administration of withaferin A (5 – 20 mg/kg, ip), however, did not alter the basal corticosterone as well as ACTH levels in pair-fed animals (data not shown).

## DISCUSSION

The main observations of this study are that withaferin A, an active constituent of WS, has an inhibitory effect on alcohol abstinence signs in rats. In line with the earlier reports, in current findings, we have observed the approximately 9-15 g/kg/day up to 15 days, which further produces the physical dependence in rats.<sup>29,30</sup> In the present study, very high blood ethanol levels ( $174.44 \pm 8.15$  mg/dl) observed just before ethanol withdrawal indicates an adequate quantity of ethanol consumed ( $13.6 \pm 1.3$  to  $15.8 \pm 2.1$  g/kg) for the induction of abstinence signs. Pilot studies from our laboratory as well as earlier published studies have demonstrated that ethanol withdrawal displays its peak behavioral and biochemical signs following 24 h of ethanol withdrawal. As depicted in Table 1, ethanol withdrawal in animal resulted insignificant decline in the blood ethanol concentration. In our study, ethanol abstinence was characterized by increased scores of stereotyped behaviors, motor hyperactivity, behavioral despair and anxiety-like behavior in EPM test. Our previous findings that ethanol withdrawal anxiety<sup>18</sup> and depression<sup>17</sup> in rodents support the development of psychological dependence. Negative reinforcement in ethanol withdrawal is one of the potential reason for ethanol dependence and reinstatement.<sup>31,32</sup> Similarly, dysphoria like the state is very common following the withdrawal of substances of abuse like ethanol, nicotine, and morphine and has been demonstrated in several animal models of depression.<sup>33,35</sup> This negative emotional state of ethanol withdrawal has been suggested a key factor in the maintenance of ethanol addiction (i.e., alcohol self-administration to prevent withdrawal).<sup>36</sup>

WS has been widely investigated for their diverse pharmacological activities, phytochemically it contains steroidal lactones, alkaloids, flavonoids, tannin etc. Withaferin A, predominantly found in WS known as withanolide. Several publish report acclaimed its different pharmacological activities viz. anti-inflammatory, anti-stress,<sup>12,37</sup> immunomodulatory,<sup>10</sup> inhibition of cognitive deficits in Alzheimer's disease<sup>38</sup> and anxiolytic-antidepressant action in rats<sup>7</sup> without inducing any significant toxicity. In the present study, we evaluated the effects of acute and chronic injections of withaferin A on the somatic manifestations, anxiety, and depression-like effect of ethanol withdrawal. Our results demonstrate that subchronic withaferin A administration (day 15- 21) during ethanol exposure prevented the somatic expression of ethanol withdrawal as revealed by a significant decrease in the most prominent somatic signs of ethanol withdrawal and also prevented the anxiety and depression-like behavior in ethanol-withdrawn rats. However, acute injection of withaferin A 30 min prior withdrawal testing did not influence any of the ethanol withdrawal signs. It is of interest to note that withaferin A (glyco withanolide) has been reported to have both anxiolytic and antidepressant activity in rats, supporting the contention that this bioactive component of WS is the effective mood regulator.<sup>7</sup>

Activation of the hypothalamo-pituitary-adrenocortical axis (HPA) during stress is a well-known phenomenon, an increase in plasma corticosterone in rodents and cortisol in humans have been utilized as markers of withdrawal associated stress and its intensity.<sup>39-40</sup> Single ethanol withdrawal induces the maladaptive drinking symptoms like anxiety, hyperactivity etc. which have been correlated with the elevated corticotrophin-releasing factor levels.<sup>41</sup> Interestingly, these maladaptive symptoms of alcohol withdrawal have been reversed.<sup>42</sup> Therefore the CRF has been proposed as a key mediator in drug and ethanol dependence.<sup>43</sup> Current reports suggest the neuroadaptive alterations activated by chronic alcohol exposure leads to up-regulation of CRF system.<sup>44</sup> Importantly, ethanol-withdrawn animals demonstrated a marked elevation in ACTH and corticosterone levels, which was reversed by withaferin A treatment. It is likely that withaferin A by its interaction with the central stress regulating system might have attenuated ethanol withdrawal syndrome in rats.<sup>45</sup> However, the role of other proposed biological targets of WS like GABA and opioids receptors needs further investigation. Thus, withaferinA-induced anti-stress effect and stabilization of HPA axis activity could have facilitated the inhibitory effect of withaferin A on ethanol withdrawal syndrome.

In conclusion, this study demonstrated that prolong administration of withaferin A during exposure to ethanol attenuate the ethanol withdrawal-induced somatic hyperexcitability, anxiety, and dysphoria like state in chronically ethanol-exposed animals. These findings strongly support further investigation of withaferin A and other bioactive components of WS in alcohol addiction. The data clearly projects withaferin A as a new potential therapeutic intervention in alcohol abuse associated complications.

## CONFLICT OF INTEREST

The authors have no conflict of interest.

## ABBREVIATIONS

**WS:** *Withania somnifera*; **HPA:** Hypothalamic Pituitary adrenal; **EPM:** Elevated Plus Maze; **FST:** Forced Swim Test; **EAS:** Ethanol Abstinence Score; **ACTH:** Adreno-Corticotropic Hormone.

## ACKNOWLEDGEMENT

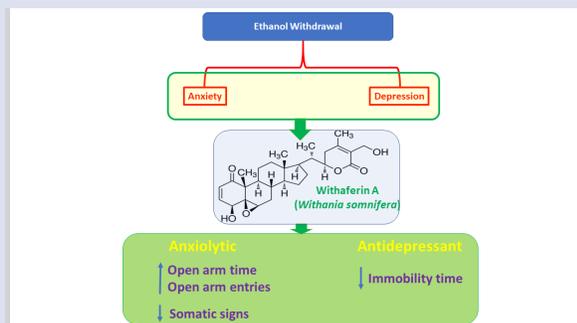
The work was funded under the institutional research grant.

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## GRAPHICAL ABSTRACT



## SUMMARY

- Alcoholism and withdrawal of its chronic intake are serious social and medical problems. There is no appropriate drug therapy which helps the patients to overcoming the withdrawal symptoms. Present study investigated the beneficial effects of withaferin A during ethanol dependence and withdrawal, hence projected new therapeutic drug molecule for the effective management of alcoholism.

**Cite this article:** Kotagale NR, Kedia A, Gite R, Rahmatkar SN, Gawande DY, Umekar MJ, Taksande BG. Withaferin A attenuates Alcohol Abstinence Signs in Rats. *Pharmacog J*. 2018;10(6):1190-5.