Research Article

Modulatory effects of *Withania somnifera* on depression and neurochemical alterations during ethanol withdrawal in rats

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ABSTRACT

Chronic ethanol consumption causes alcohol use disorders, leading to depressive-like behavior, cognitive deficits, and neurochemical dysregulation. Ethanol withdrawal disrupts GABAergic and glutamatergic neurotransmission, promoting neuroadaptations that exacerbate behavioral and physiological deficits. *Withania somnifera* (WS) (Ashwagandha), rich in withanolides, exhibits antioxidant, anti-inflammatory, and neuroprotective properties. In this study WS leaf extract effect on depression brought on by ethanol withdrawal in male Sprague Dawley rats weighing 200–250 g were evaluated. There were six groups of rats: Control, disease control, standard (MK-801, intraperitoneally, 0.1 mg/kg), and WS treatment groups (orally, 50, 100, 200 mg/kg). For a period of 28 days, a graded ethanol liquid diet was followed by withdrawal. Open-field and forced swim tests were used for behavioral evaluations, while brain tissues were analyzed for Pro-inflammatory cytokines (Tumor necrosis factor-alpha, interleukin-6), oxidative stress markers (malondialdehyde, nitrite, glutathione, catalase), neurotransmitters (GABA, glutamate, dopamine), and hippocampal histology. The data were assessed using a one-way analysis of variance and the Bonferroni *post hoc* test ($P < 0.05$). Ethanol withdrawal induced depressive-like behavior, neurotransmitter imbalance, oxidative stress, elevated cytokines, and hippocampal degeneration. WS treatment dose-dependently reversed these effects, with the 200 mg/kg dose producing maximal neuroprotective outcomes comparable to MK-801. These results indicate that WS leaf extract possesses neuroprotective, antidepressant-like, antioxidant, and anti-inflammatory activities, restoring behavioral, neurochemical, and histological parameters, underlining its possible as a natural therapeutic for ethanol withdrawal management.

Keywords: Depression, ethanol withdrawal, neuroprotection, neurotransmitters, oxidative stress, *Withania somnifera* on

Introduction

Alcohol is one of the most prevalent used psychoactive substances in the world and still one of the most abused. According to World Health Organization (WHO) estimates, approximately 76.3 million individuals worldwide are affected by alcohol use disorders (AUDs), contributing to nearly 1.8 million deaths annually. More recent statistics indicate that by 2024, an estimated 400 million individuals globally were living with AUD, reflecting a growing public health challenge.[1] As per the 2018 WHO Global Status Report on Alcohol and Health, around 3 million deaths worldwide in 2016 were caused by alcohol, accounting for 5.3% of all fatalities.[2] Chronic alcohol consumption is strongly associated with multiple neuropsychiatric complications, including impaired memory, learning, recognition, and a higher prevalence of major depressive disorder.[3]

When people with alcohol dependence suddenly cut back on or stop using alcohol, they can experience alcohol withdrawal syndrome. This kind of condition is characterized by hyper excitability of glutamatergic synaptic transmission, particularly in the amygdala, leading to anxiety, irritability, and negative affective states that often trigger relapse.[4] Withdrawal symptoms usually develop within 6–24 h of cessation and might vary from minor tremors and anxiety to potentially fatal side effects, including seizures and delirium tremens.[5]

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Approximately 5–10% of severe withdrawal cases can be fatal. AUD is therefore recognized as a chronic, relapsing brain disorder, wherein the alcohol's rewarding effects are mediated primarily by dopaminergic activation in the mesolimbic pathway, while withdrawal is associated with profound reductions in dopamine function. Pre-clinical and clinical studies suggest that diminished dopamine neurotransmission contributes significantly to the comorbidity of depression during withdrawal.[6]

At the molecular level, ethanol interacts with multiple neural targets, such as glycine, serotonin, NMDA, GABA_A, and nicotinic acetylcholine receptors.[7] Chronic and repeated cycles of drinking and withdrawal lead to long-term neuroadaptations in these systems, producing a state of allostatic dysregulation.[8] This results in anxiety, depression, and other negative emotional states that serve as reinforces for relapse and perpetuate the cycle of dependence.[9] Ethanol exerts acute effects by potentiating inhibitory cys-loop ligand-gated ion channels, including GABA_A receptors and glycine, while inhibiting excitatory ionotropic glutamate receptors.[10] Prolonged heavy drinking, however, produces neurotoxic outcomes, including motor and behavioral impairment, cognitive decline, and ultimately neuronal cell death.[11] These changes involve multiple brain regions, including the reticular activating system, medulla, frontal lobe, hippocampus, cerebellum, and prefrontal cortex. Ethanol neurotoxicity is linked with impaired neurotransmitter signaling, activation of neuroinflammatory pathways, and overproduction of reactive oxygen species (ROS). Collectively, these alterations lead to brain dysfunction and neuronal degeneration.[12]

Withdrawal from ethanol further aggravates these disturbances. Clinical manifestations include tremors, seizures, hallucinations, and autonomic hyperactivity.[13] Neurochemically, withdrawal states are characterized by upregulation of NMDA receptors and downregulation of GABAergic signaling, leading to excitotoxicity.[14] Dopamine dysregulation further compounds depressive symptoms. Adolescent exposure to alcohol presents an additional risk factor, as it interferes with neurodevelopment, causing long-term structural and functional brain alterations.[11] Mechanistic studies reveal that adolescent ethanol exposure induces immature synaptic activity, enhanced NMDA receptor signaling in CA1 pyramidal cells, and epigenetic modifications, such as reduced Arc genes and histone acetylation of brain-derived neurotrophic factor, contributing to mood and cognitive disturbances.[15]

Among the neurotransmitter systems affected, glutamatergic signaling plays a central role in ethanol-induced neurobehavioral alterations.[16] Ethanol consumption suppresses glutamate uptake, antagonizes NMDA receptor function, and impairs GABAergic neurotransmission, ultimately creating an imbalance between excitation and inhibition. Chronic ethanol use is further associated with astrocytic dysfunction, oxidative stress, neuroinflammation, and progressive neurotoxicity. These neuroadaptive processes underlie both acute withdrawal symptoms and long-term vulnerability to psychiatric disorders.[17]

Given the limitations of present pharmacological treatments for alcohol withdrawal and the associated side effects, there is growing interest in plant-based alternatives with neuroprotective and antidepressant potential.[18] *Withania somnifera* (WS) (Linn.) Dunal, often referred to as ashwagandha, this plant is used extensively in Ayurvedic medicine. It has a variety of bioactive components, including sitoindosides, withanosides, withanolides, and withaferin A.[19]These compounds have a variety of range of pharmacological properties, notably neuroprotection, antioxidant activity, anti-inflammatory effects, and modulation of neurotransmission. Withanolides are particularly abundant in the leaves and have been shown to inhibit NMDA receptor–mediated excitotoxicity, primarily via allosteric modulation of the GluN1-GluN2B receptor subunits. Such activity protects against calcium-mediated neuronal damage, mitochondrial dysfunction, lipid peroxidation, and oxidative DNA injury. Furthermore, Ashwagandha's adaptogenic and antidepressant-like properties have been demonstrated in both clinical and pre-clinical studies, supporting its use in managing stress-related and depressive disorders.[20]

Chronic alcohol consumption disrupts the balance between GABA and glutamate, leading to elevated glutamate and reduced GABA levels during withdrawal, which exacerbates excitatory symptoms and increases the risk of depression. WS has been identified as a promising candidate to counteract these effects through its ability to restore neurotransmitter balance, attenuate oxidative stress, and reduce neuroinflammation.[21]

Materials and Methods

Animals

Adult male Sprague Dawley rats (200–250 g) were procured and acclimatized for 7 days under standard laboratory conditions (12 h light/dark cycle, 22 ± 2°C, 50–60% humidity) with free access to food and water. All experimental procedures were approved by the Institutional Animal Ethics Committee and conducted in accordance with CPCSEA guidelines for the care and use of laboratory animals.

Drugs and chemicals

Absolute ethanol, MK-801 (dizocilpine maleate), and WS leaf extract were used. WS extract was prepared and standardized for withanolide content before dosing. All other chemicals and reagents were of analytical grade and procured from reputed suppliers.

Route of administration

During the study, ethanol was administered by oral route, the standard drug MK-801 (0.1 mg/kg) was given by intraperitoneal route (i.p.), and the treatment drug W.S. leaves extract (50, 100, and 200 mg/kg) was given orally. All the drugs were dissolved in sterile isotonic saline solution.

Experimental design and treatment protocol

Male Sprague Dawley rats (n = 6 per group) were randomly allocated into six groups: Control (saline), disease control (ethanol

withdrawal), standard (MK-801, 0.1 mg/kg, i.p.), and three treatment groups receiving WS extract (50, 100, or 200 mg/kg, p.o.). Animals were maintained under standard housing conditions with *ad libitum* access to a modified liquid diet (MLD) containing ethanol (2.4–7.2% v/v); controls received an isocaloric sucrose diet. Ethanol administration was withdrawn on day 28, and treatments were given 30 min before behavioral assessments conducted at 24 and 48 h post-withdrawal. Locomotor activity (open field test [OFT]) and depressive-like behavior (forced swim test [FST]) were evaluated. At the end of the study, animals were euthanized, and brains were collected for biochemical and histopathological analyses.

Experimental groups and treatment protocol

Rats were randomly assigned to six groups ($n = 6$ each) [Figure 1].

Group I (Control): Received a MLD without ethanol for 28 days; behavioral assessment was performed on day 30.

Group II (Negative Control): Received MLD without ethanol for 7 days, followed by ethanol in MLD (2.4%, 4.8%, and 7.2% v/v) from days 8–28; behavioral assessment on day 30.

Group III (Standard): Same as Group II, with MK-801 (0.1 mg/kg, i.p.) administered during ethanol withdrawal (days 28–31).

Group IV (WS-50): Same as Group II, with WS extract (50 mg/kg, p.o.) during withdrawal.

Group V (WS-100): Same as Group II, with WS extract (100 mg/kg, p.o.) during withdrawal.

Group VI (WS-200): Same as Group II, with WS extract (200 mg/kg, p.o.) during withdrawal.

Behavioral assessment

OFT

Locomotor activity was evaluated using a transparent Plexiglas open field apparatus (60 × 60 cm). Each rat was placed in the center and allowed to explore freely for 5 min. Behavior was recorded and analyzed using a video tracking system. The apparatus was cleaned with 70% ethanol between trials.

FST

Depressive-like behavior was assessed in a glass cylindrical chamber (50 cm height, 30 cm diameter) filled with water (23°C, 40 cm depth). Following OFT, each rat was subjected to a 5-min swimming session. Sessions were video recorded, and active swimming versus immobility time was analyzed to evaluate behavioral despair.

Biochemical assessments

Following behavioral tests, animals were euthanized with pentobarbital sodium overdose. Brains were excised, rinsed in isotonic saline, weighed, and homogenized (10% w/v) in phosphate buffer (0.1 M, pH 7.4). Homogenates were centrifuged (10,000 × g, 15 min, 4°C), and supernatants were collected for biochemical estimations using a double-beam UV–Visible spectrophotometer.

Neurotransmitter estimations

GABA

Tissue homogenates were extracted with ethanol, separated by paper chromatography using n-butanol: aceticacid:water (4:1:5), visualized with 0.5% ninhydrin, and quantified at 570 nm. Results were expressed as μmol/g tissue.

Glutamate

Homogenates were deproteinized with perchloric acid, neutralized with phosphate buffer, incubated on ice, filtered, and

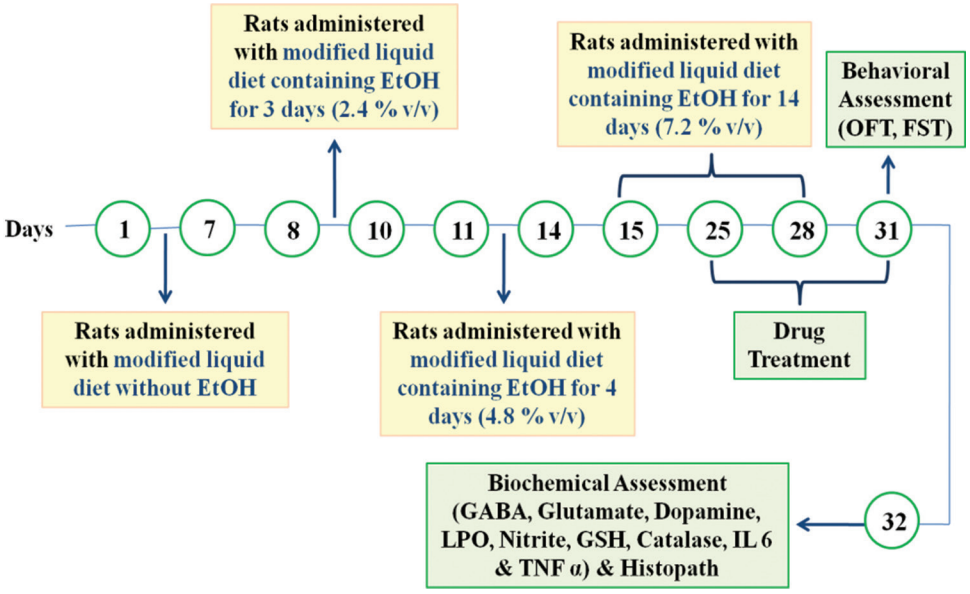

Figure 1: Experimental protocol

absorbance recorded at 340 nm. Results expressed as μmol/g tissue.

Dopamine

Supernatants were reacted with ferric chloride and potassium ferricyanide, and absorbance was measured at 735 nm. Values expressed as μmol/g tissue.

Oxidative stress markers

Lipid peroxidation (malondialdehyde [MDA] levels)

Supernatants were reacted with thiobarbituric acid after protein precipitation with trichloroacetic acid, heated, and measured at 532 nm. Results expressed as nmol MDA/mg protein.

Nitrite

Supernatants were incubated with freshly prepared Griess reagent, and absorbance was measured at 548 nm. Results expressed as μmol/mg tissue.

Reduced glutathione (GSH)

Samples reacted with DTNB in phosphate buffer, and absorbance was recorded at 412 nm. Results expressed as μmol/g tissue.

Catalase

Enzyme activity was measured in phosphate buffer by monitoring H_2O_2 decomposition at 240 nm. Activity was expressed as U/mg tissue.

Neuroinflammatory marker analysis

Interleukin-6 (IL-6) estimation

IL-6 levels in brain homogenates were quantified using a commercial rat IL-6 ELISA kit (GENLISA™). Standards were prepared through serial dilution (250–8000 pg/mL) and run in duplicate with samples. Following addition of Biotin-conjugated detection antibody and incubation at 37°C, plates were washed and treated with Streptavidin–horseradish peroxidase (HRP). Colorimetric detection was achieved using TMB substrate, and absorbance was measured at 450 nm within 30 min of reaction termination.

Tumor necrosis factor-alpha (TNF-α) estimation

TNF-α concentrations were determined using a rat TNF-α ELISA kit (GENLISA™). Samples and standards were added in triplicate, incubated with Biotinylated detection antibody, followed by Streptavidin–HRP. After washing, the TMB substrate was added, and the reaction was stopped with an acid solution. Optical density was recorded at 450 nm. Concentrations were calculated from standard curves and expressed as pg/mg of brain tissue.

Histopathological analysis

Brain tissues were fixed in 10% neutral buffered formalin for 72 h, processed through graded alcohols, cleared in xylene, and embedded in paraffin. Sagittal sections (4 μm) were cut using a rotary microtome and stained with hematoxylin and eosin for general morphology and Nissl stain (toluidine blue) to assess neuronal integrity. Hippocampal regions were examined under a microscope, and images were captured using a Full HD camera at 40× magnification.

Statistical analysis

Data were expressed as mean ± SEM. Statistical comparisons between groups were made using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. A $P < 0.05$ was considered statistically significant.

Results

Behavioral assessment

OFT

Ethanol withdrawal resulted in a significant reduction in exploratory behavior compared with control rats, as reflected by both the time spent in the central arena and the total distance travelled [Figures 2 and 3]. One-way ANOVA followed by Bonferroni's multiple comparison test revealed significant group differences [$F(5,25) = 16.92$, $P < 0.001$].

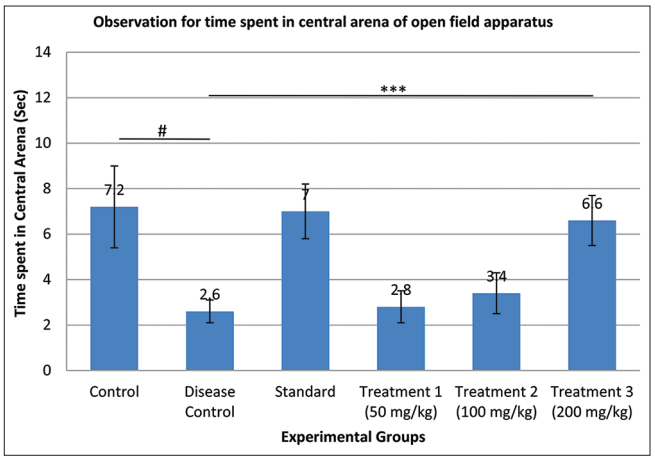

Figure 2: Effect of *Withania somnifera* leaf extract and standard drug MK-801 on time spent in the central arena during ethanol withdrawal. Data are mean ± SEM ($n = 5$). $^{\#}P < 0.001$ versus control, $^{***}P < 0.001$ versus withdrawal group

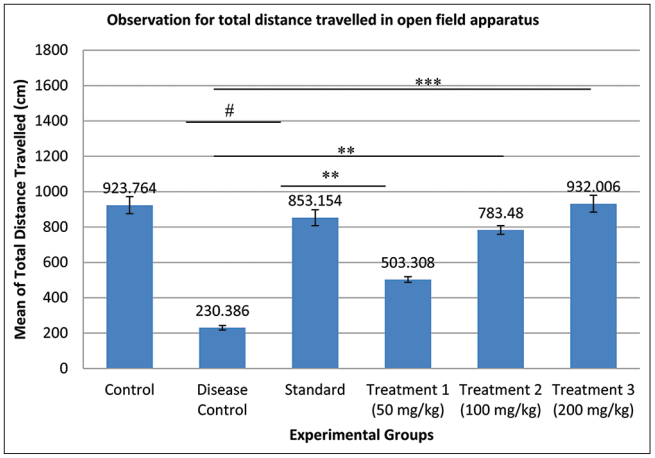

Figure 3: Effect of *Withania somnifera* leaf extract and standard drug MK-801 on locomotor activity (distance travelled) during ethanol withdrawal. Data are mean ± SEM ($n = 5$). $^{\#}P < 0.001$ versus control, $^{**}P < 0.01$ 100 mg/kg), $^{***}P < 0.001$ (200 mg/kg) versus withdrawal group

Treatment with WS leaf extract produced a dose-dependent reversal of withdrawal-induced deficits. At 200 mg/kg, WS significantly increased the time spent in the central arena (6.6 ± 1.1 s) and restored locomotor activity (932.0 ± 47.5 cm), values comparable to control rats (7.2 ± 1.8 s; 923.8 ± 48.3 cm). The standard drug MK-801 showed similar efficacy. Lower doses (50 and 100 mg/kg) produced partial improvements, with the 100 mg/kg group demonstrating significant recovery in locomotor activity ($P < 0.01$ vs. withdrawal group).

These findings indicate that WS alleviates ethanol withdrawal–induced depressive-like behavior, with the 200 mg/kg dose showing effects comparable to the reference drug.

FST

One-way ANOVA followed by *post hoc* Bonferroni's test revealed that administration of the standard drug MK-801 (0.1 mg/kg) and WS leaves extract (50, 100, and 200 mg/kg) during the withdrawal phase significantly reduced depression-like behavior in the OFT compared to the ethanol withdrawal group [$F(5,25) = 11.60$, $P < 0.05$]. Ethanol-withdrawal rats showed a significant increase in depressive behavior compared to controls. In the FST, the disease control group exhibited a marked increase in immobility time (79.06 ± 12.2 s) relative to controls (38.86 ± 7.6 s), confirming depression-like behavior. Treatment with MK-801 reduced immobility time to 38.08 ± 6.6 s, while WS leaves extract produced a dose-dependent decrease at 50, 100, and 200 mg/kg (57.88 ± 8.3 s, 42.64 ± 7.9 s, and 37.66 ± 6.3 s, respectively). The 200 mg/kg dose showed the greatest effect, comparable to MK-801, indicating a robust antidepressant-like activity of WS leaves extract in ethanol withdrawal-induced depression [Figure 4].

Biochemical assessment

Brain neurotransmitter level estimation

GABA neurotransmitter level in brain tissue

Ethanol withdrawal significantly reduced GABA levels in rat brain tissue compared to the control group (Disease Control: 4.5 ± 0.4 µmol/g vs. Control: 16.5 ± 1.6 µmol/g), indicating disruption of inhibitory neurotransmission during withdrawal. Oral administration of WS leaf extract at 50, 100, and 200 mg/kg dose-dependently increased GABA concentrations. The 50 mg/kg dose produced a moderate increase (9 ± 1 µmol/g), while 100 mg/kg restored GABA to 12 ± 1.1 µmol/g. The highest dose (200 mg/kg) almost fully restored GABA levels (15.5 ± 1.5 µmol/g), comparable to the standard treatment group (13 ± 0.9 µmol/g). These results suggest that WS effectively counteracts ethanol-induced GABA depletion in a dose-dependent manner, contributing to its neuroprotective and antidepressant-like effects.

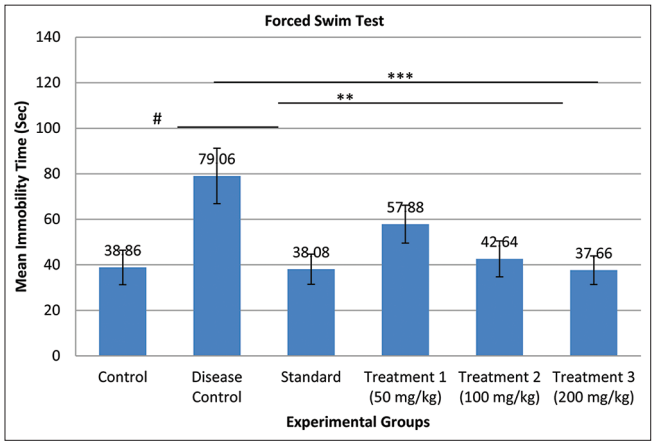

Figure 4: Effect of *Withania somnifera* extract and MK-801 on immobility time in the forced swim test during ethanol withdrawal. Data are expressed as mean ± SEM ($n = 5$). $^{\#}P < 0.001$ versus control; $^{**}P < 0.01$, $^{***}P < 0.001$ versus ethanol withdrawal group

Glutamate neurotransmitter level in brain tissue

Ethanol withdrawal caused a marked increase in glutamate levels compared to controls (Disease Control: 67.5 ± 6.74 µmol/g vs. Control: 29.5 ± 6.74 µmol/g), reflecting excitotoxicity and overactivation of excitatory pathways. WS leaf extract treatment significantly decreased glutamate concentrations in a dose-dependent manner. The 50 mg/kg dose showed a minimal reduction (68.0 ± 6.74 µmol/g), whereas 100 mg/kg reduced glutamate to 49.5 ± 6.74 µmol/g. The 200 mg/kg dose effectively normalized glutamate levels to near-control values (35.0 ± 6.74 µmol/g), similar to the standard treatment (39.5 ± 6.74 µmol/g). These findings indicate that WS mitigates ethanol-induced excitotoxicity, thereby protecting neuronal integrity during withdrawal.

Dopamine neurotransmitter level in brain tissue

Dopamine levels were significantly reduced in ethanol-withdrawn rats compared to control rats (Disease Control: 0.09 ± 0.01 µmol/g vs. Control: 0.275 ± 0.2 µmol/g), consistent with withdrawal-associated deficits in reward and mood regulation. WS leaf extract treatment dose-dependently increased dopamine concentrations. The 50 mg/kg dose increased dopamine to 0.165 ± 0.1 µmol/g, and the 100 mg/kg dose further increased it to 0.205 ± 0.2 µmol/g. The highest dose (200 mg/kg) restored dopamine to 0.275 ± 0.2 µmol/g, comparable to the standard treatment (0.265 ± 0.2 µmol/g). This indicates that WS effectively normalizes dopaminergic neurotransmission, contributing to its antidepressant-like effects during ethanol withdrawal [Table 1].

Oxidative stress markers level

Lipid peroxidation level in brain tissue

Ethanol withdrawal significantly increased lipid peroxidation, as indicated by elevated MDA levels in the brain compared to control rats (Disease Control: 7.5 ± 1.5 nmol/mg vs. Control: 1.65 ± 0.2 nmol/mg). Oral administration of WS leaf extract at 50, 100, and 200 mg/kg dose-dependently decreased MDA levels. The 50 mg/kg dose reduced MDA to 5.8 ± 1.2 nmol/mg, the 100 mg/kg dose to 4.75 ± 1.3 nmol/mg, and the 200 mg/kg dose restored MDA to 4.75 ± 1.3 nmol/mg, comparable to the standard treatment (4.5 ± 1.1 nmol/mg). These results indicate that WS effectively attenuates ethanol-induced lipid peroxidation [Table 2].

Nitrite level in brain tissue

Ethanol withdrawal significantly increased lipid peroxidation, as indicated by elevated MDA levels in the brain compared to

Table 1: Estimation of brain neurotransmitter level

Groups	GABA neurotransmitter in brain (µmol/g)	Glutamate neurotransmitter in brain tissue (µmol/g)	Dopamine neurotransmitter in brain tissue (µmol/g)
Control	16.5±1.6	29.5±6.74	0.275±0.2
Disease Control	4.5±0.4#	67.5±6.74#	0.09±0.01#
Standard	13±0.9*	39.5±6.74***	0.265±0.2**
Treatment 1 (50 mg/kg)	9±1	68.0±6.74	0.165±0.1
Treatment 2 (100 mg/kg)	12±1.1*	49.5±6.74**	0.205±0.2*
Treatment 3 (200 mg/k	15.5±1.5**	35.0±6.74***	0.275±0.2**

Data are presented as mean±SEM (n=6) and analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. Statistical significance is indicated as follows: P<0.05 (*), P<0.01 (**), and P<0.001 (***) versus disease control. Disease control, while (#) comparison versus the control group

Table 2: Estimation of oxidative stress markers level

Groups	Lipid peroxide (nmol/mg)	Nitrite (µmol/mg)	Reduced glutathione (nmol/mg)	Catalase (U/mg)
Control	1.65±0.2	180±14.3	26.5±12.6	35.325±14.7
Disease Control	7.5±1.5#	280.5±18.2#	15.5±7.4#	10.05±2.7#
Standard	4.5±1.1**	183.5±14.4**	25±11.3*	32.33±12.4**
Treatment 1 (50 mg/kg)	5.8±1.2*	245.5±16	22±12.7	16.115±5.5
Treatment 2 (100 mg/kg)	4.75±1.3**	220±15.5	24±13.6	24.55±3.4*
Treatment 3 (200 mg/kg)	4.75±1.3**	185.5±14.4*	26±13.7*	30.97±10.2**

Data are presented as mean±SEM (n=6) and analyzed using one-way analysis of variance followed by Tukey's *post hoc* test. Statistical significance is indicated as follows: P<0.05 (*), P<0.01 (**), and P<0.001 (***) versus disease control. Disease Control, while # comparison versus the control group

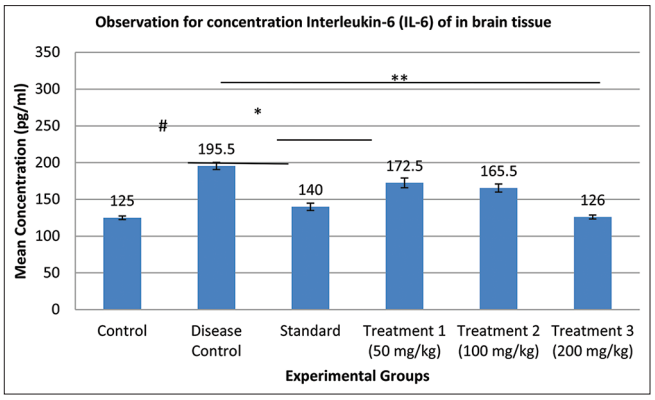

Figure 5: Effect of *Withania somnifera* leaf extract (50, 100, and 200 mg/kg orally) on ethanol withdrawal-induced changes in interleukin-6 levels. Data are expressed as mean ± SEM (n = 5). Statistical significance: $^{\#}P < 0.01$ versus control; $^{*}P < 0.05$, $^{**}P < 0.01$ versus ethanol withdrawal (one-way analysis of variance with Bonferroni *post hoc* test)

control rats (Disease Control: 7.5 ± 1.5 nmol/mg vs. Control: 1.65 ± 0.2 nmol/mg). Oral administration of WS leaf extract at 50, 100, and 200 mg/kg dose-dependently decreased MDA levels. The 50 mg/kg dose reduced MDA to 5.8 ± 1.2 nmol/mg, the 100 mg/kg dose to 4.75 ± 1.3 nmol/mg, and the 200 mg/kg dose restored MDA to 4.75 ± 1.3 nmol/mg, comparable to the standard treatment (4.5 ± 1.1 nmol/mg). These results indicate that WS effectively attenuates ethanol-induced lipid peroxidation [Table 2].

Reduced GSH level in brain tissue

Ethanol withdrawal significantly increased lipid peroxidation, as indicated by elevated MDA levels in the brain compared to control rats (Disease Control: 7.5 ± 1.5 nmol/mg vs. Control: 1.65 ± 0.2 nmol/mg). Oral administration of WS leaf extract at 50, 100, and 200 mg/kg dose-dependently decreased MDA levels. The 50 mg/kg dose reduced MDA to 5.8 ± 1.2 nmol/mg, the 100 mg/kg dose to 4.75 ± 1.3 nmol/mg, and the 200 mg/kg dose restored MDA to 4.75 ± 1.3 nmol/mg, comparable to the standard treatment (4.5 ± 1.1 nmol/mg). These results indicate that WS effectively attenuates ethanol-induced lipid peroxidation [Table 2].

Catalase level in brain tissue

Ethanol withdrawal significantly increased lipid peroxidation, as indicated by elevated MDA levels in the brain compared to control rats (Disease Control: 7.5 ± 1.5 nmol/mg vs. Control: 1.65 ± 0.2 nmol/mg). Oral administration of WS leaf extract at 50, 100, and 200 mg/kg dose-dependently decreased MDA levels. The 50 mg/kg dose reduced MDA to 5.8 ± 1.2 nmol/mg, the 100 mg/kg dose to 4.75 ± 1.3 nmol/mg, and the 200 mg/kg dose restored MDA to 4.75 ± 1.3 nmol/mg, comparable to the standard treatment (4.5 ± 1.1 nmol/mg). These results indicate that WS effectively attenuates ethanol-induced lipid peroxidation [Table 2].

Estimation of neuroinflammatory markers level

IL-6 level in brain tissue

Ethanol withdrawal significantly increased IL-6 levels in the brain compared to control rats (Disease Control: 195.5 ± 4.8 pg/mL vs. Control: 125 ± 2.5 pg/mL). Oral administration of WS leaf extract at doses of 50, 100, and 200 mg/kg dose-dependently reduced IL-6 levels. Specifically, IL-6 levels decreased to 172.5 ± 6.6 pg/mL with 50 mg/kg, 165.5 ± 5.6 pg/mL with 100 mg/kg, and 126 ± 2.7 pg/mL with 200 mg/kg, comparable to the standard treatment (140 ± 5 pg/mL) [$F(4, 25) = 27.57$, $P < 0.05$] [Figure 5].

TNF-α level in brain tissue

Ethanol withdrawal significantly decreased TNF-α levels in the brain compared to control rats (Disease Control: 70 ± 0.6 pg/mL vs. Control: 122.5 ± 2.8 pg/mL). Oral administration of WS leaf extract at doses of 50, 100, and 200 mg/kg dose-dependently increased TNF-α levels. Specifically, TNF-α levels increased to 84.5 ± 1.2 pg/mL with 50 mg/kg, 82 ± 0.9 pg/mL with 100 mg/kg, and 120.5 ± 2.3 pg/mL with 200 mg/kg, comparable to the

standard treatment (112.5 ± 1.8 pg/mL) [F (4, 25) = 12.87, $P < 0.05$] [Figure 6].

Histopathological study

Microscopic examination of the hippocampal region was performed to evaluate the effects of ethanol withdrawal and WS leaf extract treatment. Analysis at 40× magnification revealed significant histopathological alterations in disease control rats. Ethanol withdrawal induced neuronal degeneration characterized by shrinkage of neuronal cell bodies, irregular cellular arrangement, and increased glial cell proliferation, indicating an inflammatory response. In addition, vacuolization and disruption of normal hippocampal cytoarchitecture were observed, reflecting cellular stress and neurotoxicity associated with chronic alcohol exposure. Treatment with WS leaf extract (50, 100, and 200 mg/kg) demonstrated dose-dependent neuroprotective effects, with restoration of neuronal morphology, improved cellular organization, and reduced glial proliferation, comparable to the standard treatment. These findings corroborate the biochemical results and suggest a protective role of WS against ethanol-induced hippocampal damage [Figure 7].

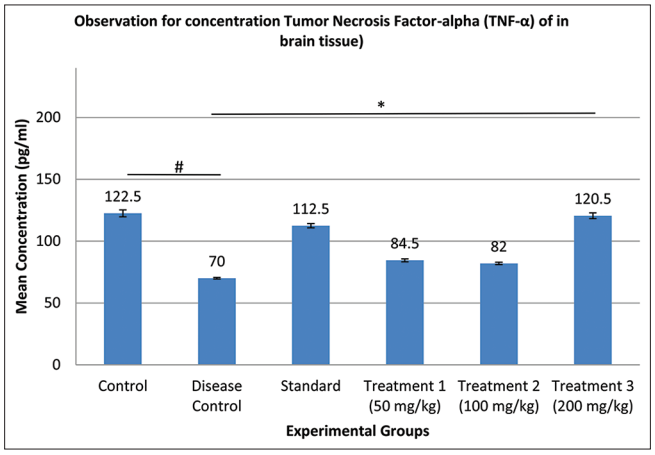

Figure 6: Effect of *Withania somnifera* leaf extract (50, 100, and 200 mg/kg orally) on ethanol withdrawal-induced changes in tumor necrosis factor-alpha levels. Data are expressed as mean ± SEM ($n = 5$). Statistical significance: $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus ethanol withdrawal (one-way analysis of variance with Bonferroni *post hoc* test)

Discussion

The present study provides comprehensive evidence that WS leaf extract exerts robust neuroprotective and antidepressant-like effects during ethanol withdrawal in rats. Ethanol withdrawal is known to induce a spectrum of neurobehavioral and neurochemical disturbances, including heightened anxiety, depressive-like behaviors, impaired locomotor activity, oxidative stress, dysregulation of neurotransmitter systems, and neuroinflammation. Chronic ethanol exposure disrupts the balance of excitatory and inhibitory neurotransmitters, particularly decreasing GABA and serotonin levels while altering dopamine concentrations, which contributes to withdrawal-associated mood disturbances and cognitive deficits. In this study, these neurochemical imbalances were dramatically corrected by administering WS leaf extract, especially with a 200 mg/kg dosage, which had effects similar to those of the NMDA receptor antagonist MK-801, a common reference for neuroprotection in withdrawal models.[22]

WS treatment notably increased GABA and serotonin levels while normalizing dopamine concentrations in alcohol-dependent rats, indicating its capacity to counteract neurotransmitter disturbances associated with ethanol withdrawal. These results align with earlier findings in which WS exhibited neuromodulatory effects, restoring neurotransmitter homeostasis in models of alcohol dependence and other neurobehavioral disorders.[23] Furthermore, in models of Parkinsonism, WS has been shown to improve motor function and dopamine levels, reinforcing its role in maintaining neurotransmitter equilibrium and neuromotor coordination. In the present study, WS administration also attenuated glutamate-induced excitotoxicity, a key mechanism underlying ethanol withdrawal-induced neuronal damage, highlighting its ability to modulate excitatory pathways alongside inhibitory neurotransmitters.[24]

Oxidative stress is a well-established consequence of ethanol withdrawal, arising from excessive generation of reactive nitrogen

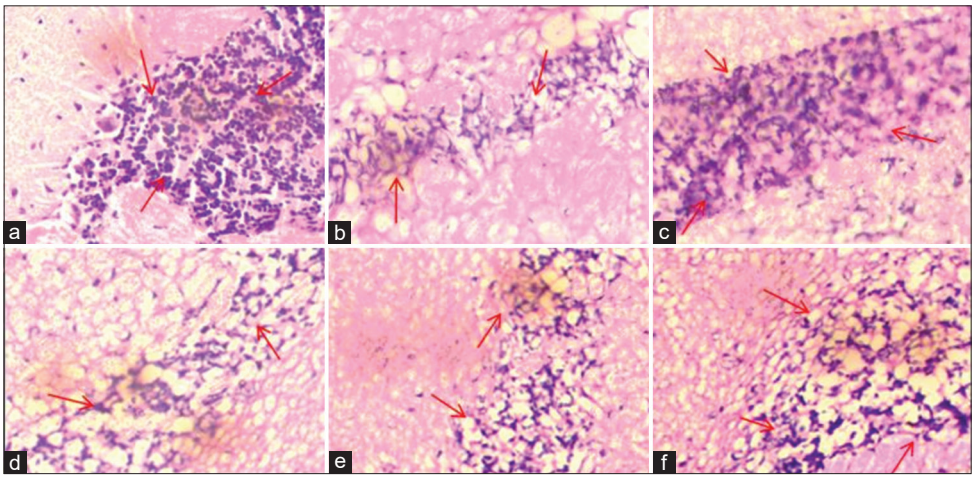

Figure 7: Histopathological changes in the ethanol withdrawal rats compared the control rats at ×40 magnification. (a) Control group, (b) disease control group, (c) Standard group, (d) Treatment group (50 mg/kg), € Treatment group (100 mg/kg), and (f) Treatment group (200 mg/kg)

species and ROS, which harm neural tissue's lipids, proteins, and nucleic acids. The present study clearly demonstrated WS's antioxidant properties, as reflected by decreased MDA and nitrite levels, along with increased catalase and GSH activity. These results align with previous studies demonstrating that WS enhances endogenous antioxidant defenses and reduces peroxidation of lipid in rodent models of neurobehavioral and oxidative stress-related disorders.[25]

Along with its antioxidant effects, WS exhibited significant anti-inflammatory activity. The study observed a reduction in ethanol-withdrawn rats' levels of pro-inflammatory cytokines, such as TNF-α and IL-6.[26] Neuroinflammation is a critical contributor to withdrawal-induced neurotoxicity, and the attenuation of inflammatory markers by WS suggests its potential to limit glial activation and inflammatory cascades. These findings corroborate earlier studies reporting similar anti-inflammatory effects of WS in models of cerebral ischemia and neurodegeneration.[27]

Histopathological evaluation further supported the neuroprotective role of WS. WS-treated rats showed preserved hippocampal architecture, with reduced neuronal shrinkage, lower gliosis, and maintenance of overall neuronal integrity compared to ethanol-withdrawn controls. This structural preservation underscores the capacity of WS to prevent against ethanol-induced neurodegeneration, complementing its biochemical and behavioral effects.[28]

Collectively, the present findings demonstrate that WS leaf extract provides multifaceted neuroprotection during ethanol withdrawal. By restoring neurotransmitter balance, enhancing antioxidant defenses, reducing neuroinflammation, and preserving neuronal morphology, WS addresses both the biochemical and structural consequences of ethanol withdrawal.[29] The 200 mg/kg dose emerged as the most effective, yielding outcomes comparable to MK-801, indicating its possible as a plant-derived therapeutic agent. These findings support the increasing amount of data indicating WS as a safe, natural, and effective intervention for managing ethanol withdrawal-induced neurobehavioral impairments and mood disorders.[30]

Conclusion

WS leaf extract effectively mitigates ethanol withdrawal-induced depression, oxidative stress, neuroinflammation, and neuronal damage in a dose-dependent manner, at 200 mg/kg, showing efficacy comparable to the standard NMDA receptor antagonist MK-801. The extract could be used as a natural, plant-based therapeutic agent to treat the neurobehavioral and biochemical issues linked to alcohol withdrawal because of its neuroprotective, antioxidant, and anti-inflammatory qualities, offering a promising adjunct or alternative to conventional pharmacotherapy.

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