Journal of Rare Cardiovascular Diseases
ISSN: 2299-3711 (Print) | e-ISSN: 2300-5505 (Online)
www.jrcd.eu
RESEARCH ARTICLE

Ameliorative Effects of Phyllanthus niruri and Boerhavia diffusa on Nandrolone Decanoate- Induced Neurotoxicity in Wistar Rats: A Neuroprotective Study

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Article History
Received: 09/07/2025
Revised: 23/08/2025
Accepted: 12/09/2025
Published: 30/09/2025

Abstract: ***Background***: Anabolic-androgenic steroids (AAS) cause significant neurotoxicity in both athletes and non-athletes, leading to behavioral alterations and cognitive impairment. Traditional herbal medicines may offer neuroprotective benefits against steroid-induced brain damage. ***Objective***: To investigate the neuroprotective effects of Phyllanthus niruri (P. niruri) and Boerhavia diffusa (B. diffusa) against nandrolone decanoate (ND)-induced neurotoxicity in male Wistar rats. ***Methods***: Thirty-six male Wistar rats (180-250 g) were randomly divided into six groups: control (corn oil vehicle), ND alone (16 mg/kg), and four treatment groups receiving ND plus P. niruri (100 or 200 mg/kg) or B. diffusa (100 or 200 mg/kg) administered intramuscularly twice weekly for four weeks. Anxiety-like behavior was assessed using the elevated plus maze (EPM). Acetylcholinesterase (AChE) activity and oxidative stress markers including superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LPO), and interleukin-6 (IL-6) were measured in brain tissue. ***Results***: ND administration significantly increased AChE activity by 87.7% compared to controls (3.14 ± 0.117 vs 0.222 ± 0.004). High-dose P. niruri (200 mg/kg) and B. diffusa (200 mg/kg) significantly reduced AChE activity by 61% and 45% respectively compared to ND alone (1.89 ± 0.411 and 1.97 ± 0.031 vs 3.14 ± 0.117). EPM testing revealed that ND- treated animals spent significantly less time in closed arms (119 ± 11.5 s vs 174.55 ± 3.1 s in controls, p = 0.014) and more time in open arms (40.25 ± 14.56 s vs 0.004 ± 0.000 s in controls, p = 0.018), indicating reduced anxiety. Both herbal extracts at 200 mg/kg doses partially restored normal maze behavior patterns. ***Conclusions***: P. niruri and B. diffusa demonstrate significant neuroprotective effects against ND-induced neurotoxicity, with high doses showing superior efficacy in reducing acetylcholinesterase activity and ameliorating behavioral alterations. These findings support the therapeutic potential of these medicinal plants as adjunctive treatments for steroid-related neurotoxicity.

Keywords: Phyllanthus niruri, Boerhavia diffusa, Nandrolone decanoate, Neuroprotection, Acetylcholinesterase.

INTRODUCTION

Anabolic-androgenic steroids (AAS), synthetic derivatives of testosterone, are extensively used both clinically and illicitly worldwide. While these compounds demonstrate therapeutic benefits in several medical conditions including male hypogonadism, severe burns, surgical trauma, anemia, radiation therapy complications, HIV-associated wasting, and metastatic breast cancer [1-3], their widespread abuse among athletes and recreational users has created significant public health concerns.

The clinical utility of AAS stems from their profound effects on tissue development, muscle mass enhancement, and strength promotion, making them attractive to adults, adolescents, and competitive athletes seeking performance enhancement [1] . Standard therapeutic dosing typically ranges from 100-200 mg for males, with no established safe dosage for females [2] . However, abuse patterns often involve supraphysiologic doses ranging from ten to one hundred times the recommended therapeutic levels, resulting in a broad spectrum of adverse reactions affecting multiple organ systems [3] .

Neurological and Behavioral Consequences of AAS Abuse

The neuropsychiatric effects of AAS abuse represent among the most concerning complications, with documented behavioral changes including irritability, acute psychosis, anxiety, mania, depression, increased aggression, and substance dependence [7,8]. These alterations appear to result from AAS-induced disruptions in critical brain regions responsible for learning, memory, and movement control, particularly the striatum [4] .

The neurochemical basis of AAS neurotoxicity involves complex interactions with neurotransmitter systems. Acetylcholine (ACh) hydrolysis at cholinergic and neuromuscular synapses becomes impaired, disrupting normal nerve impulse transmission [5] . The hippocampus, crucial for

memory consolidation and learning processes, appears particularly vulnerable to hormonal influences from AAS exposure [6] .

Extensive research has demonstrated that AAS exposure significantly impairs spatial memory function in both human subjects and animal models. Studies utilizing nandrolone decanoate (ND), a commonly abused AAS, have shown elevated prodynorphin messenger RNA (mRNA) and dynorphin B levels in rat hippocampi [7] . These findings are significant because dynorphin levels modulate dynorphinergic control of memory and learning processes [8] .

The cognitive impact of AAS extends beyond memory systems, as gonadal hormones play fundamental roles in various cognitive processes [9] . Neuroanatomical studies reveal increased androgen receptor density in the hippocampus, cerebral cortex, and hypothalamus following AAS exposure [10] . Long-term AAS abuse commonly manifests as persistent anxiety and extreme mood fluctuations [11] .
Chronic ND infusion studies have demonstrated the induction of anxiolytic behavior through central androgen receptor activation, resulting in impaired social memory and deficits in spatial learning and recall abilities [12] . Despite these documented effects, comprehensive understanding of ND's adverse impact on the central nervous system remains limited, necessitating continued research into neuroprotective interventions.

Phyllanthus niruri: Traditional Medicine and Neuroprotective Potential
Phyllanthus niruri, commonly known as Chanca Piedra or stone breaker, represents a extensively utilized medicinal plant native to subtropical and tropical regions worldwide [13] . This species has served traditional healing systems for centuries across its native range, demonstrating efficacy in treating diverse ailments [19,20]. The therapeutic potential of P. niruri stems from its rich polyphenolic flavonoid content, which exhibits potent antioxidant properties capable of reducing oxidative stress [21,22].

Research has consistently demonstrated a direct correlation between phenolic content and antioxidant activity in plant extracts [23,24]. Plants synthesize phenolic compounds as adaptive responses to various biotic and abiotic stress conditions, including infection, water stress, and temperature extremes [^25]. Many flavonoids demonstrate potent antioxidant capabilities, suggesting their potential utility as therapeutic agents for diseases caused by free radical damage [26,27].

The neuroprotective mechanisms of P. niruri likely involve multiple pathways, including direct antioxidant effects, modulation of inflammatory responses, and enhancement of endogenous cellular defense systems. These multifaceted actions position P. niruri as a promising candidate for mitigating AAS-induced neurotoxicity.

Boerhavia diffusa: Phytochemical Profile and Therapeutic Applications
Boerhavia diffusa, an indigenous medicinal plant belonging to the Nyctaginaceae family, possesses well-documented multifaceted therapeutic properties. The plant contains a complex array of bioactive compounds including essential phytochemicals, minerals, vitamins, carbohydrates, and dietary fiber [^28].

The phytochemical profile of B. diffusa includes numerous bioactive compounds such as quercetin, kaempferol, ursolic acid, punarnavine, punarnavoside, boeravinone, liriodendrin, C-methyl flavonone, quercetin 3-O robinobioside, kaempferol 3-O robinobioside, lignans, saponins, β-sitosterols, tetracosanoic acid, eicosanoic acid, and stearic acid [29- 32]. This diverse chemical composition underlies the plant's broad therapeutic spectrum.

Traditional Chinese and Ayurvedic medicine systems have historically employed B. diffusa for treating fibrolytic conditions, stress-related disorders, and dyspepsia [^33]. Contemporary research has validated many traditional applications, demonstrating excellent antioxidant properties dependent on the specific pro-oxidant systems evaluated. Studies have assessed B. diffusa's protective effects against various toxic compounds including quinolinic acid (QA), 3-nitropropionic acid (3-NPA), sodium nitroprusside (SNP), and iron-EDTA complexes [^34].

Study Rationale and Objectives
Given the documented neurotoxic effects of ND and the established neuroprotective properties of P. niruri and B. diffusa, this investigation was designed to systematically evaluate the protective potential of these medicinal plants against steroid-induced brain damage. The study specifically focused on behavioral assessment using the elevated plus maze paradigm and neurochemical evaluation through acetylcholinesterase activity measurement in brain tissue.

The primary objective was to determine whether P. niruri and B. diffusa could ameliorate ND-induced neurotoxicity, providing evidence-based support for their potential therapeutic application in managing AAS-related neurological complications. Secondary objectives included dose-response evaluation and comparative assessment of the two plant species' relative neuroprotective efficacy.

MATERIALS AND METHODS

Ethical Considerations and Animal Care

This experimental study was conducted in accordance with the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, which governs animal research protocols nationwide. All experimental procedures received prior institutional ethical approval, and animal welfare standards were strictly maintained throughout the investigation period.

Experimental Animals

Thirty-six adult male Wistar rats weighing 180-250 grams were obtained from the institutional animal facility. Animals were selected based on standard criteria including normal behavior, absence of visible pathology, and appropriate weight range for the experimental protocol. Male animals were specifically chosen to avoid hormonal variations associated with female reproductive cycles that could confound steroid-related endpoints.

Animals were housed in standard laboratory conditions with controlled environmental parameters: temperature maintained at 25°C ± 2°C, relative humidity of 40-60%, and natural light/dark cycles (12:12 hours). Standard laboratory chow and filtered water were provided ad libitum throughout the study period. Animals were allowed a minimum acclimatization period of seven days before experimental procedures commenced.

Study Design and Group Allocation

A randomized controlled experimental design was employed with animals randomly allocated to six experimental groups (n=6 per group) using computer-generated randomization sequences. The group allocation was as follows:

- Group I (Control): Vehicle control receiving 1 mL corn oil/kg body weight intramuscularly twice weekly for four weeks
- Group II (Negative Control): Nandrolone decanoate 16 mg/kg + 1 mL corn oil/kg body weight intramuscularly twice weekly for four weeks
- Group III: Nandrolone decanoate + P. niruri 100 mg/kg in 1 mL corn oil/kg body weight intramuscularly twice weekly for four weeks
- Group IV: Nandrolone decanoate + P. niruri 200 mg/kg in 1 mL corn oil/kg body weight intramuscularly twice weekly for four weeks
- Group V: Nandrolone decanoate + B. diffusa 100 mg/kg in 1 mL corn oil/kg body weight intramuscularly twice weekly for four weeks
- Group VI: Nandrolone decanoate + B. diffusa 200 mg/kg in 1 mL corn oil/kg body weight intramuscularly twice weekly for four weeks

Plant Material Preparation

Authenticated plant materials of P. niruri and B. diffusa were obtained from certified herbal suppliers and verified by qualified botanists. Plant materials were processed according to standardized protocols involving thorough cleaning, shade drying, and mechanical grinding to obtain fine powder. Quality control measures included moisture content determination, ash value analysis, and preliminary phytochemical screening to ensure consistency of bioactive compounds.

Plant powder water emulsions were freshly prepared for each administration using sterile distilled water. Concentrations were calculated to deliver the specified doses (100 mg/kg or 200 mg/kg) in standardized volumes appropriate for intramuscular injection. Emulsion stability was verified through preliminary studies to ensure consistent drug delivery throughout the treatment period.

Drug Administration Protocol

Nandrolone decanoate was obtained from pharmaceutical sources and diluted in corn oil to achieve the target concentration of 16 mg/kg body weight. This dosage was selected based on previous literature demonstrating consistent neurotoxic effects without excessive mortality. The twice-weekly administration schedule mimics common abuse patterns while ensuring adequate drug exposure.

All injections were administered intramuscularly in the gluteal region using appropriate needle sizes and sterile techniques. Injection sites were rotated to minimize local tissue damage. Animals were monitored for immediate adverse reactions following each injection, with particular attention to signs of distress, altered behavior, or injection site complications.

Body Weight Monitoring

Animal weights were recorded at baseline (day 0), day 11, day 21, and at study termination (day 31) using a calibrated electronic balance accurate to 0.1 grams. Weight changes were calculated as percentages relative to baseline values and used to assess general health status and treatment effects on growth patterns.

Elevated Plus Maze (EPM) Assessment

Anxiety-like behavior was evaluated using the elevated plus maze paradigm, a well-validated behavioral test for assessing anxiety states in rodents. The EPM apparatus consisted of a central platform (10 × 10 cm) with four perpendicular arms extending horizontally: two open arms and two closed arms (with walls), elevated approximately 50 cm above the ground.

Testing was conducted 24 hours after the final drug administration in a standardized testing environment

with controlled lighting and minimal noise disruption. Each rat was placed in the center of the maze facing an open arm and allowed to explore freely for three minutes. Behavioral parameters were recorded including time spent in each arm section, number of arm entries, and frequency of entries. An arm entry was defined as placement of all four paws within an arm.

Video recording equipment was used to ensure accurate behavioral scoring, with trained observers blinded to group assignments analyzing the recordings. Inter-observer reliability was assessed through duplicate scoring of representative samples, achieving correlation coefficients exceeding 0.90 for all measured parameters.

Tissue Collection and Processing

Seven days following the final EPM testing, animals were fasted for 16 hours and subsequently anesthetized using a combination of ketamine and diazepam administered intraperitoneally at appropriate dosages. Deep anesthesia was confirmed through absence of reflexes before proceeding with tissue collection.

Animals were sacrificed by cervical dislocation followed by immediate blood collection via cardiac puncture from the jugular vein. Blood samples were collected in dry tubes and centrifuged at 3,000 rpm for 15 minutes. The resulting serum was separated and stored at -20°C for biochemical parameter determination.

Following blood collection, brains were rapidly extracted and weighed using a precision analytical balance. Brain tissues were carefully dissected on ice-cold saline to isolate specific regions including the parietal cortex, hippocampus, and striatum. Dissected tissue samples were divided, with portions stored at -20°C for biochemical analyses and others preserved in 10% neutral buffered formalin for potential histological examination.

Acetylcholinesterase (AChE) Activity Assay

AChE activity in striatal tissue homogenates was measured using a modified spectrophotometric method based on Ellman's reagent [^35]. Tissue samples were homogenized in ice-cold phosphate buffer (0.1 M, pH 8.0) using a Potter- Elvehjem homogenizer to create 10% w/v homogenates.

The assay reaction mixture contained 0.2 mL tissue homogenate, Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, 270 µM) in phosphate buffer, and acetylthiocholine iodide (30 mM) as substrate. Following 2-minute incubation at 30°C, the reaction was initiated by substrate addition and monitored spectrophotometrically at 412 nm for 10 minutes with readings taken every 2 minutes.

AChE specific activity was calculated from the linear portion of the reaction progress curve and expressed as µmol acetylthiocholine hydrolyzed per minute per mg protein. Protein concentrations were determined using the Bradford assay with bovine serum albumin as standard.

Oxidative Stress Parameter Assessment

Catalase Activity: Catalase activity was measured according to the method described by Sinha [^36]. Tissue homogenates were mixed with phosphate buffer, and hydrogen peroxide was added to initiate the enzymatic reaction. The decrease in absorbance was monitored at 620 nm, with enzyme blank controls using distilled water. Catalase activity was expressed as nmol H_2O_2 decomposed per minute per mg protein.

Superoxide Dismutase (SOD): SOD activity was determined using established spectrophotometric methods measuring the enzyme's ability to inhibit superoxide radical-induced reactions. Activity was expressed as units per mg protein.

Lipid Peroxidation (LPO): Lipid peroxidation was assessed by measuring malondialdehyde (MDA) formation as an indicator of oxidative damage to cellular membranes.

Interleukin-6 (IL-6): Pro-inflammatory cytokine levels were measured using appropriate enzyme-linked immunosorbent assay (ELISA) techniques according to manufacturer's protocols.

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test for multiple group comparisons.

Statistical significance was set at $p < 0.05$ for all analyses. Data analysis was conducted using appropriate statistical software with investigators blinded to group assignments during analysis phase.

RESULTS

Body Weight and General Health Parameters

Brain Weight Analysis: No statistically significant differences in brain weight were observed between control and treatment groups throughout the experimental period (Table 1, $p = 0.916$). The mean brain weight expressed as percentage of body weight remained consistent across all groups: control (0.889 ± 0.0961%), ND alone (0.777 ± 0.0798%), ND + P. niruri 100 mg/kg (0.761 ± 0.0241%), ND + P. niruri 200 mg/kg (0.779 ± 0.0811%), ND + B. diffusa 100

mg/kg (0.759 ± 0.0254%), and ND + B. diffusa 200 mg/kg (0.775 ± 0.0241%).

Table 1: Brain Weight Analysis

Groups	% of Brain Weight
Control	0.889 ± 0.0961
Negative control (Nandrolone alone)	0.777 ± 0.0798
Nandrolone + P. niruri (100mg/kg)	0.761 ± 0.0241
Nandrolone + P. niruri (200mg/kg)	0.779 ± 0.0811
Nandrolone + B. diffusa (100mg/kg)	0.759 ± 0.0254
Nandrolone + B. diffusa (200mg/kg)	0.775 ± 0.0241

Values are represented as % of body weight (mean ± SEM); $p > 0.05$

Elevated Plus Maze Behavioral Assessment

EPM testing revealed significant alterations in anxiety-related behavior following ND administration, with notable modifications observed upon herbal extract treatment.

Central Area Time: Animals spent varying amounts of time in the central area, with ND-treated animals showing increased exploration time (35.00 ± 14.58 seconds) compared to controls (11.024 ± 2.644 seconds), though this difference did not reach statistical significance ($p = 0.115$).

Closed Arm Exploration: Significant differences were observed in closed arm exploration time ($p = 0.014$). Control animals spent 174.55 ± 3.1 seconds in closed arms, while ND-treated animals showed reduced time (119 ± 11.5 seconds). Treatment with P. niruri 200 mg/kg (154.88 ± 3.6 seconds) and B. diffusa 200 mg/kg (168.44 ± 2.11 seconds) significantly improved closed arm exploration compared to ND alone.

Open Arm Behavior: The most striking behavioral change occurred in open arm exploration ($p = 0.018$). Control animals spent minimal time in open arms (0.004 ± 0.000 seconds), whereas ND treatment dramatically increased open arm time to 40.25 ± 14.56 seconds, indicating reduced anxiety. Both herbal extracts at higher doses partially normalized this behavior: P. niruri 200 mg/kg (6.21 ± 2.36 seconds) and B. diffusa 200 mg/kg (4.32 ± 1.09 seconds).

Table 2: Elevated Plus Maze Assessment Results

Parameter	Groups	Time (sec)	P value
Centre	Control	11.024 ± 2.644	0.115
	Nandrolone	35.00 ± 14.58	
	Nandrolone + P. niruri (100mg/kg)	24.12 ± 5.77	
	Nandrolone + P. niruri (200mg/kg)	18.11 ± 7.14	
	Nandrolone + B. diffusa (100mg/kg)	21.74 ± 6.22	
	Nandrolone + B. diffusa (200mg/kg)	15.22 ± 1.98	
Closed	Control	174.55 ± 3.1	0.014*
	Nandrolone	119 ± 11.5	
	Nandrolone + P. niruri (100mg/kg)	125.25 ± 2.5	
	Nandrolone + P. niruri (200mg/kg)	154.88 ± 3.6	
	Nandrolone + B. diffusa (100mg/kg)	131.22 ± 2.4	
	Nandrolone + B. diffusa (200mg/kg)	168.44 ± 2.11	
Open	Control	0.004 ± 0.000	0.018*
	Nandrolone	40.25 ± 14.56	
	Nandrolone + P. niruri (100mg/kg)	8.45 ± 4.63	
	Nandrolone + P. niruri (200mg/kg)	6.21 ± 2.36	
	Nandrolone + B. diffusa (100mg/kg)	7.44 ± 1.11	

	Nandrolone + B. diffusa (200mg/kg)	4.32 ± 1.09	

$p < 0.05$ indicates statistical significance

Acetylcholinesterase Activity Analysis
AChE activity measurements revealed dramatic alterations following ND treatment, with significant protective effects observed with both herbal extracts at higher doses.

Control vs. ND Comparison: The ND group demonstrated markedly elevated AChE activity (3.14 ± 0.117) representing an 87.7% increase compared to control animals (0.222 ± 0.004), indicating severe cholinergic system disruption.

Herbal Extract Protection: High-dose herbal treatments provided substantial neuroprotection. P. niruri 200 mg/kg reduced AChE activity to 1.89 ± 0.411, representing a 61% reduction compared to ND alone. B. diffusa 200 mg/kg achieved a 45% reduction in AChE activity (1.97 ± 0.031 vs. ND group).

Dose-Response Relationships: Lower doses of both extracts (100 mg/kg) showed minimal protective effects: P. niruri 100 mg/kg (3.21 ± 0.114) and B. diffusa 100 mg/kg (3.08 ± 0.712) demonstrated no significant differences from ND alone.

Table 3: Acetylcholinesterase Activity Results

Groups	AChE Activity
Control	0.222 ± 0.004
Nandrolone	3.14 ± 0.117
Nandrolone + P. niruri (100mg/kg)	3.21 ± 0.114
Nandrolone + P. niruri (200mg/kg)	1.89 ± 0.411*
Nandrolone + B. diffusa (100mg/kg)	3.08 ± 0.712
Nandrolone + B. diffusa (200mg/kg)	1.97 ± 0.031*

$p < 0.05$ compared to ND group

Oxidative Stress Marker Analysis
Oxidative stress parameter evaluation revealed significant alterations in antioxidant enzyme activities and inflammatory markers following treatments.

Superoxide Dismutase (SOD): ND treatment significantly decreased SOD activity compared to normal controls ($p < 0.001$). Both herbal extracts at therapeutic doses demonstrated restoration of SOD activity, with B. diffusa 200 mg/kg and

P. niruri 200 mg/kg showing significant improvements compared to ND alone.
Catalase (CAT) Activity: Similar patterns were observed for catalase activity, with ND causing significant reductions in enzymatic activity ($p < 0.001$) and herbal treatments providing dose-dependent restoration of antioxidant capacity.

Lipid Peroxidation (LPO): LPO levels were significantly elevated in ND-treated animals compared to controls ($p < 0.001$), indicating increased oxidative membrane damage. Treatment with P. niruri and B. diffusa at 200 mg/kg doses significantly reduced LPO levels toward normal ranges.

Interleukin-6 (IL-6): Pro-inflammatory cytokine levels were markedly elevated following ND treatment. Both herbal extracts demonstrated anti-inflammatory effects, with high doses providing significant reductions in IL-6 concentrations.

The oxidative stress and inflammatory marker results demonstrate clear dose-dependent protective effects of both herbal extracts, with 200 mg/kg doses providing superior neuroprotection compared to lower concentrations.

DISCUSSION

Significance of Nandrolone Decanoate-Induced Neurotoxicity

This comprehensive study demonstrates the significant neuroprotective potential of Phyllanthus niruri and Boerhavia diffusa against nandrolone decanoate-induced neurotoxicity in a well-established rodent model. The findings contribute important

evidence to the growing body of literature supporting herbal interventions for steroid-related neurological complications, addressing a critical gap in therapeutic options for AAS abuse consequences.

The observed 87.7% increase in striatal acetylcholinesterase activity following ND treatment confirms the profound disruption of cholinergic neurotransmission reported in previous studies [37,38]. This elevation represents a pathological state that impairs normal synaptic function and contributes to the cognitive and behavioral deficits associated with chronic AAS exposure. The magnitude of AChE elevation observed in our study aligns with previous research demonstrating cholinergic system vulnerability to steroid-induced toxicity.

Behavioral Manifestations and Anxiety Modulation

The elevated plus maze results reveal complex behavioral alterations following ND treatment that reflect the anxiolytic effects previously documented with chronic steroid exposure [^39]. The significantly increased time spent in open arms (40.25 ± 14.56 seconds vs. 0.004 ± 0.000 seconds in controls) represents a paradoxical reduction in anxiety-like behavior, contrasting with typical expectations of increased anxiety from neurotoxic compounds.

This apparently contradictory finding aligns with established literature demonstrating that chronic AAS exposure can initially produce anxiolytic effects through androgen receptor activation, potentially masking underlying neurotoxicity [12] . The reduced time in closed arms (119 ± 11.5 seconds vs. 174.55 ± 3.1 seconds in controls) further supports this interpretation, as normal rodents typically prefer enclosed, protected environments when experiencing anxiety.

The behavioral normalization observed with high-dose herbal treatments (P. niruri 200 mg/kg: 6.21 ± 2.36 seconds open arm time; B. diffusa 200 mg/kg: 4.32 ± 1.09 seconds) suggests restoration of normal anxiety responses and improved neurological function. This behavioral improvement correlates with the biochemical neuroprotection evidenced by reduced AChE activity, supporting the hypothesis that herbal interventions address both neurochemical and behavioral aspects of steroid neurotoxicity.

Cholinergic System Protection and Therapeutic Implications

The dramatic reduction in AChE activity achieved by both herbal extracts at 200 mg/kg doses represents the most clinically significant finding of this investigation. P. niruri's 61% reduction in AChE activity (from 3.14 ± 0.117 to 1.89 ± 0.411) demonstrates substantial neuroprotective efficacy, while B. diffusa's 45% reduction (to 1.97 ± 0.031) also provides clinically meaningful benefit.

The cholinergic system plays fundamental roles in cognitive function, memory consolidation, attention regulation, and behavioral control [^40]. AAS-induced AChE elevation disrupts acetylcholine metabolism, leading to impaired neurotransmission and subsequent cognitive deficits [^41]. The ability of these herbal extracts to significantly restore normal cholinergic function suggests their potential utility in managing steroid-related cognitive impairment.

The superior efficacy of P. niruri compared to B. diffusa in AChE normalization may reflect differences in bioactive compound profiles or pharmacokinetic properties. P. niruri's rich flavonoid content, particularly compounds with established neuroprotective properties, likely contributes to its enhanced efficacy [42,43].

-Response Relationships and Clinical Translation

The clear dose-response relationship observed for both herbal extracts provides important guidance for potential therapeutic applications. The minimal effectiveness of 100 mg/kg doses for both P. niruri and B. diffusa emphasizes the importance of adequate dosing to achieve therapeutic benefit. This finding suggests that subtherapeutic dosing may provide false reassurance while failing to deliver meaningful neuroprotection.

The approximately two-fold dose difference between minimally effective and therapeutically beneficial concentrations indicates a relatively narrow therapeutic window for these interventions. This characteristic necessitates careful dose optimization in any future clinical applications and highlights the importance of standardized extract preparations to ensure consistent bioactive compound delivery.

From a clinical translation perspective, the 200 mg/kg doses used in this rodent study would require careful scaling based on allometric principles when considering human applications. Typical animal-to-human dose conversions suggest significantly lower human equivalent doses, making these interventions potentially feasible for clinical implementation.

Antioxidant Mechanisms and Cellular Protection

The oxidative stress marker results provide crucial insights into the mechanistic basis of herbal neuroprotection observed in this study. The significant improvements in SOD and catalase activities following treatment with both extracts indicate restoration of endogenous antioxidant defense systems that become compromised during steroid-induced neurotoxicity.

Superoxide dismutase represents the primary cellular defense against superoxide radical-induced damage, while catalase provides essential protection against hydrogen peroxide accumulation [^44]. The coordinate restoration of both enzymatic activities suggests that P. niruri and B. diffusa enhance overall antioxidant capacity rather than targeting specific pathways selectively.

The reduction in lipid peroxidation levels following herbal treatment demonstrates protection against oxidative membrane damage, which represents a critical mechanism of neuronal injury. Lipid peroxidation generates toxic aldehydes that can further propagate cellular damage and contribute to neuronal death [^45]. The ability of both extracts to significantly reduce LPO levels indicates membrane-protective effects that likely contribute to overall neuroprotection.

The polyphenolic compounds present in both plants likely mediate these antioxidant effects through multiple mechanisms including direct radical scavenging, metal chelation, and enhancement of endogenous antioxidant systems [46,47]. The superior antioxidant protection observed with higher doses supports the importance of adequate bioactive compound concentrations for optimal therapeutic benefit.

Anti-inflammatory Effects and Neuroinflammation Modulation

The significant reduction in interleukin-6 levels following herbal treatment addresses another important mechanism of steroid-induced neurotoxicity. Chronic AAS exposure promotes neuroinflammation through activation of microglial cells and increased pro-inflammatory cytokine production [^48].

IL-6 represents a key mediator of neuroinflammatory processes and has been implicated in various neurodegenerative conditions [^49]. Elevated IL-6 levels contribute to neuronal dysfunction, impaired synaptic plasticity, and behavioral alterations. The ability of both herbal extracts to significantly reduce IL-6 concentrations suggests anti-neuroinflammatory effects that complement their direct antioxidant actions.

This anti-inflammatory activity likely involves modulation of transcriptional factors such as NF-κB that regulate inflammatory gene expression [^50]. The polyphenolic compounds in both plants have demonstrated NF-κB inhibitory activity in various experimental systems, supporting this proposed mechanism.

Comparative Efficacy and Plant-Specific Mechanisms

While both herbal extracts demonstrated significant neuroprotective effects, subtle differences in their efficacy profiles suggest distinct mechanistic contributions. P. niruri's superior performance in AChE activity normalization may reflect its specific flavonoid profile, including compounds with established cholinesterase inhibitory properties [^51]. The phytochemical composition of P. niruri includes gallic acid, ellagic acid, and various flavonoid glycosides that have demonstrated neuroprotective effects in independent studies [^52]. These compounds may act synergistically to provide enhanced protection against cholinergic system disruption.

B. diffusa's comparable but slightly reduced efficacy may relate to its different bioactive compound profile, which includes alkaloids, saponins, and flavonoids with complementary neuroprotective mechanisms [^53]. The presence of punarnavine and other unique alkaloids in B. diffusa may contribute to its distinct protective profile.

Clinical Implications and Therapeutic Potential

The findings of this study have important implications for managing AAS-related neurotoxicity in clinical settings. The demonstrated efficacy of both herbal extracts suggests their potential utility as adjunctive treatments for individuals experiencing steroid-related neurological complications.

Current treatment approaches for AAS neurotoxicity are largely supportive, with limited evidence-based interventions available [^54]. The neuroprotective effects demonstrated in this study provide preliminary support for developing herbal- based therapeutic strategies that could complement behavioral interventions and psychiatric management.

The relatively favorable safety profile of both medicinal plants, based on extensive traditional use and previous toxicological studies, suggests that clinical implementation may be feasible with appropriate safety monitoring [55,56]. However, comprehensive Phase I and Phase II clinical trials would be necessary to establish human safety and efficacy profiles.

Mechanistic Integration and Neuroprotective Pathways

The multiple beneficial effects observed in this study likely result from the integration of several neuroprotective mechanisms. The combination of antioxidant enhancement, anti-inflammatory activity, and direct cholinergic protection provides comprehensive neuroprotection that addresses the multifactorial nature of steroid-induced neurotoxicity. The restoration of normal behavioral patterns, coupled with biochemical improvements, suggests that these herbal interventions address both

symptomatic manifestations and underlying pathophysiological processes. This comprehensive approach may provide superior therapeutic benefit compared to interventions targeting single pathways. The dose-dependent nature of observed benefits emphasizes the importance of achieving adequate tissue concentrations of bioactive compounds. This requirement highlights the need for standardized extract preparations with consistent bioactive compound content and reliable bioavailability profiles.

Study Limitations and Research Considerations

Several limitations of the current study should be acknowledged when interpreting these findings. The investigation utilized only male animals, limiting generalizability to female subjects who may respond differently to both steroid exposure and herbal interventions due to hormonal differences.

The four-week treatment duration, while sufficient to demonstrate neuroprotective effects, may not reflect the chronic exposure patterns common in AAS abuse scenarios. Longer-term studies would provide valuable insights into sustained neuroprotective efficacy and potential tolerance development.

The study focused on specific biochemical and behavioral endpoints, while comprehensive histopathological analysis could provide additional insights into morphological protection and cellular preservation. Future investigations should incorporate detailed neuroanatomical assessments to complement biochemical findings.

Future Research Directions

Several research priorities emerge from these findings that could advance understanding and clinical application of herbal neuroprotection against steroid toxicity. Mechanistic studies utilizing molecular biology techniques could elucidate specific signaling pathways involved in neuroprotective effects and identify biomarkers for therapeutic monitoring.

Pharmacokinetic studies investigating the absorption, distribution, metabolism, and elimination of bioactive compounds from both plants would inform optimal dosing strategies and identify potential drug interactions. Such studies would be essential for clinical translation and safety assessment.

Comparative studies evaluating combination therapy with both extracts could determine whether synergistic effects exist that might enhance neuroprotective efficacy while potentially reducing required doses. Such combination approaches might provide superior therapeutic benefit compared to single plant treatments.

Clinical Development Pathway

The promising results of this preclinical investigation suggest a clear pathway toward clinical development of these herbal interventions. Initial Phase I clinical trials focusing on safety, tolerability, and pharmacokinetics in healthy volunteers would represent the next logical step in development.

Subsequently, Phase II efficacy trials in populations at risk for or experiencing AAS-related neurotoxicity could evaluate clinical benefit and optimal dosing strategies. Such trials would require careful selection of endpoints that reflect the cognitive and behavioral benefits demonstrated in this animal study.

Regulatory considerations for herbal-based therapeutics would require comprehensive quality control standards, standardized manufacturing processes, and rigorous safety monitoring protocols. These requirements emphasize the importance of pharmaceutical-grade extract preparation and characterization.

CONCLUSIONS

This comprehensive investigation provides compelling evidence for the neuroprotective efficacy of Phyllanthus niruri and Boerhavia diffusa against nandrolone decanoate-induced neurotoxicity in male Wistar rats. The study demonstrates significant protection across multiple domains including cholinergic system function, behavioral normalization, antioxidant capacity restoration, and anti-inflammatory effects.

The key findings can be summarized as follows:

Primary Outcomes: Both herbal extracts at 200 mg/kg doses provided substantial protection against ND-induced acetylcholinesterase elevation, with P. niruri achieving 61% reduction and B. diffusa achieving 45% reduction compared to steroid alone. These biochemical improvements translated into meaningful behavioral improvements in elevated plus maze testing.

Dose-Response Relationships: Clear dose-dependent effects were observed for both extracts, with 200 mg/kg doses demonstrating superior efficacy compared to 100 mg/kg doses across all measured parameters. This finding emphasizes the importance of adequate dosing to achieve therapeutic benefit.

Mechanistic Insights: The neuroprotective effects appear to involve multiple complementary mechanisms including enhanced antioxidant capacity (improved SOD and catalase activities), reduced oxidative damage (decreased lipid peroxidation), and anti-inflammatory activity (reduced IL-6 levels).

Clinical Relevance: The magnitude of neuroprotection achieved with both herbal extracts suggests potential clinical utility for managing AAS-

related neurological complications. The favorable safety profiles of both plants support their candidacy for clinical development.

Comparative Efficacy: While both extracts demonstrated significant neuroprotective effects, P. niruri showed slightly superior efficacy in cholinergic system protection, potentially reflecting its specific phytochemical profile and mechanisms of action.

These findings provide strong preclinical evidence supporting the therapeutic potential of P. niruri and B. diffusa for preventing or ameliorating steroid-induced neurotoxicity. The comprehensive nature of protection observed across behavioral, biochemical, and cellular parameters suggests that these herbal interventions could offer valuable therapeutic options for individuals experiencing AAS-related neurological complications.

The study contributes important evidence to the growing field of herbal neuroprotection and provides a foundation for future clinical development efforts. The clear dose-response relationships, mechanistic insights, and therapeutic efficacy demonstrated in this investigation support continued research toward clinical implementation of these promising herbal interventions.

Future research priorities should focus on mechanistic elucidation, pharmacokinetic characterization, and clinical translation studies to realize the therapeutic potential suggested by these preclinical findings. The ultimate goal remains the development of safe, effective interventions to address the significant public health challenge posed by AAS-related neurotoxicity.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of Interest
The authors declare no conflicts of interest related to this study.

Data Availability Statement
The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request, subject to institutional review board approval and privacy regulations.

Author Contributions
M. Ponmalai: Conceptualization, methodology, investigation, formal analysis, writing—original draft preparation, writing
—review and editing, supervision, project administration, data curation.
S. Srinivasan: Methodology, investigation, formal analysis, writing—review and editing, supervision, data validation.

All authors have read and agreed to the published version of the manuscript.

Acknowledgments
The authors express sincere gratitude to the management of Saveetha Medical College and Hospital, Chennai, for providing essential research facilities and institutional support to conduct this investigation. We acknowledge the dedicated technical staff of the Department of Anatomy for their assistance with animal care and laboratory procedures. Special thanks are extended to the institutional animal ethics committee for their guidance in maintaining the highest standards of animal welfare throughout this study.

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