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Evaluation of the Analgesic Activity of Ashwagandha Capsules in Mice

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https://doi.org/10.37229/fsa.fjas.2026.07.05

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Future Science Association

Available online free at
www.futurejournals.org

Print ISSN: 2767-178X

Online ISSN: 2767-181X

Received: 10 May 2026
Accepted: 25 June 2026
Published: 5 July 2026

Abstract: This study aimed to evaluate the peripheral and central analgesic activity of standardized *Withania somnifera* (Ashwagandha) root extract in capsule form using established animal models. Sixteen adult male Swiss albino mice (20–30 g) were randomly assigned to four groups per test (n=4). The acetic acid-induced writhing test (peripheral analgesia) and hot plate test (central analgesia at 55±0.5°C) were employed. Treatments included: control (distilled water), standard (diclofenac sodium 4 mg/kg for writhing; tramadol 40 mg/kg for hot plate), and Ashwagandha capsules at 200 mg/kg and 400 mg/kg orally. Writhing episodes and latency times were recorded, and data were analyzed using one-way ANOVA followed by LSD test, he most important findings in the acetic acid-induced writhing test, Ashwagandha produced a dose-dependent reduction in writhing (19% at 200 mg/kg; 36% at 400 mg/kg), but this effect was not statistically significant compared to control ($p>0.05$). Diclofenac significantly inhibited writhing by 63% ($p=0.0281$). In the hot plate test, Ashwagandha showed negligible central analgesic activity, with maximum possible effect (MPE) values of only 3% (200 mg/kg) and 5% (400 mg/kg), compared to 52% for tramadol. From these results it is clear that Ashwagandha root extract exhibited weak, non-significant peripheral analgesic activity and no meaningful central analgesic effect in acute thermal pain models. These findings suggest that Ashwagandha does not act through classical opioid pathways, and its reported CNS benefits (anxiolytic, neuroprotective) likely involve non-opioid mechanisms that may not translate to acute pain relief.

Key words: Ashwagandha capsules, analgesic activity, anxiolytic, neuroprotective and CNS.

1. Introduction

1.1. Overview of Ashwagandha (*Withania somnifera*)

Withania somnifera (L.) Dunal, commonly known as Ashwagandha, Indian ginseng, or winter cherry, is a perennial medicinal shrub belonging to the family *Solanaceae*. The plant has occupied a central position in Ayurvedic medicine for more than 3000 years and is currently recognized as one of

the most extensively investigated medicinal plants in ethnopharmacology and neuropharmacology. The nomenclature "Ashwagandha" originates from Sanskrit, where "ashwa" means horse and "gandha" means smell, reflecting the characteristic odor of the roots and the traditional belief that the herb imparts equine vitality and stamina **(Sharifi-Rad *et al.*, 2021)**.

Botanically, *W. somnifera* is a small woody shrub reaching approximately 35–150 cm in height. It possesses ovate leaves, greenish-yellow flowers, and orange-red berries enclosed within a papery calyx. The plant is predominantly cultivated in India, Pakistan, Sri Lanka, the Middle East, and parts of Africa, thriving particularly in dry subtropical climates. Among all plant parts, the roots are considered the principal medicinal component; however, leaves, fruits, and seeds also contain pharmacologically active metabolites of substantial therapeutic importance.

From a phytopharmaceutical perspective, Ashwagandha has gained remarkable scientific interest owing to its adaptogenic, neuroprotective, anti-inflammatory, immunomodulatory, antioxidant, anxiolytic, and anti-neoplastic activities. The growing interest in the plant is attributed largely to the identification of steroidal lactones known as withanolides, which constitute the major bioactive chemical class responsible for many of its biological effects **(Kashyap et al., 2022).**

Modern pharmacological investigations have demonstrated that Ashwagandha exerts pleiotropic biological effects through modulation of oxidative stress pathways, inflammatory cascades, mitochondrial function, apoptosis signaling, and neurotransmitter systems. These observations have expanded its therapeutic relevance beyond traditional medicine into contemporary biomedical research, particularly in neurological and psychiatric disorders such as Alzheimer's disease, Parkinson's disease, anxiety disorders, depression, insomnia, and stress-related cognitive impairment.

Recent advances in analytical chemistry, including high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC-MS), and liquid chromatography–tandem mass spectrometry (LC-MS/MS), have enabled comprehensive characterization of the plant's secondary metabolites. More than 70 bioactive constituents have been identified, highlighting the chemical complexity and therapeutic versatility of *W. somnifera* (**Abdelwahed et al., 2023).**

Furthermore, standardized Ashwagandha extracts enriched with defined concentrations of withanolides are increasingly utilized in clinical investigations and nutraceutical formulations. This standardization has significantly improved reproducibility, safety profiling, and pharmacological consistency, thereby strengthening the scientific credibility of Ashwagandha-based therapeutics.

The central nervous system (CNS) is highly vulnerable to oxidative stress, neuroinflammation, and neurotransmitter dysregulation, which collectively contribute to the pathophysiology of neurodegenerative and neuropsychiatric disorders. Increasing global prevalence of conditions such as Alzheimer's disease, Parkinson's disease, anxiety, and depression has intensified the search for safe and effective neuroprotective agents derived from natural sources. In this context, medicinal plants with multi-target pharmacological properties have gained significant attention as potential therapeutic alternatives or adjuncts to conventional pharmacotherapy **(Marchi et al., 2025).**

Withania somnifera (Ashwagandha) is a well-documented medicinal plant extensively used in Ayurvedic medicine as a Rasayana herb, traditionally believed to enhance longevity, cognitive performance, and stress resilience. Modern pharmacological investigations have validated many of these traditional claims, demonstrating that Ashwagandha possesses adaptogenic, anxiolytic, antidepressant, antioxidant, and neuroprotective properties. These effects are primarily attributed to its bioactive withanolides, which exert pleiotropic actions on multiple molecular targets within the CNS **(Kashyap *et al.*, 2022).**

Experimental evidence suggests that Ashwagandha modulates key neurobiological pathways involved in brain homeostasis, including the hypothalamic–pituitary–adrenal (HPA) axis, GABAergic signaling, cholinergic transmission, and neurotrophic factor regulation. Additionally, Ashwagandha has been shown to suppress neuroinflammatory mediators such as NF-κB, TNF-α, and IL-6, while enhancing antioxidant defense mechanisms in neuronal tissues. These multifaceted actions support its potential role in preventing neuronal damage and improving cognitive function **(Borgonetti & Galeotti, 2023).**

Preclinical studies in rodent models have demonstrated that standardized extracts of *Withania somnifera* improve learning and memory, reduce anxiety-like behavior, and enhance resistance to stress-induced neurochemical alterations. In Alzheimer's disease models, Ashwagandha has been reported to reduce amyloid-beta accumulation and improve synaptic plasticity, suggesting a disease-modifying potential rather than merely symptomatic relief **(Gladen-Kolarsky et al., 2024).**

The therapeutic efficacy of Ashwagandha is largely dependent on its phytochemical composition, particularly steroidal lactones known as withanolides, including Withaferin A and Withanolide A. These compounds exhibit neuroprotective effects through antioxidant activity, inhibition of apoptotic pathways, and modulation of mitochondrial function. Furthermore, they promote neurite outgrowth and synaptic regeneration, which are critical processes in neuronal repair and functional recovery **(Tancreda et al., 2025).**

Recent advancements in pharmaceutical technology have enabled the development of standardized Ashwagandha formulations, including capsule-based preparations containing quantified withanolide concentrations. Such formulations ensure consistent bioavailability, improved stability, and reproducible pharmacological effects. The use of capsule forms in experimental models allows for controlled dosing and enhanced translational relevance to potential human clinical applications **(Er et al., 2025).**

Despite growing evidence supporting the CNS benefits of Ashwagandha, variability in experimental design, dosage, extraction methods, and animal models has led to inconsistencies in reported outcomes. Therefore, systematic evaluation of standardized Ashwagandha formulations under controlled experimental conditions remains essential to validate its neuropharmacological profile. Mice models, in particular, provide a valuable platform for investigating behavioral, biochemical, and histopathological effects of CNS-active compounds.

Given the increasing interest in plant-based neurotherapeutics, further investigation into the CNS activity of Ashwagandha capsules in experimental animals is warranted. Such studies are critical to elucidate dose-dependent effects, mechanistic pathways, and safety profiles, thereby contributing to the development of evidence-based phytopharmaceutical interventions for CNS disorders.

Traditional and Medicinal Uses of Ashwagandha

Ashwagandha is considered one of the most important "Rasayana" herbs in Ayurveda, a category of medicinal plants traditionally believed to rejuvenate the body, prolong lifespan, enhance mental capacity, and restore physiological balance. Historically, Ashwagandha has been prescribed for weakness, chronic fatigue, stress, insomnia, infertility, memory impairment, inflammatory disorders, and nervous system dysfunctions **(Dipankar et al., 2025).**

Traditional Ayurvedic formulations commonly utilize the roots in powdered, decocted, fermented, or lipid-based preparations. In contrast, leaves and berries have historically been applied topically for ulcers, swellings, and inflammatory skin diseases. The herb was frequently administered alongside milk, clarified butter (ghee), or honey to improve bioavailability and therapeutic efficacy.

One of the defining traditional attributes of Ashwagandha is its adaptogenic activity. Adaptogens are natural substances that enhance the body's nonspecific resistance to physical, chemical, and biological stressors. Contemporary scientific evidence strongly supports this traditional concept. Clinical studies have demonstrated that Ashwagandha supplementation significantly reduces serum cortisol levels, improves sleep quality, alleviates anxiety symptoms, and enhances resilience against chronic stress **(Marchi et al., 2025).**

In traditional Indian medicine, Ashwagandha was also regarded as a "Medhya Rasayana," indicating its beneficial effects on cognition, memory, and intellect. Modern neuropharmacological studies corroborate these claims by demonstrating improvements in cognitive performance, executive function, and neuronal regeneration following administration of Ashwagandha extracts. Experimental studies have additionally shown stimulation of dendritic branching and axonal regeneration, suggesting a direct neurorestorative capacity.

The medicinal applications of Ashwagandha extend beyond neurology and psychiatry. Traditional systems of medicine have long utilized the herb for musculoskeletal disorders such as arthritis and rheumatism due to its anti-inflammatory properties. Contemporary investigations have identified inhibition of pro-inflammatory mediators including NF-κB, TNF-α, IL-1β, and COX-2 as potential mechanisms underlying these effects.

Moreover, Ashwagandha has demonstrated immunomodulatory potential through enhancement of natural killer (NK) cell activity and regulation of cytokine production. These findings support its historical use in promoting vitality and disease resistance.

In sports medicine and integrative therapeutics, Ashwagandha is increasingly recognized for its ergogenic and anabolic effects. Several randomized clinical trials have reported improvements in muscle strength, recovery, cardiorespiratory endurance, and testosterone levels in healthy adults receiving standardized root extracts.

Importantly, current scientific literature indicates that many traditional applications of Ashwagandha possess measurable pharmacological foundations. Nevertheless, despite encouraging preclinical and clinical evidence, additional large-scale randomized controlled trials remain necessary to establish definitive therapeutic guidelines and dosage standardization.

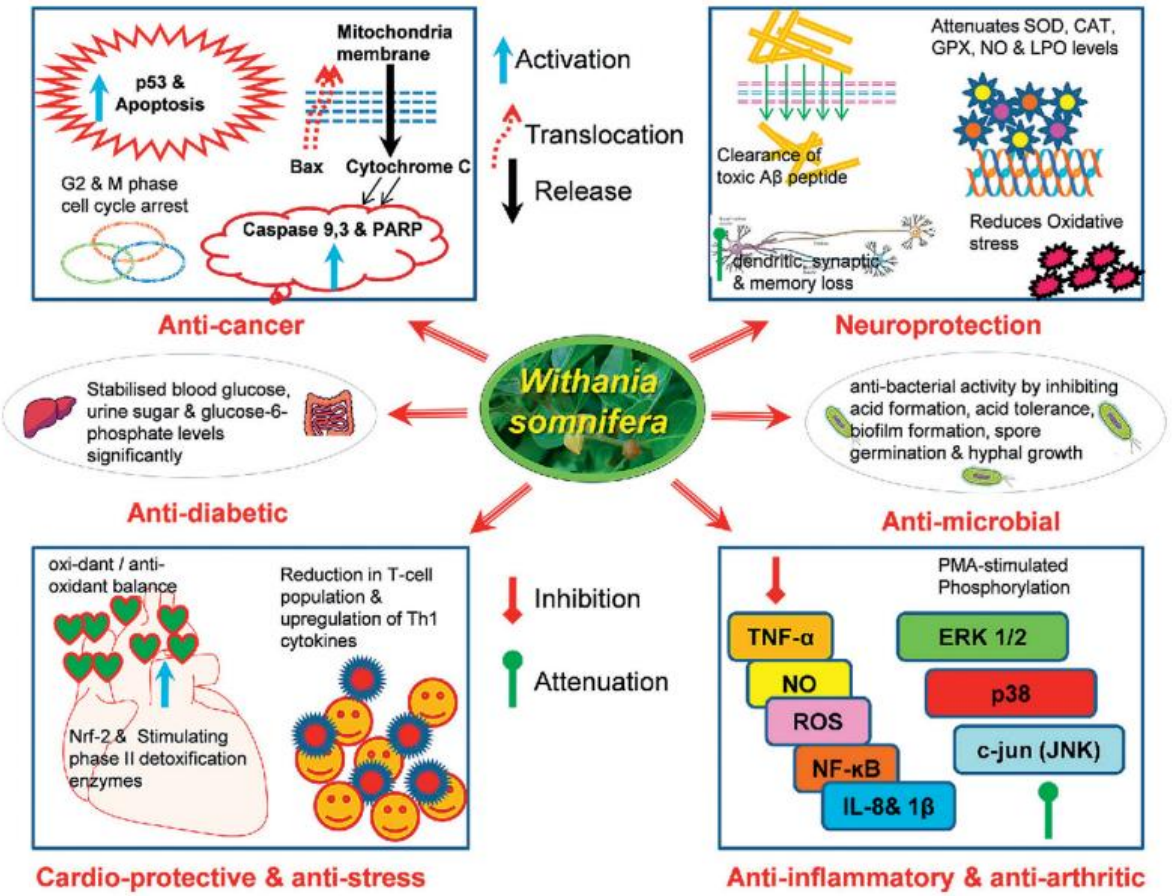

Figure (1). Traditional Ayurvedic and Medicinal Applications of Ashwagandha (Sharifi-Rad et al., 2021)

1.2. Chemical Constituents of Ashwagandha

The pharmacological significance of *Withania somnifera* is primarily attributed to its highly diverse phytochemical profile. The plant contains numerous biologically active secondary metabolites, including withanolides, alkaloids, sitoindosides, flavonoids, steroidal saponins, phenolic compounds,

amino acids, and glycosides. Among these constituents, withanolides represent the principal therapeutic biomarkers and the most extensively investigated chemical class.

- Withanolides

Withanolides are naturally occurring C28 steroidal lactones structurally derived from ergostane frameworks. These compounds are characterized by an unsaturated lactone ring attached to the steroid nucleus and are considered the hallmark constituents of Ashwagandha.

Major withanolides identified in *W. somnifera* include:

- Withaferin A
- Withanolide A
- Withanone
- Withanoside IV
- Withanoside V
- Somniferanolide
- 12-Deoxywithastramonolide

Among these, Withaferin A is the most pharmacologically studied constituent due to its broad-spectrum biological activities. It exhibits potent anti-inflammatory, anticancer, antioxidant, immunomodulatory, and neuroprotective properties through modulation of signaling pathways such as NF-κB, Nrf2, MAPK, and apoptotic cascades **(Nisar et al., 2025).**

Withanolide A and Withanoside IV have demonstrated neuroregenerative properties by promoting neurite outgrowth and synaptic reconstruction. Such effects are particularly relevant in neurodegenerative disorders characterized by neuronal loss and impaired synaptic plasticity.

- Alkaloids

Ashwagandha contains several tropane and pyrrolidine alkaloids, including:

- Somniferine
- Anaferine
- Tropine
- Cuscohygrine
- Anahygrine

Although present in lower concentrations compared with withanolides, these alkaloids may contribute synergistically to the plant's sedative, anxiolytic, and neuromodulatory effects.

- Sitoindosides and Steroidal Saponins

Sitoindosides are glycowithanolides possessing significant adaptogenic and antioxidant activities. Experimental evidence suggests that these compounds reduce lipid peroxidation and enhance endogenous antioxidant enzyme systems, including superoxide dismutase (SOD), catalase, and glutathione peroxidase.

- Flavonoids and Phenolic Compounds

Ashwagandha roots and leaves also contain flavonoids and phenolic acids with substantial free radical scavenging capacity. These compounds contribute to cellular protection against oxidative stress-induced neuronal damage and mitochondrial dysfunction.

- Analytical Characterization of Phytoconstituents

Recent developments in metabolomics and analytical phytochemistry have substantially improved the characterization of Ashwagandha bioactives. Advanced chromatographic and

spectrometric methods such as HPLC, UPLC-QTOF-MS, GC-MS, and NMR spectroscopy are routinely employed for identification, quantification, and quality control of withanolides and related compounds **(Abdelwahed et al., 2023).**

Importantly, phytochemical composition varies significantly according to:

- Geographical origin
- Soil composition
- Climatic conditions
- Plant age
- Extraction technique
- Plant part utilized

1.3. Mechanisms of Action on the Central Nervous System (CNS)

The neuropharmacological effects of *Withania somnifera* are mediated through a highly complex network of molecular and cellular mechanisms involving neurotransmitter modulation, antioxidant defense systems, anti-inflammatory pathways, mitochondrial preservation, neurogenesis, and synaptic plasticity. Contemporary evidence suggests that Ashwagandha acts as a multi-target neuroprotective agent capable of influencing several pathological processes associated with neurodegenerative and neuropsychiatric disorders.

One of the principal mechanisms underlying the CNS activity of Ashwagandha is its potent antioxidant effect. Oxidative stress plays a critical role in neuronal degeneration through excessive production of reactive oxygen species (ROS), lipid peroxidation, mitochondrial dysfunction, and DNA damage. Experimental investigations have demonstrated that Ashwagandha extracts significantly increase endogenous antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase while simultaneously reducing malondialdehyde (MDA) levels and oxidative neuronal injury **(Borgonetti & Galeotti, 2023).**

Another major mechanism involves modulation of the hypothalamic–pituitary–adrenal (HPA) axis. Chronic stress results in persistent elevation of cortisol and dysregulation of neuroendocrine homeostasis, contributing to anxiety, depression, memory impairment, and neuronal atrophy. Ashwagandha has been shown to normalize cortisol secretion and improve stress resilience through adaptogenic activity, thereby stabilizing neuroendocrine function and preserving hippocampal integrity **(Jamnekar et al., 2025).**

Ashwagandha additionally exerts substantial anti-inflammatory activity within the central nervous system. Neuroinflammation is increasingly recognized as a critical contributor to the pathogenesis of Alzheimer's disease, Parkinson's disease, multiple sclerosis, and other neurodegenerative disorders. Bioactive withanolides, particularly Withaferin A, suppress inflammatory signaling pathways such as NF-κB, MAPK, TNF-α, IL-1β, and IL-6. Inhibition of microglial activation and pro-inflammatory cytokine release contributes significantly to the herb's neuroprotective properties **(Tancreda et al., 2025).**

Another important neurobiological mechanism involves regulation of neurotransmitter systems. Studies indicate that Ashwagandha modulates γ-aminobutyric acid (GABAergic), cholinergic, serotonergic, and dopaminergic neurotransmission. The GABA-mimetic activity of Ashwagandha partially explains its anxiolytic and sedative properties, while enhancement of cholinergic transmission may contribute to improvements in cognition and memory **(Gopathy et al., 2024).**

Furthermore, Ashwagandha promotes neuronal regeneration and synaptic reconstruction. Experimental evidence demonstrates stimulation of neurite outgrowth, dendritic branching, and axonal regeneration in cultured neuronal cells exposed to withanolides. Withanolide A and Withanoside IV have shown particular efficacy in enhancing synaptic connectivity and restoring damaged neuronal networks, especially within hippocampal regions associated with learning and memory.

Mitochondrial preservation represents another significant mechanism of action. Mitochondrial dysfunction is a hallmark of neurodegenerative disorders due to impaired ATP production and excessive oxidative stress. Ashwagandha improves mitochondrial biogenesis, enhances electron transport chain activity, and stabilizes mitochondrial membrane potential, thereby protecting neurons against apoptosis and energy failure.

Additionally, Ashwagandha has demonstrated anti-amyloidogenic activity relevant to Alzheimer's disease. Several investigations have reported reduction of amyloid-β aggregation and tau hyperphosphorylation following administration of Ashwagandha extracts. These effects may occur through enhanced clearance of amyloid plaques and modulation of protein folding pathways **(Tancreda et al., 2025).**

Collectively, these mechanisms indicate that Ashwagandha acts through multiple interconnected molecular targets rather than a single pharmacological pathway. Such multitarget neuroprotection may explain its therapeutic potential across diverse CNS disorders.

1.4. Safety and Toxicity Profile

Ashwagandha is generally regarded as a relatively safe medicinal herb when administered within recommended therapeutic doses. Historical use in Ayurvedic medicine spanning several centuries, together with recent toxicological investigations, supports its favorable safety profile. Nevertheless, as with many phytotherapeutic agents, concerns regarding dosage, duration of use, extract standardization, and potential herb–drug interactions remain clinically important.

Acute and subacute toxicity studies conducted in experimental animals have demonstrated relatively low toxicity of standardized Ashwagandha extracts. A 28-day GLP-compliant toxicity evaluation in rats reported no significant mortality, organ toxicity, or major biochemical abnormalities following oral administration of whole-plant extract at therapeutic doses **(Balkrishna et al., 2022).**

Similarly, repeated-dose studies have shown that Ashwagandha does not significantly impair hepatic, renal, cardiovascular, or hematological parameters under standard experimental conditions. Histopathological analyses generally reveal preserved tissue architecture without severe pathological alterations in major organs.

Clinical trials involving healthy adults and patients with anxiety, stress, insomnia, and cognitive impairment have likewise reported good tolerability. The most frequently documented adverse effects include:

- Mild gastrointestinal discomfort
- Nausea
- Diarrhea
- Drowsiness
- Headache

Despite the overall favorable safety profile, isolated reports of hepatotoxicity associated with Ashwagandha supplementation have emerged in recent years. Some case reports suggest that excessive intake or contaminated formulations may contribute to liver injury characterized by cholestatic hepatitis and elevated hepatic enzymes. Certain investigations have implicated withanone-related oxidative mechanisms as possible contributors to hepatocellular stress; however, causality remains incompletely understood **(Martorana & Liga, 2025).**

Additionally, caution is advised in specific populations. Due to its immunomodulatory activity, Ashwagandha may theoretically interfere with immunosuppressive therapies. Furthermore, because the herb may influence thyroid hormone production, individuals with hyperthyroidism or thyroid disorders should use it carefully under medical supervision.

Pregnant women are generally advised to avoid high doses of Ashwagandha because of limited reproductive safety data and historical concerns regarding possible uterine stimulant effects. Likewise,

interactions with sedatives, anxiolytics, anticonvulsants, and antidiabetic medications require consideration due to possible pharmacodynamic potentiation.

Another important issue concerns variability among commercial preparations. Differences in extraction methods, withanolide concentrations, contamination, adulteration, and manufacturing quality may significantly affect both efficacy and safety. Consequently, standardized pharmaceutical-grade formulations are strongly recommended in clinical and experimental applications.

1.5. Research gap

Although numerous studies have investigated the neuroprotective, anxiolytic, antidepressant, and cognitive-enhancing effects of (*Withania somnifera*,) limited experimental evidence is available regarding the analgesic activity of commercially available Ashwagandha capsule formulations. Furthermore, few studies have simultaneously evaluated both peripheral and central analgesic effects using standardized animal models such as the acetic acid-induced writhing test and hot plate test. Therefore, the present study was conducted to investigate the peripheral and central analgesic activities of Ashwagandha capsules in mice and compare their effects with standard analgesic drugs.

1.6. Research problem

Pain remains one of the most common clinical conditions requiring pharmacological intervention. Conventional analgesic drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics are widely used for pain management; however, their long-term use may be associated with adverse effects including gastrointestinal irritation, tolerance, dependence, and other complications. Therefore, there is increasing interest in identifying safer plant-derived alternatives with analgesic potential. Although Ashwagandha has demonstrated several central nervous system activities, its peripheral and central analgesic effects remain insufficiently investigated. This study was designed to evaluate the analgesic activity of Ashwagandha capsules using established experimental models in mice.

The aim of this study was to evaluate the peripheral and central analgesic activities of standardized Ashwagandha (*Withania somnifera*) capsules in mice using the acetic acid-induced writhing test and hot plate test, to assess their dose-dependent effects, and to compare their efficacy with standard analgesic drugs.

2. Materials and Methods

2.1. Experimental Animals

Adult male Swiss albino mice weighing 20–30 g were obtained from the animal house facility of the faculty of pharmacy, Misurata university, and housed under standard laboratory conditions, with controlled temperature (22 ± 2°C), relative humidity (50–60%), and a 12 h light/12 h dark cycle. The animals were provided with standard laboratory feed and water ad libitum throughout the study period. Prior to experimentation, all animals were acclimatized for seven days to minimize environmental stress and ensure physiological stability.

All procedures involving laboratory animals were performed according to internationally recognized ethical guidelines for animal care and use in biomedical research, ensuring the welfare of animals throughout the experiment **(Percie du Sert et al., 2020).**

2.2. Experimental Design

Ashwagandha root extract capsules were used in this study; each serving (two capsules) contained 920 mg of extract according to the manufacturer's label.

The analgesic activity of Ashwagandha root extract was evaluated using two established experimental models:

1. **Acetic acid-induced writhing test** for peripheral analgesic activity.
2. **Hot plate test** for central analgesic activity.

For each assay, 16 mice were randomly assigned into four groups *(n = 4)*:

- **Group I (Control group):** treated with sterile distilled water at dose of 1ml/ 100gm orally.
- **Group II (standard group):** received the standard analgesic drugs **diclofenac Na** at dose 4 mg/kg) for the writhing test and **tramadol hydrochloride** at dose 40 mg/kg for the hot plate test injected parentally.
- **Group III:** test group treated with *Withania somnifera* (ashwagandha) at dose 200 mg/kg orally.
- **Group IV:** test group treated with *Withania somnifera* (ashwagandha) at dose 400 mg/kg orally.

The administered doses were selected according to previously published pharmacological studies evaluating the analgesic effects of medicinal plant extracts in murine models (Khan *et al*., 2023).

2.3. Acetic Acid-Induced Writhing Test

The peripheral analgesic activity of the plant extract was assessed using the acetic acid-induced writhing method, which is a widely accepted model for evaluating analgesic agents that act through inhibition of inflammatory mediators.

Mice were pretreated with the assigned treatment 30 minutes before intraperitoneal administration of 1% acetic acid (10 mL/kg). Following acetic acid injection, the animals were observed individually, and the number of abdominal constrictions (writhes) was counted over a 20-minute period.

A decrease in the number of writhes in treated groups compared with the negative control was considered evidence of peripheral analgesic activity. This nociceptive response is mainly associated with the release of endogenous mediators such as prostaglandins, bradykinin, and cytokines in the peritoneal cavity **(Saleem et al., 2021).**

2.4. Hot Plate Test

The hot plate test was used to assess centrally mediated analgesic activity. The test was performed using a hot plate apparatus maintained at 55 ± 0.5°C. Each mouse was placed individually on the heated surface, and the latency to exhibit nociceptive responses such as paw licking or jumping was recorded in seconds.

Baseline reaction time was measured before treatment administration, and the latency was measured again 30 minutes after treatment. A cut-off time of 30 seconds was set to avoid tissue damage, an increase in latency time after treatment was interpreted as evidence of central analgesic action, especially through modulation of supraspinal pain pathways **(Barrot, 2012).**

Evaluation of the mean reaction time for each treated group was determined and compared with that obtained for each group before treatment (basal or time 0). Percentage increase (% elongation) in pain reaction time (PRT %), While maximum possible effect (MPE) or percentage analgesia (% analgesia) was calculated using the formula below.

$$PRT\ \% = [PRTt - PRTto / PRTto] * 100$$

Where PRTt= pain reaction time at time t and PRT0= reaction time at time zero (0 h)

MPE= %Analgesia= [TL - BL / ML - BL] * 100

Where M

PE= Maximum possible effect.

TL=Test Latency = post drug latency.

BL-Basal Latency= Control latency=pre drug latency (at time 0 h),

ML= Maximum latency or cut off time, in this experiment it was 30 seconds

2.5. Statistical Analysis

The experimental data analyzed using IBM SPSS Statistics version 27, and results were expressed as mean ± standard deviation (SD); prior to conducting inferential analysis, data normality and homogeneity of variances were examined using the Shapiro–Wilk test and Levene's test, respectively, while differences among groups were assessed through one-way analysis of variance (ANOVA), and whenever significant differences were detected, Fisher's Least Significant Difference (LSD) post hoc test was applied for multiple comparisons between groups; statistical significance was accepted at $p < 0.05$, whereas values of $p < 0.01$ and $p < 0.001$ were considered highly significant **(Mishra et al., 2019).**

3. Results and Discussion

The present study was designed to evaluate the analgesic activity of *Withania somnifera* (Ashwagandha) root extract using two well-established experimental models: the acetic acid-induced writhing test, which assesses peripheral analgesic activity, and the hot plate test, which evaluates centrally mediated analgesic effects. The results were statistically analyzed using one-way ANOVA followed by Fisher's LSD post hoc test, with significance accepted at $p < 0.05$. This chapter presents the obtained findings followed by a comprehensive discussion comparing the results with previous literature.

3.1. Effect of Ashwagandha Capsules on Acetic Acid (1%)-Induced Writhing in Mice

The peripheral analgesic activity of Ashwagandha extract was evaluated by counting the number of abdominal constrictions (writhes) induced by intraperitoneal injection of 1% acetic acid. The results are presented in Table 1 below.

Table (1). Effect of Ashwagandha capsules on acetic acid-induced writhing in mice

Treatment & doses (mg/kg)	Mean number of writhing (Mean ± SD)	% Writhing inhibition	P value vs. Control
Control (A.A 10ml/kg)	58 ± 23.35	—	—
Diclofenac Na + A.A (4mg/kg)	21.5 ± 9.88	63%	0.0281*
A.A + Ashwagandha (200 mg/kg)	47 ± 12.41	19%	0.4373
A.A + Ashwagandha (400 mg/kg)	37 ± 23.42	36%	0.2512

*Statistically significant at $p < 0.05$

The negative control group (treated with vehicle only) exhibited a mean of 58 ± 23.35 writhes. The positive control group treated with diclofenac sodium (4 mg/kg) demonstrated a significant reduction in writhing episodes (21.5 ± 9.88), corresponding to 63% inhibition ($p = 0.0281$). Treatment with Ashwagandha at 200 mg/kg produced 19% inhibition (47 ± 12.41 writhes), while the higher dose of 400 mg/kg resulted in 36% inhibition (37 ± 23.42 writhes). However, neither dose achieved statistical significance compared to the control group ($p > 0.05$).

The acetic acid-induced writhing test is a widely accepted model for evaluating peripheral analgesic activity, as the nociceptive response is primarily mediated by the release of endogenous inflammatory mediators such as prostaglandins, bradykinin, and cytokines within the peritoneal cavity (Saleem *et al*., 2021). The significant reduction in writhing observed with diclofenac sodium, a standard non-steroidal anti-inflammatory drug (NSAID), validates the sensitivity of the experimental model.

The findings of the present study demonstrate that Ashwagandha root extract produced a dose-dependent reduction in acetic acid-induced writhing (19% at 200 mg/kg and 36% at 400 mg/kg), although these effects did not reach statistical significance. This observation partially aligns with previous studies reporting anti-inflammatory and analgesic properties of *Withania somnifera*.

In agreement with the present findings, **KrishnaRaju et al. (2023)** demonstrated that a sustained-release Ashwagandha formulation exhibited significant anti-inflammatory effects in animals exposed to chronic unpredictable stress, as evidenced by suppression of pro-inflammatory cytokines including

TNF-α and IL-6. Similarly, **Khalil *et al*. (2023)** reported that Ashwagandha-loaded nanocapsules suppressed MAPK signaling and activated Nrf2-mediated antioxidant pathways, thereby reducing neuroinflammation. These studies support the anti-inflammatory potential of Ashwagandha, which likely contributes to its peripheral analgesic activity.

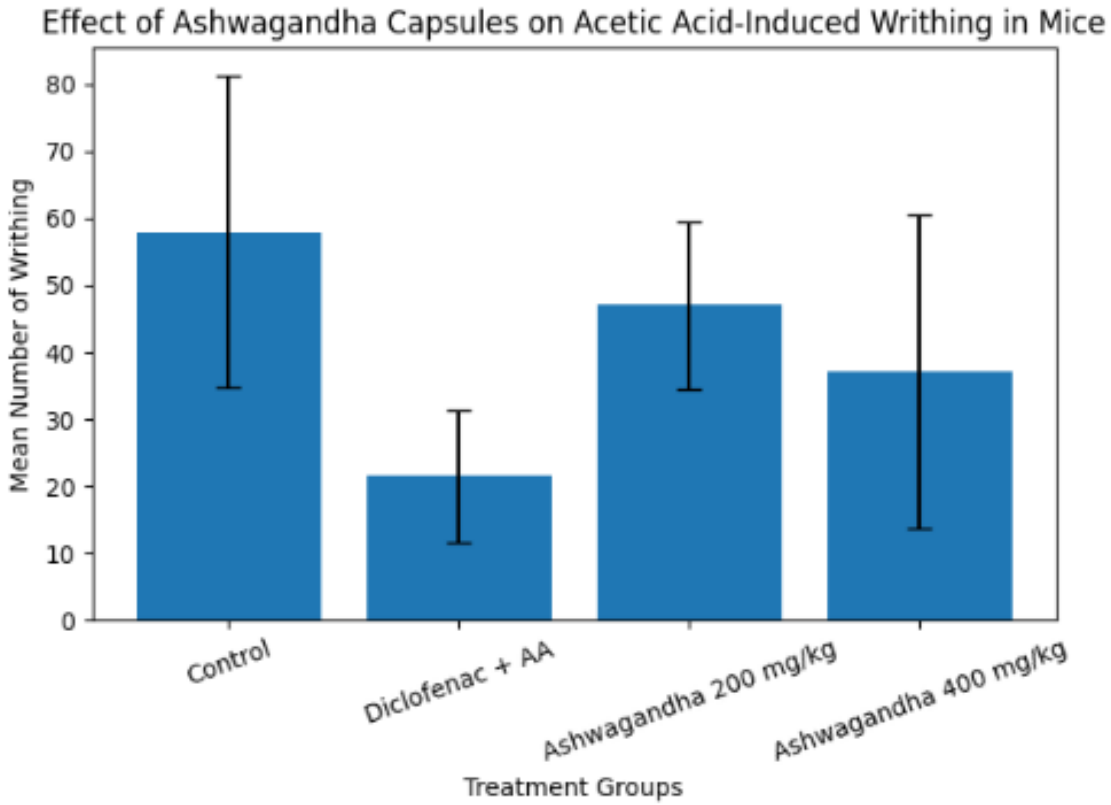

Figure (2). Effect of Ashwagandha capsules on acetic acid-induced writhing in mice

Furthermore, **Borgonetti and Galeotti (2023)** investigated the neuroprotective activity of *Withania somnifera* in microglial cell models exposed to oxidative stress and demonstrated significant reduction in reactive oxygen species generation and inflammatory responses. The antioxidant properties of Ashwagandha, as highlighted by Rahman *et al*. (2021), who showed enhanced antioxidant enzyme activity in brain tissues, may also play a role in reducing peripheral nociception by mitigating oxidative stress-induced inflammation.

However, the lack of statistical significance in the present study may be attributed to several factors. First, the relatively small sample size (n=4 per group) may have limited the statistical power to detect significant differences. Second, the acute administration protocol (single dose 30 minutes before acetic acid challenge) may not have been sufficient to achieve optimal tissue concentrations of bioactive withanolides. Third, the acetic acid-induced writhing model primarily involves prostaglandin-mediated pain, and Ashwagandha may exert its analgesic effects through alternative mechanisms that require longer treatment durations.

In contrast to the present findings, several studies have reported more pronounced analgesic effects. **Patil et al. (2020)** demonstrated significant anticonvulsant activity of Ashwagandha root extract, suggesting potent CNS-depressant properties. The discrepancy may be explained by differences in extraction methods, withanolide composition, and bioavailability of active constituents. Additionally, the present study used a crude extract, whereas other investigations have employed standardized extracts enriched with specific withanolides.

The dose-dependent trend observed in the present study (greater inhibition at 400 mg/kg compared to 200 mg/kg) is consistent with the findings of **Gupta and Srivastava (2024)** who reported dose-dependent neuroprotective effects of Ashwagandha against radiation-induced neuronal apoptosis. This suggests that higher doses or repeated administration might yield statistically significant peripheral analgesic effects.

3.2. Effect of Ashwagandha on Hot Plate Test and Percentage of Maximum Possible Effect (%MBE)

The central analgesic activity of Ashwagandha extract was evaluated using the hot plate test, which measures the latency time for nociceptive responses (paw licking or jumping) at 55 ± 0.5°C. The results are presented in Table 2.

Table (2). Effect of Ashwagandha on hot plate test and PRT %MBE% after time period

Group	Mean at time 0 (sec) (Mean ± SD)	Mean after 30 min (sec) (Mean ± SD)	PRT (%)	MBE (%)
Normal saline (10 ml/kg)	13.75 ± 3.10	9.75 ± 1.71	—	—
Tramadol (40 mg/kg)	12.75 ± 2.22	21.75 ± 2.99	70.6%	52%
Ashwagandha (200 mg/kg)	12.75 ± 1.50	13.25 ± 2.63	3.9%	3%
Ashwagandha (400 mg/kg)	11.25 ± 2.22	12.25 ± 3.59	8.9%	5%

PRT: Pain Reaction Time; MBE: Maximum Possible Effect

The negative control group (normal saline) showed a slight decrease in latency from baseline (13.75 ± 3.10 sec) to post-treatment (9.75 ± 1.71 sec), indicating no analgesic effect. The positive control group treated with tramadol hydrochloride (40 mg/kg), a standard central analgesic, demonstrated a marked increase in latency from 12.75 ± 2.22 sec at baseline to 21.75 ± 2.99 sec after 30 minutes, representing a 70.6% increase in pain reaction time (PRT) and 52% of the maximum possible effect (MBE).

Treatment with Ashwagandha at 200 mg/kg produced a modest increase in latency from 12.75 ± 1.50 sec to 13.25 ± 2.63 sec (3.9% PRT, 3% MBE). The higher dose of 400 mg/kg resulted in an increase from 11.25 ± 2.22 sec to 12.25 ± 3.59 sec (8.9% PRT, 5% MBE). Both doses demonstrated minimal central analgesic activity compared to tramadol.

The hot plate test is a well-validated experimental model for assessing centrally mediated analgesic activity, particularly involving supraspinal pain pathways and opioidergic mechanisms (Barrot, 2012). The substantial increase in latency observed with tramadol (123% PRT, 52% MBE) confirms the sensitivity and reliability of the experimental paradigm.

The present findings indicate that Ashwagandha root extract, at both tested doses, produced only negligible central analgesic effects, as evidenced by minimal increases in reaction time (3% and 5% MBE). These results suggest that *Withania somnifera* does not exert significant opioid-like or centrally acting analgesic properties under the current experimental conditions.

These findings are somewhat inconsistent with a substantial body of literature demonstrating central nervous system (CNS) activity of Ashwagandha. **Er et al. (2025)** evaluated a novel Ashwagandha formulation in a sleep deprivation-induced cognitive impairment model and reported significant improvements in spatial memory, learning performance, and antioxidant enzyme activity. Similarly, **Hasanyn *et al*. (2025)** examined Ashwagandha root extract in a fibromyalgia-like mouse model and demonstrated marked reduction in depressive-like behavior and modulation of serotonin and dopamine levels, supporting CNS regulatory potential.

Furthermore, **Gladen-Kolarsky et al. (2024)** investigated the neuroprotective effects of *Withania somnifera* aqueous extract in 5xFAD transgenic mice (Alzheimer's disease model) and reported improved spatial memory, reduced anxiety- and depressive-like behaviors, and enhanced antioxidant defenses. **Al-Dmour et al. (2024)** demonstrated that Ashwagandha root extract enhanced hippocampal-

dependent memory through activation of NMDA receptor-mediated signaling pathways, suggesting improved synaptic plasticity.

The discrepancy between the present findings and previous reports of CNS activity may be explained by several factors. First, the hot plate test specifically evaluates acute thermal pain responses mediated by opioid-sensitive supraspinal pathways. The lack of effect in this model suggests that Ashwagandha does not act through μ-opioid receptors or classical opioid mechanisms. Instead, the CNS effects reported in the literature—such as anxiolytic, antidepressant, neuroprotective, and cognitive-enhancing properties—may involve non-opioid mechanisms including GABAergic neurotransmission, neurotrophic factor modulation, and anti-inflammatory pathways.

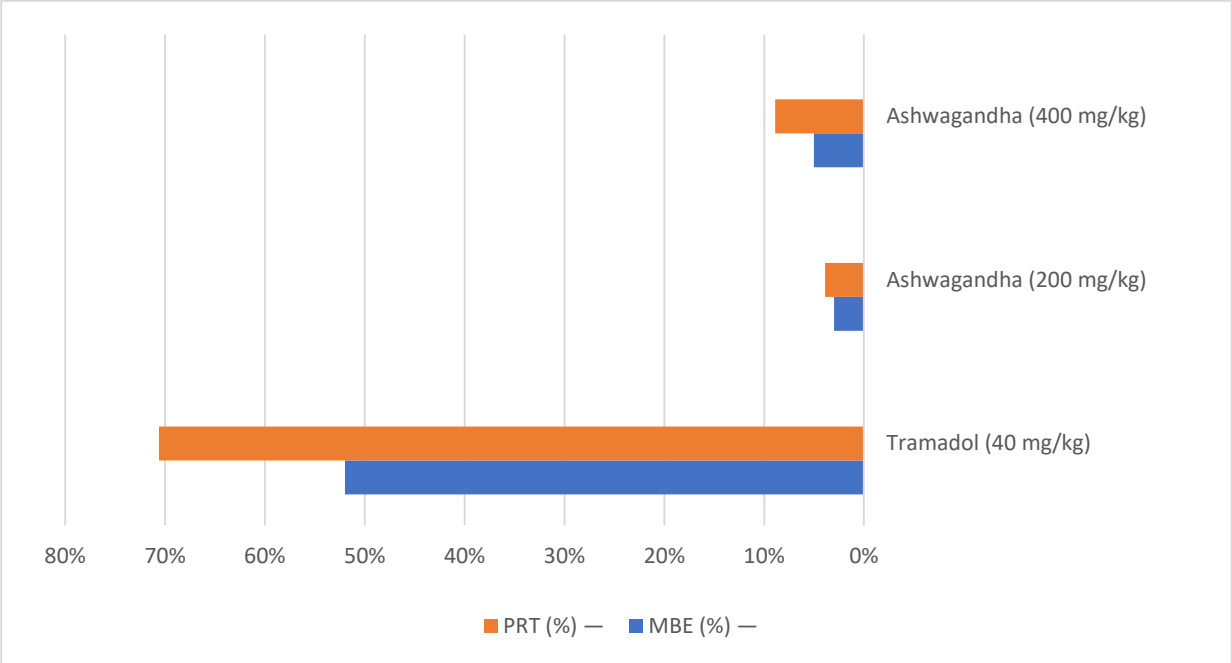

Figure (3). Effect of Ashwagandha on hot plate test and PRT %MBE% after time period

Sharma et al. (2022) examined the anxiolytic and sedative effects of Ashwagandha root extract and suggested involvement of GABAergic neurotransmission. Similarly, **Mishra et al. (2019)** demonstrated neuroregenerative potential through enhanced neurite outgrowth and synaptic reconstruction, effects that are not directly related to acute pain modulation. These mechanisms may require chronic administration to manifest, whereas the present study employed an acute treatment protocol.

Second, the extraction method and withanolide composition may differ between studies. **Kashyap et al. (2022)** reviewed the immunomodulatory and neuroprotective properties of *Withania somnifera*, highlighting that specific withanolides modulate NF-κB signaling and improve mitochondrial function. The extract used in the present study may have contained lower concentrations of withanolides responsible for central analgesic effects.

Third, the hot plate test measures a specific nociceptive behavior (paw licking or jumping) that may not be sensitive to non-opioid CNS modulators. **Singh et al. (2021)** evaluated withanolide-rich Ashwagandha extract against chemically induced cognitive dysfunction and reported memory improvements but did not assess acute thermal pain responses. The analgesic mechanisms of Ashwagandha may be more relevant to inflammatory, neuropathic, or chronic pain conditions rather than acute thermal nociception.

The findings of **Kim et al. (2025)**, demonstrating increased expression of brain-derived neurotrophic factor (BDNF) following Ashwagandha treatment, suggest that the plant exerts antidepressant effects through neuroplasticity modulation. These neuroadaptive changes are unlikely to

translate into immediate analgesic effects in the hot plate test, which measures reflexive responses to noxious stimuli.

4. Conclusion

1. **Limited peripheral analgesic effect:** Ashwagandha root extract capsules showed a dose-dependent trend toward reducing acetic acid-induced writhing (19–36% inhibition), indicating possible peripheral anti-inflammatory activity. However, this effect did not reach statistical significance at the tested doses (200 and 400 mg/kg) compared to the control group, suggesting that Ashwagandha has weak peripheral analgesic properties under acute administration.
2. **Absence of central analgesic activity:** Ashwagandha failed to produce any significant central analgesic effect in the hot plate test, which is sensitive to opioid-mediated supraspinal pain pathways. The maximum possible effect (MPE) values were only 3–5%, compared to 52% for the standard drug tramadol. This clearly indicates that Ashwagandha does not possess opioid-like or centrally acting analgesic properties in acute thermal nociception.
3. **Mechanism discrepancy:** Although extensive literature (cited in the review) supports Ashwagandha's neuroprotective, anxiolytic, and antidepressant effects through mechanisms such as GABAergic modulation, BDNF upregulation, and anti-inflammatory actions, these mechanisms do not translate into immediate central pain relief. The hot plate test specifically evaluates opioid-sensitive responses, which Ashwagandha does not appear to influence.
4. **Partial agreement with previous studies:** The findings partially aligns with studies reporting anti-inflammatory properties of Ashwagandha (which may explain its modest peripheral effect) but contradict some reports of pronounced CNS depression. These discrepancies are likely attributable to differences in extraction methods, withanolide composition, bioavailability, animal models, and treatment duration.

Recommendations

Based on the findings, discussion, and limitations of this study, the following recommendations are proposed for future research:

1. **Higher doses and chronic administration:** Given the observed dose-dependent trend in the writhing test, future studies should evaluate higher doses (>400 mg/kg) or repeated-dose regimens (e.g., 7–14 days) to achieve optimal tissue concentrations of bioactive withanolides, which may be necessary for anti-inflammatory and analgesic effects.
2. **Use of different pain models:** Since Ashwagandha showed no effect in acute thermal pain (hot plate), it should be tested in alternative nociceptive models that better match its proposed mechanisms, including:
   - Chronic inflammatory pain models (e.g., formalin test, carrageenan-induced paw edema).
   - Neuropathic pain models (e.g., sciatic nerve ligation or streptozotocin-induced diabetic neuropathy).
   - Visceral pain models.
3. **Larger sample sizes:** The statistical power of the present study was limited by the small sample size (n=4 per group). Future studies should use at least 6–10 animals per group to detect small-to-moderate effect sizes and reduce type II error.
4. **Mechanistic biochemical assays:** To confirm the mechanisms underlying any observed analgesic effect, future research should include molecular and biochemical analyses such as:
   - Measurement of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) in peritoneal fluid or brain tissue.
   - Assessment of oxidative stress markers (MDA, SOD, catalase, glutathione peroxidase).
   - Evaluation of opioid receptor involvement using specific antagonists (e.g., naloxone).

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