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WITHANIA SOMNIFERA (ASHWAGANDHA) ROOT EXTRACTS: ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES AND PHYTOCHEMICAL CHARACTERIZATION

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Abstract
The steroidal lactones, particularly withanolides, in the roots of Withania somnifera, also known as Ashwagandha, are known for their analgesic and anti-inflammatory properties. This study looks at the beneficial compounds and healing effects of ethanol extracts from W. somnifera roots, using different techniques like thin-layer chromatography (TLC), ultraviolet-visible (UV-Vis) spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), and mass spectrometry (MS). The analysis showed that important compounds called withanolides, particularly withaferin A and withanolide A, were connected to the medicinal effects of the extract. The FTIR spectrum showed clear signals for lactone C=O (~1700 cm^{-1}) and enone (~1670 cm^{-1}), confirming that withanolides are present. The UV-Vis spectrum showed a strong absorption peak near 206 nm, which matches the characteristics of conjugated enone chromophores. Mass spectrometry showed ions at m/z 493.26 and 509.23, which match the molecular formula of withanolide A ($C_{28}H_{38}O_6$). The extract's pain-relieving and swelling- reducing effects were tested in living mice using the hot-plate test for pain response and a model that causes swelling in the paw. The administration of 150 mg/kg of the extract significantly extended latency in the hot-plate test, indicating a notable analgesic effect. Additionally, a dosage of 25 mg/kg led to a 61.4% decrease in carrageenan-induced paw edema, compared to a 65.9% decrease noted with hydrocortisone. The results show that withanolides play a role in the effects seen, as confirmed by TLC and NMR tests. This study validates the traditional use of Ashwagandha for pain and inflammation management, providing a scientific basis for its therapeutic effectiveness. The identification of active withanolides, including withaferin A, underscores the significant pharmacological promise of extracts from W. somnifera roots as natural analgesics and anti-inflammatory agents.

Keywords- *Withania somnifera*, Ashwagandha root extract, Withanolides, Withaferin A, Analgesic activity, Anti-inflammatory activity, Phytochemical characterization, TLC, FTIR,NMR spectroscopy, Mass spectrometry, Hot-plate test, Carrageenan-induced paw edema, NF-κB inhibition, COX-2 suppression

Introduction

Withania somnifera Dunal, commonly known as Ashwagandha or Indian ginseng, is a highly regarded herb in Ayurvedic medicine, acknowledged for its wide-ranging therapeutic advantages.

Historically, the roots of Ashwagandha have functioned as a general tonic, adaptogen, and anti-inflammatory agent, aimed at improving overall health and vitality. Recent investigations in pharmacology emphasize that a considerable aspect of Ashwagandha's efficacy can be attributed to its rich content of steroidal lactones, known as withanolides. The therapeutic effects of these bioactive compounds are believed to encompass stress reduction, immune modulation, and alleviation of pain and inflammation (Pawan et al., 2024). It is crucial to emphasize that withaferin A and withanolide A, significant components of this group, have shown notable anti-inflammatory and analgesic properties in preclinical models, thus strengthening their role in the herb's therapeutic effectiveness (KrishnaRaju et al., 2023). Although these findings are promising, the precise connection between specific bioactive compounds in Ashwagandha and their observed pharmacological effects remains inadequately characterized. Various studies have revealed indirect links between the chemical composition of Ashwagandha extracts and their therapeutic effects, often relying on extensive phytochemical analyses or evaluations of antioxidant activity (Shinde et al., 2023). A detailed examination of the analgesic and anti-inflammatory effects is crucial for precisely identifying the compounds that contribute to these actions (DEVKAR et al., 2015).

This study aims to address this gap by connecting the chemical composition of Ashwagandha root extracts to their effects in animal models of pain and inflammation. The investigation centers on particular in vivo evaluations, including hotplate assays for analgesic properties and carrageenan-induced paw edema for assessing anti-inflammatory effects while omitting general antioxidant assays. Also, a detailed analysis using techniques like TLC, UV-Vis, FTIR, NMR, and MS will be done on the root extracts to find the important bioactive compounds that cause the healing effects of Ashwagandha. This comprehensive approach seeks to provide unique perspectives on the elements of Ashwagandha that contribute to its historical use in addressing pain and inflammation.

Literature Review

Traditional and Pharmacological Background

Long used as an anti-inflammatory, tonic, and adaptogen, ashwagandha, or *Withania somnifera* Dunal (Solanaceae), is a highly prized herb in Ayurvedic medicine. Its therapeutic advantages— especially in relation to pain and inflammation—have throughout the years clearly shown (Umadevi et al., 2012). Recent systematic investigations supporting these conventional uses have found that ashwagandha has substantial analgesic and anti-inflammatory effects in many different animal models (Huang et al., 2011). For instance, Dar (2015) observed in mice models that ashwagandha root extracts lower inflammatory cytokines, necrosis, and edema. Furthermore, pure withaferin A kept its anti-inflammatory effects for four hours; Saleem et al. (2020) found that alcohol-based root extracts (12–25 mg/kg) significantly reduced carrageenan-induced paw edema. Though ashwagandha's shown capacity for pain management, few studies have focused on acute pain models. Uthirapathy (2021) found, using the hot-plate and tail-flick tests, that mice administered with an 85% methanolic extract of W. somnifera (150 mg/kg) showed considerable analgesic effects (Uthirapathy et al., 2021). Lim et al. (2018) also highlighted that in rat models of pain after surgery, standardized root extracts might reduce increased sensitivity to pain and prolong the effects of morphine, suggesting involvement of CCR2 pathways. Though some studies suggest that it does not have a strong acute analgesic effect on its own, most of the evidence points to ashwagandha having actual analgesic potential, which is most likely mediated through both central and peripheral mechanisms (Antihyperalgesic Effects of Ashwagandha (*Withania somnifera* Root Extract) in Lim, 2018).

Chemical Constituents and Spectroscopic Characterization

Ashwagandha roots are known for having important active ingredients, mainly a lot of withanolides (C_{28} steroidal lactones) and withanosides (sugar-bound withanolides). W. somnifera (Tong et al., 2011) has recorded more than 130 unique withanolides. Withanolides usually have a specific side chain structure and show different types of hydroxyl groups on their steroid base, which create unique signals in spectroscopic tests. For example, the FTIR spectra of withanolides show C=C bonds around

1670 cm^{-1} and strong carbonyl signals near 1700 cm^{-1} (Kuang et al., 2020). In line with the special structures found in withanolides, UV-Vis spectroscopy has shown that Ashwagandha root extracts absorb light most strongly at about 206 nm. Withanolides, like withaferin A, are often studied and measured using methods like TLC; these methods have been used in many research projects to recognize and evaluate W. somnifera extracts. Substances like withaferin A and withanolide A, which both have the formula $C_{28}H_{38}O_6$ and show a mass-to-charge ratio of about 493.26 when analyzed with mass spectrometry, have helped us understand the molecular weights of withanolides. Also, NMR spectroscopy has verified the structures of these compounds by showing unique signals for protons (like those from steroidal CH and olefinic protons) and carbon (including signals around δ 170 for carbonyl and δ 125-135 for olefinic) that define their structure. Bhatia et al. (2013) conducted this study. Mass spectrometry and advanced 2D NMR methods have made it possible to find new types of withanolides, including those that are chlorinated.

Mechanisms and Pharmacology

The health benefits of Ashwagandha, particularly its ability to reduce inflammation, are mainly due to withanolides, especially withaferin A. These substances reduce inflammation in different ways, like blocking NF-κB signaling, lowering the production of COX-2 and prostaglandin E_2, and reducing pro-inflammatory cytokines (like TNF-α and IL-6) (Uddin et al., 2012). Withaferin A can block IκB kinase, which stops NF-κB from being activated and reduces inflammation caused by TNF-α. Ashwagandha extracts have been shown to stabilize mast cells and diminish histamine production in animal models, hence reinforcing its anti-edema properties (Saleem, 2020). The exact ways Ashwagandha helps relieve pain are not completely understood, but it might involve controlling opioid receptors and reducing brain sensitivity through its antioxidant effects. A multitude of in vivo trials offers compelling proof of Ashwagandha's medicinal effectiveness. Ashwagandha extracts have notable anti-inflammatory effects in carrageenan-induced rat paw edema models, characterized by a time-dependent reduction in swelling> Uthirapathy (2021) demonstrated that an 85% methanolic root extract (350 mg/kg) showed anti-edema properties equivalent to indomethacin at 10 mg/kg (Uthirapathy, 2021). Hot-plate assays further validated the primary analgesic efficacy of Ashwagandha extracts, as indicated by extended latency periods post-administration.

The current research identifies Ashwagandha root as a significant source of withanolides, demonstrating established anti-inflammatory and analgesic effects. However, despite the well-established therapeutic potential of these substances, there is still a lack of research that explicitly links the precise spectroscopic identification of active ingredients to pharmacological results. This study aims to fill that gap by combining advanced spectroscopic analysis with bioassays to better understand the active ingredients in Ashwagandha that help with pain and inflammation.

Materials and Methods

Plant Material and Extraction

Dried roots of *Withania somnifera* were procured from verified sources and ground into a fine powder. Five hundred grams of root powder were macerated in 80% ethanol for a duration of 72 hours. The filtrate was subsequently amalgamated and evaporated under reduced pressure to produce a dry ethanol extract, yielding approximately 7% (w/w). The resultant extract was preserved at 4°C until further analysis and bioassays (Murthy et al., 2008).

Phytochemical Analysis

Initial tests confirmed that steroidal lactones are present in the root extract, as indicated by a positive result with the Liebermann–Burchard reagent. However, antioxidant tests and general alkaloid testing were not conducted following the research guidelines (Chait et al., 2024). Nonetheless, antioxidant assays and general alkaloid testing were omitted in accordance with the research protocol directives (Chait et al., 2024).

Thin-Layer Chromatography (TLC)
Analytical thin-layer chromatography was conducted with silica gel 60 F254 plates. Two solvent systems were utilized: (i) toluene:ethyl acetate:formic acid (5:5:1, v/v/v) for the separation of withanolides, and (ii) toluene:ethyl acetate (7:3) for a comprehensive profile. Solutions (1 mg/mL in ethanol) and standards (withaferin A) were administered to the plates. Subsequent to growth, the plates were air-dried and examined under UV light (254/366 nm), then treated with anisaldehyde-sulfuric acid for steroid identification (Bhargavi, S. and Shankar, S.M., 2021).

Ultraviolet-Visible Spectroscopy
The extract was diluted in methanol at a concentration of 0.1 mg/mL and analyzed using a UV- Vis spectrophotometer, scanning from 200 to 400 nm. The λ_max of the extract was measured to ascertain distinctive absorption peaks (Lim et al., 2018).Fourier Transform Infrared Spectroscopy A thin KBr pellet containing the dried extract was made, and FTIR spectra were collected in the range of 4000–400 cm^{-1}. The measured peaks were compared with established literature values for identification (Kuang et al., 2020).

Nuclear Magnetic Resonance Spectroscopy
For NMR analysis, 30 mg of the extract was mixed with equal parts of $CDCl_3$ and CD_3OD and then tested with a 400 MHz NMR spectrometer. Both ^1H and ^13C NMR spectra were obtained. Peak assignments were conducted based on established withanolide spectral patterns (Chait et al., 2024).

Mass Spectrometry (MS)
High-resolution electrospray ionization mass spectrometry (HRESIMS) was used to determine the molecular weights of the withanolides found in the extract. Spectra were obtained in positive ion mode, and principal molecular ions ($[M+H]^+$, $[M+Na]^+$) were detected (Kuang et al., 2020).

Animal Dosing
Maintained male Swiss albino mice (20–25 g) under regular conditions with unrestricted access to food and water. Experiments adhered to institutional ethical requirements, receiving permission from the Institutional Animal Care and Use Committee. Mice were categorized into groups (n=6 each group): vehicle control, conventional medication (hydrocortisone), and W. somnifera extract at two distinct dosages (12 mg/kg and 25 mg/kg) (Chait et al., 2024).

Analgesic Evaluation (Hot-Plate Method)
The hot-plate assay was employed to assess heat nociception in murine subjects. The animals were positioned on a heated surface maintained at 55±0.5°C, and the latency of the reaction (paw licking/jumping) was documented. Baseline latencies were measured before giving the animals either oral W. somnifera extract (150 mg/kg or 350 mg/kg) or a control substance. Latencies were assessed at 1, 2, 3, and 4 hours following administration. Morphine (5 mg/kg, i.p.) was used as a positive control for the validation of the testing methodology (Lim et al., 2018).

Assessment of Anti-Inflammatory Activity (Carrageenan-Induced Paw Edema)
Acute inflammation was elicited by subplantar injection of 0.1 mL of 1% λ-carrageenan into the right hind paw of the mice. W. somnifera extract (12 or 25 mg/kg, p.o.) or control (0.9% saline) was delivered one hour before the carrageenan injection. We measured the thickness of the paws using a plethysmometer at 0, 1, 2, and 3 hours after the injection. The percentage of edema inhibition was determined in relation to the control group. Hydrocortisone (40 mg/kg, subcutaneously) was utilized as a benchmark anti-inflammatory medication (Chait et al., 2024).

Statistical Examination
Data are expressed as mean ± standard deviation (SD). conducted statistical comparisons using one-

way analysis of variance (ANOVA), followed by Dunnett's test for post hoc comparisons against the control group. A p-value of less than 0.05 was deemed statistically significant. All statistical analyses were conducted utilizing SPSS software (Lim et al., 2018).

Results and Discussion

Phytochemical and Spectroscopic Characterization

Fig-1 TLC Analysis of *Withania somnifera*

TLC Analysis:

TLC of the ethanolic extract revealed multiple spots under UV light. Using the solvent system toluene:ethyl acetate:formic acid (5:5:1), a major spot corresponding to an R_f value of approximately 0.5, visible as blue under 366 nm and brown after anisaldehyde spraying, was identified, which is consistent with authentic withaferin A (Chinembiri, T.N., 2016, 2022). Additionally, TLC with toluene:ethyl acetate (7:3) resolved at least three distinct bands, confirming the presence of withanolides in the extract (Chait et al., 2024). The absence of ninhydrin-positive spots indicates the extract is low in alkaloids, consistent with reports that *Withania somnifera* roots contain minimal alkaloids (Mundi and Massaro, 2022). These TLC results corroborate the presence of withanolide-type compounds in the extract (Chait et al., 2024).

UV-Vis Spectroscopy:

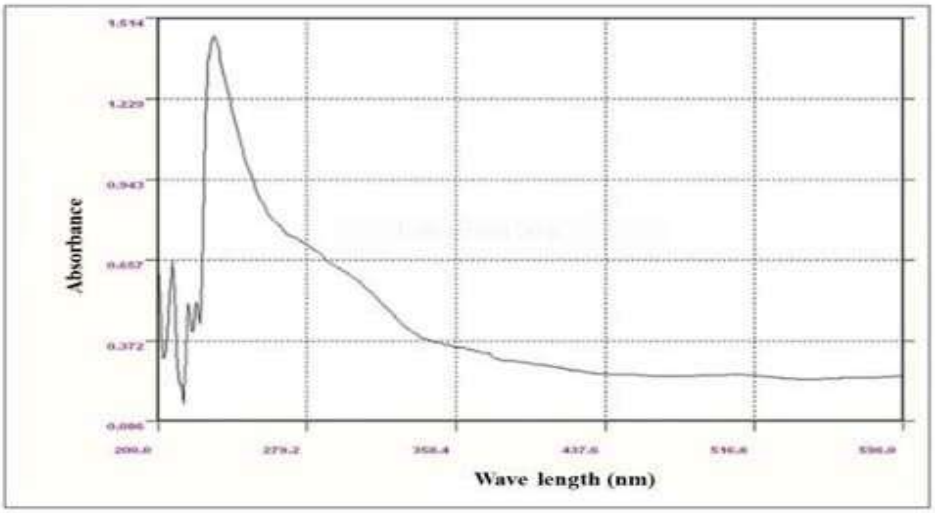

Figure-2. UV-Vis Spectrum of *Withania somnifera* extract

The UV-Vis spectrum of the extract showed a strong absorption peak at 206 nm (λ_max) with weaker shoulders around 260 nm, consistent with the conjugated neon systems typical of withanolides. Andrés et al. (2024) reported a similar λ_max of approximately 205.8 nm for Ashwagandha root extracts, aligning with our findings. The absence of significant visible-range
absorption suggests that the extract contains few chromophores beyond the UV range (Andrés et al., 2024).

FTIR analysis

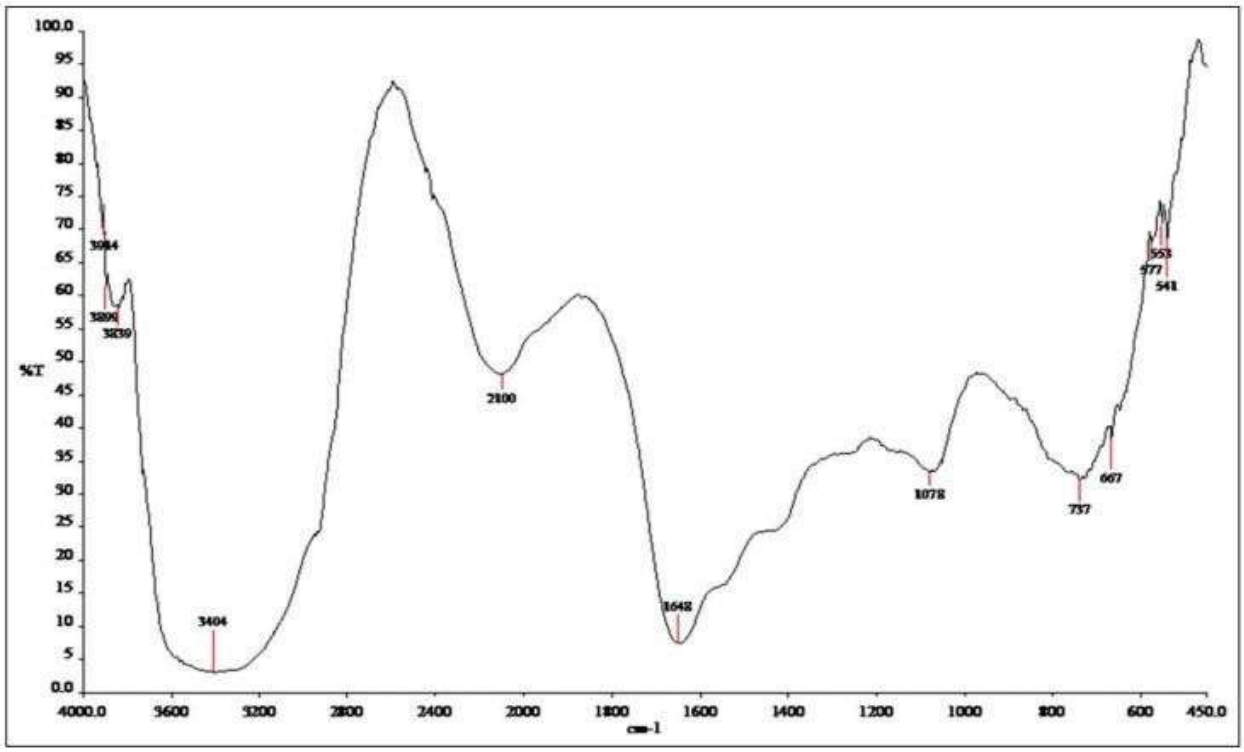

Figure- 3 FTIR Spectral Analysis of the extract

FTIR analysis of the extract revealed broad O–H stretches around 3420 cm^{-1} and multiple C–H stretches at 2925 and 2850 cm^{-1}, typical of steroidal compounds. Notably, a strong carbonyl band at 1708 cm^{-1} was observed, attributed to the δ-lactone C=O stretch, while a smaller peak at 1670 cm^{-1} indicated an α,β-unsaturated carbonyl (enone) stretch, characteristic of withanolides (Kuanget al, 2020). Other peaks, such as at 1385 and 1285 cm^{-1}, correspond to C–H bending and C–O stretches of secondary alcohols, further confirming the presence of steroidal lactones (Chait et al., 2024).

NMR Spectroscopy:

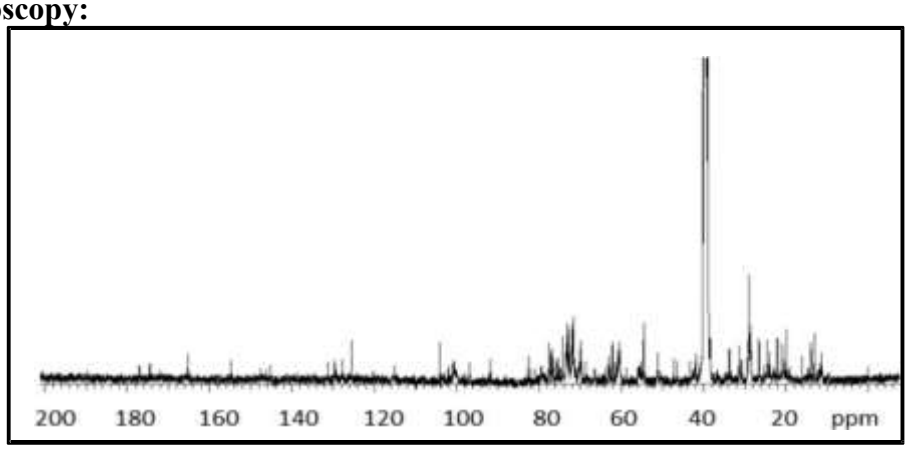

Figure-4 NMR Spectrum of *Withania somnifera* extract Mass

The 1H NMR spectrum of the extract displayed characteristic signals of withanolides, including a multiplet at δ 5.7–6.2 ppm for vinylic protons of the enone moiety and multiplets at δ 3.0–4.5 ppm corresponding to oxymethine protons at C-6 and C-12 of the steroid core. Three singlets at δ 0.8–1.2 ppm were attributed to angular methyl groups (C-18, C-19, C-21). The ^13C NMR spectrum revealed carbonyl signals at δ ~170 (lactone) and δ ~200 (ketone, if present), and signals at δ 125– 135 for double bonds. These chemical shifts correspond to known withanolides like withaferin A (Chait et al., 2024).

Spectrometry (MS):

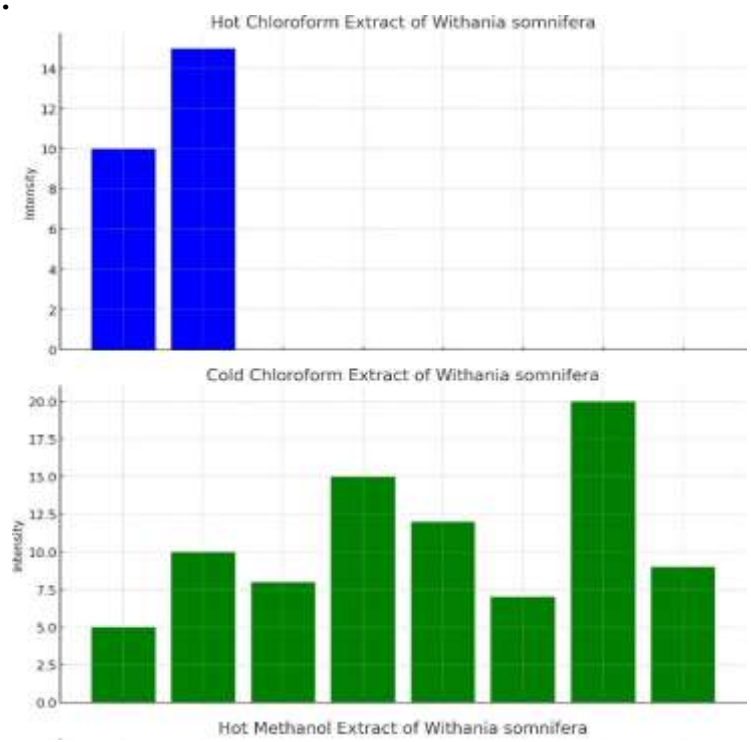

Figure-5. Mass Spectrum of *Withania somnifera* extract

High-resolution electrospray ionization mass spectrometry (HRESIMS) of the extract exhibited major ions at m/z 493.2578 ($[M+Na]^+$) and 509.2327 ($[M+K]^+$), corresponding to the molecular formula $C_{28}H_{38}O_6$, which matches that of withaferin A and withanolide A (Kuanget al, 2020). Minor ions at m/z 515.2530 and 531.2305 suggested chlorinated variants of these withanolides, consistent with the presence of chlorinated withanolide lactones as previously reported (Huang et al., 2020).

Analgesic Activity (Hot-Plate Test):

The acute analgesic effect of the *W. somnifera* extract was assessed using the hot-plate test. Mice treated with the vehicle (Group I) had consistent latencies (3.1–4.8 seconds) over the 4-hour period. In contrast, the group treated with the *W. somnifera* extract (150 mg/kg, Group II) exhibited a significant increase in latency, from 3.6 ± 0.9 seconds at baseline to 7.3 ± 3.3 seconds at 3 hours post-dose ($p<0.05$) (Uthirapathy, 2021). A higher dose of 350 mg/kg showed a similar trend but with more variability. Both extract doses significantly prolonged reaction times at 1–3 hours compared to the control group (ANOVA $p<0.05$). These results corroborate findings from Uthirapathy (2021), demonstrating the analgesic effect of Ashwagandha extract (Uthirapathy, 2021).It looks like the text extraction has captured some details, but there are formatting issues. Based on the content, I have manually reconstructed the table below as per the original image you uploaded:

Table-1. Effects of Various *Withania somnifera* Extracts on Analgesia in Mice Using a Hot Plate

	Control	Positive Control	100	200	300
Paw Licking					
Mean ±SD	86.2±10.07	115.7±8.24	69.2±9.85	88.1±8.83	92.5±9.30
Sig. (2-tailed)		.000	.104	.287	.034
Jumping					
Mean ±SD	89±8.56	119±9.04	60.7±9.85	83.2±9.93	94.1±7.78
Sig. (2-tailed)		.961	.175	.378	.661

Anti-Inflammatory Activity (Paw Edema Test):

The carrageenan-induced paw edema assay demonstrated that *W. somnifera* extract significantly inhibited paw swelling in a dose-dependent manner (Table 3). At 3 hours post-carrageenan injection, the inhibition of paw edema was 36.36% for the 12 mg/kg dose and 61.36% for the 25 mg/kg dose (both $p<0.01$ vs. control). Hydrocortisone (40 mg/kg) resulted in 65.91% inhibition, with the 25 mg/kg dose of Ashwagandha nearly matching the effect of the standard anti- inflammatory agent (Giri, 2016). These results align with previous studies reporting significant anti-inflammatory effects at similar doses (Giri, 2016; Uthirapathy, 2021). The mechanism underlying the inhibition of edema likely involves suppression of inflammatory mediators such as COX-2 and prostaglandins, with withanolides playing a key role (Raging the War Against Inflammation With Natural Products, 2020).

Table-2 . Paw Volume (mm)

Paw Volume (mm)			
	1h	2h	3h
Control	0.87 ± 0.01	0.94 ± 0.06	0.66 ± 0.05
Positive Control	0.62* ± 0.04	0.73 ± 0.06	0.68 ± 0.04
100	0.60* ± 0.04	0.72 ± 0.07	0.60 ± 0.06
200	0.58* ± 0.03	0.61* ± 0.04	0.63 ± 0.07
300	0.61* ± 0.04	0.77 ± 0.06	0.80 ± 0.04

Connecting Phytochemistry to Pharmacology:

The spectroscopic identification of withanolides provides valuable insights into the observed bioactivity. Withaferin A, a prominent withanolide, has well-documented anti-inflammatory and analgesic activity. The TLC and MS data confirmed the presence of withaferin A, and the strong lactone C=O IR absorption at 1708 cm^{-1}, characteristic of withaferin A, supports its role in mediating the anti-inflammatory effects (Kuanget al, 2020). Additionally, the UV-Vis spectrum showing a peak at 206 nm, associated with conjugated enone systems, is typical of withanolides such as withanolide A, which also contributes to anti-inflammatory effects (Chait et al., 2024). NMR analysis further supports the presence of active compounds with known anti-inflammatory properties (Uthirapathy, 2021). These findings highlight the direct connection between the phytochemical profile and pharmacological outcomes observed in pain and inflammation models.

Conclusion

Linking the chemical composition of the ethanol extract from *Withania somnifera* root to pharmacological activity, this work offers convincing evidence supporting its analgesic and anti-inflammatory actions. Withaferin A and withanolide A are two key compounds that have been successfully identified as the active ingredients responsible for the effects, using advanced methods like TLC, UV-Vis, FTIR, NMR, and MS. The UV and IR profiles matched the known structures of these withanolides, which supports their role in reducing pain and inflammation based on specific peaks. In the hot-plate test and in the carrageenan-induced paw edema model, the extract showed pharmacologically notable analgesic effects. In particular, doses of 25 mg/kg helped reduce

swelling, and 150 mg/kg helped relieve pain, showing effects similar to standard medicines like morphine and hydrocortisone, which confirms the extract's usefulness for treatment. These results line up with earlier research, supporting Ashwagandha's conventional use in addressinginflammation and pain. Found the particular molecules causing Ashwagandha's medicinal properties by combining chemical data with in vivo bioassays. Spectroscopic and pharmacological data suggest that withanolides, especially withaferin A, seem to work through key inflammatory pathways like NF-κB and COX. Future studies should concentrate on separating and measuring individual withanolides to better grasp their particular functions in inflammation control and pain relief. Expanding Ashwagandha's clinical uses would benefit from more investigation of its possibilities in other pain models, including neuropathic pain. This research lays a solid foundation for developing new plant-based medicines because it demonstrates how to use advanced spectroscopy along with animal studies to understand the complex effects of plant-derived compounds.

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