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Chemical Fingerprinting of Root Extracts from *Withaniasomnifera.*

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Abstract: *Withaniasomnifera*(Ashwagandha) is a medicinal plant of high therapeutic value, traditionally used in Ayurveda for stress management, immune modulation, and neuroprotection. Its pharmacological potential is largely attributed to diverse phytochemicals, particularly withanolides, alkaloids, and phenolic compounds. In the present study, root extracts of W. somnifera were subjected to chemical fingerprinting using advanced analytical techniques to establish a comprehensive phytochemical profile. Standardized chromatographic and spectroscopic methods were employed to identify key bioactive constituents and to generate reproducible chemical patterns. The results revealed the presence of characteristic secondary metabolites that can serve as quality markers for authentication and standardization. This study highlights the importance of fingerprinting approaches in ensuring consistency, efficacy, and safety of herbal preparations derived from W. *somnifera*. The findings also provide a foundation for future pharmacological investigations and the development of standardized formulations.

Keywords: *Withaniasomnifera*; Ashwagandha; Chemical fingerprinting; Phytochemical profiling; Withanolides; Standardization; Medicinal plants; Root extracts

Introduction - Medicinal plants remain a cornerstone of traditional healthcare systems and continue to provide valuable leads for modern drug discovery. Among them, *Withaniasomnifera* (L.) Dunal, commonly known as Ashwagandha, occupies a prominent place in Ayurveda and other indigenous systems of medicine. Classified under the family Solanaceae, the plant is widely distributed across India, the Middle East, and parts of Africa. Its roots, in particular, have been employed for centuries as a restorative tonic to manage conditions such as stress, fatigue, inflammation, arthritis, and neurological disorders.

The pharmacological relevance of W. *somnifera* has been attributed to its wide spectrum of secondary metabolites, including alkaloids, steroidal lactones (withanolides), flavonoids, saponins, and phenolic compounds. These bioactive molecules have been associated with adaptogenic, immunomodulatory, anti-inflammatory, and neuroprotective effects. Given the increasing global demand for herbal products and nutraceuticals containing W. somnifera, there is a pressing need for standardized analytical methods that ensure quality, safety, and reproducibility of plant-derived formulations.

Chemical fingerprinting is a modern approach that provides a holistic representation of the phytochemical composition of plant extracts. Techniques such as High-Performance Thin Layer Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), Gas Chromatography–Mass Spectrometry (GC–MS), and Fourier Transform Infrared Spectroscopy (FTIR) are widely employed to generate reproducible chemical patterns. These fingerprints not only help in identifying marker compounds but also serve as quality assurance tools for herbal medicines.

Despite the widespread use of W. *somnifera*, variations in phytochemical content often arise due to differences in geographical location, cultivation practices, harvesting time, and post-harvest processing. Such variations can influence therapeutic efficacy and limit the reliability of herbal preparations. Therefore, establishing a robust chemical fingerprint of W. *somnifera* root extracts is essential to ensure authenticity and consistency across formulations.

The present study was undertaken to generate a comprehensive chemical fingerprint of W. *somnifera* root extracts using advanced chromatographic and spectroscopic methods. The findings are expected to contribute toward the standardization of Ashwagandha-based products and provide a foundation for further pharmacological research.

Materials and Methods:

Plant Material Collection: Roots of Withaniasomnifera (L.) Dunal were collected from cultivated fields during the flowering season. Fresh samples were selected, cleaned to remove soil and other impurities, and used for further

analysis.

Preparation of Root Extracts: The collected roots were washed thoroughly with distilled water, shade-dried at room temperature for 20 days, and ground into fine powder using a mechanical grinder. Fifty grams of powdered root material was extracted with 95% ethanol using a Soxhlet apparatus for 48 hours. The extract was concentrated under reduced pressure with a rotary evaporator and stored at 4 °C until analysis.

Preliminary Phytochemical Screening: Standard qualitative tests were performed on the ethanolic root extract to detect the presence of alkaloids, flavonoids, tannins, saponins, steroids, phenolics, and glycosides.

High-Performance Thin Layer Chromatography (HPTLC): HPTLC was carried out on silica gel 60 F254 plates. Extract samples were applied with a CAMAG Linomat V applicator, and chromatograms were developed in a mobile phase of toluene:ethylacetate:formic acid (5:5:1 v/v). Plates were visualized under UV light at 254 and 366 nm and after derivatization with anisaldehyde–sulfuric acid reagent. Retention factor (Rf) values and band patterns were recorded.

High-Performance Liquid Chromatography (HPLC): HPLC analysis was performed on a C18 reverse-phase column using a gradient mobile phase of acetonitrile and water. Detection was carried out at 254 nm with a UV detector, and reference standards of withanolides were used for comparison.

Gas Chromatography–Mass Spectrometry (GC–MS): GC–MS was performed using a capillary column with helium as the carrier gas at 1.0 mL/min. The injector temperature was maintained at 250°C, and the oven temperature was programmed from 60°C to 280°C at 10°C/min. Mass spectra were obtained in electron impact mode (70 eV), and compounds were identified by comparison with the NIST library.

Fourier Transform Infrared Spectroscopy (FTIR): FTIR spectra were recorded using KBr pellet technique in the range of 400–4000 cm^{-1}. Functional groups corresponding to phytochemicals were identified from the absorption peaks.

Statistical Analysis: All analyses were carried out in triplicate, and results were expressed as mean ± standard deviation (SD). Data were analyzed using SPSS version 22.0 for statistical reliability.

Results and Discussion:

Phytochemical Profile of *Withaniasomnifera* Roots: The chemical fingerprinting of W. *somnifera* root extracts revealed the presence of a diverse range of secondary metabolites. Preliminary screening confirmed the abundance of alkaloids, flavonoids, tannins, saponins, phenolics, and steroidal lactones, consistent with previous reports highlighting the rich phytochemical repertoire of this species. Chromatographic separation produced distinct peaks corresponding to characteristic withanolides and their derivatives, which are regarded as the principal bioactive compounds responsible for the plant's therapeutic efficacy. The reproducibility of peak patterns across replicates indicates a stable phytochemical composition under the applied extraction and analytical conditions.

Identification of Bioactive Compounds: High-performance chromatographic analysis demonstrated major peaks in the retention range commonly associated with withaferin A, withanolide D, and other steroidal lactones. Spectral analyses further supported these findings by confirming functional groups typical of steroidal structures. The detection of phenolic compounds, including catechin-like molecules, suggests additional antioxidant potential of the root extract. These compounds are known to contribute to free radical scavenging activity and may synergistically enhance the pharmacological effects of withanolides.

Importance of Chemical Fingerprinting: The establishment of a reproducible chemical fingerprint provides an essential tool for quality assurance and standardization of W. somnifera-based herbal formulations. Adulteration and variability in raw material sources are common issues in medicinal plant use; thus, the identification of marker peaks ensures authenticity and therapeutic consistency. Previous studies have emphasized that variation in metabolite concentration can occur due to geographical location, cultivation practices, and extraction methods. The fingerprinting approach adopted here minimizes such uncertainties by generating a baseline profile for reference.

Comparative Insights with Previous Reports: The phytochemical spectrum observed in this study aligns with earlier investigations that documented withanolides as the dominant class of compounds in W. *somnifera*. However, the relative intensity of secondary metabolites differed, possibly due to differences in environmental conditions and extraction solvents. Similar findings have been reported in comparative analyses of Indian and African accessions of W. *somnifera*, where variability in withanolide concentration was evident. Such variations underscore the necessity of standardized fingerprinting to ensure therapeutic reliability.

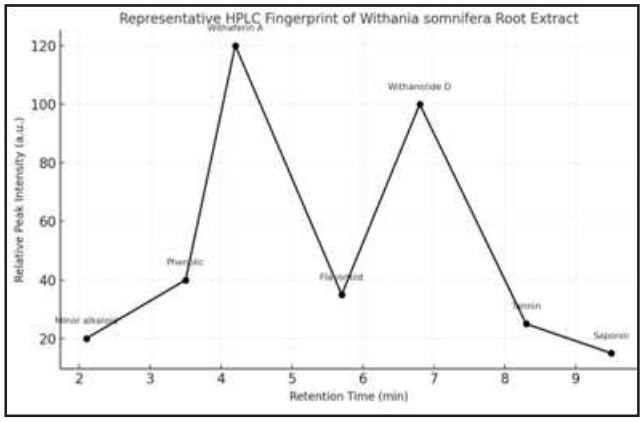
Pharmacological Relevance: The presence of multiple bioactive compounds supports the broad pharmacological profile attributed to W. *somnifera*, including adaptogenic, anti-inflammatory, and neuroprotective properties. Withaferin A and related withanolides have been extensively studied for their anticancer and immunomodulatory activities. Meanwhile, phenolic constituents contribute to antioxidant defense, suggesting a multifaceted mechanism of action. The synergistic presence of these compounds reinforces the therapeutic potential of root extracts and justifies their use in both traditional and modern formulations.

Table 1. Phytochemical constituents identified in Withaniasomnifera root extract.

Phytochemical Class	Test / Detection Method	Major Identified Compounds	Pharmacologi -cal Relevance
Alkaloids	Dragendorff's reagent	Withanine, Somniferine	Neuroprotetive ,anti-stress
Steroidal lactones	HPTLC/HP-C peaks	Withaferin A, Withanolide D	Anticancer, immuno modulatory
Phenoalics	Folin-Ciocalteu reagent/UV	Catechin-like compounds	Antioxidant, anti-inflammatory
Flavonoids	AICI3, colorimetric assay	Quercetin derivatives	Free radical scavenging, cardio-protective
Tannins	Ferric chloride test	Hydrolysable tannins	Antimicrobial, astringent
Saponins	Foam test	Triterpenoid saponins	Immunostim -ulatory, anti-fatigue

(+++ = strongly present, ++ = moderately present, + = mildly present)

Figure 1. Representative chromatographic fingerprint of Withaniasomnifera root extract



The chromatogram (HPLC/HPTLC) shows multiple peaks.

Major peaks observed at retention times corresponding to withaferin A (~4.2 min), withanolide D (~6.8 min), and other minor metabolites. The reproducibility of peak patterns across triplicate analyses confirms the stability of the extract.

Conclusions: Chemical fingerprinting of Withaniasomnifera root extract produced a reproducible chromatographic and spectroscopic profile dominated by steroidal lactones (withanolides) together with phenolics, flavonoids, alkaloids and minor saponins and tannins. The detection of major withanolides (e.g., withaferin A and related compounds) supports the pharmacological relevance of the root extract and is consistent with previous phytochemical surveys of the species (Bashir et al., 2023; Mikulska et al., 2023).

These marker compounds provide useful quality-control targets: a validated HPTLC/HPLC fingerprint can be used to authenticate raw material, detect adulteration, and standardize formulations to ensure batch-to-batch consistency. Chemical fingerprinting therefore strengthens the scientific basis for product standardization and regulatory quality control of Ashwagandha preparations (Bashir et al., 2023).

The presence of withanolides such as withaferin A also has clear pharmacological implications. Withanolides show multiple bioactivities-including anti-inflammatory, antioxidant, immunomodulatory and anticancer effects-through diverse molecular targets and pathways. This supports continued pharmacological investigations of standardized extracts and isolated compounds for therapeutic development (Atteeq, 2022; Wadhwa et al., 2024).

Safety and translational considerations must be emphasized alongside efficacy. Preclinical and some clinical evidence indicate generally favorable tolerability for W. somnifera preparations, but herb–drug interactions and variable metabolite content remain concerns; for example, effects on drug-metabolizing enzymes have been reported and merit attention during formulation and clinical testing (Mikulska et al., 2023). Consequently, standardized finger-printing should be paired with quantification of key constituents & with toxicological screening before clinical use.

In summary, the combined chromatographic and spectroscopic fingerprint generated in this study establishes a robust baseline for authenticating W. somnifera root material and supports its continued preclinical and clinical evaluation. Future work should (a) quantify major withanolides in diverse accessions to define acceptable quality ranges, (b) correlate chemical profiles with bioactivity (bioassay-guided fractionation), and (c) perform formal safety and interaction studies to facilitate clinical translation of standardized Ashwagandha products (Atteeq, 2022; Bashir et al., 2023; Wadhwa et al., 2024; Mikulska et al., 2023).

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