Phytochemical Analysis of Withania Somnifera: Isolation and Identification of Withaferin-a using TLC, HPLC, MASS and NMR

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***Abstract*—This thesis explores the analytical isolation and identification of withanolides from the tertiary roots of Withania somnifera (Ashwagandha) using High-Performance Liquid Chromatography (HPLC) coupled with photodiode array detection (PDA), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. The study emphasizes pharmaceutical analysis for herbal standardization, detailing principles of UV-Vis spectroscopy, HPLC method development. Extraction protocols involved sequential aqueous-methanol reflux, partition separation with ethyl acetate and butanol, and column fractionation using silica gel with gradient elution. Key findings include enriched withanolide fractions (e.g., up to 13.39% total withanolides in PCWS2507010), confirmed by HPLC quantification, MS fragmentation (e.g.,$[M+H]^+$ at m/z 472.3 for Withaferin A), and ^{1}H NMR assignments matching literature data. The work demonstrates a pilot-scale method for reproducible isolation, addressing regulatory compliance and quality control in herbal pharmaceuticals.**

***Index Terms*— PCWS2507010, HPLC, NMR, Mass spectrometry, Withania somnifera.**

I. INTRODUCTION

Pharmaceutical analysis plays a pivotal role in ensuring the quality, safety, and efficacy of medicinal products, particularly in the context of herbal medicines derived from natural sources. This discipline encompasses a wide array of techniques aimed at identifying, quantifying, and characterizing active pharmaceutical ingredients (APIs) and their metabolites, while also detecting impurities, adulterants, or variations in composition that could compromise therapeutic outcomes. In the realm of herbal drugs, which often exhibit complex matrices with multiple bioactive compounds, analytical methods are essential for standardization to achieve reproducibility and regulatory compliance [1,2]. Standardization involves not only botanical authentication but also chemical profiling through advanced instrumentation, ensuring that herbal formulations meet pharmacopoeial standards such as those outlined by the World Health Organization (WHO) or the Indian Pharmacopeia and United States Pharmacopeia (USP) [3]. A fundamental principle underlying many analytical techniques in pharmaceutical analysis is the interaction between matter and electromagnetic (EM) radiation. The EM spectrum spans a broad range of wavelengths, from gamma rays (shortest wavelength, highest energy) to radio waves (longest wavelength, lowest energy), with key regions exploited in detection methods including ultraviolet (UV, 10–400 nm), visible (400–700 nm), infrared (IR, 700 nm–1 mm), and microwave spectra [4,5]. These interactions such as absorption, emission, scattering, or reflection provide fingerprints of molecular structures, enabling qualitative and quantitative analysis. For instance, in absorption spectroscopy, molecules absorb specific wavelengths corresponding to electronic, vibrational, or rotational transitions, following Beer's Law ($A = \varepsilon cl$, where A is

absorbance, ε is molar absorptivity, c is concentration, and l is path length), which forms the basis for concentration determinations in pharmaceutical samples [6].

1.1. Available analytical methods

Available detection methodologies in pharmaceutical analysis leverage different portions of the EM spectrum to achieve high sensitivity and specificity. UV-Visible (UVVis)spectroscopy, operating in the UV and visible regions, is widely used for detecting chromophoric groups in drugs, such as conjugated systems in steroids or alkaloids, due to its simplicity and cost-effectiveness [6]. Infrared (IR) spectroscopy, including Fourier Transform Infrared (FTIR), targets the mid-IR region (2.5–15 μm) to identify functional groups through vibrational modes, making it invaluable for structural elucidation and impurity profiling in herbal extracts [7,8]. Raman spectroscopy, based on inelastic scattering in the visible to near-IR range, complements IR by providing information on symmetric bonds and is particularly useful for non-destructive analysis of solid pharmaceuticals or aqueous samples, as it minimizes water interference [9–11]. More advanced hyphenated techniques, such as mass spectrometry (MS) coupled with chromatography, extend detection by ionizing molecules and analyzing mass-to-charge ratios, often in tandem with UV or fluorescence detectors for enhanced selectivity [12].

These methods are critical for herbal medicines, where matrix effects from polyphenols, tannins, or polysaccharides can obscure signals, necessitating robust validation per ICH guidelines (e.g., Q2(R1) for specificity, linearity, accuracy, precision, and robustness) [17].

Analytical tools employed in the detection and standardization of medicines integrate these detection principles with separation techniques to handle complex samples. High Performance Liquid Chromatography (HPLC), a cornerstone of pharmaceutical analysis, separates compounds based on their partitioning between a mobile phase (e.g., methanol-water gradients) and a stationary phase (e.g., C18 columns), often coupled with UV-diode array detection (DAD) or MS for identification [17,18]. Other tools include Gas Chromatography GC) for volatile compounds, Thin Layer Chromatography (TLC) for preliminary screening, and Nuclear Magnetic Resonance (NMR) for detailed structural confirmation, though less common in routine standardization due to cost. In herbal drug evaluation, these tools facilitate marker compound quantification, adulterant detection (e.g., via LC-MS for synthetic drugs in botanicals), and stability studies, ensuring batch-to-batch consistency [19]. Emerging techniques like Mass Spectrometry Imaging (MSI) offer spatial distribution analysis of bioactive compound sinplant tissues, bridging phytochemistry with pharmaceutical quality control [20].

1.2. Principles of UV-Visible Spectroscopy at the Molecular Level

UV-Visible (UV-Vis) spectroscopy is a fundamental analytical technique in pharmaceutical analysis that exploits the interaction of molecules with electromagnetic radiation in the ultraviolet (190–400 nm) and visible (400–800 nm) regions of the spectrum. At the molecular level, this technique is based on the absorption of photons, which induces electronic transitions within molecules, providing insights into their structure, concentration, and chemical properties. In the context of this thesis, UV-Vis spectroscopy is integral to High-Performance Liquid Chromatography with Photodiode Array Detection (HPLC-PDA) for detecting and quantifying withanolides in Withania somnifera root extracts, as these steroidal lactones exhibit characteristic UV absorption due to their conjugated systems [21].

1.2.1. Molecular Basis of UV-Vis Spectroscopy

Atthe molecular level, UV-Vis spectroscopy involves the excitation of electrons from a lower-energy molecular orbital (typically the highest occupied molecular orbital, HOMO) to a higher-energy orbital (typically the lowest unoccupied molecular orbital, LUMO). These transitions occur in molecules containing chromophores functional groups with delocalized electrons, such as conjugated double bonds (e.g., C=C, C=O) or aromatic rings, which absorb UV or visible light. Withanolides, for instance, possess conjugated lactone rings and double bonds that absorb strongly around 220–250 nm, making UV-Vis spectroscopy ideal for their detection.

The energy required for an electronic transition corresponds to the energy difference (ΔE) between the HOMO and LUMO, as described by the equation: This relationship indicates that shorter wavelengths (higher energy, UV region) correspond to larger energy gaps, typically associated with $\pi \rightarrow \pi^*$ or $n \rightarrow \pi^*$ transitions in

organic molecules. The main types of electronic transitions relevant to UV-Vis spectroscopy include:

1) σ→σ Transitions:

Occur in molecules with single bonds (e.g., C–C, C–H), requiring high-energy UV light (< 200 nm). These are less relevant for withanolides due to their high energy and the vacuum UV requirement, which is not practical in standard HPLC-PDA setups.

2) n→σ Transitions:

Involve non-bonding electrons (e.g., on oxygen or nitrogen in C=O or N–H groups) moving to an antibonding σ* orbital. These transitions occur at longer wavelengths (150–250 nm) but are typically weak in intensity.

3) π→π Transitions:

Common in conjugated systems (e.g., dienes, aromatic rings, or α, β-unsaturated carbonyls in withanolides), these involve electrons in π-orbitals moving to π* orbitals. They occur in the 200–400 nm range and produce strong absorption bands, making them critical for detecting withanolides.

4) n→π Transitions:

Involve non-bonding electrons moving to π* orbitals, often in carbonyl or heteroatom-containing compounds. These transitions appear at longer wavelengths (250–300 nm) but have lower molar absorptivity (weaker peaks) compared to π→π* transitions.

In withanolides, the conjugated double bonds and lactone rings primarily contribute

to π→π* transitions, resulting in strong UV absorption around 220–250 nm, which is detectable by the PDA detector in HPLC systems. The absorption intensity follows Beer's Law (A=εcl A = \varepsilon c l A=εcl), where absorbance (A A A) is proportional to the molar absorptivity (ε \varepsilon ε), concentration (c c c), and path length (l l l), enabling quantitative analysis[21].

1.2.2. Instrumentation and Molecular Interaction

A UV-Vis spectrophotometer consists of a light source (deuterium lamp for UV, tungsten lamp for visible), a monochromator or g rating to select specific wavelengths, a sample holder (cuvette or flow cell in HPLC), and a detector (photodiode or photomultiplier tube). In HPLC-PDA, the flow cell allows continuous monitoring of eluting compounds. When light passes through the sample, molecules with appropriate chromophores absorb photons, exciting electrons to higher energy states. The detector measures the intensity of transmitted light, and the difference between incident and transmitted light yields the absorbance spectrum.

The PDA detector's advantage lies in its ability to record a full UV-Vis spectrum for

each eluting peak, allowing identification by comparing spectra to standards or libraries and assessing peak purity by checking for consistent spectral profiles across a peak. For withanolides, this is critical, as co-eluting matrix components (e.g., polyphenols) in Withania somnifera extracts can interfere, and PDA's multi-wavelength detection ensures specificity.

1.2.3. Factors Influencing UV-Vis Absorption

Several molecular and environmental factors affect UV-Vis absorption:

- Conjugation Length: Extended conjugation (e.g., multiple double bonds in withanolides) lowers the energy gap, shifting absorptiontolongerwavelengths (bathochromic shift).
- Substituents: Electron-donating or withdrawing groups (e.g., –OH, –OCH_3) on chromophores modify the electron density, altering λ_max and intensity.
- Solvent Effects: Polar solvents can stabilize or destabilize electronic states, causing hypsochromic (blue) or bathochromic (red) shifts. For HPLC, mobile phases like methanol or acetonitrile are optimized to maintain consistent spectra.
- pH: Protonation or deprotonation of chromophores alters their electronic properties, affecting absorption. Acidic modifiers (e.g., 0.1% formic acid) in HPLC mobile phases stabilizestructure.

1.3. Advancement from UV-Visible Spectroscopy to Photodiode Array Detection

The evolution of UV-Visible (UV-Vis) spectroscopy into advanced detection systems

like Photodiode Array Detection (PDA) represents a significant milestone in analytical chemistry, particularly within High-Performance Liquid

Chromatography (HPLC) applications [22]. Traditional UV-Vis detectors in HPLC, such as fixed-wavelength or variable-wavelength detectors (VWD), originated from standalone UV-Vis spectrophotometry principles, where light absorption at a single or selectable wavelength was measured to detect eluting compounds based on their chromophoric properties. These early detectors, developed in the 1970sand1980s, reliedon mercury or deuterium lamps for UV light and photomultiplier tubes or single photodiodes for signal conversion, providing reliable but limited data primarily absorbance at one wavelength per run. While effective for quantitative analysis adhering to Beer's Law, they lacked the capability for real-time spectral scanning, making peak identification and purity assessment challenging in complex matrices like herbal extracts [23].

The advancement to PDA detectors addressed these limitations by integrating semiconductor technology with UV-Vis principles, enabling simultaneous multi wave length detection [24]. Pioneered in the late1980sandearly1990s, PDA technology evolved from the need for faster, more comprehensive spectral data in chromatography. Unlike scanning UV-Vis detectors, which mechanically scan wavelengths sequentially (taking seconds per spectrum and risking peak distortion in fast separations), PDA employs a linear array of photodiodes typically 512 to 1024 diodes arranged after a diffraction grating[25].This setup disperses the entire UV-Vis spectrum (190–800 nm) across the array, allowing each diode to capture a narrow wavelength band (e.g., 1–2 nm resolution)simultaneously[26]. As a result, full spectra can be acquired in milliseconds, providing a three-dimensional chromatogram (retention time, wavelength, absorbance) without compromising chromatographic resolution [27].

At the molecular level, PDA builds directly on UV-Vis electronic transitions (e.g., $\pi \rightarrow \pi^*$ in withanolides), but enhances data richness by capturing the entire absorption profile in real time. This advancement facilitates peak deconvolution, where overlapping peaks can be resolved spectrally even if not chromatographically, and enables library matching for compound identification critical for withanolides with similar structures but distinct spectral fingerprints [28]. Furthermore, PDA's ability to monitor multiple wavelengths aids in method development, such as selecting optimal detection wavelengths for sensitivity while avoiding matrix interferences in Withania somnifera extracts [29].

II. LITERATURE REVIEW

Withania somnifera (L.) Dunal, commonly known as Ashwagandha, is a key medicinal plant in Ayurvedic and pharmaceutical formulations, valued for its withanolides (e.g., withaferin A, withanolide A, withanolide D) and withanosides, which exhibit adaptogenic, anti-inflammatory, and anticancer properties [77]. High-Performance Liquid Chromatography (HPLC) and its variants (e.g., UHPLC, RP-HPLC) are essential for method development, validation, quantification, and isolation of these bioactive compounds in raw extracts, roots, and various formulations such as capsules, tablets, semisolid preparations (e.g., lehyam), and herbal supplements. Literature emphasizes stability-indicating methods compliant with ICH Q2(R1) guidelines, often hyphenated with detectors like PDA, DAD, or MS for enhanced specificity [78].

a) Gupta et al., 2018 “Efficacy of Withania somnifera on seminal plasma metabolites of infertile males: A proton NMR study at 800 MHz. J Ethnopharmacol”. Developed a reliable reverse-phase high-performance liquid chromatography with photodiode array detection (RP-HPLC-PDA) method for the quantification of withaferin A, 12 deoxy with astromonolide, and withanolide A in Withania somnifera roots and commercial formulations, such as capsules and powders. The method employed a C18 column with an isocratic acetonitrile:water (phosphoric acid-adjusted) mobile phase at a flow rate of 1 mL/min, detecting analytes at 227 nm with retention times ranging from5 to15 minutes Extraction was perform educing chloroform/ethylacetatereflux, yielding robust validation parameters, including excellent linearity ($r^2 > 0.999$), precision (RSD < 2%), and accuracy (98–102% recovery), though limits of detection (LOD) and quantification (LOQ) were not specified. The study quantified withanolide content in the range of 0.5–2.5% in commercial formulations, demonstrating the method’s effectiveness for quality control and detection of adulteration in ashwagandha-based product (82).

b) Sreeja et al., 2021 "Journal of Pharmaceutical Sciences and Research" developed a reverse-phase high-performance liquid chromatography with diode array detection (RP-HPLC-DAD) method to quantify withaferin A in ashwagandhadi lehyam, a semisolid formulation derived from Withania somnifera. The method utilized an Eclipse XDB C18 column (4.6 × 150 mm, 5 µm) with an isocratic acetonitrile: phosphate buffer (35:65, pH-adjusted) mobile phase at a flow rate of 1.8 mL/min, maintained at 32°C, and detection at 227 nm, achieving a retention time of 5.015 minutes. Extraction involved methanol reflux (75 mL, 30 minutes, repeated thrice) followed by 0.22 µm filtration, ensuring high purity. The method demonstrated excellent validation parameters, including linearity (0.0106–0.17 mg/mL, r^2 = 0.9999), precision (RSD < 1.5%), specificity (no interference), and highly sensitive limits of detection (LOD) and quantification (LOQ) at 0.003 µg/mL and 0.01 µg/mL, respectively. The study quantified 0.092% withaferin A in the lehyam, establishing the method's utility for standardization and quality assurance of ashwagandha-based therapeutic formulations [83]

c) Waters et all., 2024 "The Science of What's Possible n.d." Waters (2024) developed an effective method using the Alliance iS HPLC system with a C18 column and an isocratic phosphate buffer-based mobile phase, detected at 227 nm, to quantify withaferin A, withanolide A/B, 12-deoxywithastramonolide, and withanoside A/B in USP root reference material and commercial tablets/drops of Withania somnifera. The method employed ultrasound-assisted extraction with a solvent mixture of ethanol: methanol: water (60:30:10) for 30 minutes, achieving robust validation parameters, including excellent linearity (r^2 > 0.999), repeatability (RSD < 1%), resolution (> 2), and tailing factor (< 1.5), compliant with AOAC 2015.17 standards. The study reported withanolide content ranging from 0.2–0.8% in USP root material, while commercial supplements showed variable concentrations (< 0.1–0.8%), highlighting inconsistencies and underscoring the method's applicability for regulatory compliance and quality control in ashwagandha-based products [84].

d) Sharma et al, 2020 "Investigating 11 Withanosides and Withanolides by UHPLC-PDA and Mass Fragmentation Studies from Ashwagandha (Withania somnifera)". Developed a robust ultra-high-performance liquid chromatography with photodiode array detection (UHPLC-PDA) method to quantify 11 withanosides and withanolides, including withaferin A and withanolide A, in Withania somnifera root extract (WSE). Utilizing a C18 column with a gradient acetonitrile: water (formic acid) mobile phase at a flow rate of0.4 mL/min and detection across 200–400 nm, the method analyzed a standardized extract, though specific extraction details were not provided. Validation parameters demonstrated excellent linearity (5–500 ng/mL, r^2 > 0.998), precision (RSD < 3%), accuracy (95–105% recovery), and a limit of detection (LOD) ranging from 1–5 ng/mL. The study reported withaferin A content of 0.5–1.2 mg/g, highlighting the method's utility in supporting quality control for formulations and bioactivity studies of ashwagandha-based products [85].

III. AIM AND OBJECTIVE

3.1. AIM Of the Dissertation

The aim of this thesis is to analytically isolate and identify withanolides from the tertiary roots of Withania somnifera using High-Performance Liquid Chromatography (HPLC) as the primary analytical tool, coupled with advanced spectroscopic and chromatographic techniques for structural characterization. The study seeks to establish chromatographic fingerprinting profiles, quantitatively track the distribution of key phytochemicals across fractions in commercial-scale operations, and evaluate the efficacy of a USP-validated pilot-scale method for reproducible isolation without further validation.

3.2. Objectives of the Thesis

1. Optimized Extraction and Analytical Isolation: To develop an analytically robust extraction protocol for isolating withanolides and associated phytochemicals from the tertiary roots of Withania somnifera, employing solvent-based fractionation and preparative HPLC to achieve high-purity isolates at a pilot scale.

2. HPLC-Based Chromatographic Fingerprinting: To generate precise HPLC inger printing profiles of crude extracts and fractions, enabling the identification and differentiation of withanolides and other phytochemicals based on retention times, peak areas, and spectral characteristics for analytical standardization.

3. Quantitative Tracking of Phytochemical Distribution: To quantitatively monitor the movement and partitioning of withanolides and common phytochemicals across fractions during commercial-scale operations, using HPLC to assess concentration shifts and ensure consistency in large-scale processing.

IV. MATERIALS AND EXPERIMENTAL METHODS

Below is a detailed table listing the equipment and chemicals/solvents used for the isolation and identification of withanolides from Withania somnifera, including equipment make where applicable, along with their specifications. The table incorporates items from the provided list and additional equipment/chemicals relevant to the plan of work, ensuring alignment with the experimental requirements.

Preparation Of Methanolic Extract From withania somnifera tarr

The 100 kg of Withania somnifera tertiary roots were loaded into the reflux percolation
reactor (1000 Lcapacity, SS-304) and subjected to sequential aqueous-methanol extraction.

Water Extraction:

The roots were extracted with deionized distilled water in a 1:5 w/v ratio (500 L) for the first wash and 1:4 w/v ratio (400 L) for the second wash, under reflux at 80–90°C for 4 hours per wash, with continuous stirring (Remi stirrer at 500 RPM) to enhance mass transfer. After each wash, the extract was filtered through muslin cloth filters (100–200 µm) followed by vacuum filtration to remove particulates. The filtrates from both washes were concentrated to a thick paste using a vacuum concentrator (50– 60°C) and subjected to spray drying process (SPD) with inlet temperature of 180–200°C and outlet temperature of 80–90°C with the yield of cyclone= 5kg, and chamber =7.7kg

Methanol Extraction:

The marc (residue) from the water extraction was subjected to three successive washes with commercial-grade methanol in a 1:4 w/v ratio (400 L per wash), under reflux at 60–70°C for 3 hours per wash, with continuous stirring (500 RPM). The filtrates were pooled, filtered as described above, and concentrated to a thick paste using rotary evaporators (40–50°C) under vacuum, with solvent recovery. The total percentage yield from the methanol extraction was 14.3% (14.3 kg thick paste).

Both the water extract and methanol extract were done HPLC analysis. The
chromatograms for the water extract (Figure 1) and methanol extract (Figure 2) are given
below.

Partition Separtion:

- From the result found in methanol extract withanolides were enriched.
- For further enrichment we are going for partition separation.
- For this we are taking 1.8 kg of methanol extract and dissolve in water make TS
- up to 80.
- Now we need to add the 4L of ethyl acetate shake well and keep it a side for few

minutes until it forms two layers.

- Then collect the ethyl acetate layer and water layer separately.
- For the water layer again, we need to do follow same process with ethyl acetate

this should be done for two time. Mix the ethyl acetate layer and dry in water bath.

For the water layer we need to do butanol wash, for three times by collecting

HPLC analysis

Preparation of standard solution:

Accurately weight 5mg of standard mixture of withanolides and transfer in into 50 ml of
clean dry volumetric flask. Add 30 ml of HPLC grade methanol, sonicate for 15 minutes
and dilute to the final volume (50 ml) with same methanol.

Preparation of sample solution:

Accurately weight 5mg of sample and transfer it into a 50 ml of clean and dry volumetric

flask and add 30 ml of HPLC grade Methanol and sonicate for 15 minutes and dilute to
the final volume (50 ml) with same methanol
Filtration:
Take a fresh 0.5 um syringe filter. Pipette 2ml of the standard solution, into a syringe and
pass it through the filter, collect the filtrate in an HPLC vial for analysis. Discard the first
few drops of filtrate to ensure complete removal of any particulates. Repate the same
process for the sample also.
Sample injection:
Place the vials in the HPLC instrumentation and command the system for the run, before
that save the procedure in the system. Run time was 35 minutes. After the completion of
run time integrate the peak aera and save the chromatogram, and calculate the assay.s

1. TLC analysis was performed on the fractions and mixed the fraction which exhibits the same Rf pattern combined.
2. Then the fractions were submitted for the HPLC analysis, i.e, (PCWS2505002, PCWS2505003, PCWS2505004, PCWS2505005, PCWS2505006, PCWS2505007, PCWS2505008)
3. Based on the chromatogram profile, we mixed the fractions of PCWS2505003, PCWS2505004, PCWS2505005, and PCWS2505008.
4. The mixed fraction was subjected to the next column chromatogram.

Crystallisation:

- To achieve the purer compound, we done the crystallisation with the

methanol.

- Sample PCWS2508010 has the only peaks i.e, Withanolide A and Withanolide B.\
- In chromatogram Withanolide A has the aera normalisation of 70.92%.
- The HPLC of the PCWS2508010 was given below:
- Sample code: PCWS2508010
- Fraction: F2
- Enriched compounds: Withanolide A and Withanolide B
- Assay: 7.3

So, to remove the other unwanted peak we done crystallisation with the

- By maintain temperature of 60 C, for 30 min.
- After that we kept the sample in the cool room. To form the crystallisation.
- This process is done for 3 times to remove other impure compounds.
- After the 3rd crystallisation found the white pure Withanolide A compounds. This sample is identified by the HPLC, NMR and mass.

V. RESULTS AND DISCUSSION

1. Yield data

Solvent used for partition separation	Yield
Initial methanol extract	1.8kg
Ethyl acetate layer	745.76g
Butanol	327g
Water layer	698g

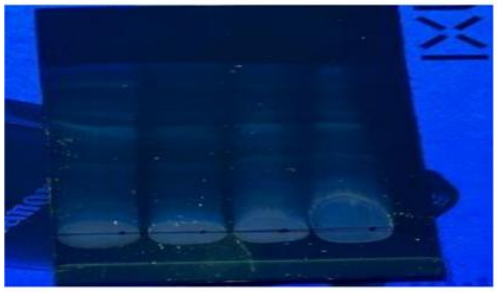
Table 9: Partition yield of methanol extract

Sample code	Description	Yield
PCWS2505002	hexane fraction	48g
PCWS2505003	10% meoH- TLC-1	28g
PCWS2505004	TLC-2	135g
PCWS2505005	TLC-3	39g
PCWS2505006	TLC-4	19g
PCWS2505007	TLC-5	11g
PCWS2505008	Perti plate sample	6g

Table 10: Yield of column1

SAMPLE CODE	Description	Yield
PCWS2508009	F1	-
PCWS2508010	F2	2g
PCWS2507001	F3&4	2.5g
PCWS2507002	F5-6	3 g
PCWS2507003	F7-9	7 g
PCWS2507004	F10	5 g
PCWS2507005	F11-14	4.5 g
PCWS2507006	F15-20	2.1 g
PCWS2507007	F21-23	6g
PCWS2507008	F24-27	17.6g
PCWS2507009	F28-30	8.8g
PCWS2507010	F31-38	9.9g
PCWS2508001	F39-40	10.7g
PCWS2508002	F41	41.4g
PCWS2508003	F42-45	5.9g
PCWS2508004	F46-49	2g
PCWS2508005	F50-60	17.8g

2. TLC Data



366nm

3. Column fractionation data

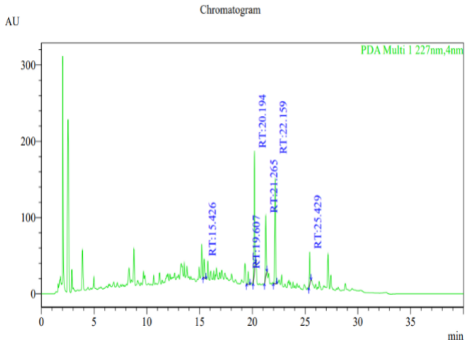


Fig-7 water extract

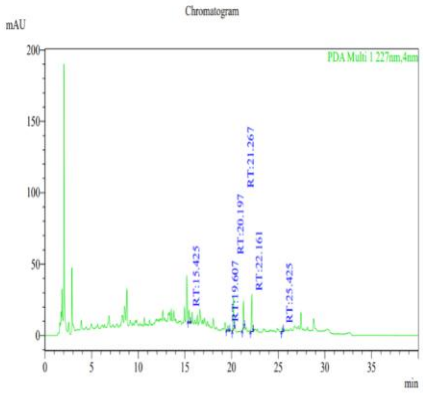


Fig-8 methanol extract

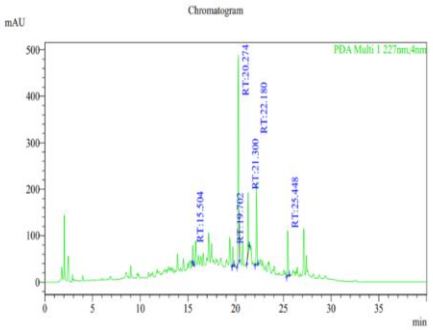


Fig-9 : Methanol extract

Mass data

The x-axis represents m/z values from approximately 60 to 500 Da, and the y-axis shows relative intensity. The dominant peak at m/z 472.3 corresponds to the protonated molecular ion $[M+H]^+$ of Withaferin A ($C_{28}H_{38}O_6$, exact mass 470.2716 Da; observed $[M+H]^+ \approx 471.28$ Da, with the slight shift to 472.3 attributable to instrument resolution or nominal mass display). This confirms the identification of Withaferin Aas the primary analyte. Supporting fragment ions include:

- m/z 453.4: Likely $[M - H_2O + H]^+$, resulting from dehydration of the lactone ring.
- m/z 417.3: Possible loss of a neutral fragment (e.g., C_3H_6O from the side chain).
- m/z 360.2: Characteristic ion from ring opening or loss of acetyl group.
- m/z 340.4: Common diagnostic fragment for withanolides, indicating loss of the
- side chain.
- m/z 274.3 and 259.2: Ions from sequential losses in the steroidal backbone.
- m/z 242.2: Smaller fragment from the A/B ring cleavage.

The spectrum matches reported ESI-MS/MS patterns for WithaferinA, where the precursor ion is m/z 471 and key fragments include m/z 299, 281, and 175 (though not all are prominent here, suggesting a softer fragmentation or full-scan mode). The high intensity of the $[M+H]^+$ peak indicates good ionization efficiency in positive ESI mode, typical for withanolides with their polar oxygen functionalities. This data supports the quantitative detection of Withaferin Ain the sample, with no significant interfering peaks, implying high purity at this

scan.[114] The accompanying extracted ion chromatogram (EIC) at the bottom (likely for m/z 471 or 472) shows a sharp peak around 4-5 minutes retention time (RT), confirming chromatographic separation from matrix components. The scan label "472.3 Scan ES+" and date (28-03-2022) suggest this is from anAgilent or similar system in scan mode, with average spectra (AV500) for noise reduction.

NMR data

This is a ^{1}H NMR spectrum recorded at 500 MHz (AV500 label), in a deuterated solvent ($CDCl_3$ common for withanolides), with chemical shifts (δ) in ppm on the x-axis and

intensity on the y-axis. The spectrum aligns with the proton environment of Withaferin A, a steroidal lactone with a complex polycyclic structure, including an α,β-unsaturated ketone, epoxide, and acetylated side chain. Key observations:

- δ 0.5-2.0 p pm: Multiple overlapping signals (e.g., 0.87, 1.02, 1.15) indicate methyl protons (CH_3) and methylene protons (CH_2) of the steroidal skeleton, typical for the A-D rings.
- δ 2.0-3.0 p pm: Peaks around 2.2-2.5 ppm suggest methine protons (CH) near the epoxide (C-5,6) or acetyl group (C-27), with coupling patterns hinting at adjacent protons.
- δ 3.0-4.0 p pm: Signals (e.g., 3.2-3.5 ppm) correspond to protons on oxygenated carbons, likely the C-5 or C-6 epoxide protons, a diagnostic feature of Withaferin A.

VI. SUMMARY

the thesis provides a comprehensive framework for analyzing Withania somnifera, starting with an introduction to pharmaceutical analysis techniques, including UV-Vis spectroscopy, HPLC-PDA, MS, and NMR, grounded in electromagnetic interactions and Beer's Law. Literature review covers HPLC methodologies for withanolide quantification, regulatory monographs (USP/IP), and commercial standardization. Aims focus on isolating withanolides via optimized extraction and fractionation, with objectives including chromatographic fingerprinting and structural characterization. The plan of work outlines literature review, solvent selection, pilot-scale extraction, and multi-step fractionation. Materials include equipment like Shimadzu HPLC and Bruker NMR, with solvents such as methanol and ethyl acetate. Experimental methods detail methanolic extraction (14.3%yield), partition separation, and two-column fractionations yielding enriched samples (e.g., 41.4 g from F31-38 with high withanosides). Results show TLC patterns, HPLC chromatograms indicating peak withaferin A and withanolides, MS confirming molecular ions and fragments, and NMR proton assignments for Withaferin A, validating the compound's presence and purity.

VII. CONCLUSION

This thesis successfully establishes an analytical pipeline for isolating and characterizing

withanolides from Withania somnifera tertiary roots, leveraging HPLC-PDA for separation, MS for molecular confirmation, and NMR for structural elucidation. The optimized extraction and fractionation yielded high-purity fractions, with Withaferin A identified as a dominant compound through diagnostic spectral data., the study enhances herbal standardization, ensuring reproducibility and regulatory compliance amid growing market demand for Ashwagandha products. Future work could explore scalability to industrial levels and bioactivity correlations, bridging traditional Ayurvedic uses with modern pharmaceutical quality control. Overall, these findings contribute to safer, efficacious herbal medicines by addressing matrix complexities and variability in bioactive content.

REFERENCE

[1] Muyumba NW, Mutombo SC, Sheridan H, Nachtergael A, Duez P. Quality control of herbal drugs and preparations: The methods of analysis, their relevance and applications. Talanta Open 2021; 4:100070. https://doi.org/10.1016/J.TALO.2021.100070.

[2] Pratiwi R, Dipadharma RHF, Prayugo IJ, Layandro OA. Recent Analytical Method for Detection of Chemical Adulterants in Herbal Medicine. Molecules 2021; 26:6606. https://doi.org/10.3390/MOLECULES26216606.

[3] Edo GI, Obasohan P, Makia RS,Abiola O T, Umelo EC, Jikah AN, et al. The use of quality control parameters in the evaluation of herbal

drugs. Areview. Discover Medicine 2024 1:1 2024; 1:1–15. https://doi.org/10.1007/S44337-024-00177-6.

[4] Electromagnetic spectrum analysis | Research Starters | EBSCO Research n.d. https://www.ebsco.com/research-starters/chemistry/electromagnetic-spectrum-analysis (accessed September 15, 2025).

[5] Electromagnetic Spectroscopy - an overview | ScienceDirect Topics n.d. https://www.sciencedirect.com/topics/medicine-and-dentistry/electromagnetic-spectroscopy (accessed September 15, 2025).

[6] Absorption Spectroscopy in Pharmaceutical Analysis n.d. https://www.terrauniversal.com/blog/absorption-spectroscopy-in-pharmaceutical-analysis (accessed September 15, 2025).

[7] Comparison of Optical Spectroscopy Techniques for Pharmaceutical Analysis American Pharmaceutical Review - The Review of American Pharmaceutical Business & Technology n.d. https://www.americanpharmaceuticalreview.com/Featured-Articles/618205- Comparison-of-Optical-Spectroscopy-Techniques-for-Pharmaceutical-Analysis/ (accessed September 15, 2025).

[8] Deidda R, Sacre PY, Clavaud M, Coïc L, Avohou H, Hubert P, et al. Vibrational spectroscopy in analysis of pharmaceuticals: Critical review of innovative portable and handheld NIR and Raman spectrophotometers. TrAC Trends in Analytical Chemistry 2019; 114:251–9. https://doi.org/10.1016/J.TRAC.2019.02.035.

[9] Saleh TA. Pharmaceutical Characterization and Detection Using Surface- Enhanced Raman Scattering. International Archives of Clinical Pharmacology 2017;3. https://doi.org/10.23937/2572-3987.1510010.