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Research Article

PHARMACOGNOSTICAL CHARACTERIZATION AND PHYTOCHEMICAL ANALYSIS OF *WITHANIA SOMNIFERA* DUNAL ROOT FROM NORTHERN INDIA

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ABSTRACT

Objective: The present study aims to establish the pharmacognostical characterization and phytochemical standardization of *Withania somnifera* Dunal root collected from Northern India.

Methods: The root material was subjected to evaluation following the World Health Organization (WHO) and Ayurvedic Pharmacopoeia protocols. Successive extractions were carried out using petroleum ether, chloroform, alcohol, hydro-alcohol (1:1), and water. Preliminary phytochemical profiling and TLC fingerprinting were performed on solvent extracts. Safety parameters including heavy metals, aflatoxins (AFs), microbial load, and pesticide residues were assessed using validated analytical methods. The combined results were used to establish reproducible pharmacognostical and phytochemical standards for Northern Indian root material.

Results: The root showed a characteristic grayish-yellow color, bitter taste, cylindrical morphology, and fibrous fracture. Microscopical examination revealed cork cells, parenchyma, xylem vessels, and abundant starch grains. The pharmacognostic analysis showed foreign matter (0.19±0.02%), swelling index (3.9±0.3 mL/g), bitterness value (2.9±0.4 units/g), total ash (6.6±0.1%), acid-insoluble ash (0.56±0.04%), water-soluble ash (1.7±0.2%), and moisture content (4.46± 0.04%), all within acceptable limits for crude herbal materials. Extractive values were found to be highest in hydro-alcoholic hot and successive extracts, 13.46±0.21% and 19.59±0.18%, respectively. However, cold extractive value was comparatively high inaqueous extract (9.7±0.1%), which is higher than extractive values reported in several earlier studies on *W. somnifera* from other Indian regions. Preliminary phytochemical screening confirmed the presence of alkaloids, flavonoids, tannins, saponins, and steroids. TLC revealed distinct spots with retention factor (Rf) values ranging from 0.12 to 0.93. Total tannin content was 8.176 mg/g. Heavy metals, AFs, microbial contamination, and pesticide residues were all within WHO permissible limits ensuring sample safety.

Conclusion: The analytical findings establish a comprehensive pharmacognostical and phytochemical profile of *W. somnifera* root from Northern India. The comparatively high hydroalcoholic extractive values and complex TLC fingerprint of polar extracts represent useful diagnostic markers for this regional chemotype and support its safe inclusion in pharmacopeial monographs and quality control of herbal formulations.

Keywords: *Withania somnifera*, Pharmacognostical standardization, Phytochemical screening, Safety evaluation.

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INTRODUCTION

Withania somnifera Dunal, a perennial plant in the family Solanaceae, is well-known as ashwagandha throughout India and its neighboring countries [1]. It is traditionally defined as a rasayana, or rejuvenating tonic, optimizing longevity, vitality, and stress adaptation in the Ayurvedic health care system [2]. Along with these traditional uses, ashwagandharishta and chyawanprash are two classical preparations containing the root material which serves as the primary efficacious agent in the treatment of inflammatory conditions, fatigue, insomnia, and mental burnout [3]. Recent pharmacological research has reproduced some of the ancient indications in particular regard to the anti-stress and neuroprotective actions attributed to the withanolides, alkaloids, and flavonoids [1,2]. The standardization of root material through pharmacognostical and phytochemical investigations is becoming increasingly relevant with the greater use of plant material globally with possible or potential adulteration possible in terms of authenticity and medicinal benefit.

The pharmacological characteristics of *W. somnifera* are attributed to a diverse range of components present in its chemically diverse root. The primary biologically active constituents include alkaloids, flavonoids, saponins, phenolic constituents, and withanolides, a class of C-28 steroidal lactones with ergostane linkages [4]. Of these compounds, withanosides IV–V, withaferin A, and withanolide A, all considered key chemical compounds for quality and standardization testing, have gained recognition [5]. These compounds are considered to support adaptogenic, neuroprotective, anti-inflammatory, and immunomodulating effects, possibly through the regulation of oxidative stress and inflammation pathways [6,34].

The authenticity and integrity of the raw material play an essential role in the clinical efficacy of natural products like Withania. Pharmacological activity can vary significantly due to differences in shape, phytochemical constituents, and storage conditions, resulting in varying clinical efficacy [7]. Reports, for instance, indicate that Ashwagandha root may sometimes be adulterated or replaced with other morphologically similar plant components, thus reducing the withanolide concentration and quality [8]. As a result, the World Health Organization (WHO) and pharmacopeial authorities have recommended detailed diagnostic standards involving, for example, chemical profiling for marker compounds, physicochemical parameters, and both macroscopic and microscopic characteristics [9]. In addition, incorporating safety evaluations like heavy metal estimation, aflatoxin (AF) determination, microbial load, and pesticide residue analysis ensures conformity with international quality guidelines [10].

Nevertheless, several studies have reported inconsistencies in the physicochemical parameters, extractive profiles, microscopic characteristics, and phytoconstituent composition of *W. somnifera*

roots sourced from different regions of India. Uddin *et al*. documented variations in major phytochemical groups and withanolide distribution, while Dhankhar *et al*. demonstrated considerable regional differences in HPTLC fingerprints and pharmacognostic markers. These interregional disparities underscore the need to establish standardized, region-specific reference parameters to ensure reliable authentication and consistent quality of crude root material.

Most of the existing contemporary research focuses on either pharmacological assays or quantification of the primary withanolides, but rarely do assessments of pharmacognostical, physicochemical, or safety parameters from medicinal plants receive adequate assessment [11,12].

Thus, the study aims to systematically conduct a comprehensive pharmacognostical and phytochemical standardization of *W. somnifera* Dunal root, addressing both qualitative and quantitative aspects, to try and resolve the inconsistencies [13,14]. The assessment includes macroscopic and microscopic identification, ash values, extractive values, pH, moisture content, and fluorescence testing, in addition to phytochemical screening, TLC profiling, and safety testing for heavy metals, AFs, microbial load, and pesticide residues. This multiparametric approach will aim to generate baseline reference standards and authentic diagnostic markers to effectively inform pharmacopeial inclusion and support global quality assurance of herbal products based on ashwagandha [15].

To develop reproducible quality standards, the current study thoroughly analyzes the root of *W. somnifera* through joined pharmacognostical and phytochemical analysis.

METHODS

Collection and authentication of plant material

The plant material was procured from the local market of New Delhi, India. The collected plant parts were thoroughly cleaned to remove adhering soil and stored under dry, contamination-free conditions until further pharmacognostical and phytochemical evaluation. The plant material was taxonomically identified and authenticated as *W. somnifera* Dunal (Ashwagandha Root), family Solanaceae, by Dr. H. B. Singh, Ex-Taxonomist, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, presently serving as Chief Scientist, Department of Herbology, Amil Pharmaceutical Pvt. Ltd., New Delhi. The voucher number is AMIL/Testing Lab/0523/17 for future reference.

Preparation of samples

The authenticated parts of *W. somnifera* Dunal were washed thoroughly with running tap water to remove soil and foreign matter, followed by rinsing with distilled water. The roots were cut into small pieces and shade-dried at room temperature (28±2°C) until a constant weight was obtained. The dried herb was coarsely powdered using a mechanical grinder, passed through sieve No. 60 to obtain a uniform particle size, and preserved into airtight amber glass containers protected from light and moisture.

Macroscopic and organoleptic evaluation

The macroscopic characteristics of *W. somnifera* Dunal were recorded with respect to shape, size, color, taste, surface characteristics, texture, and fracture. The organoleptic properties were evaluated manually to establish sensory identity and confirm conformity with authentic pharmacognostical standards [16]. Observations were documented under adequate natural light, and each parameter was compared with standard morphological descriptions available in recognized monographs to ensure specimen authenticity.

Microscopic evaluation

Transverse section

For microscopical studies, thin freehand transverse sections were taken using the sharp razor blade. Sections were cleared by warming with few drops of chloral hydrate and were mounted temporarily in glycerin for the microscopical observation [16].

Powder microscopy

For the powder microscopical studies, the coarse root powder (passed through sieve no. 60) was mounted on the glass slide using glycerin. Observing the different sections of material under the compound microscope and the photographs of microscopy were captured following the standard protocols [16,17].

Determination of foreign matter

A 100 g sample of plant material was accurately weighed and spread in thin layer on clean surface. The sample was examined under adequate illumination for foreign matter either by visual inspection, using a magnifying lens or with the help of an appropriate sieve according to the requirements. The separated fraction of foreign matter was weighed and the percentage of total foreign matter was calculated with reference to the air-dried sample weight [17].

Physicochemical parameters

Ash values

Ash values are important indices for the assessment of inorganic materials, such as carbonates, silicates, oxalates, and phosphates. Upon ignition, organic matter is eliminated as carbon dioxide, leaving behind inorganic residues. Ash value is an essential characteristic of a drug, and with the help of this parameter, we can detect the extent of adulteration as well as establish the quality and purity of the drug. The acid-insoluble ash consists primarily of silica and indicates contamination by earthy material while the water-soluble ash is used to estimate the amount of soluble inorganic elements [17].

Total ash

About 2 g of air-dried powdered drug was taken in a previously ignited and tarred silica crucible. The sample was spread in a fine even layer in the tarred crucible and incinerated gradually at 500–600°C until a uniform white residue free from carbon was obtained. It is then cooled in desiccators for 30 min and weighed. The incineration process was continued to get a constant weight. The percentage of total ash was calculated and noted with reference to the air-dried drug sample [17].

Acid-insoluble ash

The total ash obtained (as described above) was boiled gently with 25 mL dilute hydrochloric acid (HCl) (6N) for 5 min. The insoluble portion was collected on an ashless filter paper, washed with hot water, and incinerated at a temperature of 500–600°C. The incineration process was continued to get a constant weight. The percentage of acid-insoluble ash was calculated and noted with reference to the air-dried drug sample [17].

Water-soluble ash

The total ash obtained was dissolved in 25 mL of distilled water and boiled for 5 min. The insoluble part was collected on an ashless filter paper, washed with hot water, and ignited at 500–600°C to constant weight. By subtracting the weight of the insoluble part from that of the total ash, the weight of the soluble part of the ash was calculated. The process was repeated to get a constant weight. The percentage of water-soluble ash was calculated and noted with reference to the air-dried drug sample [17].

Extractive values

Hot extraction

4 g of air-dried drug was separately refluxed in a glass-stoppered conical flask with 100 mL of each solvent, petroleum ether (60–70°C), chloroform, alcohol, alcohol: water (1:1), and water for 6 h. The extracts were cooled, filtered, and 25 mL of each filtrate was evaporated to dryness in a tarred flat-bottom dish on a water bath. The residues were dried in a desiccator for 30 min and weighed. The process was

repeated to get a constant weight. The percentage of extractive matter was calculated and noted with reference to air-dried drug sample [17].

Cold extraction

4g of air-dried drug was separately macerated in a glass-stoppered conical flask with 100 mL of each solvent, petroleum ether (60–70°C), chloroform, alcohol, alcohol: water (1:1), and water for 24 h, with intermittent shaking, followed by a final standing period of 18 h. The extracts were filtered, and 25 mL of each filtrate was evaporated to dryness in a tarred flat-bottom dish on a water bath. The residues were dried in a desiccator for 30 min and weighed. The process was repeated to get a constant weight. The percentage of extractive matter was calculated and noted with reference to air-dried drug sample [17].

Successive solvent extraction

The coarse powdered drug was packed in a Soxhlet apparatus and extracted sequentially with solvents, petroleum ether (60–70°C), chloroform, alcohol and alcohol: water (1:1), water. Extraction with each solvent was carried out about 72 h. The resulting extracts were concentrated under reduced pressure and weighed after complete solvent removal. The extractive value was calculated using the formula:

$$\text{Extractive value } (\text{mg/g}) = \frac{\text{Weight of extract}}{\text{Quantity of initial plant material taken}} \times 100$$

Moisture content

The moisture content of the plant material was determined to estimate the amount of water and volatile components present in the sample (i.e., water drying off from the drug). 10g of dried material was placed on a tarred evaporating dish and dried at 105ºC for 1 h. The dish was then cooled in a desiccator and reweighed. The process was repeated at 1-h intervals until a constant weight was obtained [17].

Preliminary phytochemical screening

Screening of the air-dried drug for various phytochemical constituents was carried out using standard methods [16-18,35].

Detection of alkaloids

- Mayer's test: 2 mL of the extract was treated with 2 mL of Mayer's reagent. Formation of cream yellow precipitate indicates alkaloids
- Dragendorff's test: 2 mL of the extract was treated with 2 mL of Dragendorff's reagent. Appearance of reddish-brown precipitate confirms alkaloids
- Hager's test: 2 mL of the filtrate was treated with 1–2 mL of Hager's reagent. Yellow crystalline precipitate indicates alkaloids
- Wagner's test: 2 mL of the filtrate was treated with 1–2 mL of Wagner's reagent. Reddish-brown precipitate shows the presence of alkaloids
- Tannic acid test: 2 mL extract was treated with 2 mL of tannic acid solution. Buff-colored precipitate confirms alkaloids.

Detection of carbohydrates

- Molisch's test: 1 mL of the test solution was mixed with 2 mL of Molisch reagent followed by the addition of 1 mL of concentrated sulfuric acid (H_2SO_4). Formation of a violet–purple ring indicates the presence of carbohydrates
- Fehling's test: Boiled 1 mL of test solution with 1 mL of Fehling's solution A and 1 mL of Fehling's solution B on a water bath. Brick-red precipitate confirms reducing sugars
- Benedict's test: Mixed 2 mL of the Benedict's reagent with 2 mL of the test solution. Boiled in a water bath. The appearance of an orange-red precipitate indicates reducing sugars.

Detection of glycosides

- Keller–Killiani Test: 2 mL extract was treated with 1 mL glacial acetic acid containing a drop of ferric chloride ($FeCl_3$) solution, followed by the addition of 1 mL H_2SO_4. Formation of a brown ring confirms the deoxy sugars of cardiac glycosides
- Legal's test: To 1 mL of extract, 1 mL of pyridine, and 1 mL of sodium nitroprusside were added and alkalinized with NaOH. Pink to blood-red color indicates cardiac glycosides
- Baljet test: 1 mL of the extract was reacted with sodium picrate solution. The development of an orange color indicates cardiac glycosides
- Borntrager's test: 2 mL extract was boiled with 1 mL dilute H_2SO_4 for 5 min, cooled, filtered, and shaken with chloroform. The chloroform layer was separated and treated with dilute ammonia. A pink to red coloration confirms anthraquinone glycosides
- Modified Borntrager's test: 2 mL extract was boiled with $FeCl_3$ solution and conc. HCl for 5 min, cooled, and extracted with chloroform. The chloroform layer was treated with dilute ammonia. Rose-pink color indicates cardiac glycosides
- Foam test: 2 mL extract was vigorously shaken with 5 mL distilled water for 2 min and left undisturbed for 10 min. Persistent froth for 10 min confirms saponin glycosides.

Detection of tannins and phenolic compounds

- $FeCl_3$ test: Mixed 2 mL of the test solution with few mL of 5% $FeCl_3$ solution. Blue-black or green coloration denotes tannins or phenolics
- Lead acetate test: Mixed 2 mL test solution with 1 mL of lead acetate solution. Appearance of white-yellow precipitate confirms tannins
- Acetic acid test: 1 mL of 1% acetic acid was added to 2 mL extract. Red coloration shows phenolic compounds
- Dilute nitric acid (HNO_3) test: Mixed 2 mL of the test solution with dilute HNO_3. Red coloration indicates phenolic compounds
- Potassium dichromate ($K_2Cr_2O_7$) test: 1 mL of 1% $K_2Cr_2O_7$ was added to 2 mL extract. Dark brown or green coloration indicates tannins.

Detection of flavonoids

- Zinc chloride ($ZnCl_2$) test: Few drops of $ZnCl_2$ solution were added to 2 mL extract. Yellow coloration indicates flavonoids.
- Shinoda test: 2 mL extract was added with magnesium ribbon fragments and 1–2 drops of conc. HCl. Pink-red coloration confirms flavonoids
- Lead acetate test: Mixed 2 mL of the test solution with 1 mL of 10% lead acetate. Yellow precipitate indicates flavonoids.

Detection of steroids and triterpenoids

- Liebermann Burchard Test: 2 mL extract was dissolved in chloroform, 2 mL acetic anhydride was added, followed by 1–2 drops of conc. H_2SO_4. Bluish-green color confirms steroids/triterpenoids.

Detection of mucilage

- Ruthenium red test: Few drops of Ruthenium red solution were added to a small portion of extract. Appearance of pink or red coloration indicates mucilage.

Detection of starch

- Iodine test: 2 drops of iodine solution were added to 2 mL of extract. Blue or black coloration confirms starch.

Fluorescence analysis

The powder was observed as such and after treatment with concentrated acids (H_2SO_4, HCl, HNO_3), their aqueous dilutions, and various organic solvents such as methanol, chloroform, petroleum ether, acetone, and sodium hydroxide solution. Each sample was observed under visible light, ultraviolet (UV) rays (254 nm and 365 nm). The color of the emitted radiation was observed and noted [19,20].

Volatile-oil determination

The volatile oil content of drug was determined by hydrodistillation using the Clevenger apparatus by distilling the drug with a mixture of water and glycerine. The distillate was collected in a graduated receiver tube, allowing automatic separation of the aqueous layer. The content of the volatile oil was directly expressed as a percentage v/w [17].

Thin-layer chromatography (TLC) profiling

The drug extract was subjected to TLC for phytochemical profiling. TLC plates were prepared by using silica gel G, air dried, and activated by heating in hot air oven at 105°C for 1 h. The extract was dissolved in respective solvents and applied on TLC plates by means of a capillary tube, a few cm above from the edge of plate. Plates were then developed in TLC chamber, previously saturated with different spot systems. Colorless components were detected using visualizing agent [21,22]. Rf values were calculated using the formula:

$$\text{Rf value} = \frac{\text{Distance travelled by the solute}}{\text{extract Distande travelled by solventn}} \times 100$$

Bitterness value

Preparation of stock diluted quinine hydrochloride solutions

0.1 g of quinine hydrochloride was dissolved in sufficient safe drinking water to produce 100 mL. Further, dilute 5 mL of this solution to 500 mL with safe drinking water. This stock solution of quinine hydrochloride contains 0.01 mg/mL. 9 test tubes were used for the serial dilution for the initial test.

Preparation of stock and diluted solutions for plant material

The solution was prepared as specified in the test procedure for the given plant material. 10 test tubes were used for the serial dilution for the test as indicated in Tables 1 and 2.

Method

After rinsing the mouth with safe drinking water, 10 mL of the most diluted solution was tasted by swirling it in the mouth near the base of tongue for 30 sec. If no bitter sensation was felt in the mouth, after 30 s, the solution was spat out and waited for 1 min to ascertain whether this was due to delayed sensitivity. Then rinsed with safe drinking water and the next higher concentration was tested after 10 min. The threshold bitter concentration refers to the lowest concentration at which bitterness persisted for 30 s. Between tests, the mouth was rinsed thoroughly until no residual taste remained.

The bitterness value (units/g) was calculated using the formula:

$$\text{Bitterness value} = \frac{2000 \times c}{a \times b}$$

where,

a = concentration of stock solution (mg/mL)

b = volume (mL) of test solution producing threshold bitterness,

c = amount (mg) of quinine hydrochloride producing equivalent bitterness [17].

Swelling index

1 g of the plant material was placed in a 25 mL glass-stoppered measuring cylinder, followed by addition of 25 mL of distilled water. The resulting mixture was shaken thoroughly every 10 min for 1 h, then allowed to stand for 3 h at room temperature. The final volume occupied by the swollen material was measured, and the swelling index was expressed in mL/g of the sample. The mean value of three determinations was recorded [17,20].

Foaming index

About 1 g of coarse powder was weighed accurately and transferred to a 500 mL conical flask containing 100 mL of boiling water and maintained at moderate boiling for 30 min. It was cooled and volume was made up to 100 mL. The decoction was poured into 10 mL stoppered test-tubes in successive portions of 1 mL, 2 mL, 3 mL, etc. and adjusted the volume of liquid in each tube with water to 10 mL. Tubes were shaken lengthwise for 15 s (two shakes per second) and allowed to stand for 15 min and the height of the foam was measured [17,20].

The foaming index (FI) was calculated using the formula:

$$\text{Foaming index} = \frac{1000}{a}$$

where,

a = the volume (mL) of decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

Hemolytic activity

25 mL of blood was collected from 10 rats immediately after sacrifice and mixed thoroughly. This blood was centrifuged until complete sedimentation of red blood cells (RBCs) occurred. The supernatant was discarded, and the packed RBCs were washed and suspended in 150 mL of normal saline to obtain the RBC suspension. After this, 5 test tubes were taken for each herb for the hemolysis test, and 5 mL of RBC suspension was transferred to all five test tubes and then treated with (a.) 0.5 mL of normal saline, (b.) 0.5 mL of 1% drug solution, and (c.) 0.5 mL of 1% test solution [17].

AF evaluation

Sample preparation

AFs were extracted from 50 g of sample using methanol: deionized water (80:20 v/v) and mixed for 30 min at 120 rpm using a shaker. 20 mL of filtered solution was diluted 4:1 with Phosphate Buffer Solution (pH 7.4). The diluent was centrifuged at 3400 rpm for 15 min, and filtered using a nylon membrane filter (pore size, 0.45 µm).

Table 1: Serial dilution for the initial test to determine bitterness value (standard)[1]

Solution used	Tube Numbers								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Drinking water	5.8	5.6	5.4	5.2	5.0	4.8	4.6	4.4	4.2
Stock solution quinine HCl (mL)	4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8
Quinine HCl in 10 mL solution (mg/mL)	0.042	0.044	0.046	0.048	0.050	0.052	0.054	0.056	0.058

Table 2: Serial dilution for the second test to determine bitterness value (Test sample)[2]

Solution used	Test tube numbers									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Drinking water (mL)	9.0	8.0	7.0	6.0	5.0	4.0	3.0	2.0	1.0	0.0
Stock sol. of herbal drug examined (mL)	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0

1 Table 1 shows the serial dilution of quinine hydrochloride standard used to determine the threshold bitterness concentration

2 Table 2 shows the serial dilution of the plant extract stock solution used to determine the threshold volume.

Affinity chromatography

50 mL clarified extract was passed through an Aflaclean™ (LCTech, Germany) immunoaffinity column at flow rate of 1 mL/min. After the AF molecules were bound, the column was washed with deionized water to remove unbound materials. AFs were eluted using 2 mL of methanol and evaporated at 50 °C under nitrogen. The sample was injected into the high-performance liquid chromatography (HPLC) for quantitative determination of AF.

HPLC analysis of AFs

AF quantities of standards and samples were determined using HPLC with fluorescent detection. Separation was achieved on a C18 column using water: methanol:acetonitrile (60:30:15, v/v/v) at a 1.2 mL/min flow rate. AFs were detected at excitation wavelength 365 nm and emission wavelength 440 nm, with retention between 8 and 17 min for a total run time of 25 min.

Microbial contamination determination

1 g of powdered drug was suspended in 50 mL of sterile distilled water. The suspension was shaken for a sufficient period of time so as to allow maximum mixing. The suspension was filtered through a sterile membrane, and the filtrate was used as the stock solution. Series dilutions (1:1, 1:10, 1:100) were prepared and 1 mL of each was separately inoculated (with the spreading method) on a nutrient agar medium (Table 3) and incubated at 37°C for 24 h. After 24 h, the Petri plates with clearly visible colonies were taken and number of colonies was determined using colony counter. Specific pathogenic organisms, including *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, were assessed using selective media as prescribed standards [23].

The medium was autoclaved at 15 lbs per square inch pressure at 121°C [17].

Atomic absorption spectrophotometric (AAS) analysis of heavy metals

Heavy metal analysis was performed using Schimadzu AA-6300. Measurements were made using a hollow electron discharge lamp for cadmium (Cd), lead (Pb), arsenic (As), Cd, and mercury (Hg) at wavelengths of 228.80 nm, 283.31 nm, 193.70 nm, and 253.7 nm, respectively.

Instrument conditions

AAS operating parameters were optimized for each metal. For Cd and Pb, acetylene–air was used as the fuel oxidant system at 2.5:15.0 L/min. Argon–air at 5.5:15.0 L/min was found robust for the separation of As and Hg.

Standard solution

Standard stock solution of 1000 mg/L in 0.1M HCl for As and 0.5M HNO_3 for Cd, Pb, and Hg was used. From the stock solution, different standard dilutions of 5.0, 10, 15, 20, and 25 ppm were prepared using the same respective acids.

Digestion of sample

About 1–2 g of drug sample was incinerated in a muffle furnace at 600 °C for 2 h. The ash was cooled and treated with 0.1 M HCl (for As) or 0.5 M HNO_3 (for Cd, Pb, Hg). Contents were then transferred to 25 mL volumetric flask, followed by repeated washing until all the contents were removed from the crucible. Heating on water bath for 15–20 min was done to complete digestion. The mixture was filtered and made up to 25 mL with the respective acid. Blanks, standards and digested samples were aspirated using the optimized AAS parameters [24,25].

Table 3: Composition of nutrient agar media

Ingredients	Compositions
Agar	15.0%
Peptic digest of animal tissue	5.0%
Sodium chloride	5.0%
Beef extract	1.5%
Yeast extract	1.5%
pH	7.4±0.2at 25ºC
Distilled water	1000 mL

Determination of tannin contents by titrimetric method

Total tannins were quantified using the standard indigosulfuric acid potassium permanganate ($KMnO_4$) titration method. 2–3 g of drug powder was transferred to a 250 mL glass stoppered glass flask and cold macerated with 100 mL of distilled water overnight. The mixture was filtered through Whatman No. 1 paper, discarding the first 20 mL.

10 mL of the filtrate was added with 750 mL of water and 25 mL of indigo sulfuric acid solution in 1 l conical flask. It was than titrated with 0.1 N $KMnO_4$ solution. The flask was shaken vigorously till a golden yellow endpoint was reached (T2). A blank determination (T1) was also performed. $KMnO_4$ was standardized using sodium oxalate, following pharmacopeial norms. Indigo sulfuric acid solution was prepared by dissolving 1.2 g of Indigo carmine in 10 mL concentrated sulfuric acid and diluting to 200 mL of distilled water [26].

Each milliliter of 0.1 N $KMnO_4$ corresponds to 0.004157 g tannins.

Calculation:

$$\text{Quantity of total tannins} = \frac{(T2 - T1) \times N \times 0.004157 \times 1000}{W \times 0.1}$$

where,
T2 = sample titer,
T1 = blank titer,
N = actual normality of $KMnO_4$,
W = weight of sample (g)

RESULTS AND DISCUSSION

Macroscopic and organoleptic evaluation

The examined root samples of *W. somnifera* Dunal, sourced from the Northern Indian region, displayed a cylindrical, tapering morphology with a grayish-yellow exterior and fibrous fracture (Fig. 1). The odor was characteristic and the taste was distinctly bitter and acrid, typical of the genuine crude drug. These diagnostics were consistent with standard pharmacognostical monographs, confirming authentic plant identity and purity suitable for developing baseline standards. The results were presented in Table 4.

Fig. 1: Macroscopic appearance of the dried roots of *Withania somnifera* Dunal used in this study

Microscopic characteristics

The transverse section exhibited well-defined tissue zones with a multilayered cork, parenchymatous cortex, and a continuous ring of secondary xylem separated by medullary rays. Vessels with bordered pits, lignified fibers, and abundant starch grains were prominent features, serving as distinct micro-anatomical markers. Powder microscopy revealed diagnostic fragments such as xylem vessels, tracheids, cork cells, starch grains, and fibers. These structural details collectively authenticate the regional identity and support inclusion in quality assessment protocols for Northern Indian *W. somnifera*. The results were presented in Fig. 2.

Table 4: Organoleptic characters of *Withania somnifera* Dunal root[3]

S. No.	Parameters	Root
1.	Color	Grayish yellow
2.	Odor	Characteristic
3.	Taste	Bitter and acrid
4.	Size	3–5 cm long, 2 mm thick
5.	Shape	Cylindrical
6.	Surface	Plain
7.	Fracture	Slightly fibrous, short, and uneven

Physicochemical parameters

Physicochemical constants were established to define the quality and purity standards of the Northern Indian root samples. Foreign matter (0.19±0.02%) and moisture content (4.46±0.04%) were within permissible limits, confirming the absence of external contamination and low hygroscopic nature. Foaming index (<100), swelling index (3.9±0.3 mL/g), and bitterness value (2.9±0.4 units/g) further validate the intrinsic characteristics of the root, forming reproducible parameters for future pharmacopeial inclusion. The ash values, total (6.6±0.1%), acid-insoluble (0.56±0.04%), and water-soluble (1.7±0.2%) reflect appropriate inorganic content for this species. The results were presented in Table 5.

Extractive values

Extractive profiles (Table 6) revealed a marked predominance of polar phytoconstituents. Hydroalcoholic hot extracts (13.46±0.21%)

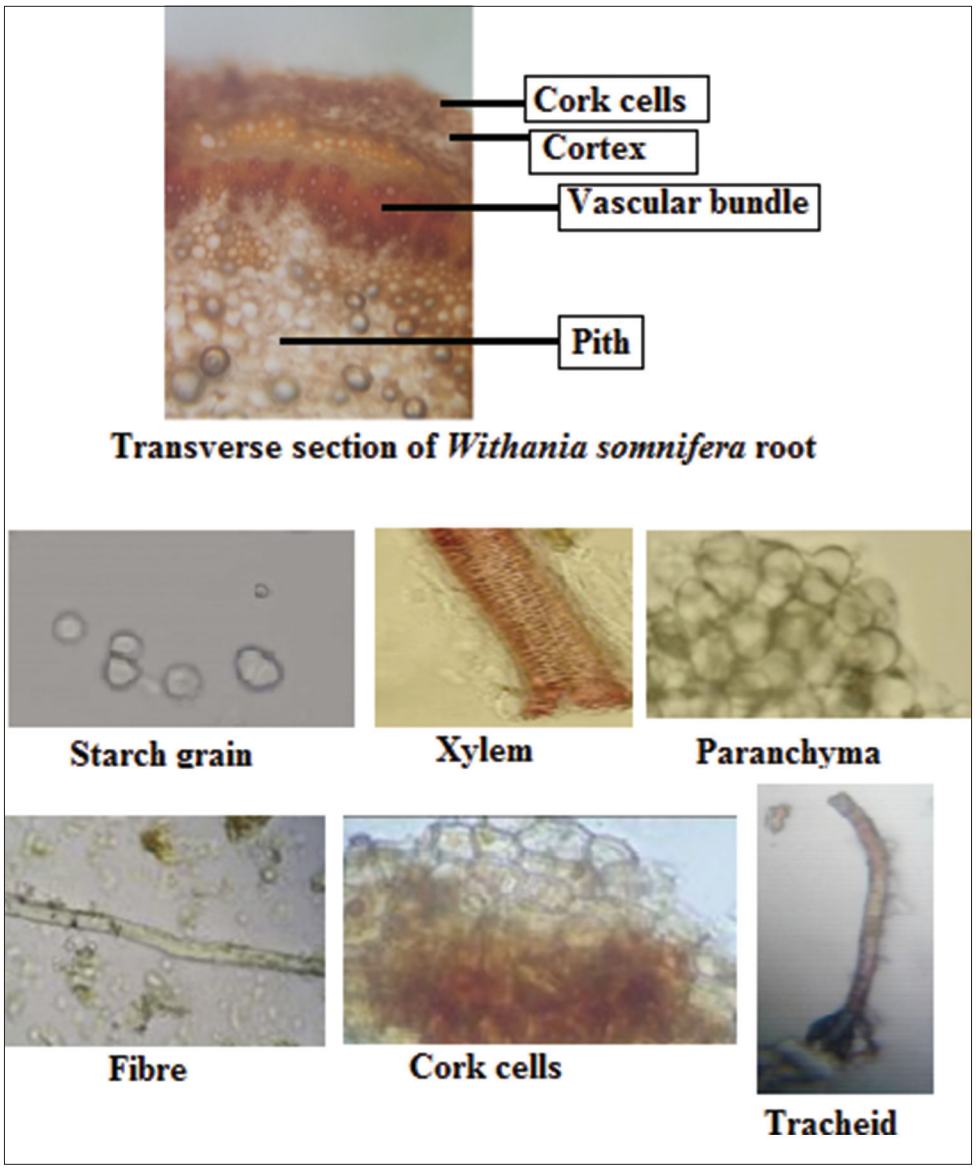

Fig. 2: Microscopic characteristics of *Withania somnifera root*

3. Observations were made by three independent evaluators

Table 5: Physiochemical parameters of *Withania somnifera* Dunal root[4]

S. No.	Parameters	Sample I	Sample II	Sample III	Average results
1.	Foreign matters (%)	0.19	0.21	0.17	0.19±0.02
2.	Moisture content (%)	4.46	4.5	4.42	4.46±0.04
3.	Foaming index	<100	<100	<100	<100
4.	Swelling index (mL/g)	3.9	4.2	3.6	3.9±0.3
5.	Bitterness value (units/g)	2.9	2.5	3.3	2.9±0.4
6.	Ash values (%)				
6.1.	Total ash	6.6	6.5	6.7	6.6±0.1
6.2.	Acid-insoluble ash	0.56	0.6	0.52	0.56±0.04
6.3.	Water-soluble ash	1.7	1.9	1.5	1.7±0.2

Table 6: Extractive values of *Withania somnifera* Dunal root in different solvent system[5]

S. No.	Parameters	Sample I % w/w	Sample II % w/w	Sample III % w/w	Average results % w/w
1.	Cold extracts				
1.1.	Petroleum ether (60–70°C)	1.8	2	1.6	1.8±0.2
1.2.	Chloroform	2.1	2.6	1.6	2.1±0.5
1.3.	Alcohol	4.2	4.6	3.8	4.2±0.4
1.4.	Hydro-alcoholic	6.2	6.8	5.6	6.2±0.6
1.5.	Aqueous	9.7	9.6	9.8	9.7±0.1
2.	Hot extracts				
2.1.	Petroleum ether (60–70°C)	2.8	3	2.6	2.8±0.2
2.2.	Chloroform	3.0	3.2	3.1	3.1±0.1
2.3.	Alcohol	8.8	8.6	9.0	8.8±0.2
2.4.	Hydro-alcoholic	13.46	13.25	13.67	13.46±0.21
2.5.	Aqueous	12.16	12.86	11.46	12.16±0.70
3.	Successive extracts				
3.1.	Petroleum ether (60–70°C)	3.46	3.98	2.94	3.46±0.52
3.2.	Chloroform	5.3	5.9	5.6	5.3±0.3
3.3.	Alcohol	12.49	6.87	5.81	8.39±3.59
3.4.	Hydro-alcoholic	19.79	19.55	19.44	19.59±0.18
3.5.	Aqueous	16.66	16.98	16.32	16.66±0.33

and hydroalcoholic successive extracts (19.59±0.18%) exhibited the highest recoveries, followed closely by aqueous extracts, indicating a strong affinity of *W. somnifera* root for polar solvents. Alcoholic extracts showed moderate yields, while chloroform and petroleum ether extracts produced comparatively lower values. The solvent-dependent yield pattern indicates chemical richness in polar metabolites such as withanolides, glycosides, and flavonoids, establishing a regional baseline extractive standard for *W. somnifera*.

Preliminary phytochemical screening

Qualitative analysis confirmed the presence of alkaloids, flavonoids, phenols, saponins, glycosides, mucilage, and carbohydrates, particularly in hydroalcoholic and aqueous extracts. Non-polar extracts demonstrated limited activity. This phytochemical diversity validates the plant's therapeutic reputation and provides confirmatory evidence for chemical standardization. The predominance of secondary metabolites in polar fractions supports the bioactive potential unique to Northern Indian material. The results were presented in Table 7.

pH evaluation

The pH of 1% and 10% aqueous solutions was found to be 6.93 and 6.95, respectively (Table 8), indicating neutral to slightly acidic nature. Such pH characteristics are desirable for maintaining stability of aqueous herbal preparations and ensuring compatibility with oral dosage forms, where near-neutral pH minimizes degradation and improves palatability. These findings confirm physicochemical uniformity in the regional sample.

Fluorescence analysis

The powdered drug displayed distinct fluorescence characteristics when treated with different reagents and observed under visible and UV (254 nm, 366 nm) light. Color variations from whitish-gray to dark brown reflect the presence of diverse phytoconstituents capable of forming chromogenic complexes upon reagent interaction. Thus, fluorescence analysis provides a rapid, non-destructive diagnostic tool for preliminary authentication and standardization purposes. The results were presented in Table 9.

Volatile oil determination

The root sample showed no detectable volatile oil content during hydrodistillation, confirming that volatile constituents are not characteristic of the Northern Indian chemotype. This observation reinforces the view that the pharmacological activity of *W. somnifera* is primarily due to non-volatile withanolides, alkaloids, and other polar metabolites rather than essential oils.

TLC profiling

TLC chromatograms of hydroalcoholic and alcoholic extracts displayed distinct multi-spot profiles with Rf values ranging from 0.12 to 0.90, while chloroform and petroleum ether extracts showed fewer spots. The complex chromatographic pattern serves as a reference fingerprint, essential for authenticity validation and interregional comparison. These data contribute to establishing a standard TLC profile for *W. somnifera* roots of Northern Indian origin for future pharmacognostical and quality control applications. The results were presented in Table10 and Fig. 3.

4. Sample I, II, and III represent individual determinations. Values are expressed as Mean ± Standard Deviation (n = 3).Foaming index is reported as a qualitative pharmacopoeial parameter and therefore SD is not applicable
5. Sample I, II, and III represent individual determinations. Values are expressed as Mean ± Standard Deviation (n = 3).

Table 7: Phytochemical screening of *Withania somnifera* Dunal root[6]

Constituents	P	C	EtOH	Aq: EtOH	Aq
Alkaloids	-	+	+	+	+
Carbohydrates					
Molisch test	-	-	+	+	+
Fehling's test	-	-	+	+	+
Benedicts test	-	-	+	+	+
Glycosides					
Cardiac glycoside					
Keller Killiani test	-	-	-	-	-
Legal test	-	-	-	-	-
Baljet test	-	-	+	+	+
Anthraquinone glycoside					
Borntrager's test	-	-	-	-	-
Mod. Bontrager	-	-	+	+	+
Saponin glycoside	-	+	+	+	+
Tannins and phenols					
5% ferric chloride	-	+	+	+	+
Lead acetate	-	+	+	+	+
Acetic acid	_	+	+	+	+
Dilute nitric acid	-	+	+	+	+
Potassium dichromate	-	+	+	+	+
Flavonoids					
Zinc chloride test	-	+	+	+	+
Shinoda test	-	+	+	+	+
Lead acetate test	-	+	+	+	+
Steroids and triterpenoids					
Liebermann test	+	+	+	+	+
Mucilage					
Ruthenium red test	-	-	+	+	+
Starch					
Iodine test	-	-	+	+	+

Table 8: Evaluation of pH of *Withania somnifera D*unal root solution

S. No.	Solution	pH
1.	1% Solution	6.93
2.	10% Solution	6.95

Hemolytic activity

The RBC suspension assay indicated that the extract of *W.somnifera* root produced no significant hemolysis compared to the positive control. The absence of hemolysis at the tested concentrations suggests that the saponin-rich root extract is safe with respect to erythrocyte membrane integrity. As hemolysis of membrane-active compounds is often concentration-dependent [27,28], these findings further support the extract's suitability for pharmaceutical use and its safety profile for Northern Indian material.

AF evaluation

The root sample of *W. somnifera* showed non-detectable levels of AFB_1, AFB_2, and AFG_1, and G_2 was detected at <0.3 ppb (Table 11). This result complies with both Chinese Pharmacopoeia limits (5 µg/kg for AFB_1 and 10 µg/kg for total AFs) and other international guidelines for herbal materials [29]. Previous surveys show that up to 70% of herbal products may contain AFs when handling and storage are poor [30]. The very low levels in the Northern Indian root material suggest good sourcing and storage practices, supporting its safety and suitability for therapeutic use.

Microbial contamination determination

The root sample showed a total aerobic count of 3486 cfu/g and a fungal load of 2798 cfu/g, both within internationally accepted limits for dried herbal materials (≤10^5 cfu/g for bacteria; ≤10^4 cfu/g for fungi) (Table 12). Pathogens including *E. coli*, *Salmonella* spp., *P. aeruginosa*, and *S. aureus* were absent, confirming microbial safety. These findings indicate that studied *W. somnifera* roots maintain acceptable hygienic quality, reflecting proper post-harvest handling and storage practices consistent with standardized herbal raw material requirements [23].

AAS analysis of heavy metals

AAS evaluation showed that the concentrations of Pb, Cd, As, and Hg were well below the permissible limits prescribed for herbal raw materials (Table 13). These low levels suggest minimal environmental or soil-related contamination and confirm that the Northern Indian material complies with established safety standards as roots typically

Table 9: Evaluation of fluorescence analysis of *Withania somnifera* Dunal root

S. No.	Reagent	Day light	254 nm	366 nm
1.	Drug powder as such	Whitish-gray	Light gray	Dark gray
2.	Drug+Conc. HCl	Dark gray	Yellowish gray	Dark brown
3.	Drug+Conc. HCl+Distilled water	Dark gray	Dark gray	Brown
4.	Drug+Conc. HNO_3	Lightgray	Brownish	Black
5.	Drug+Conc. HNO_3+Distilled water	Light brown	Brown	Black
6.	Drug+Methanol	Yellowish gray	Yellowish brown	Black
7.	Drug+Chloroform	Yellowish gray	Yellowish brown	Light brown
8.	Drug+Petroleum ether	Yellowish gray	Light brown	Brown

HCl: Hydrochloric acid, HNO_3: Nitric acid, Conc.: Concentration

Table 10: Evaluation of thin-layer chromatography profile of different extracts of *Withania somnifera* Dunal[7]

Extracts	Hydro-alcoholic	Alcoholic	Aqueous	Chloroform
Solvent system	P: C (7:93)	P: C: M (7:3:4)	P: C: M (6.5:3:0.5)	P: C: M (4.5:5.5:2)
Spots observed in ultraviolet	6	3	4	3
Spots observed in iodine	8	7	6	7
retention factor value observed	0.90, 0.71, 0.61, 0.48, 0.32, 0.25, 0.21, 0.12	0.24, 0.33, 0.47, 0.68, 0.78, 0.87, 0.92	0.3, 0.53, 0.73, 0.76, 0.84, 0.93	0.1, 0.16, 0.4, 0.52, 0.64, 0.76, 0.90

6. + = Present; – = Absent, P = Petroleum ether, C = Chloroform, EtOH = Ethanol, Aq: EtOH = Aqueous Ethanol, Aq = Aqueous solution
Mayer's, Dragendorff's, Hager's and Wagner's tests indicate the presence of alkaloids through formation of characteristic precipitates. Molisch, Fehling's, and Benedict's tests confirm carbohydrates and reducing sugars based on colour complex formation. Keller–Killiani, Legal, and Baljet tests detect cardiac glycosides via deoxy-sugar or lactone ring reactions, while Borntrager's and modified Borntrager's tests identify anthraquinone glycosides through anthracene derivatives. Foam test confirms saponins due to their surfactant nature. Ferric chloride, lead acetate, acetic acid, dilute HNO_3, and potassium dichromate tests indicate tannins and phenolic compounds by forming coloured complexes. Zinc chloride, Shinoda, and lead acetate tests detect flavonoids based on flavone and flavonol chromophore reactions. The LibermannBurchard test confirms steroids and triterpenoids by producing a greenbluish chromogen specific to sterol/triterpenoid nuclei, the structural core of withanolides. Ruthenium red test detects mucilage by staining acidic polysaccharides, while the iodine test identifies starch through the characteristic blueblack starchiodine complex
7. P = Petroleum ether; C = Chloroform; M = Methanol. All solvent ratios are expressed in v/v.

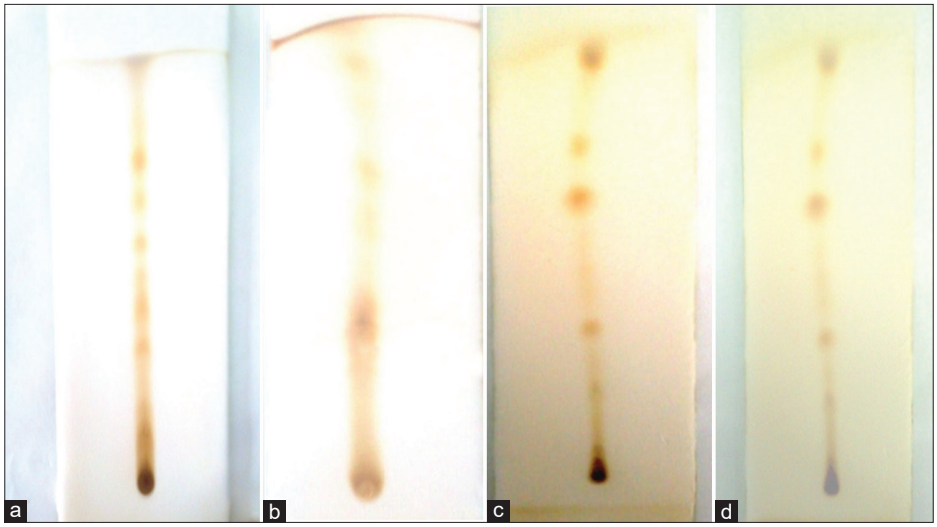

Fig. 3: Thin-layer chromatographic profiles of different extracts of *Withania somnifera* Dunal root[8]. (a) Hydro-alcoholic extract, (b) Alcoholic extract, (c) Aqueous extract, (d) Chloroform extract

Table 11: Determination of Aflatoxins in *Withania somnifera* Dunal root

S. No.	Aflatoxins	Detection Limit	Observation (In ppb)		
			Sample-1	Sample-2	Sample-3
1.	B_1	0.3 ppb	Not detected	Not detected	Not detected
2.	B_2	0.3 ppb	Not detected	Not detected	Not detected
3.	G_1	0.3 ppb	Not detected	Not detected	Not detected
4.	G_2	0.3 ppb	Less than 0.3	Less than 0.3	Less than 0.3

Table 12: Estimation of total viable aerobic count and microbial load in *Withania somnifera* Dunal root

Observations of viable aerobic count			
Total bacterial count	3486 cfu/g		
Total fungal count	2798 cfu/g		
Observations of microbial load			
Pseudomonas aeroginosa	***Staphylococcus aureus***	***Salmomella***	***Escherichia coli***
Absent/g	Absent/g	Absent/g	Absent/g

Table 13: Evaluation of heavy metals in *Withania somnifera* Dunal root

S. No.	Heavy metals	Concentration (ppm)	Mean absorbance	Actual concentration (ppm)
1.	Lead	0.137	0.0042	0.204
2.	Cadmium	0.014	0.0065	0.150
3.	Arsenic	0.070	0.0004	0.086
4.	Mercury	0.002	0.0002	0.0350

accumulate higher amounts of trace metals than aerial parts, adherence to international limits is crucial. The present findings therefore support the suitability of this sample for medicinal use and align with WHO and Indian Pharmacopoeia recommendations for heavy-metal safety in herbal drugs [9,26].

Table 14: Determination of tannin contents

S. No.	Burette reading ($KMnO_4$)		Volume of $KMnO_4$ Consumed (mL)
	Initial	Final	
1.	0.0	5.9	5.9
2.	5.9	11.8	5.9
3.	11.8	17.8	6.0
	Average reading T2		17.8/3=5.933 mL
	Blank reading T1		0.2 mL

Weight of herb=2.8242 g, Quantity of total tannins=8.1760 mg/g, Normality of ($KMnO_4$)=0.0968. $KMnO_4$: Potassium permanganate

Tannin content

The tannin content of the studied *W. somnifera* root sample was found to be 8.176 mg/g using the titrimetric method (Table 14). Quantification of tannins is an essential pharmacognostical parameter as they contribute to antioxidant potential, astringency, and overall phytochemical stability of herbal materials. In addition, variation in tannin levels may reflect differences in soil composition or processing conditions which can influence polyphenolic content in root-based crude drugs [31,32]. The value obtained in the present Northern Indian sample reflects a moderate phenolic profile consistent with typical *W. somnifera* roots and supports its phytochemical authenticity.

CONCLUSION

The present investigation provides a comprehensive and systematically generated pharmacognostical, physicochemical, phytochemical, chromatographic, and safety profile of *W. somnifera* Dunal roots collected from Northern India. This study represents one of the few detailed attempts to establish region-specific quality parameters for

8. All plates were visualized under UV light at 254 nm and 366 nm, followed by exposure to iodine vapour for non-chromophoric constituents.
(a) Hydro-alcoholic extract developed in Petroleum ether : Chloroform (7 : 93 v/v).
(b) Alcoholic extract developed in Petroleum ether : Chloroform : Methanol (7 : 3 : 4 v/v/v).
(c) Aqueous extract developed in Petroleum ether : Chloroform : Methanol (6.5 : 3 : 0.5 v/v/v).
(d) Chloroform extract developed in Petroleum ether : Chloroform : Methanol (4.5 : 5.5 : 2 v/v/v).

this widely used medicinal plant, addressing observed variations reported across different geographical sources.

The comparatively high hydroalcoholic extractive values and the complex, reproducible TLC fingerprint obtained from polar extracts emerged as distinguishing features of the Northern Indian chemotype, indicating a predominance of polar secondary metabolites. These parameters, along with defined ash values, moisture content, and diagnostic microscopic characters can serve as reliable markers for authentication, quality control, and detection of adulteration in raw materials and herbal formulations.

Safety evaluation confirmed that heavy metals, AFs, microbial load, and pesticide residues were within permissible limits, supporting the safe use of the studied material in traditional and commercial preparations. Although quantitative estimation of key marker compounds such as withaferin A and withanolide A was not performed in the present study. This limitation highlights the need for future investigations employing validated chromatographic techniques for global standardization.

Overall, the generated dataset contributes valuable baseline information for the development of pharmacopeial monographs and quality assurance protocols for *W. somnifera* derived from Northern India, and provides a scientific foundation for its consistent and safe utilization in herbal medicine.

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AUTHORS' CONTRIBUTIONS

All authors contributed to the conception and design of the study, acquisition and analysis of data, and interpretation of the findings. All authors were involved in drafting or revising the manuscript for the final version for publication.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this research work. All experimental procedures were conducted in accordance with institutional and ethical guidelines for the handling of biological materials.

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