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Ashwagandha Root Laboratory Guidance Document

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1. Purpose

Ashwagandha (*Withania somnifera*) is commonly used in dietary supplements, including powders and extracts, some of which are standardized for withanosides and withanolides. Despite its recent domestication and cultivation, reports of adulteration have been published. Therefore, ensuring the purity and efficacy of ashwagandha-based ingredients and finished products remains crucial. Instances of undeclared admixture of ashwagandha aerial parts or substitution with other species for cost-savings highlight the need for rigorous identity testing.[1] This comprehensive review, presented as a Laboratory Guidance Document, aims to assist readers in understanding the nomenclature, morphology, and anatomy of *W. somnifera*, its congeneric taxa, and potential adulterants in ashwagandha root powders and root extracts.

2. Scope

This Laboratory Guidance Document evaluates analytical methods for *W. somnifera* dried root material (Figure 1) and root extracts, but it does not exempt laboratory personnel from the need to verify method performance in their own laboratories. Adherence to accepted protocols is required, in line with regulations such as 21 CFR Part 111 (Current Good Manufacturing Practices [cGMPs] in Manufacturing, Packaging, Labeling, or Holding Operations for Dietary Supplements, in the US Code of Federal Regulations [CFR]) and 21 CFR Part 117 (cGMP, Hazard Analysis, and Risk-Based Preventive Controls for Human Food, established under the Food Safety Modernization Act [FSMA]), and by AOAC International, International Organization for Standardization (ISO), World Health Organization (WHO), and the International Council for Harmonisation (ICH). The following pharmacopeia guides, monographs, and compendia have also been used for the interpretation of this document, viz. American Herbal Pharmacopoeia (AHP), Ayurvedic Pharmacopoeia of India (API), British Pharmacopoeia (BP), European Pharmacopoeia (EP), Indian Pharmacopoeia (IP), Japanese Pharmacopoeia (JP), Pharmacopoeia of the People's Republic of China (PPRC), and United States Pharmacopeia (USP).



Figure 1. Dried roots of ashwagandha (*Withania somnifera*)

3. Common and Scientific Names

3.1 Common names:

Arabic: samu alfarakh/sim el firakh (سم الفرخ), murjan/morgaan (مرجان)[2]

Chinese: shuì qié shu (睡茄), nán fēi zuì qié (南非醉茄)[3]

English: ashwagandha, Indian ginseng, winter cherry[4]

French: ashwagandha, cerise d'hiver, coqueret somnifère, ginseng Indien[5]

German: Ashwagandha, Schlafbeere, Winterkirsche[5]

Hindi: asgandh, ashwagandha (अश्वगंधा)[6]

Italian: ashwagandha, ciliegia d'inverno, ginseng indiano[5]

Spanish: ashwagandha, cereza de invierno, ginseng indio[5]

Swedish: ashwagonda, giftiga krusbär, Indisk ginseng, vinterkörsbär[5]

Sanskrit: ashvagandha (अश्वगन्धा)[4]

3.2 Botanical family: Solanaceae

3.3 Botanical name:

Withania somnifera (L.) Dunal

Basionym: *Physalis somnifera* L.



Figure 2. *Withania coagulans* (left and center) and *Withania somnifera* (right)

3.4 Synonyms:

Larnax morrisonii (Dunal) Miers; *Physalis alpini* J.Jacq.; *Physalis flexuosa* L.; *Withania arborescens* Dunal; *Withania morisonii* Dunal.; [7] *Physalis alpini* J.Jacq.; *Physalis flexuosa* L.; *Physalis scariosa* Webb & Berthel.; *Withania arborescens* Dunal; *Withania chevalieri* A.E. Gonç.; *Withania kansuensis* Kuang & A.M.Lu; *Withania microphysalis* Suess.; *Withania morisonii* Dunal; *Withania mucronata* Chiov.; *Withania obtusifolia* Täckh.; *Withania sicula* Lojac.[7]

3.5 Classification and distribution of the genus *Withania*: Recent phylogenetic work using four genomic regions focused on assessing the taxonomy of several taxa in the tribe *Physalideae* (syn. *Physaleae*) in the family Solanaceae, including six species of *Withania*.[8,9] The sampled species formed two well-supported, geographically distinct clades, except *W. adpressa* Coss., which was grouped within *Physalis* subg. *Rydbergis*. The first clade includes *W. somnifera* (Figure 2), *W. riebeckii* Balf. f., and *W. coagulans* (Stocks) Dunal (Figure 2), found from Iran to India, Socotra, and widely across Africa and southern Europe, and clusters closely with members of the genus *Nothocestrum*. The second clade, limited to species of southwestern Europe, northwestern Africa, and the Canary Islands, includes *W. aristata* (Aiton) Pauquy and *W. frutescens* (L.) Pauquy,[9] and is closely related to the genus *Aureliana*. Though Hepper noted their distinct calyx and androecium, this study is the first to confirm their phylogenetic divergence.[10]

4. Plant Description, Phenology, Habitat, Distribution, Comparison with Congeneric Taxa, and Cultivars

4.1 Description: Ashwagandha is a perennial, branched, erect undershrub that grows 70–150 cm high (Figure 3a). It is typically harvested for medicinal use five to six months after initial planting. The roots are mainly fusiform (Figure 1) with lateral fibrous branches. Stems are terete and usually covered with a mealy, stellate hoary tomentum. The leaves are ovate to elliptic, 5-10 x 2.5-5 cm, with an acute to obtuse apex and margins that range from entire to repand. In distal leaves, the base is cuneate and short-petiolate, while in proximal leaves, it is attenuate and long-petiolate. The petioles are 2-5 cm long. Leaf surfaces are sparsely stellate and pubescent, and they are discolorous — dark green on the adaxial surface and pale green and glaucescent on the abaxial surface — with conspicuous secondary veins in 5-6 pairs. The flowers in leaf axils are greenish or vivid-yellow and typically occur in clusters of 3-5. They are sessile to pedicellate (5-10 mm) with fasciculate cymes (Figure 3b). The calyx is urceolate to cupuliform (Figure 3c), about 10 mm long, pale green, densely stellate, glaucous tomentose, with ca. 5 mm long, linear-elliptic, acute, deflexed teeth. The corolla is ca. 10 mm long, adherent to the calyx tube, and the lobes are lanceolate, acute, deflexed, pubescent on the outside, and yellowish green (Figure 3c, d). The stamen filament is linear, hyaline, ca. 4 mm long, and the anthers are broadly

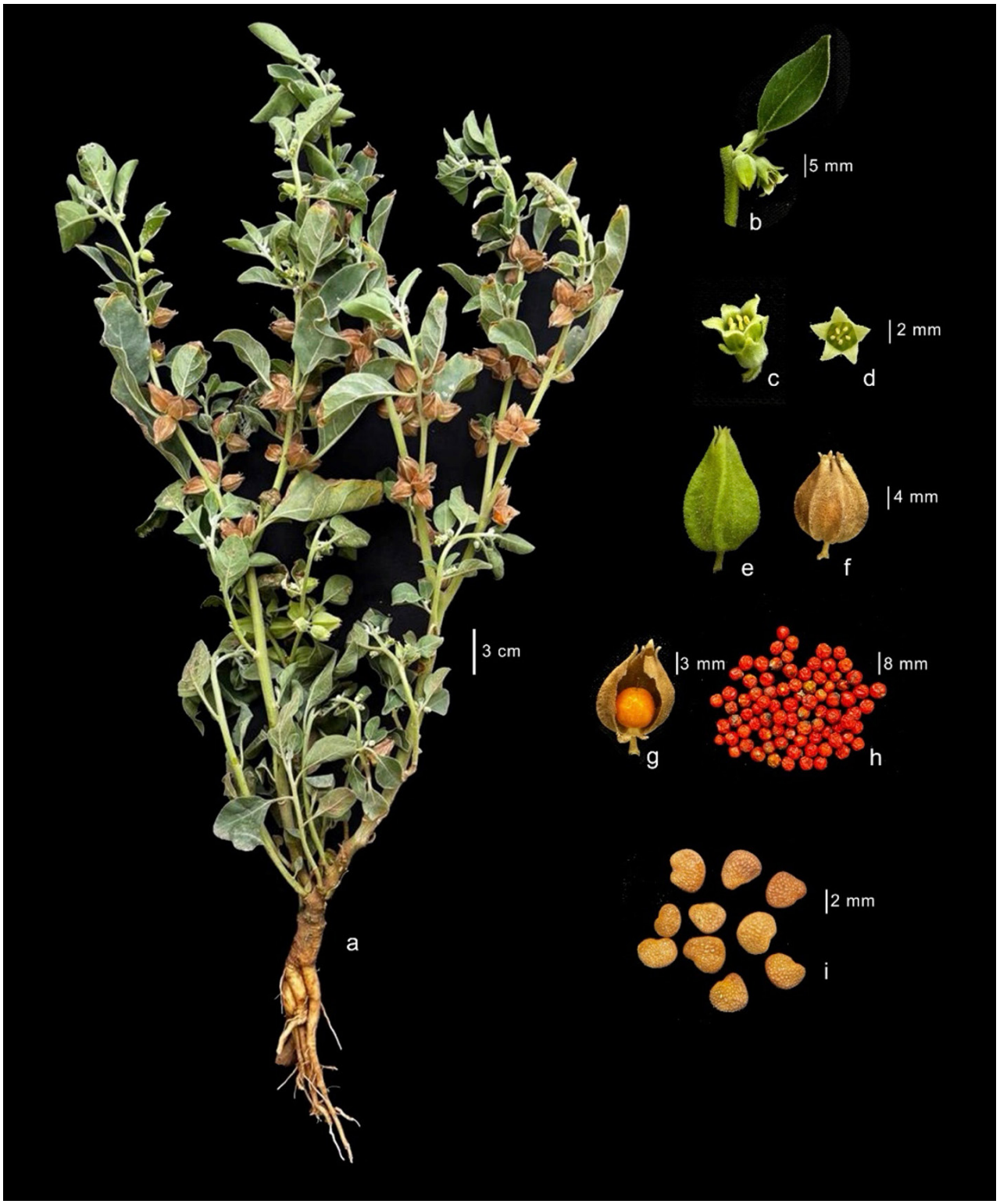


Figure 3. Morphology of *Withania somnifera*. a. Whole plant. b. Axillary fasciculate cymes, usually with 3-5, sessile to pedicellate (5-10 mm) flowers. c. Lurid-yellow flower (lateral view) with urceolate calyx tube and corolla lobes. d. Flower (frontal view) with spreading, acute corolla lobes and yellowish anthers. e. Angular-pyriform, urceolate, enlarged glaucous calyx enclosing young fruit. f. Mature berry with an ochre-yellow calyx. g. Calyx torn away, revealing the mature berry with persistent style. h. Harvested, dried berries. i. Seeds dorsiventrally flattened, discoid to reniform, and furfuraceous (covered with flaky particles).

ellipsoid to orbicular, and yellowish green. The ovary is globose, glabrous, and the style is glabrous, ca. 4 mm long. The stigma is capitate. The berries are red to pale orange, globose, glabrous, 5-8 mm in diameter (Figure 3g, h), and many-seeded. They are enclosed in an angular-pyriform, enlarged calyx measuring 18-20 x 10-12 mm, which is marginally keeled, stellate, and glaucous-pubescent (Figure 3e). At maturity, the calyx turns ochre-yellow to straw-colored (Figure 3f). The seeds are 2-3 mm in diameter, dorsiventrally flattened, discoid to reniform, pale brownish yellow, and furfuraceous (Figure 3i).

4.2 Phenology: The plant flowers after rains from October to January, or often throughout the year.

4.3 Habitat: The plant thrives in dryland habitats (Figure 4a), in fully exposed to partially shady habitats, naturalized in scrubland, and often grows invasively in pasturelands around the countryside.





Figure 4. Habitat of *Withania somnifera*. a. Fallow land habitat where *Withania somnifera* predominantly grows. b. Indian farmer holding freshly harvested *W. somnifera* plants from a 120-day-old cultivation.

4.4 Distribution: Ashwagandha is native to Old World tropics, mainly the drier regions of India and Sri Lanka, and it is found from southern Europe to central China, and from Africa to Myanmar. The plant has been introduced into South Australia.[7]

4.5 Morphological similarities with other congeneric taxa: *Withania somnifera* shares morphological similarities with *W. coagulans* in flowers and habit, but the latter differs by having glaucous, elliptic leaves, dioecious flowers, and an accrescent calyx in fruits.[11]

4.6 Cultivars: Due to its commercial importance, more than a dozen cultivars have been introduced in recent decades by Indian organizations, such as the Council of Scientific and Industrial Research (CSIR) and the Indian Council of Agricultural Research (ICAR), with the aim of improving yield, enhancing resistance to pests and pathogens, and increasing the concentration of active phytochemicals.[12] The withanolide composition has been shown to vary substantially depending on the cultivar chemotype.[13,14] Some varieties have also been studied for yield and phytochemical content in different agro-climatic zones.[15] These varieties are now commercially cultivated throughout India (Figure 4b), predominantly in the states of Madhya Pradesh, Rajasthan, Andhra Pradesh, and Telangana.[16] *Withania somnifera* has become an important cash crop, establishing India as a commercial hub for ashwagandha cultivation and production.

5. Potential Adulterants

5.1 Use of aerial parts of *Withania somnifera*: For centuries, dried roots of ashwagandha have been integral to ancient cultures and traditional Indian medicine systems, including Ayurveda, Siddha, Unani, and Sowa Rigpa. Today, ashwagandha is widely available as a dietary supplement in many countries and is recognized in various pharmacopeias and literature, which specifically highlight the use of ashwagandha roots.[12] In the past few decades, sales of *W. somnifera*-based dietary supplements have increased substantially, and adulteration of root extracts with extracts from other plant parts — including leaves, stems (Figure 5), or mixed aerial parts (locally called *panchang*, a mixture of stem, leaves, flowers, fruits, and seeds) — has become more prevalent. Several



Figure 5. Dried aerial parts (left) and dried stems (right) of *Withania somnifera* are often mixed with root material.

reports have documented that the aerial parts of ashwagandha are sometimes added to roots without being properly declared due to their lower cost compared to the dried roots.[5,17] Such adulteration is intentional and motivated by financial gain.

5.2 *Withania coagulans*: Although *W. coagulans* contains similar chemical marker compounds (e.g., withanolides) and occurs sympatrically with *W. somnifera* in arid parts of India and Pakistan, it has not been reported as an alternative or adulterant of *W. somnifera* due to its rarity and polygamous nature. In its growing range, *W. coagulans* is seen as a beneficial plant and valued for its fruits, which are harvested seasonally as a cash crop, contributing to the livelihood of local communities. The berries of the shrub are used for milk coagulation to produce cheese products such as paneer. This property is linked to the pulp and husk of the berry. The fruits of the plant are sweet and are said to be sedative, emetic, alterative, and diuretic.

5.3 Other adulterants: Fresh roots of ashwagandha have a characteristic smell and can be easily identified in the field. However, after drying, grading, and further processing, they can be mixed with roots of other plants, making visual detection difficult.[1,5] Dried roots from *Cullen corylifolium* (Fabaceae), *Mucuna pruriens* (Fabaceae), *Senna auriculata* (Fabaceae), *Trigonella foenum-graceum* (Fabaceae), *Vigna radiata* (Fabaceae), *Lepidium sativum* (Brassicaceae), *Moringa oleifera* (Moringaceae), and *Cenchrus* spp. (Malvaceae) have been reported as adulterants and have also been found in "ashwagandha" root extracts sold in the market.

5.4 Ashwagandha as an adulterant of other medicinal plants: Ashwagandha has also been reported as an adulterant of *Saussurea lappa* (syn. *Dolomiaea costus*, Asteraceae).[18] This claim may be an error, given the rarity of *Saussurea lappa*, which is included in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The confusion likely arises from the use of similar vernacular names: *kustha* for *S. lappa* (sold as *kaduwa kustha*) and for *W. somnifera* (sold as *meetha kustha*).

6 Identification of Ashwagandha Dried Roots and Genetic Diversity

6.1 Macroanatomical and organoleptic characteristics: Harvested fresh root has a primary starchy taproot that is straw colored, while the secondary roots, along with the wiry fibrous roots, are darker yellow. The diameter of dried roots ranges from 5-20 mm (sometimes up to 30 mm), and their color often varies with age, from brown to pale brown. The root surface is smooth, with lenticels, striations, and fibrous roots. It is hard, often with a mid-central cavity or a solid, starchy



Figure 6. Transverse section of a *Withania somnifera* leaf passing through the mid-rib. a. Outline with outer epidermis, mesophyll, and vascular bundles (5x). b. Branched trichome (40x). c. Trichomes and stomata on the adaxial surface of the leaf (40x).

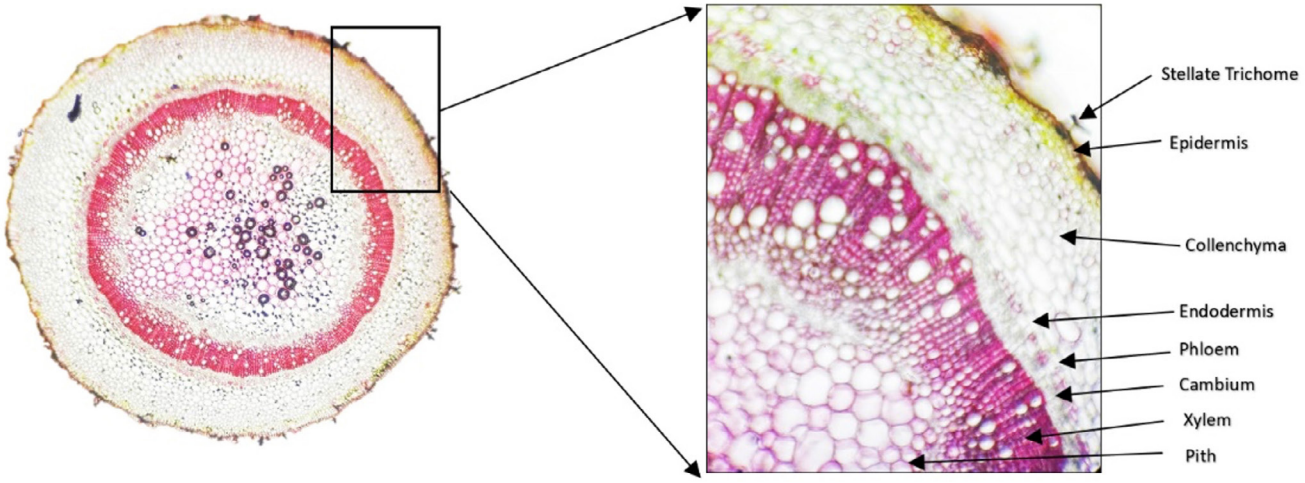


Figure 7. Transverse section of *Withania somnifera* stem (5x, left) with magnified view (10x, right).

interior that is pale brown to straw colored (Figure 8a), or white when broken. The powder of the dried root is light yellow to pale brown, bitter and acrid in taste, and has the characteristic odor of a horse.

6.2 Microanatomical characteristics: Microscopic characteristics of ashwagandha leaves and stems are shown in Figures 6 and 7. Leaf fragments can be detected by the presence of branched trichomes (Figure 6b) and anisocytic stomata (Figure 6c). The transverse section of roots (Figures 8 and 10) shows a narrow band of yellowish, crumpled cork and a 5-8-cell-layered parenchymatous cortex with occasional patches of sclereids. The cork cells are rectangular, radially flattened, non-lignified, and filled with starch grains and reddish-brown content (Figure 9c). The cork cambium has 2-4 diffused rows of cells. The secondary cortex is made up of 20-25 rows of thin-walled parenchymatous cells containing starch grains and sparse crystals of calcium oxalate. The phloem consists of sieve tubes, companion cells, and phloem parenchyma, while the vascular cambium is made of tangentially elongated parenchymatous cells. Vessels and tracheids are arranged in radial rows toward the periphery of the secondary xylem. Medullary rays are uniseriate or 2- to 3-seriate and filled with starch grains. Scattered vessels in groups are found embedded in the parenchyma. The vessels are pitted and show a scalariform thickening. Generally, the end walls are perforated. A few fibres with thick, lignified walls are also found scattered in the wood.

6.3 Strengths and limitations of macro-anatomical, micro-anatomical, and organoleptic characteristics:

Comments: Anatomical analysis is suitable and valuable for the initial identification and quality assessment of crude ashwagandha roots. However, it has limited use as a stand-alone method for highly processed materials. Key strengths and limitations for both crude and processed materials are summarized below.

Strengths:

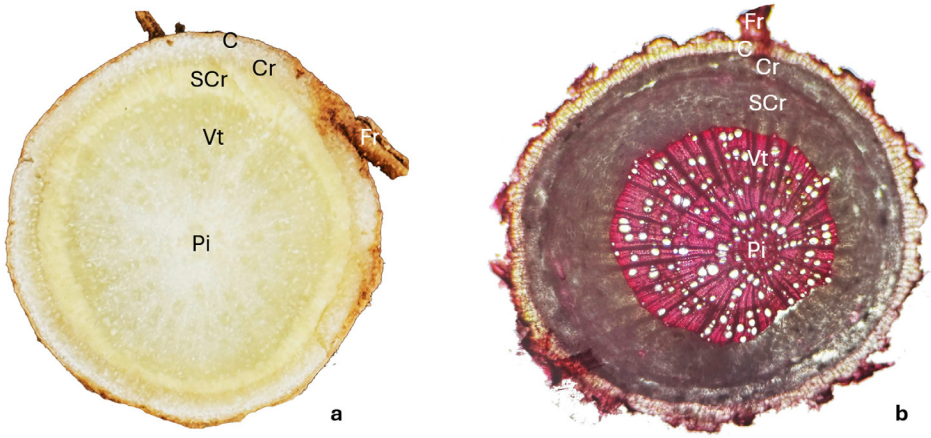


Figure 8. Transverse section of *Withania somnifera* root. a. Stereo microscope view (4x). b. Compound microscope view (5x). Abbreviations: Fr, fibrous root; C, cork; Cr, cortex; SCr, secondary cortex; Vt, vascular tissue; Pi, pith.

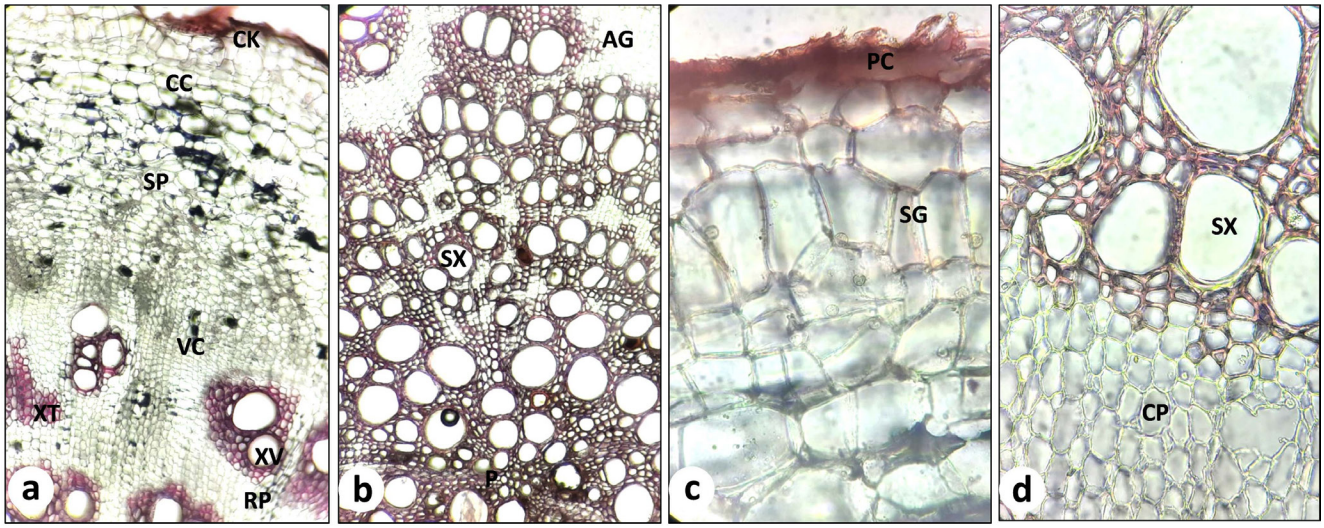


Figure 9. Magnified view of *Withania somnifera* root. a. Cortical portion (10x). b. Stelar portion (10x). c. Cork cells (40x). d. Anomalous growth (40x). Abbreviations: CK, cork; CC, cork cambium; SP, secondary phloem; VC, vascular cambium; XT, xylem tracheid; XV, xylem vessel; RP, ray parenchyma; AG, anomalous vascular cambium producing secondary xylem on both sides; SX, secondary xylem vessels; P, pith; PC, piliferous cork layer; SG, cork cells showing presence of starch grains; CP, cells of ray parenchyma differentiating into cambium showing anomalous growth.

Macroanatomical and organoleptic differentiation: The approach effectively identifies and differentiates ashwagandha roots based on physical characteristics such as color, texture, flavor, and smell. This is beneficial for quick initial identification of crude, dried roots when external features or intact material are accessible.

Microanatomical analysis: Examining microscopic features (e.g., cork structure, cortex composition, and vascular arrangements) provides robust insights into cellular-level details, which can help verify authenticity and detect adulteration in powdered materials. Parameters like the presence of simple and compound starch grains, cell wall thickness, distribution of sandy and prismatic calcium oxalate crystals, and a poorly defined and easily exfoliate periderm provide diagnostic markers for quality control, making this method useful for both crude and powdered materials.

Limitations:

Variability in natural samples: Macro- and micro-anatomical features can vary significantly due to agricultural practices, environmental factors, age, and harvesting and post-processing conditions. This may reduce the reliability of identification for crude materials.

Failure to detect adulteration for processed material: When ashwagandha roots are processed into extracts, these anatomical features are lost. For powdered

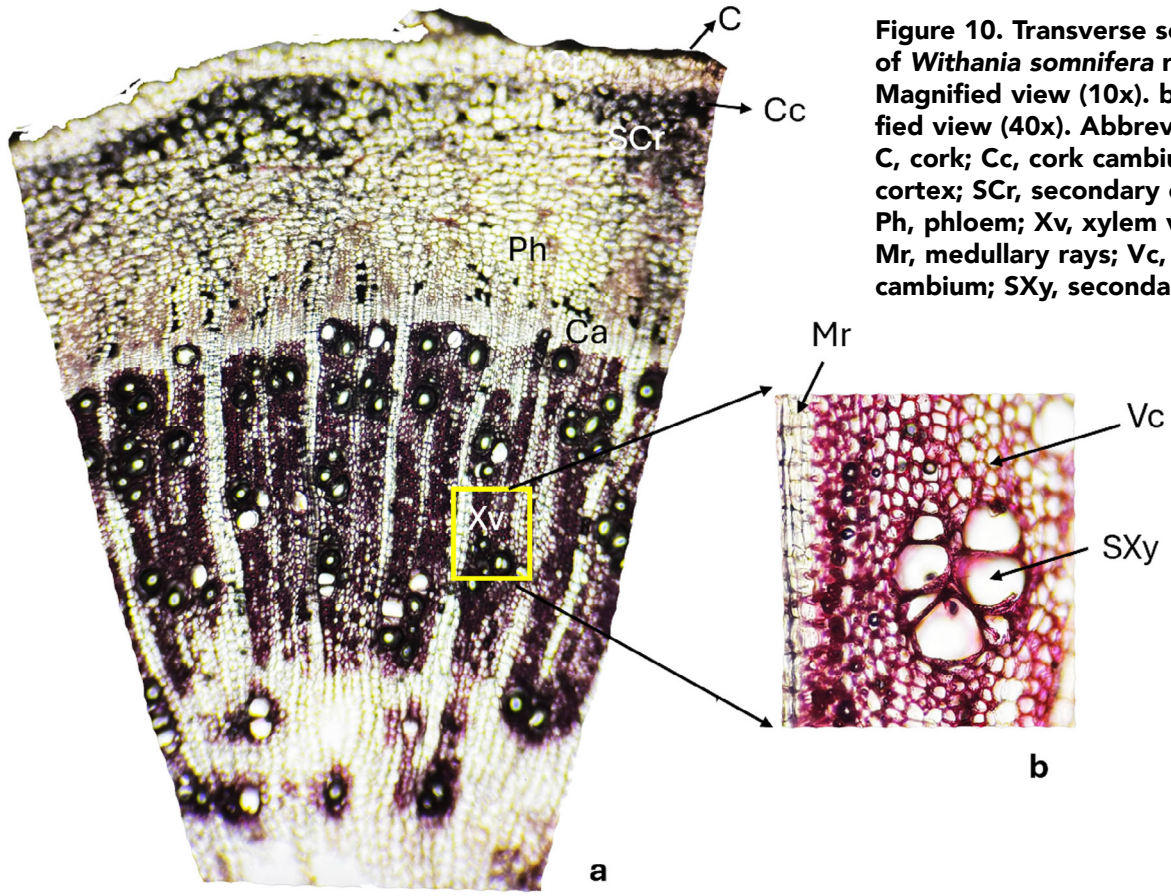


Figure 10. Transverse section of *Withania somnifera* roots. a. Magnified view (10x). b. Magnified view (40x). Abbreviations: C, cork; Cc, cork cambium; Cr, cortex; SCr, secondary cortex; Ph, phloem; Xv, xylem vessels; Mr, medullary rays; Vc, vascular cambium; SXy, secondary xylem.

materials, microscopic characteristics might be insufficient to distinguish them from roots of other species and require an orthogonal approach, such as chemical profiling or genetic analysis, to ensure accurate identification.

7. Chemical Identification and Distinction

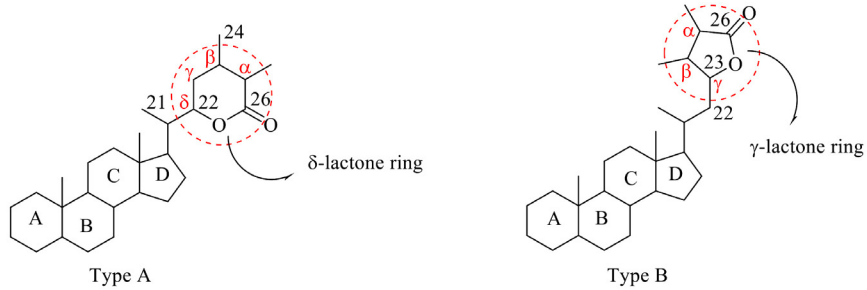
7.1 Phytochemistry of *Withania somnifera* and potential congeners and confounding materials

Withanolides: Withanolides are C-28 steroidal lactones with an ergostane skeleton. They are among the most important chemical constituents of *W. somnifera* and typically contain oxygen atoms at positions C-26 and C-22 or C-23. Based on their structure, the natural withanolides are mainly divided into two types: δ-lactone or δ-lactol and γ-lactone or γ-lactol side chains (Figure 11).[19-21] Most of the withanolides in *W. somnifera* belong to type A.

Withanolides are further classified by having either a modified or unmodified carbocyclic skeleton and/or side chain. An unmodified skeleton can be separated into withanolides with withanolides with 3α-oriented side chains and 3β-oriented side chains. Ashwagandha has only 3β-oriented substituents. The modified skeleton can be categorized into seven different classes (not all of which occur in ashwagandha): phenolic withanolides, 5–enes, 5α,6α–epoxides, 5β,6β–epoxides, 6α,7α–epoxides, 6β,7β-epoxides, and intermediate compounds.[22,23]

Constituents of *Withania somnifera*: Ashwagandha roots contain the following primary metabolites: withanolides, flavonoids, alkaloids, terpenoids, phenolic

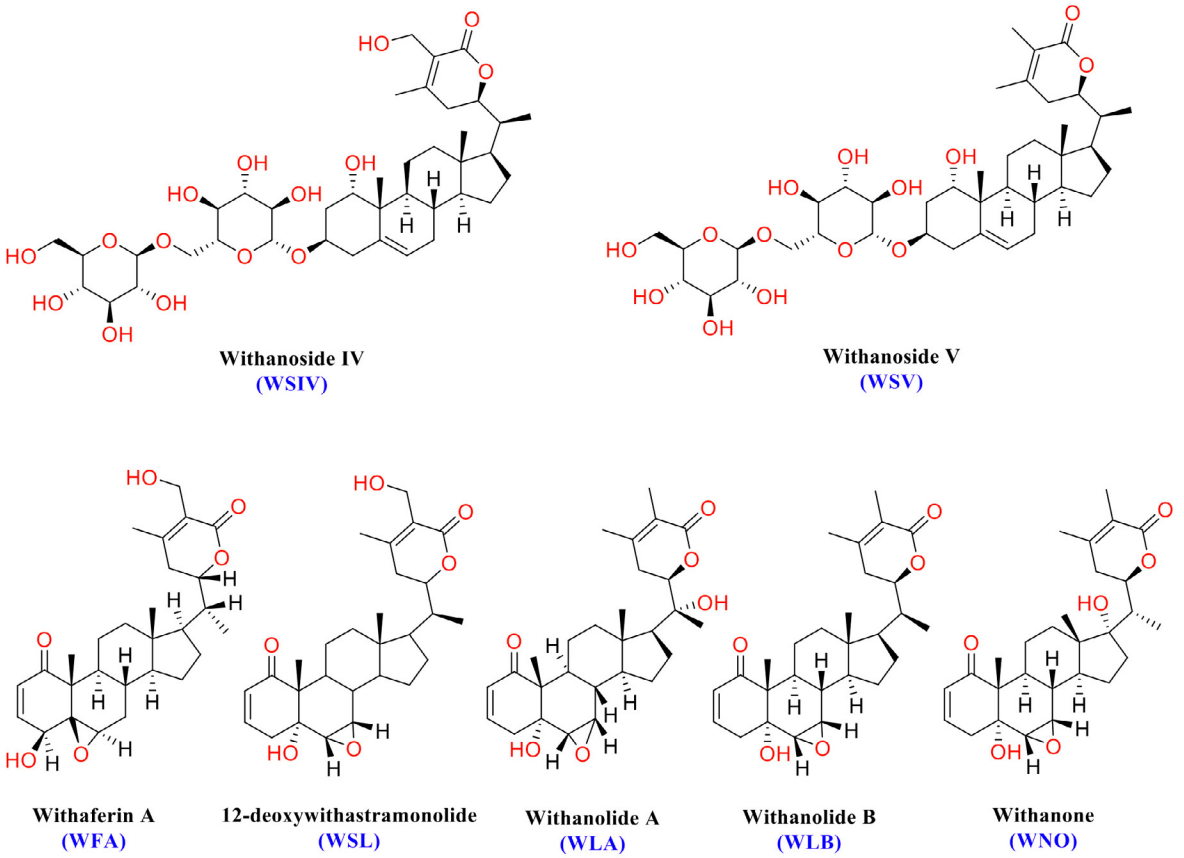
Figure 11. The structures of the two main types of withanolides.



acids, amino acids, and mono- and polysaccharides. Quantitatively, fructose and glucose (monosaccharides) and the disaccharide sucrose make up over 30% in a 50% ethanol extract of the roots. Other constituents in larger concentrations include malonic acid and the amino acid proline.[24] Many publications have documented other constituents, but quantitative data are relatively scarce. The withanolide composition depends on the chemotype,[13,14] but in general, the major withanolides are withanolide A, 12-deoxywithastramonolide, withaferin A, withanolide B, and withanone (Figure 12).[25-30] Withanolide glycosides, namely withanosides I–VII, were first isolated by Matsuda et al. from methanol extract of roots of *W. somnifera*.[31] Ashwagandhanolide, a withanolide dimer with an unusual thioether linkage, was identified in the roots in 2006 by Subbaraju et al.[32] Other major terpenoids are β-sitosterol, cholesterol, campesterol, stigmasterol, and the saponins sitoindosides VII-X.[33]

There are some withanolides that can help distinguish between aerial part and root parts of ashwagandha. Withaferin A concentrations are substantially higher in the leaves compared to the roots, according to Chandra et al[27] and Srivastava et al.[30] Hence, high concentrations of withaferin A, especially in relation to other withanolides, may indicate the presence of ashwagandha leaf. The data on specific withanolides that can help distinguish among different plant parts are limited. However, 14α,15α-epoxy-withaferin A, 2,3-dihydro-3β-*O*-sulfonyl-withaferin A, 3β-methoxy-2,3,24,25-tetradihydrowithanolide D, 5β-chloro-6α-hydroxy-27-acetylwithaferin A, and 1α,3β,20-trihydroxy-witha-5,24-dienolide, so far, have been found only in the plant's aerial parts.[34] On the other hand, a review of ashwagandha constituents by Zhang and Timmermann listed a number of withanolides that have been reported to date solely from the roots. These include glucosomniferanolide; 3α,6α-epoxy-4β,5β,27-trihydroxy-1-oxo-witha-24-enolide; coagulin Q; sitoindoside X, withacoagin (6,7-deoxy-withanolide A, erroneously named withacogin by Zhang and Timmermann); withasomniferol C; and 5α,7α-epoxy-6α,20-dihydroxy-1-oxo-witha-2,24-dienolide. The roots also contain several withanosides, such as withanosides I-III and VI-XI.[34]

Figure 12. Chemical structures of marker compounds: withanoside IV (WSIV), withanoside V (WSV), withaferin A (WFA), 12-deoxywithastramonolide (WSL), withanolide A (WLA), withanolide B (WLB), and withanone (WNO).



Flavonoids are mainly present in the leaves. Quercetin 3-*O*-robinoside-7-*O*-glucoside, quercetin 3-*O*-rutinoside-7-*O*-glucoside, and kaempferol 3-*O*-robinoside-7-*O*-glucoside have been reported as chemical markers to distinguish the leaves from the roots. According to USP monographs, their presence is limited to 0.01% in powdered root and to 0.04% in root extracts.[35,36] Phenolic acids, such as caffeic acid, ferulic acid, and benzoic acid, are found in both the roots and the leaves. The roots also contain the alkaloids anaferine, anahygrine, cuscohygrine, isopelletierine, peudotropine, tropine, and 3α-tigloyloxtropane (syn. tigloidine).[24,37]

Withanolides are the main constituents of interest in *W. somnifera*, *W. coagulans*, and *P. peruviana*. *Withania somnifera* has relatively high concentrations of withaferin A. Additionally, withanolide D has been reported in *W. somnifera* but not in *W. coagulans* or *P. peruviana*.[38]

Azhar et al. measured the total flavonoid content (TFC) and total phenolic content (TPC) using a spectrophotometric method. The flavonoid content in the leaves of *W. somnifera* was found to be 43.51 mg rutin equivalents (RE)/g, which is similar to *W. coagulans*. The concentration of flavonoids in *W. coagulans* is higher in leaves compared to stems and roots. Similarly, the flavonoid concentration of *W. somnifera* was highest in the leaves and lowest in the roots. The total phenolic content was higher in *W. somnifera* (leaves: 53.53±0.537, stem: 15.95±0.572, root: 11.60±0.350) but lower than in *W. coagulans*.[39]

Constituents of *Withania coagulans*: *Withania coagulans* is frequently referred to as "Indian cheese maker" or "paneer dodi" because of the fruits' ability to coagulate milk. Several plant parts, particularly fruits, are used in traditional medicine to treat a variety of illnesses.[40] Research on the chemistry of *W. coagulans* has focused on the whole plant or the aerial parts. An exception is the paper by Neogi et al, which detailed the identification of withacoagin as a new compound from the roots of *W. coagulans*, along with three additional withanolides: withaferin A; (20R,22R)-6α,7α-epoxy-5α,20-dihydroxy-1-oxowitha-2,24-dienolide; and (20S,22R)-6α,7α-epoxy-5α-hydroxy-1-oxowitha-2,24-dienolide.[41]

Constituents of *Physalis peruviana*: *Physalis peruviana* also contains phenolic compounds, withanolides, and carotenoids.[42] Several withanolides (i.e., withaperuvin and withaperuvins B-H) have been isolated from the roots of *P. peruviana*.[43-47] According to Frolow et al,[43] withaperuvin is the main steroidal lactone in the roots of the Varanasi variety.

These authors also reported substantial differences in composition depending on the growing region. In plants growing in southern India, withanolide E, withanolide S, and 4β-hydroxywithanolide E were the main steroidal lactones, whereas these compounds were nearly absent in plants growing in the northern state of Uttar Pradesh, which borders Nepal. Some of the withaperuvins (e.g., withaperuvin and withaperuvin C) also occurred in the aerial parts with other steroidal lactones.

28-Hydroxywithanolide E was identified in the calyces of *P. peruviana*. Lan et al examined extracts from *P. peruviana* and isolated withanolides, such as peruvianoxide, and phyperunolides A-F, from the aerial parts of the plant.[48]

7.2 Laboratory methods: Withanolides are the primary analytes of interest in ashwagandha, and most analytical methods in the literature focus on these steroidal lactones. In some instances, the analysis of flavonol glycosides is included, as their presence allows for the distinction between roots and aerial parts. The various techniques used to identify and/or quantify individual or total withanolides (sum of withanosides and withanolide glycosides) in root powder and extracts include thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography (UHPLC), liquid chromatography coupled with mass spectroscopy (LC-MS), and UV/Vis spectroscopy.

7.2.1 Thin-layer chromatography/high-performance thin-layer chromatography: Methods from the following sources were evaluated in this review: Ghoshal et al,[17] Singh et al,[5] Upton,[49] the HPTLC Association,[50] the Indian Pharmacopoeia,[51] the United States Pharmacopeia,[35] and Tomar et al.[52]

TLC or HPTLC are among the easiest and most affordable ways for the chemical identification of *Withania somnifera* raw material, extracts, finished products (capsules, tablets, standardized extracts), and classical Ayurvedic formulations. Commonly used solvents for the extraction of withanolides include ethanol, ethanol-water mixtures, methanol, and methanol-water mixtures. A wide variety of solvent combinations are used as mobile phase, although mixtures of toluene, ethyl acetate, and acetic or formic acid appear to be the most common. For safety and environmental reasons, mobile phases devoid of chloroform and toluene are preferred. Methods generally include visualization at 254 nm and at 366 nm after derivatization with either anisaldehyde-sulfuric acid or sulfuric acid. Densitometry of the developed plates can be

conducted to quantify the withanolides before and after derivatization. An indication of adulteration with leaves or stems is the presence of characteristic red bands close to the solvent front due to the presence of chlorophyll in the aerial parts. However, these chlorophyll pigments can be removed by using simple chromatographic means. A summary of method parameters is provided in Table 1.

According to the method described in the AHP monograph,[49] withaferin A and β-sitosterol were used as reference standards in an HPTLC analysis proposed for quality control. The mobile phase consists of toluene:ethyl acetate:formic acid (50:15:5, v/v/v). The method was developed with the differentiation of ashwagandha chemotypes in mind, and while distinction from other potentially adulterating species was not investigated, it seems reasonable to believe that the method is capable of doing so. The USP has a similar mobile phase using the same three solvents but a different ratio (50:45:3, v/v/v).[35] As mentioned in the Botanical Adulterants Prevention Bulletin,[5] adulteration of leaf material can be identified using an HPTLC-based method, allowing for the detection of plant pigments (i.e., chlorophylls that occur as red bands in leaf material), while in roots, these bands tend to be absent. One limitation of this HPTLC method is that it only works for methanolic or hydro-methanolic extracts and not for water extracts.[5] The Botanical Adulterants Prevention Bulletin on ashwagandha proposes a different mobile phase for the detection of chlorophyll (Table 1).

Ghoshal et al proposed an HPTLC-densitometry-based quantification technique to differentiate roots and aerial parts (Figure 13). In this validated method, kaempferol-3-*O*-robinoside-7-*O*-glucoside is used as a marker to help distinguish leaves and roots, as this compound has only been reported in the leaves.[17] The method also aids in successfully differentiating

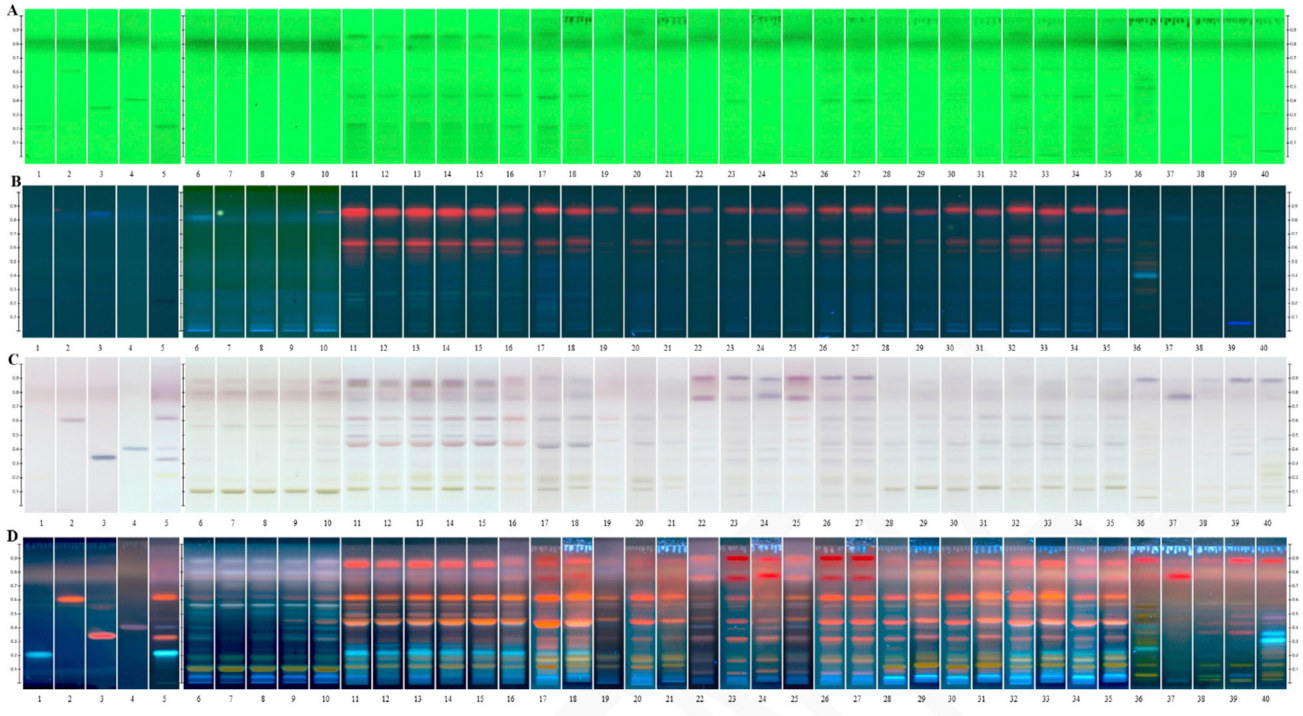


Figure 13. HPTLC fingerprint of ashwagandha raw material and potential adulterants. The "L" denotes the lane for each reference standard and sample.

L1: Kaempferol-3-*O*-robinoside-7-*O*-glucoside; L2: withaferin A; L3: withanoside IV (WS4); L4: withanoside V (WS5); L5: mixed standards; L6-L10: ashwagandha roots; L11-L15: ashwagandha leaves; L16-L18: ashwagandha aerial parts; L19-L21: ashwagandha stem; L22-L24: ashwagandha fruits; L25-L27: fruits with calyx; L28,L29: ashwagandha roots:leaves (9:1); L30,L31: ashwagandha roots:leaves (4:1); L32,L33: ashwagandha roots:leaves (1:1); L34,L35: ashwagandha roots:aerial parts (1:1); L36: *Cassia auriculata* seeds; L37: *Withania coagulans* fruit; L38: *Mucuna pruriens* white seeds; L39: *M. pruriens* black seeds; L40: *Trigonella foenum-graecum* seeds at (A) UV 254 nm, (B) UV 366 nm, (C) before derivatization, and under white light after derivatization, and (D) UV 366 nm after derivatization.

W. somnifera from other *Withania* species. Tomar et al describe a validated method for three withanolides and three phenolic acids, which was tested on roots, stems, and leaves.[52] Since none of the six markers is characteristic of leaves or stems, it is unclear how useful this method is for detecting adulteration.

The USP monograph for *Withania somnifera* root dry extract and the method of the HPTLC Association are aligned (Table 1).[35,50] The Indian Pharmacopoeia uses a relatively simple mobile phase in the monograph of powdered ashwagandha root extract for quality control purposes. Another TLC method, which is used for the identification of *W. somnifera* root extract, is mentioned in the Indian Pharmacopeia. In this method, the profiling is done using withaferin A as a reference standard.[51]

Comment: Adulteration with ashwagandha leaf or other adulterants in roots can be confirmed by the method suggested by Ghoshal et al.[17] The method uses the presence of kaempferol 3-*O*-robinoside-7-*O*-glucoside to identify aerial parts, leaves, stems, fruits with calyx, and fruits in the HPTLC analysis, separating them from root samples. The method can also distinguish ashwagandha roots from fruits/seeds of four potential confounding species; however, its ability to detect adulteration of root material in the case of these four species has not been determined. Additionally, some reports suggest that fraudulent suppli-

Table 1. Comparison of different published TLC/HPTLC methods for *Withania somnifera* constituents

Reference Compounds	Mobile Phase (v/v)	Derivatizing Reagent	Findings/Remarks	Reference
Withaferin A, β-sitosterol	Toluene: ethyl acetate:formic acid (50:15:5)	Sulfuric acid	Limited to two markers only; mobile phase contains toluene; not tested for water extracts	Upton[49]
NM	Methylene chloride: methanol:acetone:diethyl ether (100:6.5:6.5:6.5)	Vanillin-sulfuric acid reagent	Limited to methanolic or hydro-methanolic extracts only Detection of leaf material based on the presence of chlorophyll	Singh et al[5]
Withaferin A, withanolide A, withanone, benzoic acid, caffeic acid, and ferulic acid	Toluene: ethyl acetate:acetic acid (60:40:4)	Sulfuric acid (10%) followed by densitometry	Includes three phenolic acids in addition to withanolides; ability to detect adulteration unclear; mobile phase contains toluene	Tomar et al[52]
Kaempferol-3-*O*-robinoside-7-*O*-glucoside	Ethyl acetate: chloroform:methanol:water (8:3:4.4:1.8)	Anisaldehyde-sulfuric acid	For differentiation of leaves and roots sample; mobile phase contains chloroform	Ghosal et al[17]
Withanolide A, β-sitosterol	Toluene:ethyl acetate:glacial acetic acid (55:45:3)	Sulfuric acid in cold methanol	Specified for extracts containing NLT 2.5% withanolides; mobile phase contains toluene	USP Pharmacopeia,[35] HPTLC Association[50]
NM	Chloroform:methanol (9:1)	Anisaldehyde-sulfuric acid	Applies to raw material; chemical markers are not specified; mobile phase contains chloroform	Indian Pharmacopoeia[51]
Withaferin A	Toluene:ethyl acetate:formic acid (5:5:1)	Anisaldehyde-sulfuric acid	Applies to *W. somnifera* root extract; mobile phase contains toluene	Indian Pharmacopoeia[51]

Abbreviations: NM, not mentioned; NLT, not less than.

ers remove flavonoids from extracts of ashwagandha aerial parts (L. Hingorani [Pharmanza Herbal Pvt Ltd] email to S. Gafner, August 19, 2021) to make detecting adulteration more difficult, so relying solely on kaempferol 3-*O*-robinoside-7-*O*-glucoside as a marker for adulteration may not be sufficient.

7.2.2 High-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC): Methods described in the following literature were evaluated: Polcaro et al,[24] Chandra et al,[27] Girme et al,[26] Indian Pharmacopoeia,[51] the United States Pharmacopeia,[35] Mundkinajeddu et al,[53] AOAC SMPR®,[54] Alam et al,[55] and Ganzera et al.[56]

To date, most HPLC-UV/Vis-based methods follow the same or similar chromatographic conditions as in the USP monograph.[35,36] There are differences in the number and composition of target analytes (Table 2). The USP method applies to *W. somnifera* root powder, for which NLT 0.3% total withanolides are specified, and *W. somnifera* dry root extract (NLT 2.5% total withanolides). For the differentiation of roots and aerial parts, a different UV/Vis detection-based HPLC method is used. Three flavonoid glycosides (Table 2) are quantified based on relative retention time against kaempferol 3-*O*-robinoside-7-*O*-glucoside as a reference standard, with a maximum permissible content of 0.04%. Mundkinajeddu et al developed and validated an HPLC-UV/Vis method for the simultaneous analysis of the above-mentioned flavonol glycosides.[53]

The Indian Pharmacopoeia uses the same HPLC method as the USP but with slight modifications in the mobile phase.[35,51] Additionally, there are differences in the extraction protocol, with the USP approach being much less time-consuming. Another difference is the constituents included in the analysis of total withanolides: the Indian Pharmacopoeia excludes physagulin D and withaferin A, while these constituents are included in the USP monographs. However, withanosides V and VI coelute in the USP method.

Another official method was developed and validated as part of the AOAC Standard Method Performance Requirement (SMPR®) 2015.007, titled Standard Method Performance Requirement for Withanolide Glycosides and Aglycones. In 2007, a method was submitted to the AOAC Expert Review Panel on Dietary Supplements for consideration as a single-laboratory validation (SLV) and got approval as First Action Official Method℠ in 2015.[54] The methodology has been shown to work with samples (ashwagandha roots containing more than 0.2% total withanolides, and extracts containing more than 2.5% total withanolides) from a variety of different suppliers. Extracts were made using solvents of differing polarity, including water, aqueous ethanol, and methanol. Compared to the USP method, the run time of the AOAC method is 5 minutes longer and includes a minor modification of the gradient program.

A UHPLC method using both UV/Vis and triple quadrupole tandem mass spectrometric (TQ-MS/MS) detection was developed and validated (using the UV/Vis detector) by Girme et al.[26] This method includes the determination of eleven withanosides and withanolides (withanoside VII, 27-hydroxywithanone, dihydrowithaferin A, viscosalactone B, withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A, withanone, and withanolide B). The method has the same run time (40 minutes) as the USP method but a different mobile phase gradient. The addition of tandem mass spectrometry enables more robust peak identification and a more accurate constituent quantification than the detection at 227 nm, which shows several partially overlapping peaks. Considering its sensitivity and satisfactory performance, this method can be used for routine laboratory analysis of *W. somnifera* raw material, extracts, and complex finished products with other ingredients and excipients.

While the method by Girme et al[26] allows to separate eleven markers, the resolutions between 27-hydroxywithanone and dihydrowithaferin A, and withanolide A and withanone are not optimal. Also, fronting was observed in withanoside IV and 12-deoxywithastramonolide. The addition of ESI−MS/MS, which helps with constituent identification, is advantageous compared to methods relying on UV detection, but requires a substantial financial investment due to the cost of a mass spectrometer.

Chandra et al[27] and Polcaro et al[24] developed UHPLC-MS methods for the analysis of ashwagandha roots and, in the case of Chandra et al, leaves as well. If a short run time is needed, the UHPLC-MS/MS method by Chandra et al is a good choice. The validated method allows quantification of six withanolides in a 2.5-minute run using different multiple reaction monitoring (MRM) transitions for each constituent. While high withaferin A concentrations may indicate admixture of aerial parts, the ability to detect adulteration using this method needs to be further evaluated. Polcaro et al[24] published a method with a longer run time (35 minutes) but excellent separation of the withanolides and the method provides information for 24 withanolides. However, the method was tested on only one root sample, and no validation data are provided. Nevertheless, due to the large number of ashwagandha constituents identified, this method is

expected to be appropriate to detect adulteration.

In all the described methods, some overlapping peaks are present, which may hamper accurate quantification. In the USP monograph (which has similar conditions to the Indian Pharmacopoeia), the peaks of withanoside IV, physagulin D, and 27-hydroxywithanone were not well resolved in the sample chromatogram. Moreover, 12-deoxywithastramonolide coelutes with another peak present in the sample. Similarly, the method published by Koshy et al[54] has constituents coeluting with withanoside IV and withanolide A, which may lead to an inaccurate content of total withanolides. In most of these published methods, the run time was set to 40 to 45 minutes, separating about nine compounds.

Sica et al emphasised the use of a multidetector approach to characterize ashwagandha root extract. They used UHPLC-UV/Vis-CAD-HRMS to identify constituents of ashwagandha. Using a CAD detector, they quantified some peaks. All constituents above a threshold of toxicological concern were identified using HRMS and quantified. Data from this process were used by BSC (Botanical Safety Consortium), along with other chemical analyses, to identify physicochemical properties and pharmacokinetics of these constituents, as well as their safety, using computational models.[57]

Ratnasekhar et al. demonstrated that metabolomic fingerprinting combined with machine learning models can be used for determining the geographical origin, variety, tissue-specific authentication, and adulteration detection of *W. somnifera* based on orbitrap-HRAMS.[58]

7.2.3 UV/Vis spectroscopy: Ajage and Kasture[59] quantified withanolide A in a marketed product consisting of ashwagandha, ginger (*Zingiber officinale*, Zingiberaceae), and autumn crocus (*Colchicum autumnale*, Colchicaceae) using a "validated" UV/Vis spectroscopic method at 223 nm. The authors used an optimized solvent system with distilled water mixed with lime and treated with ammonia solution, which was partitioned with chloroform.

Comment: This UV/Vis method has several flaws, not the least of which is the optimization of the solvent system for alkaloids, despite withanolide A not being an alkaloid. The authors failed to use an orthogonal method such as HPLC-UV/Vis to verify the composition of the extraction product, hence it is likely that what they considered as withanolide A was composed of a myriad of compounds. Moreover, UV/Vis methods to measure individual or total withanolides are not specific enough for the measurement of the steroidal lactones, and hence, the results cannot be considered reliable. Due to the lack of specificity, UV/Vis methods can be easily fooled and are not appropriate for detecting ashwagandha adulteration.

7.2.4 Nuclear magnetic resonance spectroscopy: Two methods were retrieved and assessed for this Laboratory Guidance Document: Polcaro et al[24] and Chatterjee et al.[25]

Comment: Two nuclear magnetic resonance (NMR) methods were developed to determine the chemical composition of the roots (Polcaro et al.) or roots and leaves (Chatterjee et al.) of ashwagandha. Both publications used proton NMR experiments to assess the ingredients; however, Chatterjee et al also included bidimensional NMR experiments to confirm structures. Polcaro et al. used multivariate statistics (principal component analysis, PCA) to assess the similarity of extracts using nine different solvents. Withanolides were quantified by HPLC-MS/MS. Similarly, Chatterjee et al. used GC-MS and UHPLC-MS/MS to quantify those constituents for which NMR was not suitable. While the enormous amount of information obtained from these orthogonal methods is impressive, and these techniques can certainly help in detecting many adulterants, the need to combine two or three methods requiring expensive equipment makes such an approach impractical for use in routine quality control.

8. Conclusions

Ashwagandha is a medicinal plant valued for its benefits to strengthen the body and support resilience to mental and physical stress. It is easily recognized by its horse-like odor, greenish-yellow flowers, and red berries enclosed in an angular-pyriform, ochre-yellow, enlarged calyx. It grows mainly in dry regions across Asia, Europe, and Africa, and has become a globally important botanical. It is cultivated largely in the tropics, with increasingly large areas under cultivation due to growing demand. However, the rising popularity has led to supply shortages and an increase in adulteration, most commonly by mixing or substituting the roots with leaves and stems without proper disclosure. This requires the implementation of appropriate quality control measures. While physical identification methods are helpful for crude raw materials, extracts require the use of chromatographic and spectroscopic methods (e.g., HPTLC, HPLC-UV/Vis, HPLC-MS, and metabolomic fingerprinting, or a combination thereof) to ensure authenticity. This Laboratory Guidance Document outlines potential adulteration scenarios for ashwagandha roots and root extracts, and discusses strengths and limitations of detecting

Table 2. Comparison of different published LC methods for *Withania somnifera* constituents

Sample type	Analyte(s)	Method	Remarks/Findings	Reference
Crude raw material and extract	Withanoside IV, physagulin D, 27-hydroxywithanone, withanoside VI, withaferin A 12-deoxywithastramonolide, withanone, withanolides A and B	HPLC-UV	A widely accepted method for quality control purposes; Resolution between the withanolide A and withanone peaks should be NLT 1.0 (slight overlapping), and between the withaferin A peak and the peak corresponding to coeluting withanoside V and withanoside VI	USP monograph[35,36]
Crude aerial part and root, extracts	Quercetin 3-*O*-robinoside-7-*O*-glucoside, quercetin 3-*O*-rutinoside-7-*O*-glucoside, kaempferol 3-*O*-robinoside-7-*O*-glucoside	HPLC-UV	Aerial part and root differentiation	USP monograph,[35] Mundkinajeddu et al[53]
Crude raw material and extract	Withanoside IV, V, VI, 12-deoxywithastramonolide, withanolide A, withanolide B, withanone	HPLC-UV	Two methods required, including a separate isocratic method for withaferin A; resolution should be > 2.0	Indian Pharmacopoeia[51]
	Withaferin A	HPLC-UV	Withaferin A	
Crude raw material and extract	Withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolides A and B	HPLC-UV/PDA	Limited number of constituents; resolution between withanoside V and withaferin A is >3.0	Koshy et al[54]
Crude raw material and extract	Withanoside VII, 27-hydroxywithanone, dihydrowithaferin A, viscosalactone B, withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolides A and B	UHPLC-PDA and TQ-MS/MS	Good number of withanolides analyzed with mass spectrometric confirmation.	Girme et al 2020[26]
Crude root, leaf, fruit	Catechin, gallic acid, syringic acid, vanillic acid, *p*-coumaric acid, benzoic acid, naringenin, kaempferol	HPLC-PDA	Focused on phenolics and flavonoids; no system suitability parameters provided.	Alam et al[55]
Crude root and aerial parts	Withaferin A, withanolide D	HPLC-PDA	Only two analytes included; no system suitability parameters provided.	Ganzera et al[56]
Crude root, stem, and leaves, 26 commercial ashwagandha products	Withanolide A, withanolide B, withaferin A, withanone, withanoside IV, withanoside V	UHPLC-ESI-MS/MS	Validated UHPLC-MS/MS method with a 2.5-minute run time; no system suitability parameters provided	Chandra et al[27]
Crude roots	24 withanolides and eight alkaloids	HPLC-quadrupole/orbitrap-MS	Excellent separation and wide array of constituents analyzed. Only one sample analyzed, no validation.	Polcaro et al[24]

adulteration through morphological, anatomical, and phytochemical analyses. As this ancient herb gains global recognition for its health benefits, ensuring the availability of authentic and high-quality products is more important than ever.

9. References

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