Indian Journal of Natural Products and Resources
Vol. 17(1), March 2026, pp. 155-164
DOI: 10.56042/ijnpr.v17i1.16914

Exploring HPLC-mapped variations of marker compounds in commercially cultivated *Withania somnifera* from Andhra Pradesh, India

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Received 04 February 2025; revised received 25 February 2026; accepted 27 February 2026

The study aimed to assess five marker compounds (Withanoside IV, Withanoside V, Withaferin A, Withanolide A, and Withanone) extracted from the root of the indigenous medicinal plant Ashwagandha (*Withania somnifera*) across 125 different cultivars in Andhra Pradesh, India. This is the first report of the simultaneous determination of these markers using a novel buffer-free HPLC method. Results showed dynamic variations in marker levels across different mandals and villages, likely influenced by microclimate and soil conditions. Notably, Withanoside IV was most prevalent in Alur, Aspiri, Pattikonda, and Holagunda mandals of Kurnool, followed by Withanolide A and Withaferin A. Conversely, Withaferin A exhibited dominance in the Halharvi mandal. The integration of PCA added a deeper layer of understanding, shedding light on the interrelationships between these compounds and the regional cultivation variances of Ashwagandha in Andhra Pradesh. Based on principal component analysis (PCA), the first two components accounted for over 97.24% of the variance, with eigenvalues of 0.0083 and 0.0012, respectively. The first PCA explained a significant amount of variability. Withanoside IV, Withanoside V, and Withaferin A notably contribute to the first principal component, explaining 84.92% of its variability. Withaferin A stands out as the primary contributor to the second principal component.

Keywords: Ashwagandha (*Withania somnifera*), Cultivars, HPLC, Marker compounds, Principal component analysis

IPC code; Int. cl. (2021.01)− G01N 30/00

Introduction

Withania somnifera (L.) Dunal (Family: Solanaceae), commonly known as Ashwagandha, Winter Cherry, or Indian ginseng, is one of the most esteemed medicinal plants in the Indian traditional systems of medicine. Its therapeutic use dates back to the period of the ancient scholar Punarvasu Atreya (circa 1000 BCE), who taught medicine at Takshashila (Taxila). For more than 3000 years, Ashwagandha has occupied a prominent place in Ayurveda, Siddha, Unani, and other indigenous healthcare traditions. In classical Ayurvedic texts such as the Brihat Trayi, Ashwagandha is described as a potent *rasayana* (rejuvenative tonic) and is recommended for conditions associated with debility and chronic illness. It has been traditionally prescribed for pain, arthritis, inflammation, rheumatism, cough, dropsy, consumption, and age-related weakness. The drug is characterised as tonic, pungent, astringent, and aphrodisiac in nature. Different parts of the plant are employed therapeutically. In traditional practice, leaves are used to manage fever, inflammatory lesions, swellings, ophthalmic conditions, and syphilitic sores. The plant has also been reported to possess diverse pharmacological activities, including ringworm infection[1], inflammation[2], anxiety[3], neurological disorders[4], Parkinson's disease[5], hyperlipidemia[6], antitumor, antiangiogenic[7], anti-stress[8], cytotoxic[9], hepatotoxicity activities[10]. The leaves of this plant were known to act as an insect repellent[11]. Experimental studies further support its neurological benefits. Notably, the methanolic root extract has demonstrated significant neurite outgrowth-promoting activity in human neuroblastoma SH-SY5Y cell line[12], indicating its potential role in neuroregeneration and neurotherapeutic applications.

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Supplementary Tables and Figures are available in the online version only.

Ashwagandha cultivation is carried out on a commercial scale primarily in the districts of Ananthapur and Kurnool in Andhra Pradesh, covering approximately 10,000 hectares annually. The farming community typically procures seeds from the open market to cultivate the crop, subsequently selling the produce in the market to sustain their livelihoods. Ashwagandha is significant to the pharmaceutical and Ayush industries due to the presence of withanolide markers, prompting their procurement of the herb for drug manufacturing. The availability of these marker compounds in Ashwagandha is influenced by various factors, including the planting material used, cultivation practices employed, soil quality, environmental conditions, and irrigation techniques, among others. Systematic studies are required to map the distribution of these marker compounds across different cultivars in the districts of Ananthapur and Kurnool. Such investigations will help elucidate the influence of regional climate, soil variability, and other environmental parameters on the concentration and stability of these bioactive constituents. Understanding these influences can significantly optimise cultivation practices and enhance the quality of Ashwagandha produced in these regions.

A limited number of HPLC methods have been documented using buffer as the mobile phase to separate marker compounds, encompassing a wide spectrum of differentially functionalised phytomolecules[13,14]. Although these methods offer increased baseline resolution, they have limitations for comprehensively quantifying constituents. To overcome these limitations, limited efforts have been made to develop an analytical HPLC profile capable of simultaneously quantifying five marker compounds from *W. somnifera* along with a comparative evaluation across different geographical regions. To surmount these challenges, we've devised a reliable, reproducible, buffer-free, and efficient HPLC methodology for analysing the five marker compounds Withanoside IV, Withanoside V, Withaferin A, Withanolide A, and Withanone from *W. Somnifera* (Fig. 1). Furthermore, we aim to identify the genotype that synthesises a superior quality and quantity of bioactive Withanolides, maximising economic benefits. Addressing the variation in the content of these five marker compounds across plants from diverse geographical regions is crucial and warrants attention. This analytical method has been validated with respect to pertinent parameters to ensure enhanced data quality and accreditation.

Therefore, the objective of the present mapping study was to (a) evaluate the *W. somnifera* populations collected from different regions of Andhra Pradesh for profiling of five marker compounds, and (b) identify the genotype enriched in bioactive constituents for subsequent exploitation and value addition.

Materials and Methods

Collection of plant samples

Ananthapur and Kurnool districts are low-rainfall areas of Andhra Pradesh, where Aswagandha has been widely cultivated for the last 20 years. We

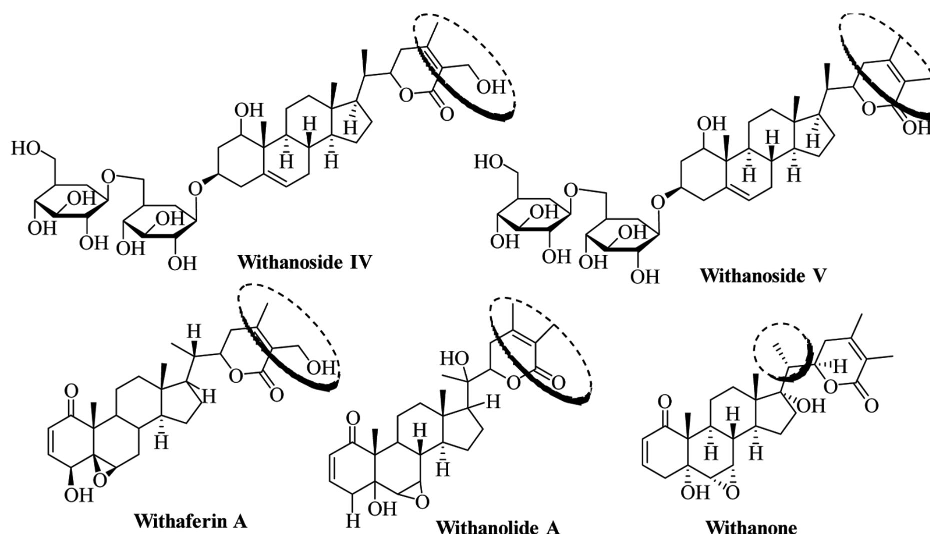

Fig. 1 — Structures of analysed marker compounds from *W. somnifera*.

selected these two districts for a mapping study of variations in marker compounds across different cultivars at varying latitudes, longitudes, and altitudes (Fig. 2, Table S1). Dr Venkat Ramana, Assistant Professor in the Department of Botany at Nizam College, Osmania University, Hyderabad, India, taxonomically authenticated the collected root samples. A voucher specimen (CIMAP-WS/1-125) was deposited at the CSIR–Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Research Centre, Hyderabad.

Kurnool district

In the mapping study conducted in Kurnool, a total of 75 samples were collected from eight mandals and eleven villages. The collection details of the root samples are presented in Table S2 and Fig. 3. Additionally, the geographical coordinates, including latitude, longitude, and altitudes of these cultivars, are provided in Table S3. Notably, Pattikonda and Aspiri mandals stand out for cultivating significant acreage dedicated to Ashwagandha within the region. The concentration of cultivars in these areas indicates a substantial cultivation footprint for this herb within the Kurnool district.

Ananthapur district

Similarly, our mapping study encompassed seven mandals across nine villages in the Anantapur district, involving a total of 50 samples. The detailed collection of root samples is outlined in Table S4 and depicted in Fig. 4. This detailed data provides an in-depth understanding of the distribution and characteristics of Ashwagandha cultivars within the Ananthapur district, offering valuable insights into this region's cultivation patterns. These collected

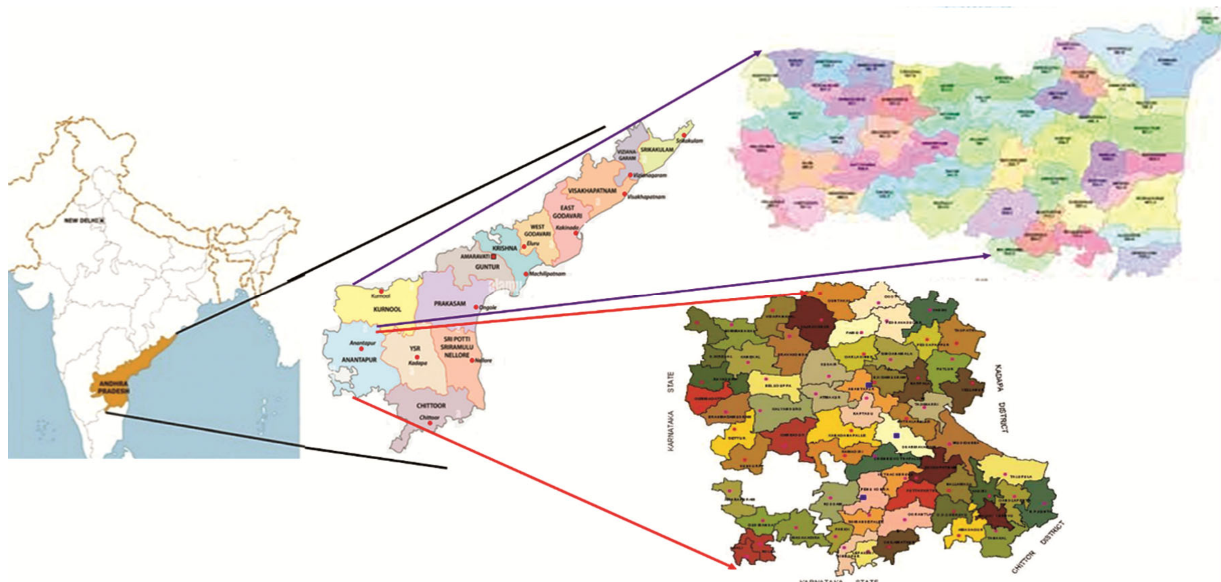

Fig. 2 — Mapping of Ashwagandha sample collection.

Fig. 3 — Collection of plant samples of cultivars in the Kurnool district.

Fig. 4 — Collection of plant samples of cultivars in Ananthapur district.

samples were shade-dried, powdered, and packed in an air-tight cover till further analysis.

Chemicals and reagents

The reference standards Withanoside IV, Withanoside V, Withaferin A, Withanolide A, and Withanone (Fig. 1) were acquired from M/S Natural Remedies Pvt. Ltd., based in Bengaluru, India. These standards played a crucial role in the development of methods and the analysis of *W. somnifera* samples. For the analysis, HPLC-grade Methanol and Acetonitrile (Merck, Bombay, India) were utilised, filtered through a Millipore filter (0.5 µm) as necessary to ensure purity and precision. Water of HPLC grade (Rankem, New Delhi, India) was also employed in the analytical process to maintain the required standards and accuracy in the analysis of *W. somnifera* samples.

Preparation of the sample solution

The powdered root (1 g) was carefully weighed and placed into a conical flask. Exactly 30 mL of methanol was added to the powdered root within the conical flask. The mixture was extracted by sonication at 45°C for 30 minutes. Following extraction, the resulting solution was filtered through filter paper into a 100 mL volumetric flask. The filtrate was returned to the conical flask. This extraction procedure was repeated twice more to ensure thorough extraction of the constituents, and the combined extracts were diluted to the final volume with methanol in a 100 mL volumetric flask. Each resulting sample solution was further filtered through a 0.45 µm membrane filter into an HPLC sample vial before being injected into the HPLC system for analysis.

Instrumentation

The HPLC analysis was conducted using a Water modular system comprising two model 1524 pumps, an automatic gradient controller, a model 717 plus injector, a model 2996 Photodiode Array Detector (PDA), and Empower software for streamlined operation. Integration of the injector, gradient controller, and chromatography manager ensured consistent and reproducible results.

Chromatographic conditions

The analysis employed a Phenomenex Sphereclone ODS (2) column (4.6 mm x 250 mm, 5 µm particle size, Made in USA). The injection volume was chosen to be 20 µL. The mobile phase consisted of two solvents: acetonitrile (solvent A) and water (solvent B). The gradient programming, performed at room temperature was initially at 5% A, changed to 10% A at 5.0 minutes at a flow-rate of 1.0 mL/ min, changed to 30% A at 6 minutes and maintained for the next 4.0 min with flow rate 1.5 mL/min, and then at 11.0 min the flow-rate was changed to 0.3 mL/ min and maintained for the next 4.0 min. The mobile phase was changed to 60% A at 20 minutes; the flow rate was 1.0 mL/min, and at 22 minutes it was changed to 0.3 mL/min and maintained for the next 13 minutes. Then, at 40 minutes, the flow rate was changed to 1.0 mL/min. Then the percentage of A was changed to 10% at 45 minutes at a flow rate of 1 mL/min, then reduced to 5% at 50 minutes with the same flow rate and continued it for another

10 minutes. The PDA wavelength scan range was set from 200 to 400 nm, recording chromatograms at 227 nm, where all five marker compounds exhibited favourable absorbance. Retention times for the standards were identified as follows: Withanoside IV (12 min), Withanoside V (25 min), Withaferin A (26 min), Withanolide A (29 min), and Withanone (30 min) (Figs. S1-S4). Calibration graphs were constructed by plotting the relative peak area, enabling quantification of these marker compounds in the samples.

RP-HPLC method validation

The method was validated in accordance with ICH guidelines. The validation method results are shown and Table 1.

Statistical analysis

Principal Component Analysis (PCA) was performed on the HPLC data using Paleontological Statistics (PAST) software version 3.15, which was used for data analysis.

Results and Discussion

The main task of this work was to develop a sensitive, selective, and accurate RP-HPLC method for the determination of five marker compounds from *W. somnifera*, with satisfactory precision, in accordance with good analytical practice (GAP). Also, study the mapping of the determination of the quantity of marker phyto-molecules in different cultivars in India.

Development of the RP-HPLC method

RP-HPLC has become the most versatile and widely used technique in the pharmaceutical industry for drug quality control and analysis. It has many applications in the field of pharmaceuticals, including the quantitative determination of drugs present either alone or in the presence of other mixture components.

Chromatographic conditions

Good separations and suitable retention time of five analytes (Withanoside IV, Withanoside V, Withaferin A, Withanolide A and Withanone) were obtained in gradient elution using Phenomenex Sphereclone ODS (2) column (4.6 mm X 250 mm, 5 µm particle size) with mobile phase consisting of acetonitrile and water. Different mobile phases have been tested to achieve the best chromatographic separation among the studied, including isocratic and gradient combination elution of methanol and water, acetonitrile and water in two different columns, *viz*. Phenomenex® Sphere Clone ODS (2) column (4.6 mm x 250 mm, 5 µm particle size) and Phenomenex® Luna C_{18} column (4.6 mm x 250 mm, 5 µm particle size). Among all the tested, Acetonitrile (A) and water (B) in gradient mode with flow rates ranging from 0.3 to 1.5 mL/min within 50 minutes analysis time resulted in better resolution with sharp peaks of *W. somnifera* root extracts while using Phenomenex Sphereclone ODS (2) column (4.6 mm x 250 mm, 5 µm particle size) column at 227 nm.

RP-HPLC Method validation

Linearity

The calibration curve showed good linearity over a wide concentration range (at 20, 40, 60, 80, and 100 µg/mL) for five standards with r^2 = 0.9941 to 0.9986. The pre-analysed samples were spiked with standards at three different concentration levels (20, 60, and 100 µg/mL) (Table 1).

Table 1 — Results of method validation for standards

Parameters	Withanoside IV	Withanoside V	Withaferin A	Withanolide A	Withanone
Linearity (r^2)	0.9986	0.9967	0.9980	0.9941	0.9983
LOD (% RSD)	1.9237	1.7947	0.8166	1.0071	1.3617
LOQ (% RSD)	1.4070	1.7620	0.3623	0.8542	1.7425
Precision (% RSD)					
Intra-day	1.8202	1.8498	1.3017	0.9797	1.0336
Inter-day	1.5369	1.6505	1.3016	0.8292	0.5654
Accuracy (% RSD)	0.8621	0.5418	0.4220	0.2116	0.2547
Stability					
24 h	0.9622	1.3999	0.8939	2.017	0.1384
48 h	1.6034	1.9160	1.0227	1.1286	1.1348
72 h	1.7846	1.7935	1.1983	1.6599	0.1513

LOD – Limit of detection; LOQ – Limit of quantitation; RSD – Relative standard deviation
The developed RP-HPLC method is gradient, simple, buffer-free, precise, and efficient, with better baseline resolution and peak separation for simultaneous quantification of five standards in the root part of *W. somnifera.*

Lower Limit of Detection (LOD) and Lower Limit of Quantification (LOQ)

The LOD and LOQ were calculated based on signal-to-noise ratio method. They were found to be 1.9247 and 1.4070 µg/mL for Withanoside IV, 1.7947 and 1.7620 µg/mL for Withanoside V, 0.8166 and 0.3623 µg/mL for Withaferin A, 1.0071 and 0.8542 µg/mL for Withanolide A, 1.3617 and 1.7425 µg/mL, respectively (Table 1).

Precision

Repeatability (intra-day) and intermediate precision (inter-day) were determined through analysis (in triplicate). Values of % RSD obtained during precision studies at all levels were ≤ 2.0 (Intra-day and inter-day were found to be 1.8202 and 1.5369 μg/mL for Withanoside IV, 1.8498 and 1.6505 μg/mL for Withanoside V, 1.3017 and 1.3016 μg/mL for Withaferin A, 0.9797 and 0.8292 μg/mL for Withanolide A, 1.0336 and 0.5654 μg/mL, respectively), which indicates that the proposed method was precise (Table 1).

Stability studies

The stability of individual sample solutions of five standards was tested at 24, 48, and 72 h after preparation. The results were calculated as the percentage of non-degraded all five standards at the specified time intervals. The samples remained stable for up to 24 h, but thereafter the percentage of the five standards gradually increased, which may be due to solvent evaporation (Table 1).

Accuracy

The accuracy of the developed method was evaluated through the analyte recovery test at three concentration levels (30, 60, and 90 μg/mL). The recoveries ranged from 85% to 96%. The mean recoveries were found to be RSD 0.8621% for Withanoside IV, 0.5418% for Withanoside V, 0.4220% for Withaferin A, 0.2116% for Withanolide A, and 0.2547% for Withanone, which is considered acceptable. Hence, the developed method was accurate (Table 1).

Mapping study on marker compounds

Chemical analysis, based on the quantification of five major bioactive molecules, from 125 root samples of *W. somnifera*, revealed significant chemical variability among the collected populations, as depicted in Figs. 5 and 6 and Table S3.

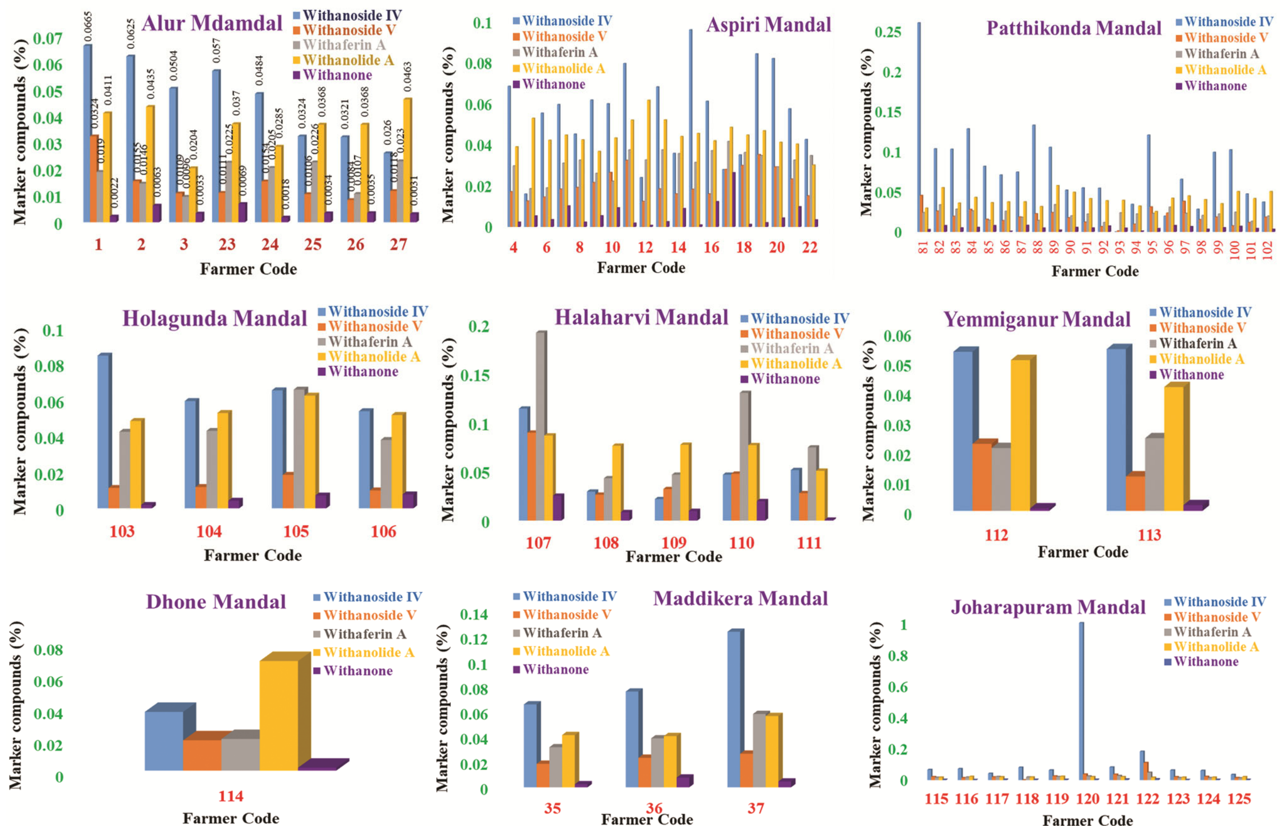

Fig. 5 — Variation of marker compounds from different cultivars of Kurnool district.

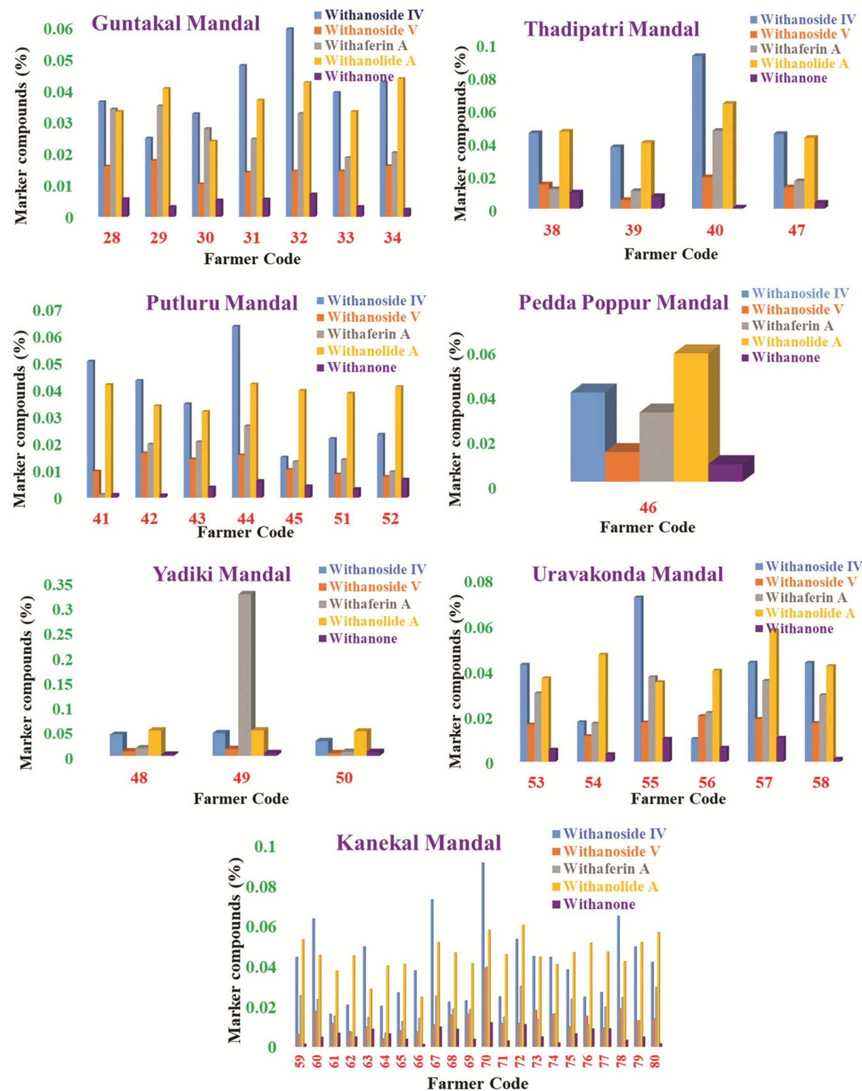

Fig. 6 — Variation of marker compounds from different cultivars of Ananthapur district.

Mapping study in the Kurnool district of Andhra Pradesh

In the Kurnool district of Andhra Pradesh, qualitative and quantitative disparities were observed among 75 different cultivars across eight mandals (Fig. 5). The marker compounds Withanoside IV [0-1.0016% (± 0.196)], Withanoside V [0.0013% (± 0.005) -0.1097% (± 0.042)], Withaferin A [0.0096% (± 0.006) - 0.1912% (± 0.066)], Withanolide A [0.0163% (± 0.006) - 0.0866% (± 0.012)], and Withanone [0.0007% (± 0.001) - 0.0264% (± 0.014))] exhibited a wide range of concentrations.

Remarkably, the root sample from Joharapuram village in Aspiri mandal (Sample 120) demonstrated a predominant presence of Withanoside IV (1.0016% (± 0.196)), surpassing all other samples in its dominance. Across Alur, Aspiri, Pattikonda, and Holagunda mandals, Withanoside IV notably prevailed, followed by Withanolide A and Withaferin A. Conversely, Withaferin A exhibited prominence primarily in the Halharvi mandal. The remaining mandals showcased varying levels of these marker compounds, indicating distinct chemical profiles among the cultivars across different regions within the district.

Mapping study in the Ananthapur district of Andhra Pradesh

In the Ananthapur district, root samples from 50 cultivars across seven mandals were analysed (Fig. 6), revealing varying concentrations of marker compounds Withanoside IV (ranging from 0.01% (± 0.010) to 0.0927% (± 0.016)), Withanoside V (ranging from 0.0039% (± 0.002) to 0.0391%

(± 0.014)), Withaferin A (ranging from 0.0012% (± 0.001) to 0.325% (± 0.043)), Withanolide A (ranging from 0.0239% (± 0.010) to 0.0637% (± 0.015)), and Withanone (ranging from 0.0008% (± 0.001) to 0.012% (± 0.005)). Across all the mandals in Anantagiri, Withanoside IV emerged as the major compound, followed by Withanolide A and Withaferin A. However, a significant observation was made in a sample collected from a farmer in Yadiki mandal (Sample 49), where Withaferin A emerged as the predominant component in the root samples. This distinctive composition in the Yadiki mandal suggests a unique chemical profile compared to those of the other mandals in the Ananthapur district (Fig. S5).

Correlations

In correlation, the individual chemical constituents of the marker compounds identified by the RP-HPLC study were also correlated among themselves (Table S5). The major constituent, Withanolide A, showed significant positive correlations with Withanone (0.3769), Withaferin A (0.3757), and Withanoside V (0.1167), and a negative correlation with Withanoside IV (-0.1875). Whereas, Withaferin A exhibited significant positive correlations with Withanoside V (0.3583) and Withanone (0.3243). Similarly, the glycoside Withanoside V showed significant positive correlation with all the constituents (Withanone: 0.3367; Withanoside V: 0.3204, and Withanolide A: 0.1167). Another glycoside, Withanoside IV, showed positive correlation with Withaferin A (0.0362) and negative correlation with Withanone (-0.0306) and Withanolide A (-0.1875), respectively.

PCA unveiling key contributors: Understanding chemical variability

The PCA revealed the two most informative principal components with eigenvalues of 0.0083 and 0.0012, respectively. Together, these two components accounted for 97.24% of the total variance in the dataset. This suggests that these two components capture a significant portion of the data's variability. The loadings of different chemical variables on the principal component are listed (Table 2). Variables like Withanoside IV, Withanoside V, and Withaferin-A have relatively high contributions to the total chemical variability, accounting for 84.92% of the variability explained by the first principal component. It appears that Withaferin A has the highest contribution to the second principal component.

Based on the PCA analysis, places were selected or categorised according to their principal component scores. Places with positive scores were 49 and 120, while places with negative scores were 93, 64, and 41 (Fig. 7). These scores contribute to the total diversity

Table 2 — The loadings of different chemical variables on the principal component are presented in this table

Traits	PC1	PC2	PC3	PC4	PC5
Withanoside IV	0.99834	-0.018762	-0.037365	0.039576	0.0033675
Withanoside V	0.048774	0.14958	0.94927	-0.25511	-0.095191
Withaferin A	0.016073	0.97473	-0.18768	-0.11959	-0.011206
Withanolide A	-0.026212	0.15944	0.22285	0.9559	-0.10234
Withanone	-0.0012337	0.041956	0.1123	0.072787	0.99012

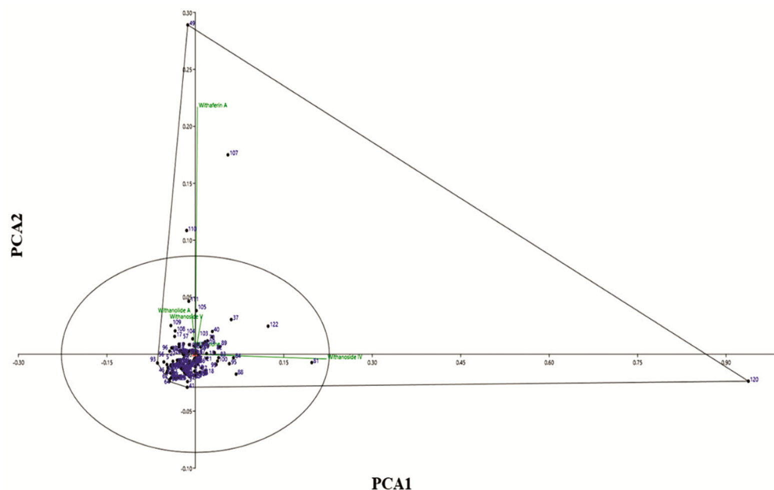

Fig. 7 — PCA analysis for major active principal components of samples collected from different ashwagandha grower fields of Andhra Pradesh.

or variability in the dataset. These places are vertex entries, which might imply that they play a significant role in the dataset's structure. In summary, PCA was used to reduce the dimensionality of a dataset and to identify the main sources of variability among different chemical characters across locations and seasons. The first two principal components explained a large portion of the variance and specific variables, and places were highlighted based on their contributions to these components. Similar kinds of studies reported by earlier researchers in *Mimosa tenuifolia*[15,16] and in *Pinellia ternate*[17].

Conclusion

The method described herein represents a significant improvement in the analysis of Withanoside IV, Withanoside V, Withaferin A, Withanolide A and Withanone in Ashwagandha. The developed method not only allows determination in root material but is also suitable for quantifying standards in leaf and stem parts of the plant. Thus, it should be useful for both scientific and commercial applications. The mapping studies revealed variations in marker compounds among root samples collected from different villages in the Kurnool and Ananthapur districts of Andhra Pradesh, with considerable differences observed between regions. In Kurnool, the sample collected from Joharapuram village in Aspiri mandal (Sample 120) showed a predominance of Withanoside IV (1.0016%) and was the most dominant among all the samples. Withanoside IV was more prevalent in Alur, Aspiri, Pattikonda, and Holagunda mandals of Kurnool, followed by Withanolide A and Withaferin A. Whereas Withaferin A was predominantly found in the Halharvi mandal. The remaining mandals showed variable amounts of the marker compounds, possibly due to red soil conditions. Similarly, in Ananthapur, all the mandals of Anantagiri showed Withanoside IV as the major compound, followed by Withanolide A and Withaferin A. But a farmer sample collected in Yadiki mandal (Sample 49) showed Withaferin A as the predominant component in the root samples, as Ananthapur district is one of the low-rainfall areas in the South and experiences higher temperatures during the plant growing period. Due to these climatic conditions, the plant has grown under stress conditions. Conclusively, the PCA can be utilised to reduce the multidimensional characteristics of medicinal plant samples into distinct groups, facilitating the identification of suitable locations for cultivation among a large number of samples. Future studies will focus on multi-seasonal validation of withanolide profiles, the integration of soil and environmental factors, and molecular characterisation of elite genotypes to develop predictive models for quality cultivation and enhanced commercial utilisation.

Acknowledgement

The authors are thankful to the Andhra Pradesh State Medicinal Plants Board (APMAPB), Vijayawada, A.P., India, for sanctioning financial assistance for the project (GAP-444).

Conflict of interest

We would like to declare on behalf of our co-authors that there is no conflict of interest in the submission of this manuscript, which is approved by all authors.

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