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Metabolic Characterization of *Withania somnifera* from Different Regions of India Using NMR Spectroscopy

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Key words
- *Withania somnifera*
- Solanaceae
- metabolic characterization
- NMR
- withanolides

Abstract

Withania somnifera (L.) Dun. (Solanaceae), known as Indian ginseng, is one of the most popular medicinal plants in India. Considering the importance and common use of this plant, it is necessary to investigate its holistic metabolite profile. However, with existing analytical methods which are based on TLC and HPLC-UV (or MS), it is difficult to obtain information of the whole range of compounds appropriately. In this study, the metabolic characterization of *Withania somnifera* leaves, stems, and roots collected in six different regions in India was performed using ^{1}H NMR spectroscopy followed by principal component analysis (PCA) and hierarchical clustering analysis (HCA). Of the parts of *Withania somnifera* analyzed in this study, the leaf was found to have the widest range of metabolites, including amino acids, flavonoids, lipids, organic acids, phenylpropanoids, and sugars, as well as the main secondary metabolites of the plant, withanolides. The ^{1}H NMR spectra revealed the presence of two groups of withanolides: 4-OH and 5,6-epoxy withanolides (withaferin A-like steroids) and 5-OH and 6,7-epoxy withanolides (withanolides A-like steroids). The ratio of these two withanolides was found to be a key discriminating feature of *Withania somnifera* leaf samples from different origins.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Withania somnifera Dunal (Solanaceae), popularly known as Indian ginseng, is one of the most commonly used ingredients of Ayurveda, Unani, and Sidha formulations [1]. Various parts of the plant – fruits, leaves, and roots – have been used for centuries to treat a variety of ailments [2–4]. The pharmacological properties of the plant include adaptogenic, anti-sedative, and anticonvulsant activities [2–4] while other therapeutical activities are also currently being investigated [5]. Most bioactivities of *W. somnifera* are believed to be due to two major groups of metabolites: steroidal alkaloids and steroidal lactones known as withanolides [6]. So far, more than 10 alkaloids and 40 steroids including withanoloids and sitoindosides have been isolated from the plant [7, 8]. The level of the diverse *W. somnifera* metabolites varies according to the part of the plant – root, leaves, or stems – very likely resulting in unique medicinal properties for each part. Also, the different growth conditions in the regions cause significant differences in the metabolic profile of the plants, since external environmental factors, including soil contents, climate, and other coexisting organisms have a considerable effect on individual metabolite content.

Considering the importance of this plant, a great deal of work directed at finding adequate methods for quality evaluation and characterization of discrete chemotypes of *W. somnifera* plants has been made, usually using conventional chromatographic methods. However, due to the inherent characteristics of these methods, quality control evaluation has been restricted to the quantitation of a few withanolides, or more precisely to withaferin A, which is an important active principle [9–11]. This is clearly insufficient. When plant material or extracts are used as herbal medicines, the objective of the method used for their quality control has to be necessarily that of verifying its identity and/or origin in the first place and then, when possible, quantifying compounds that have a direct impact on its potency. In the case of identification, the possibility of comparing the largest

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set of components or metabolites as possible with those of standard material, allows the detection of adulterations or substitutions. While chromatographic profiles are helpful, drawing a line between standard and substandard material is very hard because the number of discriminating metabolites detected is usually limited due to technical features of the classical methods as mentioned above. Thus, the possibility of counting on a method that can afford a comprehensive profile of both primary and secondary metabolites rather than a few major compounds would be a major contribution to the quality control of plant material and derivatives, providing a clearer picture of the key constituents associated to the identity of the herbal product on one hand and assisting in the choice of several compounds related to its pharmacological activity for quantitation.

Metabolomics – comprehensive metabolomic profiling in combination with multivariate analysis of the data – is considered to be a very useful technique for this purpose. We have published several reports on the successful application of nuclear magnetic resonance spectroscopy (NMR)-based metabolomics to the quality control of medicinal plants including ginseng [12], *Ephedra* species [13], *Cannabis* varieties [14], and *Ilex* species [15].

In the case of *W. somnifera*, the metabolic difference of roots and leaves was investigated employing a metabolomics approach that allowed the identification of 62 major and minor primary and secondary metabolites from leaves and 48 from roots using NMR, HPLC, and GC-MS [16].

In view of these encouraging results, the metabolomic characterization of *W. somnifera* roots, leaves, and stems of plants obtained from various locations in India was undertaken, using NMR spectroscopy combined with multivariate data analysis. Classification of the plants according to their primary and secondary metabolites content was also performed.

Materials and Methods

Materials

Leaves, stems, and roots of *W. somnifera* were collected in November 2008 from six different locations in Pune (India), such as Badgaon, Dhanukar colony, Singhgad, Warje, Karve and Hadapsar, and Gwalior (Madhya Pradesh, India). All the regions are located at a distance of about 10 km from each other, with the exception of Gwalior (Madhya Pradesh, India) that is about 900 km from Pune. The Regional Research Institute (RRI) of Pune, India authenticated the samples, and voucher specimens are kept at the Departmental Herbarium of RRI (Voucher No. 384-1 ~ 384-6). Commercial *Withania* roots used in the present study were generously provided by Green Pharmacy, Dhanvantri Ayurvedic Bhandar, Atharav Pharmaceutical, Madura Pharmacy, and United Pharmacy, all of them located in Pune, India, while the *Withania* root extracts were provided by the following Indian companies: Alchemy Chemicals, Ujjain; Amsar Pvt. Ltd., Indore; Ansar, Surat; Natural Remedies, Banglore; and Tulsi Amrit, Indore.

Solvents and chemicals

D_2O (99.0%) and CH_3OH-d_4 (99.8%) were obtained from Cambridge Isotope Laboratories, Inc. Trimethylsilane propionic acid sodium salt (TMSP) and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck. NaOD was purchased from Cortec.

Extraction

For preliminary experiments directed at testing extraction solvent efficiency, 5 mL each of chloroform, acetone, MeOH, MeOH-Water (1 : 1), and water were added to 50 mg of *W. somnifera* ground leaves and sonicated for 20 minutes. After filtering, the resulting extracts were evaporated using a rotary evaporator, and the dried extracts were redissolved in deuterium solvents; $CDCl_3$ for the chloroform extract, CH_3OH-d_4 for the acetone and MeOH extract, CH_3OH-d_4-KH_2PO_4 in D_2O buffer (1 : 1, pH 6.0) for the MeOH-water extract, and KH_2PO_4 in D_2O buffer (pH 6.0) for the water extract.

For metabolomics experiments, our in-house protocol was used [17]. Three biological replicates were used for all experiments. A sample of 50 mg of powdered material of ground roots was transferred to a centrifuge tube. A volume of 750 µL of KH_2PO_4 in D_2O buffer (with 0.01% TMSP) and 750 µL of CH_3OH-d_4 was added to the tube followed by vortexing for 1 min and sonication for 20 min. The tube was centrifuged at 13 000 rpm for 10 min at 25 °C. The supernatant (800 µL) was transferred to a 5 mm-NMR tube.

Isolation of withanolides

Dried extracts (30 g) from commercial *W. somnifera* root extracts obtained from Alchemy Chemicals were dissolved in 250 mL of deionized water and submitted to liquid-liquid fractionation with *n*-hexane, dichloromethane, and *n*-BuOH (5 × 250 mL). The dichloromethane extract was dried under reduced pressure to obtain three grams of a green-brown semisolid. Two grams of this organic extract were fractioned by column chromatography (45 × 3 cm, flow rate approx. 3 mL/min) on silica gel (40–60 µm; 1 : 50, w/w) using stepwise gradient elution from *n*-hexane, ethyl acetate to methanol. Specifically, the solvent system used was 100% *n*-hexane, *n*-hexane-EtOAc (90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90), 100% EtOAc, EtOAc-MeOH (97.5 : 2.5, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 50 : 50), and 100% MeOH. The volume eluted in each step was 150 mL and eighteen fractions were obtained and evaporated to dryness. The ^{1}H NMR spectrum of the fractions F-12 and F-14 eluted with EtOAc-MeOH (97.5 : 2.5) and EtOAc-MeOH (90 : 10), respectively, showed chloride attached withanolide (55 mg) and withaferin A (290 mg) as the major compound. Structures were also confirmed by the comparison with reported values [18,19]. The fractions F-10 (81 mg) and F-11 (22 mg) eluted with *n*-hexane-EtOAc (10 : 90) and EtOAc 100% were combined and subjected to flash chromatography (57 × 1 cm, flow rate approx. 1 mL/min) on silica gel (40–60 µm; 1 : 70, w/w) using a mixture of two system solvents A ($CHCl_3$-*n*-hexane, 74 : 24) and B (EtOAc-MeOH, 4 : 8) as the mobile phase. The solvent system applied was 100% A, A–B (99 : 1, 98 : 2, 97 : 3, 96 : 4, 95 : 5, 90 : 10, 80, 20, 50 : 50), 100% B. Aliquots of 5 mL were collected and pooled in 13 fractions according to their similarity by TLC (SiO_2, $CHCl_3$-EtOAc-MeOH-*n*-hexane, 74 : 4 : 8 : 24, vanillin phosphoric acid, and anisaldehide reagents). ^{1}H NMR spectrum of subfraction SFX-11-5 (14.8 mg) eluted with A–B (98 : 2) corresponded to withanolide A. The structure was confirmed by the comparison of ^{1}H NMR spectra with reported values [20].

NMR measurements

^{1}H NMR, 2D *J*-resolved spectra, as well as HSQC and HMBC, were recorded at 25 °C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz proton frequency) equipped with a TCI cryoprobe and Z-gradient system following our previous publication

[15]. ^{1}H-^{1}H Double-quantum filter correlation spectroscopy (DQF-COSY) spectra were acquired with presaturation (γB_1 = 50 Hz) during a relaxation delay of 1.5 s. A data matrix of 1024 × 2048 points covering 7739.4 × 7739.4 Hz was recorded with 8 scans for each increment. Data was zero filled to 2048 × 2048 points prior to States-TPPI type 2D Fourier transformation and a sine bell-shaped window function was applied in both dimensions. ^{1}H NMR spectra were submitted to further data analysis using PCA and all 2D spectra were used for the identification of metabolites.

Data analysis

The ^{1}H NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7; Bruker Biospin). Spectral intensities of ^{1}H NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.3 – δ 10.0. The regions of δ 4.7 – δ 5.0 and δ 3.34 – δ 3.28, originated from the residual signal of HDO and CH_3OH-d_4, were excluded from the analysis. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed with the SIMCA-P software (v. 12.0; Umetrics). Pareto scaling method which gives each variable a variance numerically equal to its initial standard deviation was used for PCA. Hierarchical cluster analysis (HCA) was performed by using 8, 5, and 5 PCs for leaf, stem, and root samples, respectively, based on Ward's minimum variance method.

Supporting information

Loading plot of PC1 (A) and PC3 (B) of principal component analysis of *Withania somnifera* samples of leaves, stems, and roots and the loading plot of PC1 of principal component analysis of *Withania somnifera* samples of leaves are available as Supporting Information.

Results and Discussion

The selection of an appropriate system that can potentially extract all the chemically diverse groups of metabolites that can be detected using NMR spectroscopy is a fundamental step in the development of a method for the quality control or identification of plant material. Preliminary experiments were performed in order to find the best extraction solvent for *W. somnifera* leaves. Chloroform, acetone, MeOH, MeOH-water (1 : 1), and water were tested as extraction solvents, and their NMR profiles were examined. Signals corresponding to H-2 and H-3 of withanolides were clearly detected in all extracts. Depending on the position of the -OH and epoxy substituents in the withanolide structure, the chemical shifts of H-2 and H-3 are different from each other. In the case of withanolides containing 5-OH and 6,7-epoxy as in withanolide A, H-2 and H-3 are found at δ 5.8 – δ 6.1 and δ 6.6 – δ 6.8, respectively (**Fig. 1**). However, for the compounds having 4-OH and 5,6-epoxy as in withaferin A, these signals show a ca. 0.5 ppm downfield shift as compared to those observed for withanolide A (**Fig. 1**). Among the solvents tested, MeOH-water (1 : 1) proved to extract a greater diversity of withanolides and other metabolites than any other solvent with the exception of acetone that proved to be quite efficient for the extraction of pyrazole-type alkaloids. However, due to its overall performance, MeOH-water (1 : 1) was selected for further metabolomic studies of *W. somnifera*. To simplify the sample preparation step, deuterated methanol and water were used for the extraction and samples were analyzed directly by NMR spectroscopy.

^{1}H NMR spectra of the samples were analyzed using multivariate data analysis (MVDA). For MVDA, principal component analysis (PCA) was used first because PCA is a typical unsupervised method requiring no knowledge of the data set and reduces the dimensionality of multivariate data while preserving most of the variance [21]. Initially, PCA was applied to the separation of different parts of samples such as leaves, stems, and roots of *W. somnifera*. As shown in **Fig. 2**, leaves, stems and roots are clearly separated by PC1, PC2, and PC3. Although the aim of this study was the differentiation of *W. somnifera* samples according to their regional origin, this was not clearly achieved because the variation between leaves, stems, and roots was found to be much larger than the geographical variation. As shown in **Fig. 2**, root samples have higher PC1 values than samples from other parts of the plants. From the loading plot (**Fig. 1S**) the two major metabolites that separate root samples from the others were identified as sucrose and γ-aminobutyric acid (GABA). The resonances of H-1 and H-2′ of sucrose at δ 5.41 (d, 3.6 Hz) and δ 4.16 (d, 6.5 Hz), and those of H-2 and H-3 of GABA at δ 2.30 (t, 7.5 Hz) and δ 1.90 (m) are more abundant in root samples. This can be explained by the fact that sucrose is the most common storage form of carbohydrates in roots. In the case of GABA it is thought to be the product of interactions with microorganisms in soil [22]. The separation between the leaves and the stems of *W. somnifera* was obtained by PC3 for which withanolides containing 4-OH and 5,6-epoxy groups such as withaferin A were found to be the major contributing metabolites. In the loading plot of PC3, the resonances of H-2 (δ 6.28, d, 10.0 Hz) and H-3 (δ 7.06, dd, 10.0, 5.8 Hz) of withaferin A were found to be major discriminating signals between leaf and stem samples. However, the signals of H-2 and H-3 of 5-OH and 6,7-epoxy groups as in withanolide A, were not clearly related with the leaf samples. The comparison of ^{1}H NMR spectra of leaves, stems, and roots of the plants can be seen in **Fig. 3**. Among the samples analyzed in this study, the highest diversity of metabolites was found in *W. somnifera* leaves. In addition to steroids, a great number of other metabolites including trigonelline, ferulic acid, tryptamine, and kaempferol glycosides were detected. When comparing leaf and stem samples, as shown in the PCA results, leaf samples were found to contain higher amounts of withaferin A while the levels of withanolide A in leaf and stem samples were not significantly different.

An effect of the geographical origin of the plants could not be concluded from the PCA analysis because differences were not statistically significant when compared to the variation between plant parts. Thus, as a next step, a supervised MVDA, partial least square-discriminant analysis (PLS-DA) was applied to the separation of the samples in order to see if this would allow the detection of regional variations. However, once more the differences between plant parts (leaf, stem, and root) proved to be greater, and it was concluded that the PLS-DA model could thus not be validated. Actually, geographical variations might often be difficult to detect because they could be a minor factor. For example, in our previous research on the geographical variation of *Narcissus* bulbs, we found that it was only reflected in minor PCs (combination of PC2 and PC4) [23].

To eliminate the unwanted plant part variations, PCA analysis was applied separately to each individual part of *W. somnifera*, i.e., leaf, stem, and root collected in different regions. Additionally, hierarchical clustering analysis (HCA) was applied to examine metabolomic resemblances, using the first 8 PCs (leaf), 5 PCs

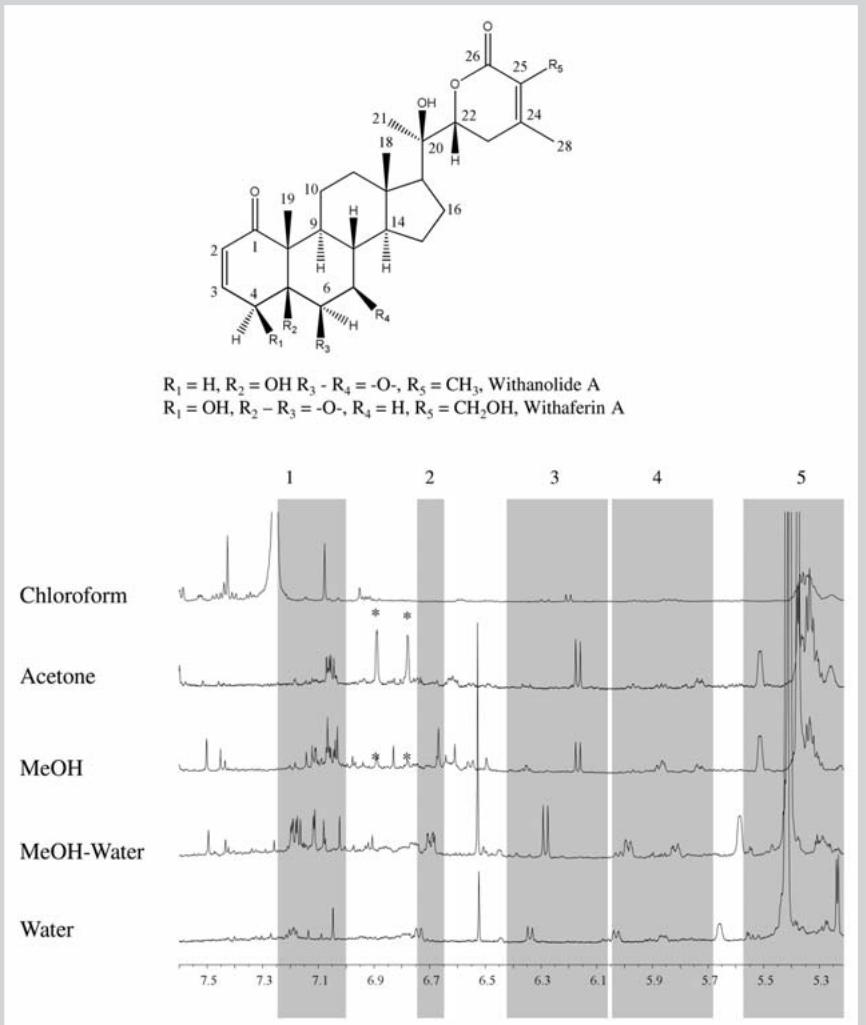

Fig. 1 Chemical structures of withanolide A and withaferin A, and ^{1}H NMR spectra of *Withania somnifera* leaves collected in Badgaon (Pune, India) extracted with chloroform (in $CDCl_3$), acetone (in CH_3OH-d_4), MeOH (in CH_3OH-d_4), MeOH-water [in CH_3OH-d_4-KH_2PO_4 in D_2O buffer (1 : 1, pH 6.0)], and water [KH_2PO_4 in D_2O buffer (pH 6.0)] in the range of δ 5.2 – δ 7.7. 1: H-3 of withaferin A; 2: H-3 of withanolide A; 3: H-2 of withaferin A; 4: H-2 of withanolide A; 5: H-1 of sucrose and a-glucose and olefinic H of lipids; *: pyrazole resonances.

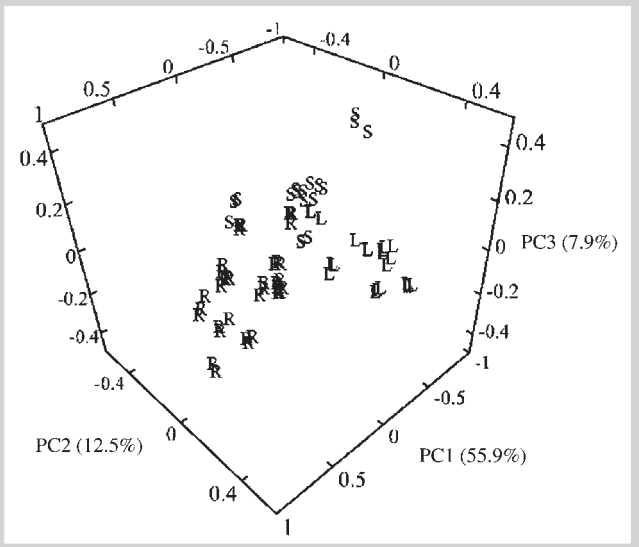

Fig. 2 Score plot of principal component analysis of *Withania somnifera* samples using PC1, PC2, and PC3. L: leaf, S: stem, R: root.

(stem), and 5 PCs (stem) obtained by PCA. In all the samples of the same part of *W. somnifera* cultivated in different regions the metabolome was clearly distinguished. In particular, the samples cultivated in Gwalior (Madhya Pradesh, India) showed a very discriminant metabolic pool in all parts of the plant employed in this study (leaf, stem, and root) as can be seen in the PCA score plot in **Fig. 4**. Although the PCA score plot is definitely a good way to visualize the difference, there is a limitation in the number of PCs employed. In cases in which more than 3 PCs affect a separation, data visualization cannot be completed by a score plot. Therefore, PCs reduced from the original ^{1}H NMR spectra, were further analyzed by hierarchical clustering analysis. It was confirmed that samples of all parts of *W. somnifera* cultivated in Gwalior were clearly distinguishable from others (**Fig. 4**). The separation of the leaves collected in Gwalior was due to a higher level of withanolides containing 4-OH and 5,6-epoxy groups as well as ferulic acid, kaempferol glycosides, sucrose, aspartate, citrate, and malate (**Fig. 2S**). Conversely, levels of withanolides containing 5-OH and 6,7-epoxy groups and glucose showed lower levels in the leaves of this accession. Ganzera and his colleagues reported that the concentrations of withaferin A and withanolide D differed in *W. somnifera* collected in Pakistan [24] while there was no change in the level of withanolides in the *W. somnifera* populations in Israel [25]. In this study, the level of withanolides was similar in all the samples with the exception of one sample.

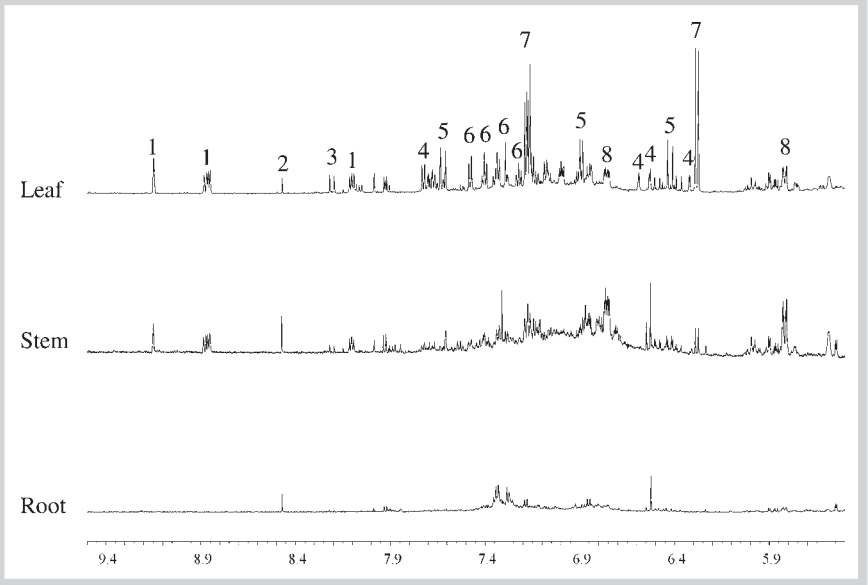

Fig. 3 ^{1}H NMR spectra of *Withania somnifera* leaves, stems, and roots collected in Gwalior (Madhya Pradesh, India) in the range of δ 5.5 – δ 9.5. 1: trigonelline; 2: formic acid; 3: adenine; 4: kaempferol glycosides; 5: ferulic acid; 6: tryptamine; 7: withaferin A; 8: withanolide A.

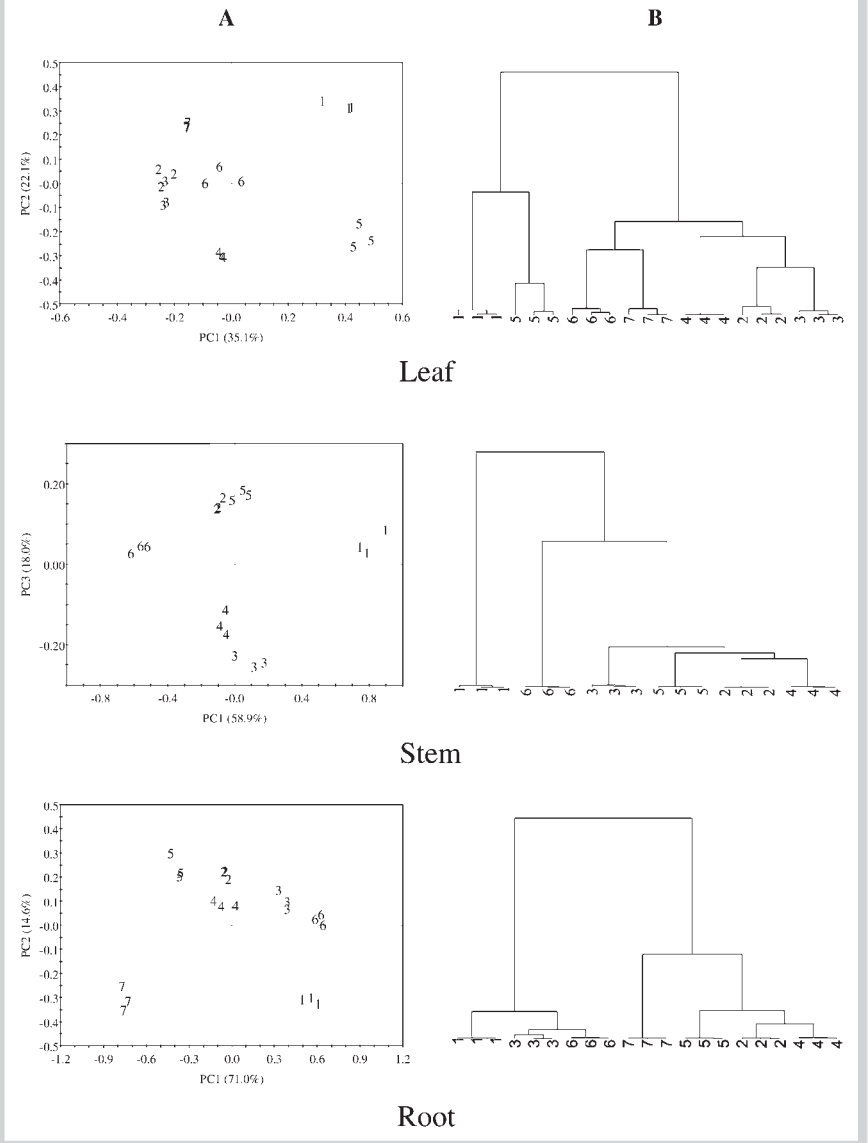

Fig. 4 Score plot of principal component analysis (**A**) and hierarchical clustering analysis (**B**, on the basis of 8, 5, and 5 PCs for leaf, stem, and root samples, respectively) of *Withania somnifera* cultivated in different regions of India. 1: Gwalior; 2: Badgaon; 3: Dhanukar colony; 4: Singhagad; 5: Warje; 6: Karve; 7: Hadapsar. Stem sample No. 7 (Hadaspar) was not measured for the analysis.

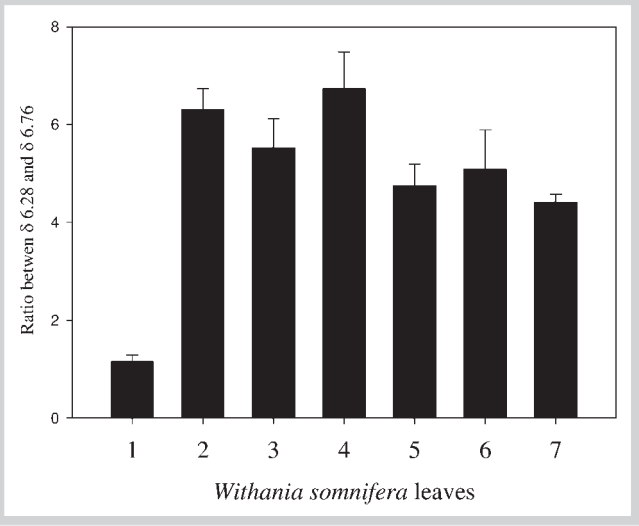

Fig. 5 Ratio of ^{1}H NMR intensities of withanolides containing 4-OH and 5,6-epoxy groups and H-2 of withanolides containing 5-OH and 6,7-epoxy groups. 1: Gwalior; 2: Badgaon; 3: Dhanukar colony; 4: Singhagad; 5: Warje; 6: Karve; 7: Hadapsar. SD values were calculated based on triplicates.

In **Fig. 5** the ratio of ^{1}H NMR intensities of withanolides containing 4-OH and 5,6-epoxy groups at δ 6.76 (m) and 5-OH and 6,7-epoxy groups at δ 6.28 (d, 10.0 Hz) is shown. As can be observed in **Fig. 5**, *W. somnifera* leaf samples collected in Gwalior exhibited an extremely low level implying that withanolides containing 5-OH and 6,7-epoxy groups might be more selectively produced than those containing 4-OH and 5,6-epoxy groups. The large difference in withanolides levels in different geographical populations of *W. somnifera* is an important issue to be solved.

In all other parts of the plant that were studied, i.e., stems and roots, the samples collected in Gwalior were clearly distinguished from others, mainly due to a high level of sucrose.

Withania somnifera is one of the most extensively used medicinal plants in Ayurvedic formulations for a variety of health-promoting effects. Accordingly, its quality control and/or phytochemical investigation are increasingly crucial. Though conventional chromatographic methods such as HPLC-UV and LC-MS have been used quite successfully for this purpose, they share inherent limitations such as lack of reproducibility and, principally, the fact that absolute quantitation is possible only with the aid of a calibration curve of each metabolite. Moreover, these methods require a relatively longer time of analysis. The use of NMR-based metabolic characterization can be a very promising solution for many of these problems.

In this study, leaves, stems, and roots collected in diverse regions of India were evaluated by ^{1}H NMR spectroscopy and multivariate data analysis. An overview of a wide range of metabolites in a sample including amino acids, flavonoids, lipids, organic acids, phenylpropanoids, steroids, and sugars, can be obtained within very short time (less than 10 min analysis time). Of the organs analyzed, the leaf exhibited the widest range of metabolites. Additionally, among the metabolites detected by ^{1}H NMR spectroscopy, the ratio between two major types of withanolides, those containing 4-OH and 5,6-epoxy groups (withaferin A-like steroids) and those containing 5-OH and 6,7-epoxy groups (withanolides A-like steroids), was found to be a marker for discriminating leaf samples.

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Conflict of Interest

No conflict of interest.

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